uPA deficiency exacerbates muscular dystrophy in *MDX* mice

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Duchenne muscular dystrophy (DMD) is a fatal and incurable muscle degenerative disorder. We identify a function of the protease urokinase plasminogen activator (uPA) in *mdx* mice, a mouse model of DMD. The expression of uPA is induced in *mdx* dystrophic muscle, and the genetic loss of uPA in *mdx* mice exacerbated muscle dystrophy and reduced muscular function. Bone marrow (BM) transplantation experiments revealed a critical function for BM-derived uPA in *mdx* muscle repair via three mechanisms: (1) by promoting the infiltration of BM-derived inflammatory cells; (2) by preventing the excessive deposition of fibrin; and (3) by promoting myoblast migration. Interestingly, genetic loss of the uPA receptor in *mdx* mice did not exacerbate muscular dystrophy in *mdx* mice, suggesting that uPA exerts its effects independently of its receptor. These findings underscore the importance of uPA in muscular dystrophy.

Introduction

Duchenne muscular dystrophy (DMD) is one of the most common X-linked lethal diseases, affecting 1 in 3,500 newborn males. DMD results from mutations in the gene coding for the protein dystrophin, which localizes at the innerface of the sarcolemma. Dystrophin associates with a large complex of membrane proteins, called the dystrophin glycoprotein complex, which is important for cell membrane integrity (Hoffman et al., 1987; Ervasti and Campbell, 1991). Without the dystrophin complex to tether the actin cytoskeleton inside the muscle cell to the extracellular matrix, forces generated by the muscle fiber result in tears of sarcolemma, leading to muscle damage (for review see Campbell, 1995). The *mdx* mouse strain is the most widely used animal model for DMD, having a nonsense mutation in exon 23, which eliminates dystrophin expression (Sicinski et al., 1989; Durbeej and Campbell, 2002). Human patients with DMD and *mdx* mice suffer from progressive muscle cell degeneration and regeneration episodes. Ultimately, however, the dystrophic muscle damage cannot be repaired any longer, and the dystrophic myofibers become gradually replaced, initially by fibrotic infiltrates and subsequently by fat tissue (Stedman et al., 1991).

DMD remains an incurable and devastating disease. Therapies based on the restoration of dystrophin expression or the administration of dystrophin$^+$ stem cells are promising but are still in the preclinical phase (Goyenvalle et al., 2004; Gregorevic et al., 2006; Montarras et al., 2005; Sampaolesi et al., 2006; Shi and Garry, 2006; Welch et al., 2007). Intense research efforts have identified muscle-specific factors regulating muscle progenitor cell (satellite cell [SC]) functions (i.e., proliferation and differentiation), which also play a key role in *mdx* muscle regeneration (e.g., Pax7, MyoD family members, etc.; Megeney et al., 1996; Sabourin et al., 1999; Seale et al., 2000; Charge and Rudnicki, 2004; Oustanina et al., 2004; Kuang et al., 2006). However, these intrinsic factors will be difficult to target throughout the musculature when developing alternative therapies to treat DMD disease.

Mounting evidence indicates a critical involvement of extrinsic factors in DMD disease progression and the recovery of

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Abbreviations used in this paper: BM, bone marrow; CK, creatine kinase; CTX, cardiotoxin; DMD, Duchenne muscular dystrophy; HE, hematoxylin/eosin; HGF, hepatocyte growth factor; SC, satellite cell; SF, scatter factor; uPA, urokinase plasminogen activator; uPAR, uPA receptor; WT, wild type.

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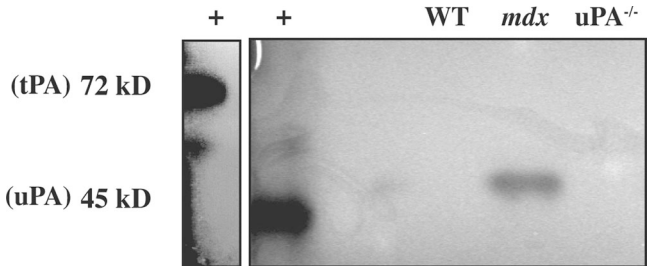

Figure 1. **Increased activity of uPA in *mdx* muscle dystrophy.** Zymographic analysis of WT, *mdx*, and uPA$^{-/-}$ muscles. Purified murine uPA (45 kD) and tPA (72 kD) were used as a control for activity (+).

injured muscles. Indeed, infiltrated inflammatory cells release several cytokines and growth factors that modulate muscle degeneration, inflammation, and regeneration (e.g., TNFα, VEGF, and nitric oxide synthase; Collins and Grounds, 2001; Wehling et al., 2001; Germani et al., 2003; Grounds and Torrisi, 2004; Chen et al., 2005, 2006; Tidball, 2005; Brunelli et al., 2007). We previously reported a critical role of the protease urokinase plasminogen activator (uPA) in the recovery of experimentally injured muscle (Lluis et al., 2001). Among the several enzymatic functions of uPA, the most classic one is the ability to convert the zymogen plasminogen into active plasmin, whose classic role is degradation of the fibrinogen end product fibrin (from here on, we refer to both by the term fibrin/ogen). By binding of uPA to its uPA receptor (uPAR), uPAR localizes the conversion of plasminogen to plasmin to the cell surface, thereby increasing pericellular proteolysis. In addition, uPAR also allows uPA to induce intracellular signaling, thereby promoting cell proliferation and migration (Blasi and Carmeliet, 2002; Mondino and Blasi, 2004). Importantly, uPA and plasmin promote inflammatory cell infiltration and repair of injured muscle, whereas the role of uPAR herein remains unclear (Lluis et al., 2001; Suelves et al., 2002). As the role of uPA and uPAR in *mdx* dystrophy remains unknown, we therefore intercrossed *mdx* mice with mice lacking either uPA (uPA$^{-/-}$) or uPAR (uPAR$^{-/-}$) and examined disease progression and its pathological features.

Results

Expression of uPA in *mdx* muscle dystrophy

We previously showed that uPA mediates the recovery of experimentally injured muscle (Lluis et al., 2001), but its role in *mdx* dystrophy remains unknown. Therefore, we first analyzed by zymography uPA activity in *mdx* muscle extracts before and after the onset of muscle degeneration. At 14 d of age (i.e., before disease onset), the activity levels of uPA were undetectable in wild-type (WT) mice and in *mdx* mice (unpublished data). In contrast, after disease onset (i.e., 30 d of age), the activity levels of uPA were increased in *mdx* muscle but not in WT muscle (Fig. 1). These changes were specific for uPA, as no lytic band corresponding to tPA (at 72 kD) was detected by zymography (Fig. 1). Thus, uPA activity is specifically increased in *mdx* dystrophic muscle during disease.

Genetic loss of uPA exacerbates *mdx* dystrophic disease

To evaluate whether uPA would affect the disease course in *mdx* mice, we intercrossed *mdx* mice with uPA$^{-/-}$ mice and phenotyped uPA$^{+/+}$*mdx* (from here on referred to as *mdx*) and uPA$^{-/-}$*mdx* littermates. Both genotypes were healthy at birth and did not show any signs of muscle injury or differences in muscle size before disease onset (14 d of age; Fig. 2, a and d; and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200705127/DC1). Beyond 3–4 wk of age, obvious signs of muscle dystrophy were detectable in *mdx* and uPA$^{-/-}$*mdx* mice. However, compared with *mdx* mice, uPA$^{-/-}$*mdx* mice suffered from a much more severe dystrophinopathy, at least up to 4 mo of age, as characterized by a more widespread and extensive myofiber degeneration and necrosis (Fig. 2 a). Indeed, uPA$^{-/-}$*mdx* muscles contained larger areas of muscle damage and significantly more clusters of degenerated myofibers ($P < 0.05$; Fig. 2 b). Furthermore, von Kossa–stained calcium deposits, which are typically found in necrotic myopathies (Franco and Lansman, 1990), were almost exclusively detected in uPA$^{-/-}$*mdx* but minimally in *mdx* muscle (Fig. 2 c). Moreover, the number of centrally nucleated fibers (indicator of muscle regeneration) was lower in uPA$^{-/-}$*mdx* than in *mdx* muscle (Fig. 2 e). Consistent with this, the mean muscle cross-sectional area and myofiber size were smaller in uPA$^{-/-}$*mdx* mice as compared with *mdx* mice (Fig. 2 d and Table S1).

To ascertain worsening in the pathology of the whole skeletal musculature, we measured the serum levels of creatine kinase (CK), a biomarker of sarcolemmal damage (Bulfield et al., 1984). Consistent with the more severe muscle degeneration, uPA$^{-/-}$*mdx* mice showed approximately twofold higher serum CK levels as compared with *mdx* mice at 2.5 mo of age (Fig. 2 f). To determine the functional status of the diseased muscle, we used grip-strength and treadmill assays. Compared with *mdx* mice, muscle strength at 2.5 mo of age was substantially decreased in uPA$^{-/-}$*mdx* mice in both assays (Fig. 2 g). Altogether, these findings provide histological, biochemical, and functional evidence that uPA deficiency aggravates muscle degeneration and attenuates regeneration in *mdx* muscle.

BM-derived uPA promotes the infiltration of inflammatory cells into *mdx* dystrophic muscle

In experimentally injured muscle, uPA is produced by SCs and by inflammatory cells (Lluis et al., 2001). Although T lymphocytes and neutrophils also infiltrate dystrophic *mdx* muscles, infiltrated macrophages appear to be the major inflammatory cell type (Fig. S1 a, available at http://www.jcb.org/cgi/content/full/jcb.200705127/DC1; Engel and Arahata, 1986; Pimorady-Esfahani et al., 1997; Spencer et al., 2001; Tidball, 2005). We first aimed to analyze the impact of uPA deficiency in the inflammatory response in *mdx* muscular dystrophy. Before disease onset (i.e., at 14 d of age), Mac-1$^+$ macrophages and T-11$^+$ T lymphocytes were rarely detected in *mdx* or uPA$^{-/-}$*mdx* muscles (Fig. 3 a). After disease onset (i.e., at 30 d of age), these inflammatory cells had infiltrated the dystrophic muscle of *mdx* mice (Fig. 3 a). However, compared with *mdx* mice, the number of infiltrated

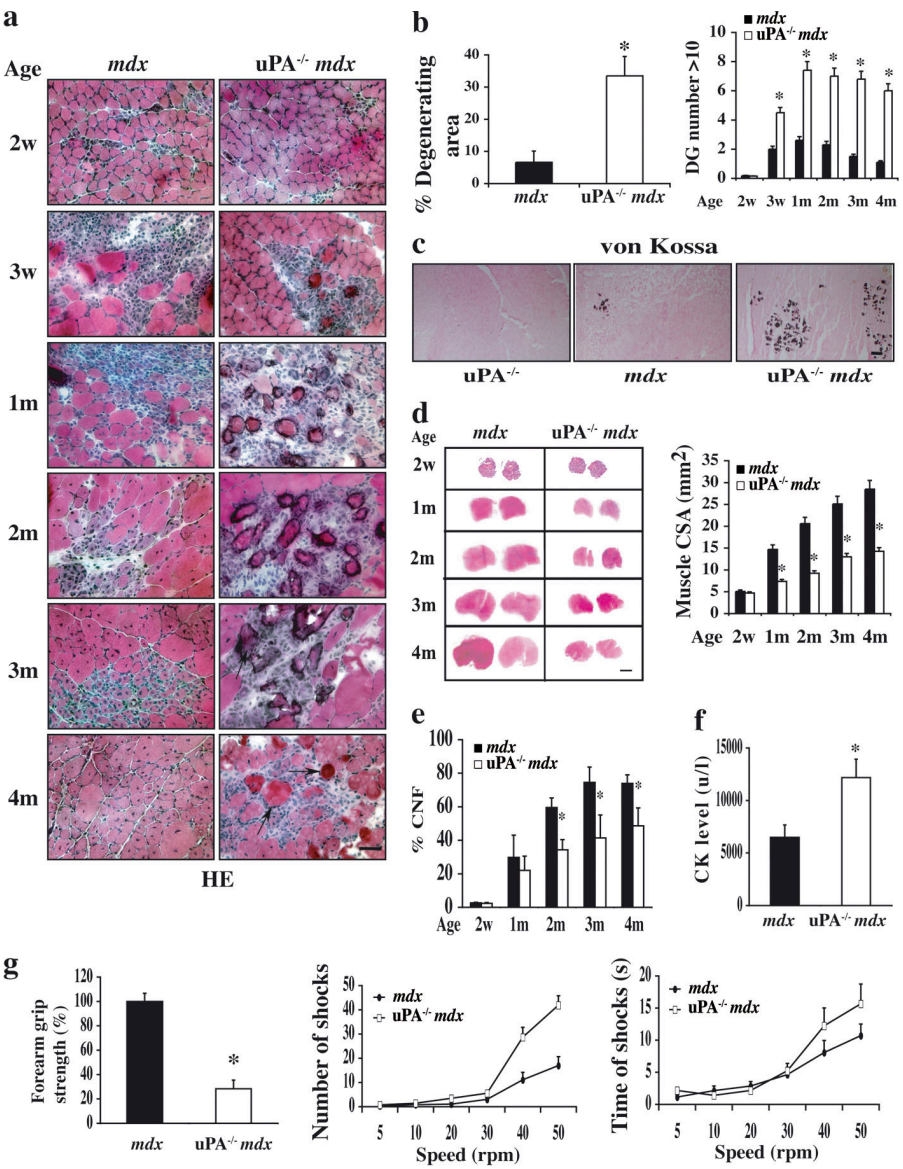

Figure 2. **uPA deficiency exacerbates *mdx* muscle degeneration.** (a) Muscle sections of *mdx* and uPA$^{-/-}$*mdx* mice of 2 and 3 wk of age and of 1–4 mo of age were stained with HE. Nonphagocytosed necrotic fibers in uPA$^{-/-}$*mdx* muscle sections are indicated with arrows. (b) Percentage of total muscle-degenerating area of 1-mo-old mice (left) and mean number of degenerating groups (DGs) containing >10 fibers per muscle section (right) at the indicated ages. (c) Von Kossa staining of muscles of uPA$^{-/-}$, *mdx*, and uPA$^{-/-}$*mdx* mice (at 1 mo of age). (d) Reduced muscle size in uPA$^{-/-}$*mdx* mice. (left) HE staining of gastrocnemius muscle sections (at the indicated ages). (right) Muscle cross-sectional area (CSA; at the indicated ages). (e) Reduced muscle regeneration in uPA$^{-/-}$*mdx* mice. Percentage of central nucleated fibers (CNF; at the indicated ages). (f) Increased muscle damage in uPA$^{-/-}$*mdx* mice. Serum CK levels in *mdx* and uPA$^{-/-}$*mdx* mice at 2.5 mo of age. (g) Reduced muscle strength in uPA$^{-/-}$*mdx* mice. Comparison of functional muscle strength between *mdx* and uPA$^{-/-}$*mdx* mice at 2.5 mo of age in grip strength (left) and treadmill assays (middle and right) as described in Materials and methods. Data are means ± SEM (error bars). $n = 10$ animals per group. *, $P < 0.05$. Bars (a and c), 50 µm; (d) 300 µm.

Mac-1$^+$ and T-11$^+$ cells in uPA$^{-/-}$*mdx* muscle was reduced up to ∼50% (Fig. 3 a). Consistent with this, the loss of uPA also reduced the number of infiltrated inflammatory cells in cardiotoxin (CTX)-injured muscle (Fig. S2 a). This was not the result of a genotypic difference in the number of circulating leukocytes in the peripheral blood (unpublished data).

This reduced infiltration and accumulation of inflammatory cells in uPA$^{-/-}$*mdx* dystrophic muscles was likely attributable to the fact that they lack the uPA needed to invade injured tissues. Indeed, when performing in vitro migration experiments, uPA$^{-/-}$*mdx* and uPA$^{-/-}$ macrophages were found to migrate less compared with control cells (Figs. 3 b and S2 b). Therefore, we evaluated whether the conditional restoration of uPA expression in the bone marrow (BM) of uPA$^{-/-}$*mdx* mice achieved via the transplantation of uPA$^{+/+}$*mdx* BM (termed uPA$^{-/-}$*mdx*$^{(\text{mdx-BM})}$ mice from here on) could revert the deficient inflammatory response. As a negative control, we transplanted uPA$^{-/-}$*mdx* BM into uPA$^{-/-}$*mdx* mice (uPA$^{-/-}$*mdx*$^{(\text{uPA}-/-\text{mdx-BM})}$ mice). We also transplanted WT BM into uPA$^{-/-}$ recipient mice (termed uPA$^{-/-(\text{WT-BM})}$ mice from here on) or into WT mice (WT$^{(\text{WT-BM})}$ mice) and induced muscle injury by intramuscular injection of CTX (supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200705127/DC1). In both experiments, we found that the transplantation of uPA-expressing BM increased the infiltration of inflammatory cells into dystrophic or injured uPA-deficient muscles. Indeed, compared with uPA$^{-/-}$*mdx*$^{(\text{uPA}-/-\text{mdx-BM})}$ mice, muscles in uPA$^{-/-}$*mdx*$^{(\text{mdx-BM})}$ mice became infiltrated with plenty of (uPA expressing) inflammatory cells (Fig. 3 c); likewise, inflammatory cells accumulated in the damaged muscle in uPA$^{-/-(\text{WT-BM})}$ mice to the levels found in WT or WT$^{(\text{WT-BM})}$ mice (Fig. S2 c). Together, these data demonstrate that uPA is critical for inflammatory cells to infiltrate the degenerating myofibers of *mdx* mice.

BM-derived uPA attenuates the degeneration of *mdx* dystrophic muscle

There is increasing evidence that the inflammatory response can promote both muscle injury and repair (Tidball, 2005;

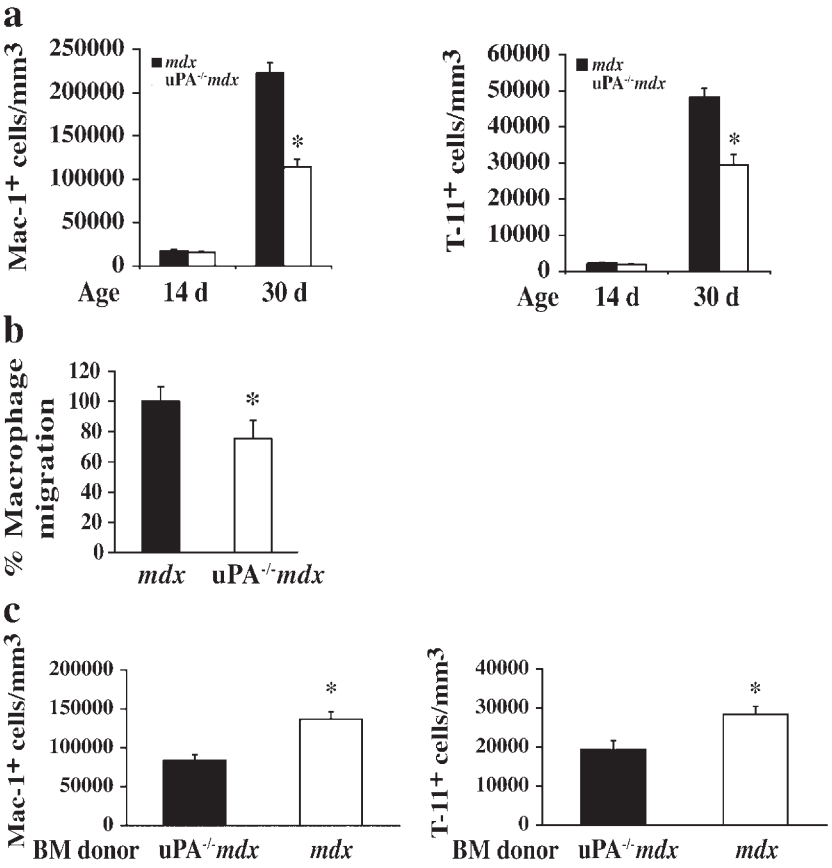

Figure 3. **BM-derived uPA promotes the infiltration of inflammatory cells into *mdx* dystrophic muscle.** (a) uPA deficiency reduces the inflammatory response in *mdx* dystrophic muscle. Number of Mac-1– and T-11–positive cells in muscle sections of *mdx* and uPA$^{-/-}$*mdx* mice at 14 d of age (i.e., before the onset of degeneration) and at 30 d of age (15 d after the onset of degeneration). (b) uPA deficiency reduces *mdx* macrophage migration in vitro. Migration assays were performed in transwells. Conditioned medium from *mdx* mouse primary SC cultures was placed in the lower chamber of the transwell. Macrophages obtained from *mdx* or uPA$^{-/-}$*mdx* mice were placed in the upper chambers. Experiments (three) were performed in duplicate. The value 100% was arbitrarily given to the number of migrating *mdx* macrophages. (c) Number of Mac-1– and T-11–positive cells in muscle sections of uPA$^{-/-}$*mdx* mice transplanted with BM from uPA$^{-/-}$*mdx* or *mdx* donor mice, respectively. Data are means ± SEM (error bars). $n = 4$ animals per group. *, $P < 0.05$.

Arnold et al., 2007; Pelosi et al., 2007; Tidball and Wehling-Henricks, 2007). Therefore, we evaluated whether the transplantation of uPA-expressing BM also attenuated muscle degeneration in *mdx* mice. Compared with uPA$^{-/-}$*mdx*$^{(\text{uPA}-/-\text{mdx-BM})}$ mice, muscles in uPA$^{-/-}$*mdx*$^{(\text{mdx-BM})}$ mice exhibited less severe signs of degeneration at 2 mo after transplantation (Fig. 4 a). Consistent with this, serum CK levels were lower in uPA$^{-/-}$*mdx*$^{(\text{mdx-BM})}$ than in uPA$^{-/-}$*mdx*$^{(\text{uPA}-/-\text{mdx-BM})}$ mice (Fig. 4 b). Thus, uPA-expressing BM-derived cells attenuate muscle degeneration in uPA$^{-/-}$*mdx* mice. Consistent with this notion, muscle damage was reduced and regeneration was rescued in uPA$^{-/-(\text{WT-BM})}$ mice at 10 d and 25 d after CTX injury, respectively, whereas degeneration persisted in nontransplanted uPA-deficient mice (Fig. 4 c).

Reduction of fibrin/ogen levels by BM-derived uPA or by ancrod treatment reduces the exacerbated degeneration of uPA$^{-/-}$*mdx* mice

We previously showed that the persistent muscle degeneration in uPA$^{-/-}$ mice after injury was mediated, at least in part, by the impaired dissolution of intramuscular fibrin/ogen deposits (Lluis et al., 2001). Therefore, we analyzed in *mdx* and uPA$^{-/-}$*mdx* muscle the extent of fibrin/ogen accumulation before and after disease onset. Before disease onset (14 d of age), fibrin/ogen was undetectable by immunostaining or Western blotting in *mdx* and uPA$^{-/-}$*mdx* muscles (unpublished data). However, at the first disease peak (30 d of age), fibrin/ogen deposits were readily detectable in muscles of both genotypes (Fig. 5 a). Importantly, however, compared with *mdx* muscles, fibrin/ogen deposition was increased in uPA$^{-/-}$*mdx* muscles up to 2.5-fold (Fig. 5 a). Interestingly, the prior transplantation of uPA-expressing BM cells attenuated this increased deposition of fibrin/ogen in uPA$^{-/-}$*mdx* mice (Fig. 5 b) and in uPA$^{-/-}$ mice challenged with CTX (Fig. S2 d).

To directly prove that the increased accumulation of fibrin/ogen mediated the exacerbated dystrophic disease in uPA$^{-/-}$*mdx* mice, we depleted the circulating fibrinogen levels by administering the defibrinogenating snake venom ancrod to uPA$^{-/-}$*mdx* mice. Daily delivery of ancrod (1 U per day) starting at 12 d after birth and continuing for 18 d thereafter effectively reduced the accumulation of fibrin/ogen in uPA$^{-/-}$*mdx* muscles (Fig. 5 c). Importantly, compared with saline, the area of degenerated muscle in uPA$^{-/-}$*mdx* mice was significantly reduced ($P < 0.05$) by ancrod therapy, indicating that the increased deposition of fibrin/ogen mediated the severe muscle dystrophy in uPA$^{-/-}$*mdx* mice (Fig. 5, d and e). In addition, compared with saline, fewer muscle groups containing >10 degenerating fibers were found in ancrod-treated uPA$^{-/-}$*mdx* mice (Fig. 5 e). However, ancrod treatment in uPA$^{-/-}$*mdx* mice failed to completely rescue the exacerbated muscle dystrophy phenotype of uPA$^{-/-}$*mdx* mice. Indeed, compared with *mdx* mice, the muscle degeneration area was still larger in ancrod-treated uPA$^{-/-}$*mdx* mice (compare Fig. 5 e with Fig. 2 b). This incomplete rescue might be attributable to the finding that inflammatory infiltration remained halted in

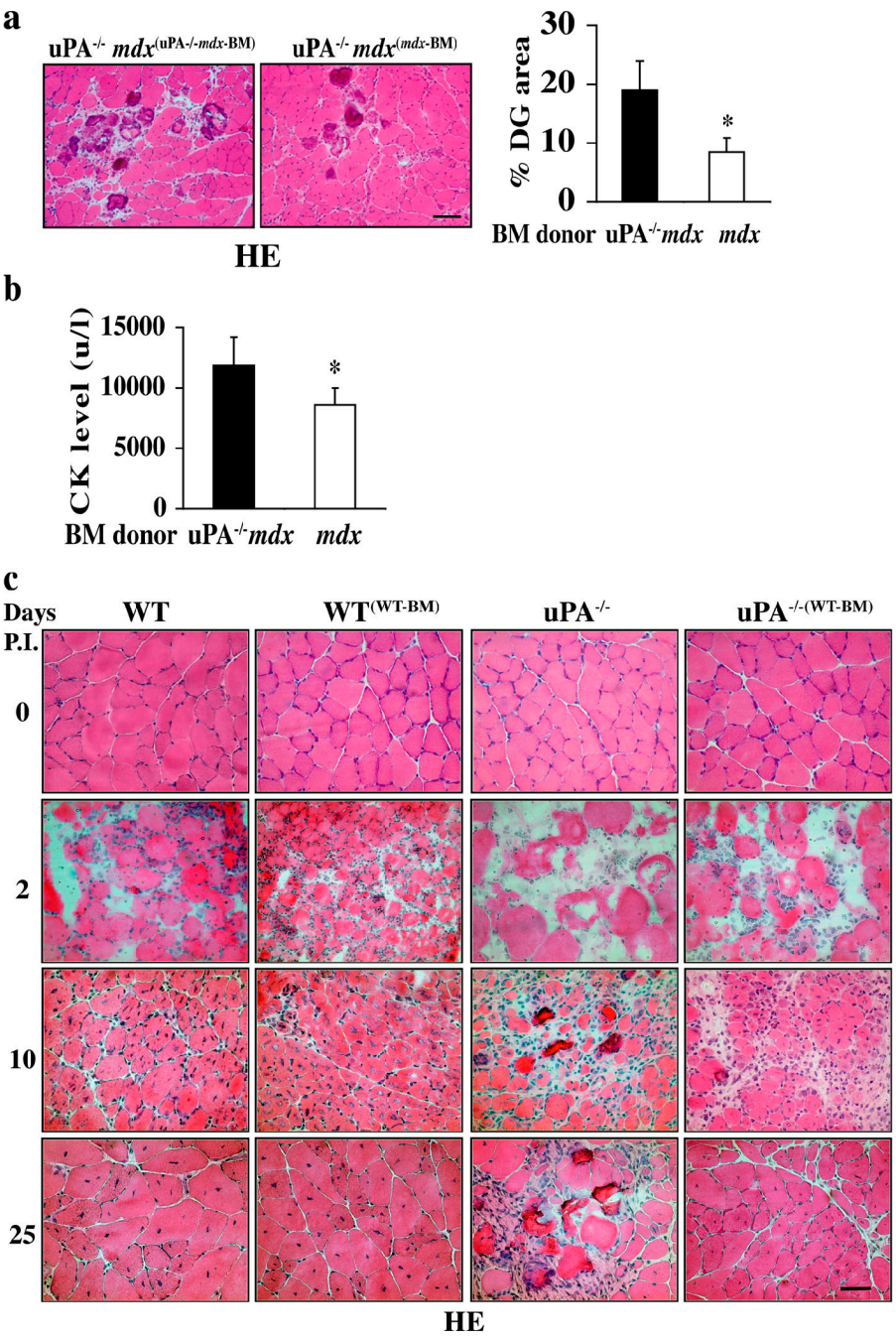

Figure 4. **Transplantation of uPA-expressing BM ameliorates the severe uPA$^{-/-}$mdx muscular dystrophy and rescues the regeneration defect in CTX-injured uPA$^{-/-}$ mice.** (a and b) BM from *mdx* or uPA$^{-/-}$*mdx* donor mice was transplanted into uPA$^{-/-}$*mdx* mice, and different muscle parameters were analyzed at 2 mo after transplantation. (a) Histological analysis. (left) HE staining of muscle sections. (right) Reduced degeneration in uPA$^{-/-}$*mdx* mice transplanted with BM from *mdx* donor mice. Percentage of total muscle-degenerating area. DG, degenerating group. (b) Reduced muscle damage in uPA$^{-/-}$*mdx* mice transplanted with BM from *mdx* donor mice as reflected by decreased serum CK levels. (c) BM transplantation rescues the regeneration defect of uPA$^{-/-}$ mice. BM from WT mice was transplanted into uPA$^{-/-}$ mice (uPA$^{-/-(WT\text{-}BM)}$) and into WT mice (WT$^{(WT\text{-}BM)}$). At 6 wk after transplantation, muscle injury was induced by CTX injection in these mice as well as in aged-matched nontransplanted WT and uPA$^{-/-}$ mice. HE staining. Data are means ± SEM (error bars). $n = 4$ animals per group. *, $P < 0.05$. Bars, 50 µm.

uPA$^{-/-}$*mdx* mice after ancrod treatment (Fig. 5 f). This result further underscores the importance of BM-derived uPA in the infiltration of inflammatory cells (Fig. 3). Thus, BM-derived uPA is required for dissolving fibrin/ogen deposits in dystrophic *mdx* muscles, but it also mediates processes independent of fibrinolysis.

BM cell–derived uPA promotes regeneration by stimulating muscle cell migration

To further study the role of uPA during muscle regeneration, we used the model of CTX-induced muscle injury, wherein regeneration can be analyzed in a more time-controlled fashion. Consistent with the *mdx* model, the loss of uPA impaired muscle regeneration in the CTX model (Fig. 4 c and supplemental material). Notably, transplantation of WT BM improved the defective muscle regeneration in uPA$^{-/-}$ mice (uPA$^{-/-(WT\text{-}BM)}$ mice), thereby highlighting the importance of BM-derived uPA in muscle repair (Fig. 4 c). However, we found no evidence of a relevant direct contribution of BM-derived uPA-expressing cells to regenerating myofibers (very few GFP-positive myofibers were detected after transplanting GFP-labeled WT BM cells; Fig. S2 e), suggesting that these cells likely promoted muscle regeneration via paracrine pathways.

During myofiber regeneration, resident SCs proliferate, migrate to, and fuse with the injured muscle fibers. As the loss of uPA in the *mdx* and CTX models reduced the number of

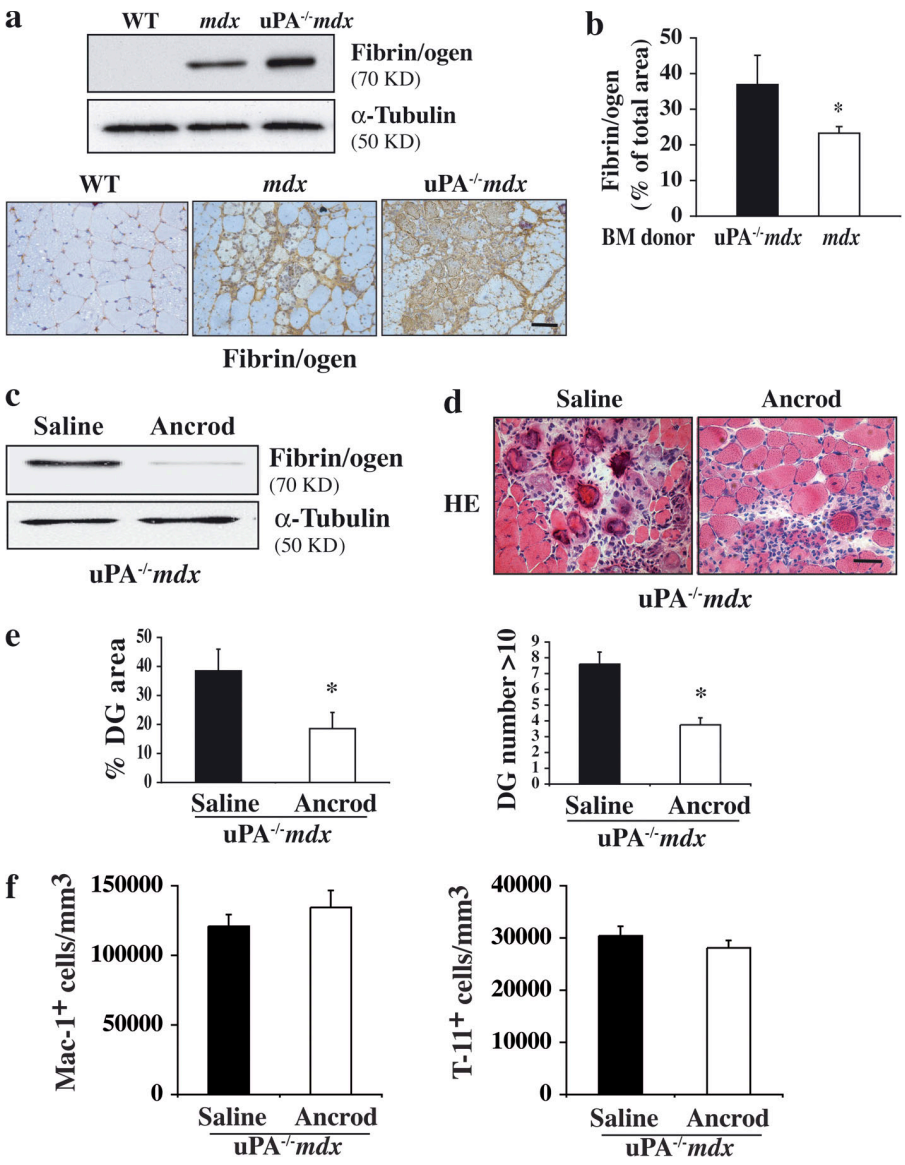

Figure 5. **Reduction of fibrin/ogen levels by BM-derived uPA or by ancrod treatment reduces the exacerbated degeneration of uPA$^{-/-}$*mdx* mice.** (a) Increased fibrin/ogen deposition in the muscle of uPA$^{-/-}$*mdx* mice. (top) Western blotting analysis of extracts of WT, *mdx*, and uPA$^{-/-}$*mdx* muscles at 30 d of age using an anti-fibrin/ogen antibody. α-Tubulin was analyzed as a loading control. (bottom) Fibrin/ogen deposition in WT, *mdx*, and uPA$^{-/-}$*mdx* muscles at 30 d of age was analyzed by immunohistochemistry. (b) Fibrin/ogen levels were quantified in muscle sections of uPA$^{-/-}$*mdx* mice transplanted with BM from uPA$^{-/-}$*mdx* or *mdx* donor mice. Fibrin/ogen was detected by immunohistochemistry, quantified, and represented as the percentage of total muscle area. $n = 7$. (c) 12-d-old uPA$^{-/-}$*mdx* mice were intraperitoneally injected daily with ancrod or with saline solution for 18 d up to 30 d of age. Comparison of fibrin/ogen levels in muscle of uPA$^{-/-}$*mdx* mice (at 30 d of age) after ancrod or saline treatment. (d) HE staining of muscle sections. (e) Percentage of the total degenerating area of muscles (left) and mean number of degenerating groups (DGs) containing >10 fibers (right) per muscle section. (f) Number of Mac-1– and T-11–positive cells in muscles of uPA$^{-/-}$*mdx* mice of 30 d of age that had been treated for 18 d with saline or ancrod starting at 12 d of age. Data are means ± SEM (error bars). $n = 4$ animals per group. *, $P < 0.05$. Bar, 50 μm.

regenerating myofibers (Figs. 2 and 4), we wondered whether uPA, which is expressed by SCs (Fig. S1 b; Lluis et al., 2001), might also affect SC functions. Activation and proliferation rates of SCs were comparable in *mdx* and uPA$^{-/-}$*mdx* muscles or in CTX-challenged WT and uPA$^{-/-}$ muscles (Fig. 6 a). Consistent with this, although uPA/plasmin mediates the activation of hepatocyte growth factor (HGF)/scatter factor (SF) and TGF-β1 (i.e., modulators of SC activation and proliferation; Naldini et al., 1992; Robertson et al., 1993; Odekon et al., 1994; Pimorady-Esfahani et al., 1997; Tatsumi et al., 1998; Yablonka-Reuveni et al., 1999; Shefer et al., 2001), the active levels of these factors were comparable in *mdx* and uPA$^{-/-}$*mdx* muscles or in WT and uPA$^{-/-}$ injured muscle (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200705127/DC1). Furthermore, SC–derived primary myoblasts from uPA$^{-/-}$ muscle showed normal proliferation and migration in vitro (Fig. 6, b–d). Interestingly, however, the addition of murine recombinant (r-uPA) stimulated the migration of WT and uPA$^{-/-}$ myoblasts in both scratch wounds and transwell assays (Fig. 6, c and d), although it failed to affect the proliferation rates (Fig. 6 b). Consistent with the promigratory effect of uPA, myoblast migration was increased in the presence of conditioned medium obtained from WT macrophage cultures (compared with nonconditioned control medium) but was only minimally stimulated by uPA$^{-/-}$ macrophage conditioned medium (Fig. 6 e). The migration in response to WT macrophage conditioned medium was abrogated when the uPA inhibitor amiloride was added (Fig. 6 e). Moreover, the absence of migration in response to uPA$^{-/-}$ macrophage conditioned medium was restored by supplementation with r-uPA (Fig. 6 e). Thus, our data suggest that macrophage-derived uPA might promote muscle regeneration by enhancing SC migration.

Dispensability of uPAR for muscle regeneration after injury and in *mdx* dystrophinopathy

By binding to uPAR, uPA is capable of exerting its proteolytic effects at the pericellular level, but it also enables uPA to

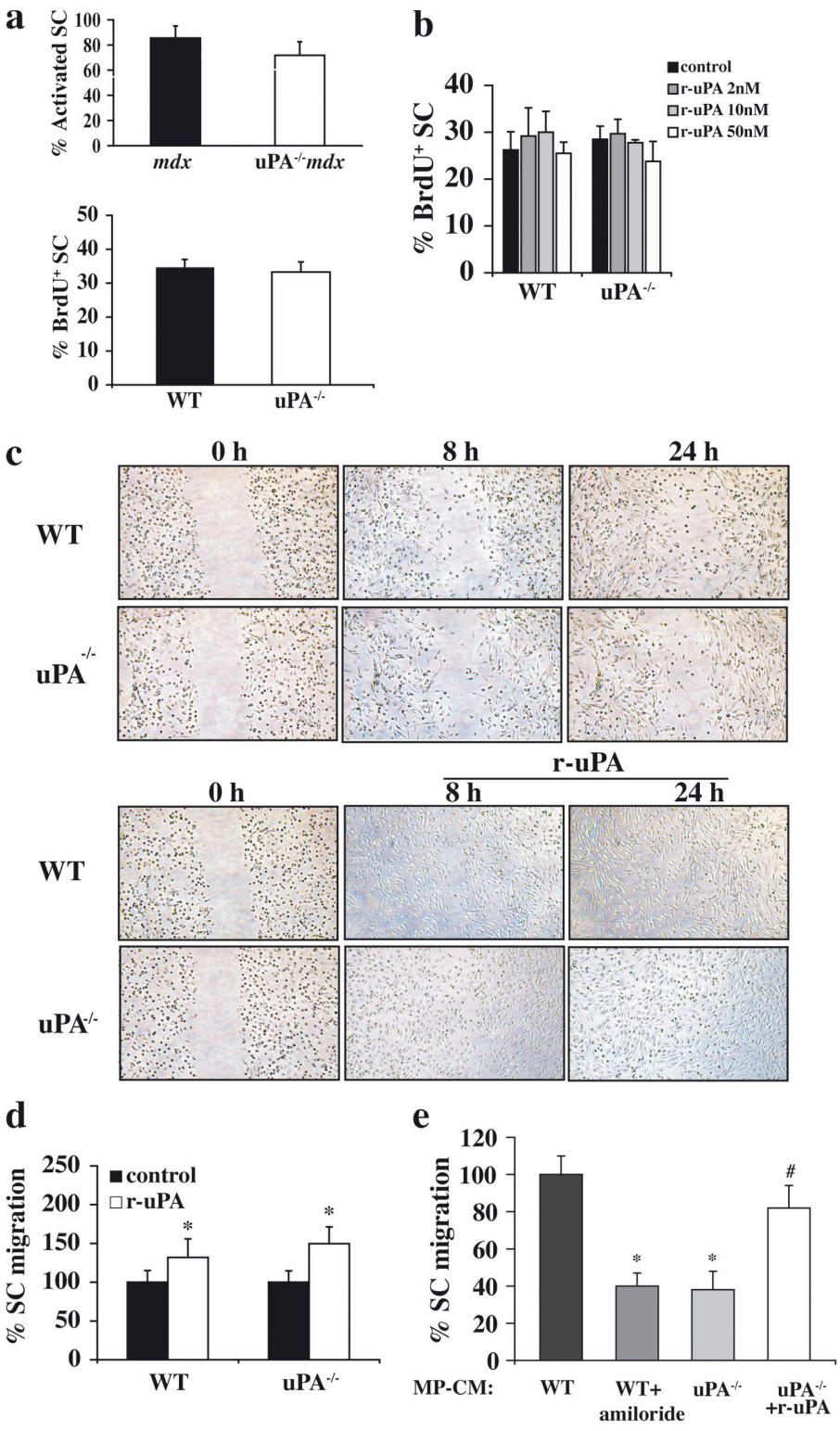

Figure 6. **Macrophage-released uPA stimulates SC migration.** (a, top) Comparative analysis of activated satellite cells (SCs) in muscles of *mdx* and uPA$^{-/-}$*mdx* mice. Freshly isolated SCs from muscles of *mdx* and uPA$^{-/-}$*mdx* mice were analyzed for the expression of CD34 (SC marker) and MyoD (activated SC marker); the percentage of activated SCs positive for both CD34 and MyoD is represented. The 100% value referred to the total number of SCs (CD34 positive). (bottom) Comparative analysis of SC proliferation in vivo. WT and uPA$^{-/-}$ muscles were injected with CTX, and, 24 h later, BrdU was injected intraperitoneally; 18 h later, SCs were harvested and cultured for 24 h. The percentage of SCs that had been BrdU labeled in vivo was determined by immunocytochemistry and counted microscopically. $n = 3$ animals per group. (b) Effect of uPA in SC proliferation. WT and uPA$^{-/-}$ SCs were cultured in growth medium, and recombinant murine uPA (r-uPA) was added when indicated. Cells were labeled with BrdU for 2 h, and BrdU incorporation was determined as in panel a. (c and d) Effect of uPA on SC migration. Cell migration assays were performed on plates or transwells coated with matrigel. (c) Wound-healing assay in vitro of WT and uPA$^{-/-}$ SCs in the absence or presence of r-uPA. Cells were photographed at 0, 8, and 24 h after wounding. (d) WT and uPA$^{-/-}$ SCs were added to the upper chamber of the transwell. 10 nM r-uPA was added to the lower chamber of the transwell when indicated. The value 100% was arbitrarily given to the number of WT SCs that had migrated. (e) Conditioned medium (CM) from WT and uPA$^{-/-}$ macrophages (MP) without or with supplementation of amiloride or r-uPA as indicated was placed in the lower chamber of the transwell, and SCs from WT mice were placed in the upper chambers. Culture medium alone was used as a reference for basal migration. Migration of SCs in WT macrophage conditioned medium conditions was given an arbitrary value of 100%. $n = 3$ experiments performed in triplicate. *, $P < 0.05$ versus WT values; #, $P < 0.05$ versus uPA$^{-/-}$ values. Data are means ± SEM (error bars).

promote cell proliferation and migration via nonproteolytic pathways (Blasi and Carmeliet, 2002; Mondino and Blasi, 2004). We found that uPAR expression was induced in muscle extracts of WT mice after CTX injury and of *mdx* mice after disease onset (Fig. 7 a). Thus, we reasoned that the role of uPA in muscle regeneration might be dependent, at least in part, on its binding to uPAR. To directly evaluate this hypothesis, we performed CTX injury in muscles of WT and uPAR-deficient mice (uPAR$^{-/-}$), crossbred the *mdx* mice into the uPAR-deficient background (uPAR$^{-/-}$*mdx* mice), and analyzed the consequences of uPAR deficiency on muscle regeneration in both models. CTX-induced muscle regeneration was indistinguishable between WT and uPAR$^{-/-}$ mice after histological analyses at 2, 10, and 25 d after injury (Fig. 7 b). Consistent with this,

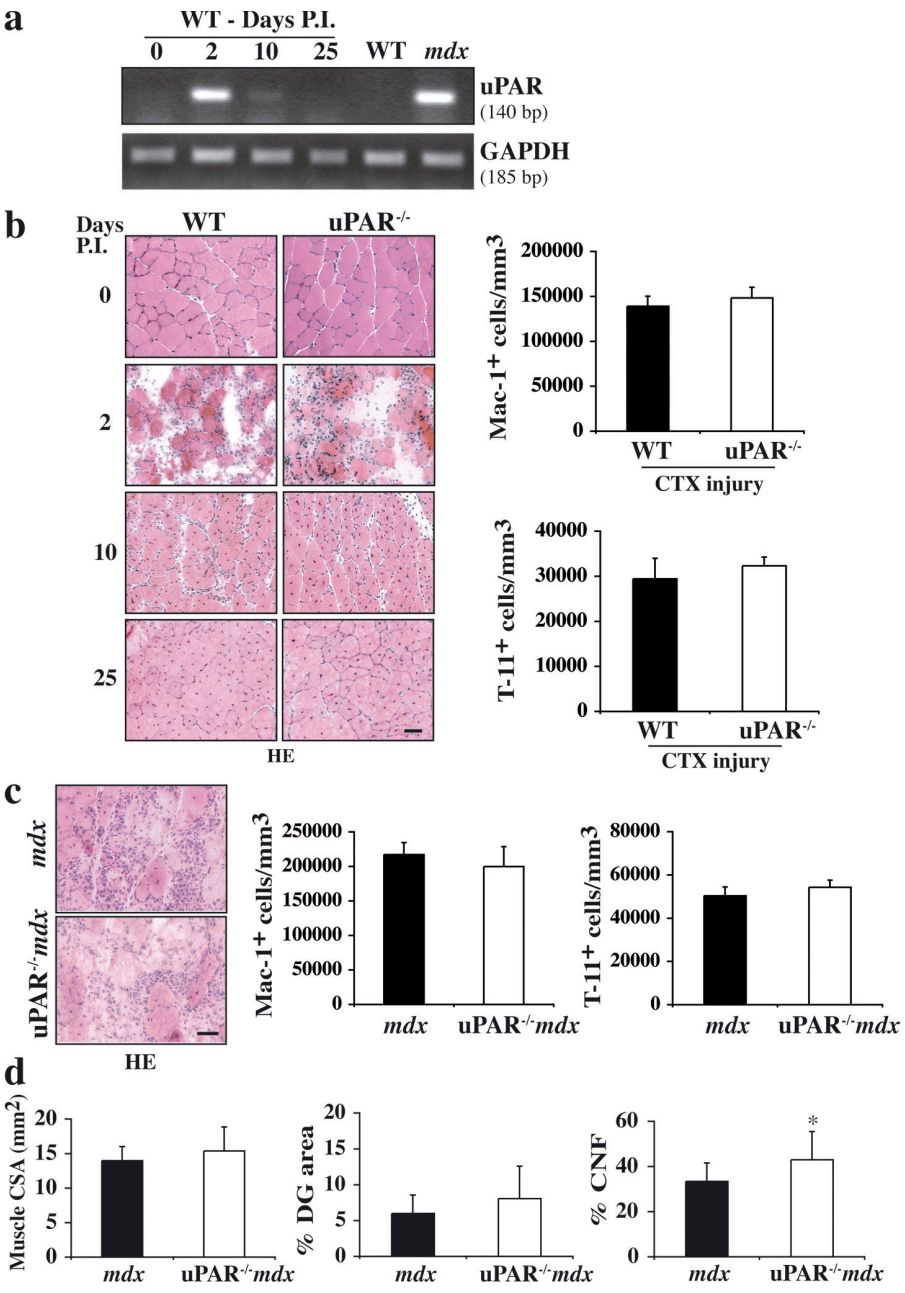

Figure 7. **uPAR is dispensable for muscle regeneration.** (a) Induction of uPAR expression in CTX-injured WT muscles and *mdx* muscle as shown by RT-PCR. (b, left) Muscle sections of control (noninjured) and CTX-injured WT and uPAR$^{-/-}$ mice stained with HE. (right) Number of Mac-1– and T-11–positive cells in muscle sections of WT and uPAR$^{-/-}$ mice at 2 d after CTX injury. (c, left) HE staining of 1-mo-old *mdx* and uPAR$^{-/-}$*mdx* mice muscle sections. (middle and right) Number of Mac-1– and T-11–positive cells in muscle sections of *mdx* and uPAR$^{-/-}$*mdx* mice at 30 d of age. (d, left) Muscle cross-sectional area (CSA) of 1-mo-old *mdx* and uPAR$^{-/-}$*mdx* mice. (middle) Percentage of total muscle-degenerating area of 1-mo-old *mdx* and uPAR$^{-/-}$*mdx* mice. (right) Percentage of central-nucleated fibers (CNF) in 1-mo-old *mdx* and uPAR$^{-/-}$*mdx* mice. Data are means ± SEM (error bars). $n = 6$ animals per group. *, $P < 0.05$. Bars, 50 μm.

the infiltration of inflammatory cells was also not affected in the absence of uPAR (Fig. 7 b). Most importantly, the muscle cross-sectional area and the extent of muscular dystrophy were also similar in *mdx* and uPAR$^{-/-}$*mdx* mice (Fig. 7, c and d). Indeed, the percentage of muscle degeneration was not different between *mdx* and uPAR$^{-/-}$*mdx* mice (Fig. 7 d). In addition, the number of infiltrated macrophages and T cells did not differ between *mdx* and uPAR$^{-/-}$*mdx* mice (Fig. 7 c). Interestingly, the percentage of centrally nucleated fibers was slightly increased in uPAR$^{-/-}$*mdx* mice (Fig. 7 d); however, SC–derived primary myoblasts from uPAR$^{-/-}$ mice presented normal proliferation and migration rates in vitro (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200705127/DC1). Altogether, these results demonstrate that uPAR is dispensable for muscle tissue remodeling during regeneration both after acute injury and in *mdx* muscle dystrophy and suggest that uPA regulates key processes during muscle regeneration in a uPAR-independent manner.

Discussion

Despite intense research efforts, DMD is still an incurable and fatal disease. The principal finding of this study is that uPA plays an important reparative role in muscular dystrophy. Indeed, uPA expression and activity increase during dystrophic disease, and the genetic loss of uPA exacerbated muscle dystrophinopathy and worsened muscle performance in the *mdx* mouse model of Duchenne's disease. Importantly, these defects in the absence of uPA were largely rescued by the transplantation of uPA-expressing BM, thus highlighting the importance of

uPA-secreting BM-derived cells in muscular dystrophy. Our data also indicated a critical role for fibrin/ogen deposits in dystrophic muscle and a crucial role for uPA to dissolve them. Notably, muscle dystrophinopathy was unaffected in the absence of uPAR, suggesting that uPA exerts its effect independently of its receptor. Thus, these results underscore the important role of muscle-extrinsic factors such as BM cell–derived uPA in DMD disease.

Our findings not only showed that uPA was produced by BM-derived cells but also that these cells required uPA for their infiltration into dystrophic muscle. Accordingly, macrophages showed reduced migration in vitro in the absence of uPA. It has long been proposed that inflammation exacerbates muscular dystrophy via the release of cytotoxic cytokines and free radicals, leading to myofiber necrosis (Spencer and Tidball, 2001; Grounds and Torrisi, 2004; Hodgetts et al., 2006; Pizza et al., 2005; Tidball, 2005), although recently, evidence has been accumulating on a positive role for inflammatory cells during muscle regeneration (Tidball, 2005; Sonnet et al., 2006; Arnold et al., 2007; Pelosi et al., 2007; Tidball and Wehling-Henricks, 2007). Indeed, we found less inflammation but increased muscle degeneration in uPA$^{-/-}$*mdx* mice, whereas the transplantation of uPA-expressing inflammatory cells rescued these degenerative defects. Thus, it is conceivable that inflammatory cells require uPA to infiltrate degenerating muscles of dystrophic mice and initiate the repair process. Indeed, macrophages might require uPA for the activation and phagocytosis of necrotic debris and for extracellular matrix remodeling. It has been demonstrated that the activation and release of prorecovery cytokines by leukocytes is reduced in uPA$^{-/-}$ mice (Matsushima et al., 1986; Sitrin et al., 1996; Gyetko et al., 2002; Abraham et al., 2003) and that uPA$^{-/-}$ leukocytes have impaired phagocytosis capacity (Gyetko et al., 2004). One potential mechanism underlying the uPA-mediated activation of leukocytes might involve mactinin, an α-actinin fragment that promotes monocyte/macrophage maturation, whose formation is mediated by uPA (Luikart et al., 2002; Luikart et al., 2006). Moreover, our data indicate that uPA-expressing inflammatory cells are required for intramuscular fibrinolysis. Collectively, we propose that uPA drives the infiltration and function of inflammatory cells required to create a beneficial environment for the repair of dystrophic muscle.

Another prerequisite for the efficient regeneration of dystrophic muscle appears to be prevention of the excessive deposition of fibrin/ogen. Indeed, in *mdx* muscle, fibrin/ogen accumulates as the disease progresses but is absent before disease onset. In the absence of uPA, both dystrophinopathy and fibrin/ogen accumulation were enhanced in *mdx* mice. Importantly, depletion of fibrinogen by ancrod treatment attenuated the severe muscle degeneration in uPA$^{-/-}$*mdx* mice. Thus, removal of fibrin/ogen deposits appears to be required for the resolution of muscle damage in *mdx* mice. Unpublished findings indeed indicated that fibrin/ogen promoted the persistent inflammation and degeneration of *mdx* muscles. Thus, by preventing excessive fibrin/ogen accumulation, uPA produced by BM-derived inflammatory cells might attenuate muscle degeneration and persistent inflammation in *mdx* mice.

Several studies have shown that both uPA and uPAR are expressed by a variety of cells of hematopoietic origin (Plesner et al., 1997; Blasi and Carmeliet, 2002; Mondino and Blasi, 2004) and that both molecules are up-regulated during severe infections, supporting a role for the uPA–uPAR system in inflammatory responses. Indeed, in uPAR-deficient mice, macrophages and neutrophils failed to infiltrate the lungs of mice in response to microbial infections (Gyetko et al., 2000; Rijneveld et al., 2002) or to migrate to the inflamed peritoneum of thioglycollate-treated mice (May et al., 1998). Therefore, we reasoned that the critical role of uPA in driving the infiltration and function of inflammatory cells during *mdx* muscle regeneration might involve uPAR. However, our results clearly showed that the loss of uPAR did not affect the degeneration/regeneration process nor did it impair the inflammatory response in dystrophic muscle, indicating that uPAR is not required for either process. Consistent with this notion, no degeneration or inflammatory phenotype was observed in uPAR-deficient mice after CTX injury. These results together with the reported observations that uPA and uPAR knockout mice have different susceptibilities to several pathogenic infections or biological processes (Carmeliet et al., 1998; Gyetko et al., 2000, 2001; Rijneveld et al., 2002; Deindl et al., 2003) indicate that uPAR and uPA may operate at different steps and may even be independent of each other.

After the clearance of degenerating myofibers by uPA^{+} inflammatory cells, muscle regeneration also appears to require uPA. Indeed, in the absence of uPA, muscle regeneration was attenuated in *mdx* and CTX-injured muscle; the transplantation of uPA-expressing BM rescued this defect. In addition, although the migratory capacity of primary myoblasts from uPA$^{-/-}$ muscle was normal, myoblast migration was enhanced in the presence of recombinant or macrophage-produced uPA. In contrast, the supplementation of r-uPA failed to affect their ability to proliferate. Thus, our results suggest that uPA derived from inflammatory cells specifically promotes the migration of muscle cells. As uPA deficiency failed to affect the activation of latent growth factors (e.g., HGF/SF or TGF-β1) in regenerating muscle in vivo, uPA might affect SC migration via alternative pathways. Unpublished findings from our group suggest that the removal of fibrin/ogen deposits promotes SC migration. Notably, these data extend previous observations that uPA promotes the migration of C2C12 immortalized myoblasts and primary human myoblasts by regulating membrane ruffling or by binding uPAR (Chazaud et al., 2000; Fibbi et al., 2001; El Fahime et al., 2002). However, we found that the genetic loss of uPAR did not affect primary myoblast migration.

DMD remains an incurable and fatal disease. No therapies correcting the primary defect in DMD (i.e., dystrophin replacement) are yet available, and current DMD therapies have a narrow therapeutic window (e.g., temporary efficacy and severe side effects). Our study shows that uPA activity, by providing an adequate inflammatory response and by promoting fibrinolysis and muscle regeneration, is beneficial in *mdx* muscle dystrophy. Notably, we and others recently demonstrated that genetic loss of the uPA inhibitor PAI-1 accelerated the recovery of CTX-injured muscle (Koh et al., 2005; Suelves et al., 2005). Thus, stimulating uPA activity may constitute a novel potential alternative for DMD disease amelioration.

Materials and methods

Generation of double mutant mice

uPA and uPAR knockout male mice (Carmeliet et al., 1994; Dewerchin et al., 1996) were crossed with *mdx* female mice (Jackson ImmunoResearch Laboratories). Male F1 mice were bred with *mdx* female mice, and their F2 heterozygous uPA and uPAR male and female offspring were intercrossed. The resulting F3 generation showed the expected Mendelian distribution of uPA-WT, uPAR-WT, and heterozygous and homozygous deficient genotypes, all of them in an *mdx* background. The uPA$^{-/-}$ and uPAR$^{-/-}$ genotypes were confirmed by PCR of tail biopsy genomic DNA as previously described (Carmeliet et al., 1994; Dewerchin et al., 1996). The *mdx* genotype was confirmed by Western blotting of muscle biopsies using an anti-dystrophin antibody (1:200; Novocastra). All animal experiments were approved by the Catalan Government Animal Care Committee.

Morphometric analysis

At selected times, muscles of WT, uPA$^{-/-}$, uPAR$^{-/-}$, *mdx*, uPA$^{-/-}$*mdx*, and uPAR$^{-/-}$*mdx* mice were removed after cervical dislocation, frozen, and stored at −80°C before analysis. 10-μm sections were collected from the midbelly of muscles and stained with hematoxylin/eosin (HE). Images were acquired with a microscope (DMR; Leica) equipped with a camera (DFC300 FX; Leica) and using 10× 0.25 NA, 20× 0.40 NA, and 40× 0.75 NA objectives (Leica). The acquisition software was the IM1000 program (Leica). The cross-sectional areas of entire muscles and myofibers were measured using the computer-assisted morphometric measurement Image 1.62c program (Scion).

Biochemical and functional assessment of muscle

Serum CK was measured with the indirect CK colorimetric assay kit and standards (Thermo Electron). For the grip strength assay, forearm grip strength was measured as tension force using a computerized force transducer (grip strength meter; Bioseb) to measure the peak force exerted by a mouse's forelimbs as its grip was broken by the experimenter pulling the mouse by the base of the tail away from the transducer (Costa et al., 1999) of the grip strength meter (Meyer et al., 1979). Three trials of three measurements per trial were performed for each animal with a few minutes resting period between trials. The mean tension force (in newtons) was calculated for each group of mice. The 100% value was arbitrarily assigned to the recorded force of *mdx* mice (Fig. 2 g). For the treadmill assay, the treadmill apparatus (Treadmill; Panlab) consisted of a belt set at a slope of 10° and varying in terms of rotational speed (5–150 rpm; Martinez de Lagran et al., 2004). At the end of the treadmill, an electrified grid was placed on which footshocks (0.6 mA) were administered whenever the mice felt off the belt. The latency to fall off the belt (time of shocks in seconds) and the number of received shocks in consecutive trials with increasing fixed rotational speeds (5, 10, 20, 30, 40, and 50 rpm) with a cut-off period of 1 min per trial were registered. Animals were trained to walk on a motor-driven treadmill belt at constant speed (5 rpm) to obtain baseline values for locomotion in the intact state.

Von Kossa staining

Muscle sections were placed in a silver nitrate solution, exposed to strong light for 30 min, and rinsed in distilled water. Sections were treated with sodium thiosulphate, rinsed in distilled water, and counterstained with neutral red. Finally, preparations were covered with aqueous mounting media and photographed.

Immunohistochemistry

The following primary antibodies were used for immunohistochemistry: anti–Mac-1 (M1/70; Hybridoma Bank), anti-T11 conjugated with fluorescein (1:50; Coulter Immunology), anti-fibrin/ogen (1:100; Nordic), anti-F4/80 (1:200; Serotec), and anti-uPA (1:20; Santa Cruz Biotechnology, Inc.). Depending on the antibody, immunohistochemistry was performed with the tyramide signal amplification cyanine 3 system (PerkinElmer) or as previously described (Lluis et al., 2001; Suelves et al., 2002). Control experiments without primary antibody demonstrated that the signals observed were specific.

Preparation of muscle extracts and Western blot analysis

Muscle extracts were prepared from gastrocnemius muscles in 100 mM Tris-HCl buffer, pH 7.6, containing 200 mM NaCl, 100 mM $CaCl_2$, and 0.4% Triton X-100. 50 μg of total protein was resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Antibody dilutions were anti-fibrin/ogen at 1:3,000 (provided by K. Dano, Finsen Laboratory, Rigshospitalet, Copenhagen, Denmark) and anti–α-tubulin at 1:4,000 (DM1A; Sigma-Aldrich).

Zymography

Zymography of muscle extracts was performed as previously described (Lluis et al., 2001). An SDS-PAGE gel was laid onto a casein gel, incubated in a humid chamber at 37°C until caseinolytic bands (corresponding to uPA or/and tPA) were visualized, and photographed.

Systemic defibrinogenation

12-d-old uPA$^{-/-}$*mdx* mice were daily injected intraperitoneally with ancrod (1 U ancrod/day; Sigma-Aldrich) or with a saline solution for 18 d and killed at 30 d of age. Muscles were dissected and frozen before analysis.

Analysis of muscle fiber degeneration and regeneration

Muscle degeneration was determined microscopically and expressed as a percentage of the total muscle area. The number of DGs (degenerating groups) that contained >10 degenerating fibers was counted in complete muscle cross sections of *mdx* and uPA$^{-/-}$*mdx* mice. Muscle fiber regeneration was determined microscopically and expressed as the percentage of total muscle fibers containing central nuclei present in the entire cross section of the muscle.

Migration assays

Macrophage migration was assayed on transwells (3-μm pore size; Beckton Dickinson). BM-derived macrophages were obtained as previously described (Celada et al., 1984) from *mdx* and uPA$^{-/-}$*mdx* mice (or from WT and uPA$^{-/-}$ mice). 5×10^4 macrophages/transwell in RPMI 1640 containing 1% FCS were added to the upper chamber of the transwell, and the conditioned medium of muscle SCs, which was previously concentrated fivefold using the Centrifugal Filter Device (Millipore), was added to the lower chamber. SC migration was performed on 8-μm pore size transwells. SCs from WT or uPA$^{-/-}$ mice (5×10^4 cells/transwell) in Hams F-10 containing 1% FCS were added to the upper chamber of transwells. Transwells were coated with matrigel before addition of the cells. When indicated, 10 nM recombinant murine uPA (Molecular Innovations) was added to the lower chamber of the transwell. Alternatively, conditioned medium of WT or uPA$^{-/-}$ macrophages, which were previously concentrated 2.5-fold and supplemented or not supplemented with 10 nM of murine r-uPA (Molecular Innovations) or 1 mM amiloride (Sigma-Aldrich), was added to the lower chamber. After 16 h of incubation at 37°C, cells on the filter's upper surface were scraped off. Then, filters were fixed in cold ethanol and stained with 5% crystal violet. Cells on the filter's lower surface were counted (12 fields per filter). Experiments were performed in triplicate.

Wound-healing assay in vitro

WT and uPA$^{-/-}$ SCs (2×10^5 cells) were plated in 12-well plates coated with matrigel (BD Biosciences). Once cells were attached to the matrix, a wound was performed across the well using a sterile pipette tip with an outer diameter of 500 μm. When indicated, 10 nM recombinant murine uPA was added to the culture media. Cells were then photographed at 0, 8, and 24 h after wounding using a microscope with 10× magnification (DMR; Leica). Experiments were performed in triplicate.

Induction of muscle regeneration

Regeneration of skeletal muscle was induced by intramuscular injection of 300 μl of 10^{-5} M CTX (Latoxan) in the gastrocnemius muscle group of the mice (Kherif et al., 1999). This concentration and volume were chosen to ensure maximum degeneration of the myofibers. The experiments were performed in right hindlimb muscles, and contralateral intact muscles were used as a control. Morphological and biochemical examinations were performed at 0, 2, 10, and 25 d after injury.

BM transplantation

Donor BM cells were obtained by flushing the femurs and tibiae of *mdx* or uPA$^{-/-}$*mdx* mice with RPMI 1640 medium (Invitrogen) and were transplanted into 4-mo-old uPA$^{-/-}$*mdx* mice after lethal irradiation (9 Gy). Alternatively, donor BM cells were obtained from WT mice and transplanted into 8-wk-old WT or uPA$^{-/-}$ mice. The reconstituting cells (5×10^6 cells) were injected intravenously into the tail of the recipient mice within 24 h after irradiation. Alternatively, donor BM cells were obtained from GFP mice (TgN-GFPU-5Nagy mice; provided by A. Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada) and were transplanted into 2-mo-old WT and uPA$^{-/-}$ mice. The mice were placed in sterile cages and fed with sterile chow until the reconstitution of BM was completed 8 wk after the transplantation. No changes in general health status were noted in the recipient mice. Regeneration of skeletal muscle in WT and uPA$^{-/-}$ mice was induced by the intramuscular injection of CTX as described in the previous section.

SC isolation and FACS analysis of activated SCs

SCs were isolated from *mdx* and uPA$^{-/-}$*mdx* mice of 2.5 mo of age as described previously (Mitchell and Pavlath, 2004). For FACS analysis, 2×10^5 SCs were used. SCs were permeabilized with 70% EtOH for 1 h at –20°C after incubation with an anti-CD34 antibody (FITC anti–mouse CD34; Ram34; BD Biosciences). Next, SCs were incubated with an anti-MyoD antibody (MyoD; Santa Cruz Biotechnology, Inc.) followed by incubation with a phycoerythrin-labeled secondary antibody (donkey anti–rabbit phycoerythrin; Abcam). Activated SCs were cells double positive for CD34 and MyoD. Experiments were performed in triplicate.

BrdU incorporation in vitro

WT, uPA$^{-/-}$, and uPAR$^{-/-}$ SCs were cultured in Ham's F-10 medium containing 20% FBS. 3.5×10^4 cells were plated in 12-well plates. When indicated, recombinant murine uPA (Molecular Innovations) was added to the culture medium at different concentrations (2, 10, or 50 nM). After 18 h, proliferating cells were labeled with 1.53 μg/ml BrdU (Sigma-Aldrich) for 2 h. BrdU-labeled cells were detected by immunocytochemistry and counted microscopically. Antibodies used for immunodetection were monoclonal rat anti-BrdU (1:500; Oxford Biotechnology) and biotin-SP–conjugated donkey anti–rat IgG (1:250; Jackson ImmunoResearch Laboratories). Experiments were performed in triplicate.

BrdU incorporation in vivo

Gastrocnemius, quadriceps, and tibialis muscles from WT and uPA$^{-/-}$ mice were injected with CTX to induce muscle regeneration. 24 h after injury, 50 mg/kg BrdU was injected intraperitoneally. 18 h later, SCs were isolated as described previously (Mitchell and Pavlath, 2004) and cultured for 24 h in Ham's F-10 medium containing 20% FBS. The percentage of SCs that had been BrdU labeled in vivo was determined by immunocytochemistry using a monoclonal rat anti-BrdU (as described above) and counted microscopically. Experiments were performed in triplicate.

RNA isolation and RT-PCR analysis

Total RNA was extracted from muscles or SCs using the commercially available Ultraspec RNA isolation system (Biotecx). For RT-PCR, 2 μg of total RNA were reverse transcribed using the first-strand cDNA synthesis kit (GE Healthcare). Amplification parameters were denaturation at 94°C for 30 s, annealing for 30 s at 50°C (uPAR) and 55°C (uPA and glyceraldehyde-3-phosphate dehydrogenase), and extension at 72°C for 30 s. Primers for the detection of reverse transcriptase products were derived from different exons to distinguish RT-PCR products from genomic DNA contaminations. Primer sequences were as follows: uPAR (5′-GTGACCCTCCAGAGCACAGAA-3′ and 5′-GCAGTGGGTGTAGTTGCAACA-3′), uPA (5′-GGCAGTGTACTTGGAGCTCCT-3′ and 5′-TAGAGCCTTCTGGCCACACTG-3′), and glyceraldehyde-3-phosphate dehydrogenase (5′-ACTCCCACTCTTCCACCTTC-3′ and 5′-TCTTGCTCAGTGTCCTTGC-3′). The expected product sizes were uPAR at 140 bp, uPA at 450 bp, and glyceraldehyde-3-phosphate dehydrogenase at 185 bp.

Measurement of activated HGF

Muscle extracts were analyzed for the presence of activated HGF by Western blotting using a goat anti–αHGF antibody (1:100; Santa Cruz Biotechnology, Inc.), which recognizes the active form (60 kD) of mouse HGF.

Measurement of activated TGF-β1

Crushed muscle extracts from *mdx* and uPA$^{-/-}$*mdx* mice and from WT and uPA$^{-/-}$ mice after CTX injury were prepared as described previously (Chen and Quinn, 1992). The presence of activated TGF-β1 was analyzed using the Quantikine TGF-β1 immunoassay kit (R&D Systems) according to the manufacturer's instructions.

Statistical analysis

All quantitative data were analyzed by *t* test. $P < 0.05$ was considered statistically significant.

Online supplemental material

Table S1 shows a comparison of the morphometric properties of gastrocnemius muscle of WT, *mdx*, and uPA$^{-/-}$*mdx* mice. Fig. S1 demonstrates that macrophages and SCs express uPA. Fig. S2 shows that BM transplantation rescues abnormal inflammatory infiltration and fibrin/ogen deposition in uPA$^{-/-}$ mice. Fig. S3 shows that uPA-deficient muscles present normal activated TGF-β1 and HGF/SF levels. Fig. S4 shows that uPAR$^{-/-}$ SCs have normal proliferation and migration rates. Supplemental material contains a description of the impaired muscle regeneration in uPA-deficient mice after CTX injury. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200705127/DC1.

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Supplemental materials

Suelves et al., http://www.jcb.org/cgi/content/full/jcb.200705127/DC1

Consistent with the *mdx* model (this study) and with experimental muscle injury induced by glycerol injection (Lluis et al., 2001), the loss of uPA was found to cause a similar muscle regeneration defect in the CTX model. At 2 d after CTX injury, muscle damage was comparable in WT and $uPA^{-/-}$ mice. At 10 d after injury, muscles of WT mice showed features of on-going regeneration, as indicated by the presence of myofibers with central nuclei (Fig. 4 c). In contrast, in $uPA^{-/-}$ mice, muscles were edematous and necrotic, and only rare and small regenerating central nucleated fibers could be detected. By 25 d after injury, muscles in WT mice were almost completely regenerated, whereas large areas of necrotic muscle fibers still persisted in $uPA^{-/-}$ mice.

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