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Fibrogenic Cell Plasticity Blunts Tissue Regeneration and Aggravates Muscular Dystrophy

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SUMMARY

Preservation of cell identity is necessary for homeostasis of most adult tissues. This process is challenged every time a tissue undergoes regeneration after stress or injury. In the lethal Duchenne muscular dystrophy (DMD), skeletal muscle regenerative capacity declines gradually as fibrosis increases. Using genetically engineered tracing mice, we demonstrate that, in dystrophic muscle, specialized cells of muscular, endothelial, and hematopoietic origins gain plasticity toward a fibrogenic fate via a TGFβ-mediated pathway. This results in loss of cellular identity and normal function, with deleterious consequences for regeneration. Furthermore, this fibrogenic process involves acquisition of a mesenchymal progenitor multipotent status, illustrating a link between fibrogenesis and gain of progenitor cell functions. As this plasticity also was observed in DMD patients, we propose that mesenchymal transitions impair regeneration and worsen diseases with a fibrotic component.

INTRODUCTION

Successful regeneration after tissue injury requires timely coordinated actions of diverse cell types. In skeletal muscle, in response to acute damage, the muscle stem cell (satellite cell) progeny gives rise to new regenerating myofibers, aided by the concerted action of specialized cells, such as infiltrating bone-marrow-derived inflammatory cells, which phagocytose tissue debris and provide pro-myogenic growth factors and cytokines; fibrogenic stromal cells such as fibroblasts and adipogenic progenitors (FAPs), which provide transient matrix support; and angiogenic cells that vascularize the newly formed muscle tissue (Abou-Khalil et al., 2010; Mounier et al., 2011). In chronically damaged muscle, however, this coordination is lost, leading to deficient regeneration (Serrano et al., 2011). In the yet incurable Duchenne muscular dystrophy (DMD), caused by loss of the myofiber protein dystrophin, successive cycles of tissue degeneration and regeneration lead to an eventual muscle regenerative failure and replacement of dystrophic muscle by fibrotic tissue, resulting in respiratory failure and early death (Mann et al., 2011; Stedman et al., 1991; Wallace and McNally, 2009).

Cell plasticity (i.e., the capacity of cells to change their phenotypic properties) is inherent to organismal development and is becoming increasingly associated with tissue remodeling in the adult (Medici and Kalluri, 2012; Nieto, 2013). Mesenchymal transitions (particularly epithelial- and endothelial-to-mesenchymal transitions, EMTs and EndMTs, respectively) are connected both to fibrotic pathologies and cancer progression of distinct etiologies, affecting organs such as liver, lung, heart, or kidney (Medici and Kalluri, 2012; Nieto, 2013; Nieto and Cano, 2012; Zeisberg and Kalluri, 2013). Lineage-tracing and fate-mapping strategies have precisely determined and quantified the source of fibrogenic cells in fibrotic kidney, underscoring the relevance of EMT, EndMT, and bone-marrow-derived cells to this organ's fibrosis (LeBleu et al., 2013). Incomplete EMT also can occur in tumors, with cells acquiring mesenchymal properties without undergoing the full EMT as it also occurs in embryos, where intermediate phenotypes have been described in different contexts (Nieto, 2011, 2013; Nieto and Cano, 2012). These incomplete transitions implicate a change in cellular functions and behavior. In skeletal muscle, studies on cell plasticity during repair are emerging. In addition to resident interstitial fibroblasts and FAPs, which are considered the major producers of the collagen-rich extracellular matrix (ECM) in injured muscle and in young dystrophic muscle (Joe et al., 2010; Mann et al., 2011; Uezumi et al., 2011, 2014), perivascular progenitor cells transiently produce collagen in response to acute muscle damage, but disappear as regeneration advances (Dulauroy et al., 2012). Similarly, depletion of macrophages or age-induced Wnt signaling in acutely injured muscle can divert vascular and myogenic cell fates, respectively (Brack et al., 2007; Zordan et al., 2014). However, whether cell plasticity occurs in dystrophic muscle and how it affects disease progression have remained elusive.

Recently, fibrogenesis from muscle cells has been reported in DMD (Biressi et al., 2014).

Here we demonstrate that specialized cells of muscular, endothelial, and hematopoietic origins acquire mesenchymal-fibrogenic traits in dystrophic muscle, with this cellular plasticity being particularly associated with advanced DMD stages. The mesenchymal-fibrogenic plasticity of these cells is induced by increasing TGFβ signaling in dystrophic muscle with aging, and results in the loss of cell identity, thus precluding normal regenerative functions. Together, our findings suggest that, during efficient tissue repair, specialized cells preserve their lineage identity by avoiding entrance into a mesenchymal-like/fibrogenic state. This protection is lost in chronic degenerative conditions such as DMD.

RESULTS

The levels of TGFβ and downstream signaling mediators (activated SMAD2/3) increase in muscle of dystrophic mdx mice with age, correlating to reduced regeneration, angiogenesis and function, and higher fibrosis extent (Ardite et al., 2012; Kharraz et al., 2014; Mann et al., 2011; Vidal et al., 2008; Figure 1A; Figures S1A and S1B). Inflammatory cells and FAPs appeared as the principal sources of TGFβ in dystrophic muscle (Figure S1C). Higher levels of this pathway also were found in muscle of wild-type (WT) mice after laceration (a severe injury model that induces persistent degeneration and more sustained fibrosis) than after cardiotoxin (CTX) injury (in which collagen-rich ECM is transient and full regeneration and muscle function are achieved rapidly) (Figures S1D and S1E). In agreement with the profibrotic role of TGFβ, exogenous delivery of TGFβ to CTX-injured WT muscle or dystrophic muscle of young mdx mice delayed regeneration and vascularization, while promoting fibrogenesis. This suggests that TGFβ inhibits myogenic and angiogenic capacity of muscle stem cell (satellite cell)-derived myoblasts and endothelial cells, respectively, while promoting matrix accumulation (Figures S1F and S1G). Consistent with this, freshly isolated WT satellite cells were unable to fuse into myotubes in differentiation medium (DM) in the presence of TGFβ (Figure S2A), correlating with gain of expression of fibrogenic genes (*αSma*, *Collagen I*, *Eda-Fibronectin*, or *Timp1*) and loss of myogenic gene expression (*Myf5* and *Pax7*) after a 10-day treatment (Figure S2B). Likewise, endothelial cells isolated from skeletal muscle could not form angiotubes in vitro after a 10-day TGFβ treatment period (Figure S2C), consistent with loss of expression of endothelial genes (*Cd31* and *Tie1*) and de novo acquisition of fibrogenic traits (Figure S2D). These results indicate that TGFβ induces the loss of identity of muscle-resident myogenic and endothelial cells by promoting their switch into matrix-producing fibrogenic cells, thus precluding their bona fide functions.

To further understand this cellular plasticity process induced by TGFβ, we performed a microarray gene expression analysis of satellite cells treated (or not) with TGFβ for 4 days (before achieving maximal levels of fibrogenic conversion, i.e., expression of *αSma* [Figure S2B]). Gene ontology functional annotation of genes upregulated in TGFβ-treated myogenic cells, compared to non-treated cells, showed enrichment in mesenchymal-fibrogenic functions (Figure S3A). Moreover, after comparison with a curated list of mesenchymal progenitor cell-specific transcripts (Kubo et al., 2009), we identified a group of mesenchymal cell-specific genes induced by TGFβ (Figure S3A). qRT-PCR analysis of a TGFβ cell-treatment kinetics experiment validated the expression of mesenchymal progenitor genes at intermediate time points (Figure 1B). These mesenchymal genes were also significantly upregulated in endothelial cells isolated from skeletal muscle in response to identical TGFβ treatment, prior to larger acquisition of fibrogenic traits (Figure 1B; see scheme in Figure 1C).

To prove whether this gain of mesenchymal gene expression translated into de novo functional cellular multipotency (i.e., potential to differentiate into distinct cellular fates), satellite cells and endothelial cells were treated with TGFβ for 3 days and further incubated with osteogenic or adipogenic medium for 7–14 days, or they continued to be treated with TGFβ for 7 extra days. Notably, cells pretreated with TGFβ showed induced expression of adipocyte, osteoblast, or fibrogenic traits under their respective differentiation regimes (Figures 1D and 1E; Figure S3B); in contrast, cells that had not been pretreated with TGFβ did not undergo any of these conversions under identical differentiation conditions (Figures 1D and 1E). These results suggest that these two specialized cell types (myogenic and endothelial cells) gain expression of mesenchymal genes during the plastic process toward a more mature fibrogenic fate in response to TGFβ; furthermore, these cells exhibit multipotency under adequate culture conditions (see scheme in Figure 1C). Of note, TGFβ was capable of inducing the expression of transcription factors and microRNAs associated with mesenchymal transitions in myogenic and endothelial cells (Figures S3C and S3D). In particular, *Mir21* induction appeared to mediate TGFβ-induced fibrogenesis in both cell types (Figure 2), reinforcing this *Mir* as a fibrogenic effector of TGFβ action (Acuña et al., 2014; Ardite et al., 2012; Kumarswamy et al., 2012).

Since TGFβ levels and signaling are elevated in aged dystrophic muscle (Figure 1A), we next investigated whether endothelial and satellite cells also undergo plastic fibrogenesis in vivo. To this end, we generated endothelial