The Fibrinolytic System in Muscle Regeneration and Dystrophy

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The Fibrinolytic System in Muscle Regeneration and Dystrophy

Memoria presentada por Berta Vidal Iglesias para optar al grado de Doctor en Ciencias de la Salud y de la Vida por la Universitat Pompeu Fabra.

Este trabajo ha sido realizado bajo la dirección de la Dra. Pura Muñoz Cánoves en el Programa de Diferenciación y Cáncer del Centro de Regulación Genómica (CRG) de Barcelona.

Berta Vidal Iglesias **Dra. Pura Muñoz Cánoves**

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ABBREVIATIONS

ANG	Angiotensin
ATF	Amino-Terminal Fragment
bFGF	basic Fibroblast Growth Factor
BMD	Becker Muscular dystrophy
BM	Bone Marrow
CK	Creatine Kinase
CM	Conditioned Medium
CMD	Congenital Muscular Dystrophy
CNF	Central Nucleated Fiber
CSA	Cross-Sectional Area
CTX	Cardiotoxin
DAB	Diaminobenzidine
DGC	Dystrophin Glycoprotein Complex
DM	Differentiation Medium
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle's Medium
DPI	Days Post-Injury
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
EMT	Epithelial-Mesenchymal Transition
EndMT	Endothelial-Mesenchymal Transition
ERK	Extracellular signal Regulated Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
Fibrin/ogen	Fibrin and/or Fibrinogen
FPRL1	Formyl Peptide Receptor-Like1
GM	Growth Medium
GPI	Glycosylphosphatydilinositol
HE	Hematoxilin / Eosin
HGF/SF	Hepatocyte Growth Factor / Scatter Factor
ICAM-1	Intracellular Adhesion Molecule-1
IL	Interleukin
IL-1ra	IL-1 Receptor Antagonist
LGMD	Limb-Girdle Muscular Dystrophy
LIF	Leukemia Inhibitory Factor

LMW	Low Molecular Weight
LPS	Lipopolisaccharide
LRP	Low-density lipoprotein (LDL)-receptor Related Protein
MCP	Monocyte Chemoattractant Protein
mdx	X-Chromosome-linked Muscular Dystrophy
MHC	Myosin Heavy Chain
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloproteinase
MRF	Muscle Regulatory Factor
NE	Nuclear Extract
NF-κB	Nuclear Factor kappa B
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NTX	Notexin
OAT	Ornithine Amino Transferase
ODC	Ornithine Decarboxylase
PA	Plasminogen Activator
PAI	Plasminogen Activator Inhibitor
PAMPs	Pathogen-associated Molecular Patterns
PDGF	Platelet-Derived Growth Factor
PI3K	Phosphoinositide-3-kinase
Plg	Plasminogen
Plm	Plasmin
PPARs	Peroxisome Proliferator-Activated Receptors
qRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
SC	Satellite Cell
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SPD	Serine Protease Domain
TGFβ	Transforming Growth Factor beta
Th	T Helper
TIMP	Tissue Inhibitor of Metalloproteinases
TLR4	Toll-Like Receptor 4
TNFα	Tumor Necrosis Factor alpha
tPA	Tissue-type Plasminogen Activator
TSA	Tyramide Signal Amplification
uPA	Urokinase-type Plasminogen Activator

uPAR	uPA Receptor
VE-Cadherin	Vascular Endothelial-Cadherin
VEGF	Vascular Endothelial Growth Factor
VN	Vitronectin
WT	Wild Type

INTRODUCTION

1. MYOGENESIS

1.1 SKELETAL MUSCLE STRUCTURE

Myogenesis refers to the formation of skeletal muscle either in embryonic development or during pathological regeneration. Skeletal muscle is a type of striated muscle, which is anchored by tendons to bone. It is used to generate skeletal movement such as locomotion and to maintain posture. Muscle cells (also called myofibers) have an elongated, cylindrical shape, and are multinucleated. The nuclei of myofibers are located in the periphery of the cell, just under the plasma membrane. Within the myofibers are myofibrils containing sarcomeres, which are composed of actin and myosin. Individual muscle fibers are surrounded by endomysium. Muscle fibers are bound together by perimysium into bundles called fascicles; the bundles are then grouped together to form muscle, which is enclosed in a sheath of epimysium (Fig. 1).

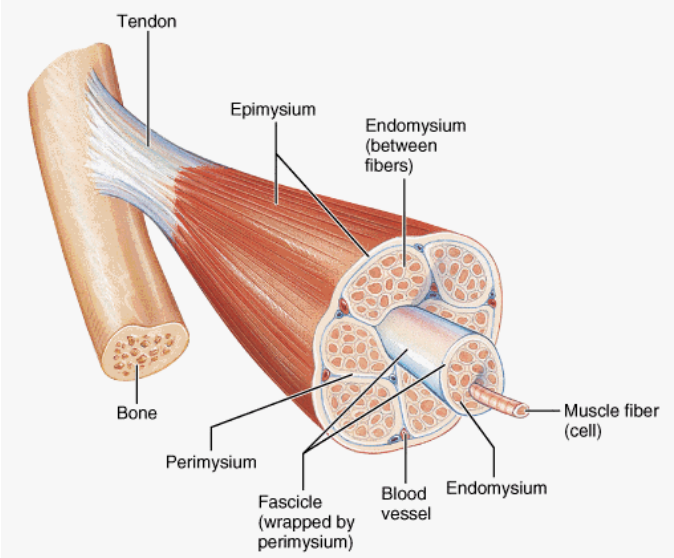

Figure 1. Skeletal muscle structure.

1.2 THE SKELETAL MUSCLE REGENERATION PROCESS

Mammalian adult skeletal muscle constitutes half of the body mass and is a stable tissue with very little turnover under normal circumstances. Nonetheless, mammalian skeletal muscle has the ability to complete a rapid and extensive regeneration process in response to severe damage. Whether the muscle injury is inflicted by direct trauma or

innate genetic defects (as in muscle dystrophies), muscle regeneration is characterized by two phases: a degenerative phase and a regenerative phase (Fig. 2).

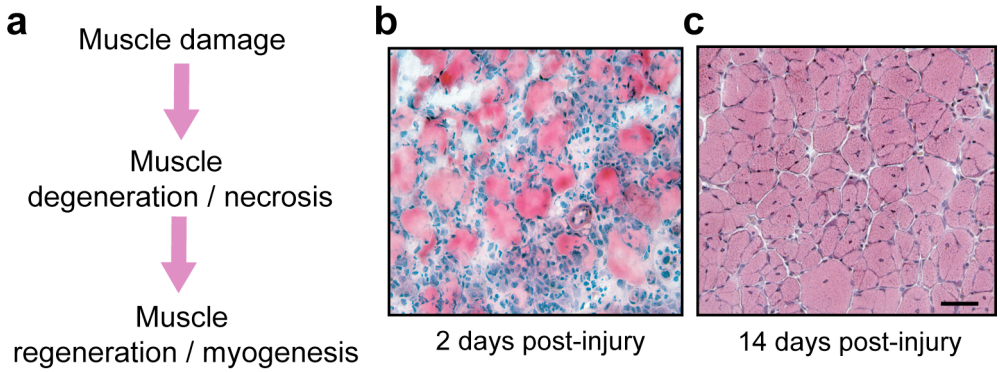

Figure 2. Skeletal muscle repair process. a. Mammalian skeletal muscle repair after damage is characterized by a degenerative phase followed by a regenerative phase. **b.** Injury to the tibialis anterior muscle by cardiotoxin (CTX) injection results in the rapid necrosis of myofibers and the activation of an inflammatory response leading to the loss of muscle architecture. **c.** Regenerating fibers, which depend on muscle satellite cells, are characterized by their small caliber and their centrally located myonuclei.

The initial event of muscle degeneration is necrosis of the muscle fibers. This event is generally triggered by disruption of the myofiber sarcolemma resulting in increased myofiber permeability. Disruption of the myofiber integrity is reflected by increased serum levels of muscle proteins, such as creatine kinase, which are usually restricted to the myofiber cytosol. Indeed, in humans and mice, increased serum creatine kinase is observed after mechanical stress of muscle and in the course of muscle degenerative diseases such as muscular dystrophies (Nicholson et al. 1979). Reciprocally, the uptake of low-molecular-weight dyes, such as Evans blue or procion orange, by the myofiber is a reliable indication of sarcolemmal damage and is also associated with strenuous exercise and muscle degenerative diseases (Hamer et al. 2002). Thus, disrupted myofibers undergo focal or total autolysis depending on the extent of the injury.

The early phase of muscle injury is usually accompanied by the activation of mononucleated cells, principally inflammatory cells and satellite cells. Some reports suggest that factors released by the injured muscle activate inflammatory cells residing within the muscle, which in turn provide the chemotactic signals to circulating inflammatory cells (Rappolee and Werb 1992). Neutrophils are the first inflammatory cells to invade the injured muscle, with a significant increase in their number at 1-6 h after myotoxin- or exercise-induced muscle damage. After neutrophil infiltration, and ~48 h

post-injury, macrophages become the predominant inflammatory cell type within the site of injury (Orimo et al. 1991). Macrophages phagocytose cellular debris and may also affect other aspects of muscle regeneration by activating satellite cells (Lescaudron et al. 1999; Tidball 2005a). Thus, muscle fiber necrosis and increased number of inflammatory cells within the damaged site are the main histopathological characteristics of the early stages following muscle injury.

Muscle degeneration is followed by a process of myofiber repair. This process starts with the activation of satellite cells (muscle stem cells), which will proliferate and expand, providing a sufficient source of new myonuclei for muscle repair. Following the proliferation phase, satellite cell-derived myoblasts will differentiate and fuse to existing damaged fibers for repair or to one another for new myofiber formation. On muscle cross-sections, the fundamental morphological characteristics to identify regenerating newly formed myofibers are their small caliber and their centrally located myonuclei (Fig. 3). Newly formed growing myofibers are often basophilic (reflecting high protein synthesis) and express embryonic/developmental forms of myosin heavy chain (MHC) (reflecting de novo fiber formation) (Hall-Craggs and Seyan 1975; Whalen et al. 1990). New myofibers mature and reach a size similar to the original one with myonuclei moving to the periphery. Under normal conditions, the regenerated muscle is morphologically and functionally indistinguishable from undamaged muscle.

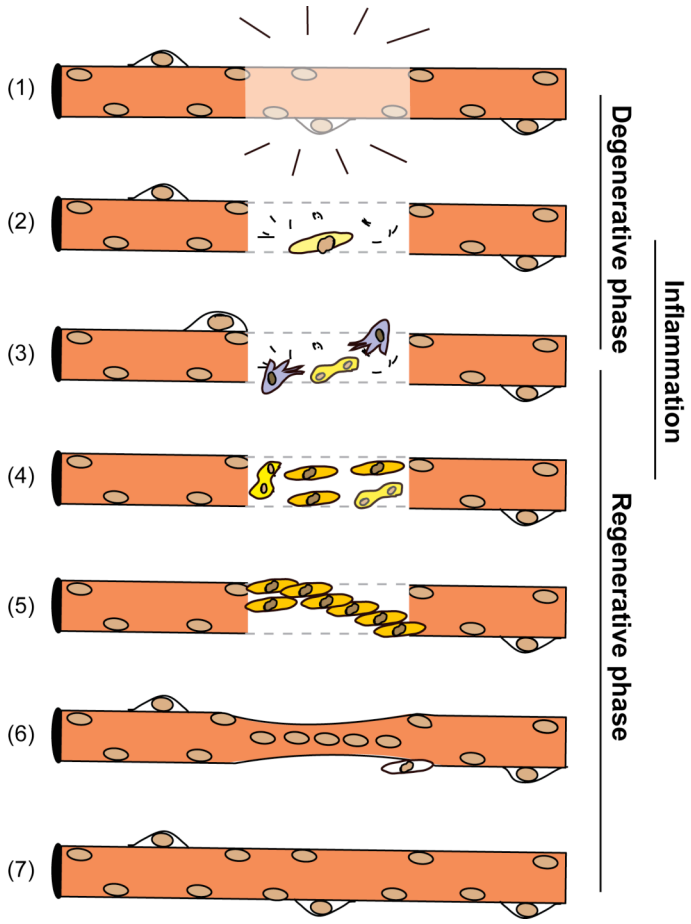

Figure 3. Injury-induced skeletal muscle regeneration. (1) Injury destroys the integrity of the myofiber plasmalemma. **(2)** The myofiber undergoes necrosis and autodigestion by intrinsic proteases. **(3)** Macrophages, derived from blood monocytes, penetrate the endomysial tube and complete the removal of necrotic debris. Satellite cells become activated and enter the cell cycle. **(4)** Satellite cells proliferate and **(5 and 6)** begin to withdraw from the cell cycle and fuse to form multinucleated myotubes. **(7)** The original myofiber and the quiescent satellite cell pool are restored.

1.3 EXPERIMENTAL MODELS OF MUSCLE REGENERATION INDUCTION

To study the process of muscle regeneration in a controlled and reproducible way, different models of muscle injury have been extensively used in mice and rats. These include intramuscular injection of myotoxins such as bupivacaine, cardiotoxin (CTX) and notexin (NTX) (Hall-Craggs 1974; Harris 1975; Couteaux et al. 1988), direct infliction of a wound by crushing and / or freezing the muscle, denervation-devascularization by transplantation of a single muscle, or repeated bouts of intensive exercise, which will trigger the process of muscle degeneration-inflammation-regeneration (Schultz et al. 1985; Lefaucheur and Sebille 1995).

Different transgenic mice with alterations in the process of muscular regeneration after injury have been described: mice deficient in transcription factors such as MyoD or Pax7 (Megeney et al. 1996; Sabourin et al. 1999; Kuang et al. 2006), mice deficient in growth factors like FGF-6 (Fibroblast Growth Factor-6) or LIF (Leukemia Inhibitory Factor) (Floss et al. 1997; Kurek et al. 1997) or mice deficient in other muscular proteins such as desmin (Smythe et al. 2001).

1.4 DUCHENNE MUSCULAR DYSTROPHY

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 (Hoffman et al. 1987), which localizes at the inner face of the sarcolemma. The gene is the largest in the human genome, encompassing 2.6 million base pairs of DNA and containing 79 exons. Approximately 60% of dystrophin mutations are large insertions or deletions that lead to frameshift errors downstream, whereas approximately 40% are point mutations or small frameshift rearrangements. The vast majority of DMD patients lack the dystrophin protein. Becker muscular dystrophy (BMD)-a

much milder form of the disease- is caused by a reduction in the amount, or alteration in the size, of the dystrophin protein (Monaco et al. 1988).

Dystrophin has a major structural role in muscle as it links the internal cytoskeleton to the extracellular matrix. The amino-terminus of dystrophin binds to F-actin and the carboxyl-terminus to the dystrophin glycoprotein complex (DGC) at the sarcolemma (Blake et al. 2002). The DGC includes the dystroglycans, sarcoglycans, dystrobrevin, syntrophins, sarcospan, caveolin and laminin (Fig. 4). Mutations in some of these components cause autosomally inherited muscular dystrophies (see Table 1) (Dalkilic and Kunkel 2003). The DGC is destabilized when dystrophin is absent, which results in diminished levels of the member proteins (Straub and Campbell 1997). This in turn leads to progressive membrane leakage and fiber damage. Thus, DMD patients exhibit progressive muscle degeneration, which is exacerbated by persistent inflammation via the production of free radicals and cytotoxic cytokines (Tidball 2005b). Myofiber loss is initially compensated by proliferation and fusion of resident satellite cells with pre-existing myofibers that thereby enlarge in size. Ultimately, however, after repetitive cycles of muscle degeneration and persistent inflammation, regeneration gradually fails as the pool of endogenous satellite cells ceases to compensate for the damaged muscle fibers and dystrophic myofibers become gradually replaced by fibrotic and fat tissue (Stedman et al. 1991). Fibrosis (intramuscular deposition of fibrillar collagens) is a hallmark of DMD. It progressively deteriorates locomotor capacity, posture maintenance, and the vital function of cardiac and respiratory muscles, thereby restricting life expectancy in this devastating disease (Yasuda et al. 2005; Radley et al. 2007).

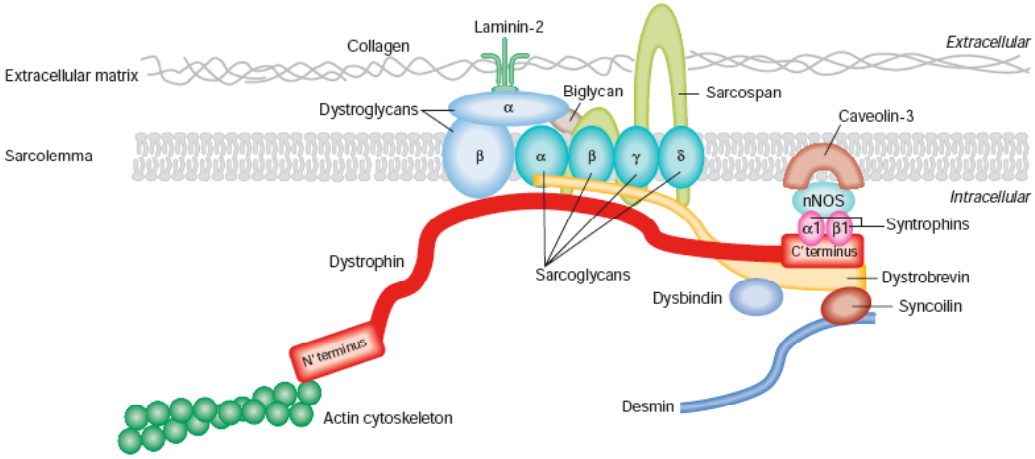

Figure 4. The dystrophin glycoprotein complex (DGC) in muscle fibers linking the internal cytoskeleton to the extracellular matrix. Dystrophin binds to the DGC through its C-terminus. The DGC is comprised of sarcoplasmic proteins (α-dystrobrevin, syntrophins and neuronal nitric oxide synthase (nNOS)),

transmembrane proteins (β-dystroglycan, the sarcoglycans, caveolin-3 and sarcospan) and extracellular proteins (α-dystroglycan and laminin). The N terminus of dystrophin binds to the cytoskeleton through filamentous (F)-Actin. (Adapted from (Nowak and Davies 2004)).

Disease	Inheritance pattern	Gene product	Mouse model
DMD/BMD	XR	Dystrophin	mdx
LGMD 1C	AD	Caveolin-3	$Cav3^{-/-}$
LGMD 2C	AR	γ-sarcoglycan	$Sgcg^{-/-}$
LGMD 2D	AR	α-sarcoglycan	$Sgca^{-/-}$
LGMD 2E	AR	β-sarcoglycan	$Sgcb^{-/-}$
LGMD 2F	AR	δ-sarcoglycan	$Sgcd^{-/-}$
CMD 1A	AR	Laminin α2	dy

Table 1. Several forms of muscular dystrophy arise from primary mutations in genes encoding components of the DGC. DMD/BMD, Duchenne/Becker muscular dystrophy; LGMD, limb-girdle muscular dystrophy; CMD, congenital muscular dystrophy; XR, X-linked recessive; AD, autosomal dominant; AR, autosomal recessive. For primary references see (Dalkilic and Kunkel 2003) and main text.

1.5 THE MDX MOUSE MODEL OF DMD

The *mdx* (X-chromosome-linked muscular dystrophy) mouse strain is the most widely used animal model for DMD, having a nonsense mutation in exon 23 of the dystrophin gene, which eliminates its expression (Sicinski et al. 1989). Though *mdx* mice and DMD patients have many genetic, biochemical and histological similarities in common, the clinical manifestations are less severe in *mdx* mice than in humans, having the mice a near-normal life span (Carnwath and Shotton 1987). The progressive muscle-wasting disease is much milder at least in certain *mdx* muscles (limb muscles) (Bulfield et al. 1984). The first necrotic wave appears around the third week and continues for about 2 months. Active and efficient regeneration seems to compensate for the repeated cycles of degeneration with an expansion of the satellite cell population, resulting in muscle hypertrophy. However, *mdx* muscle does exhibit a susceptibility to damage by lengthening (eccentric) contractions and has lower specific force (Stevens and Faulkner 2000). The most affected muscle in the *mdx* mouse is the diaphragm, which presents high levels of fibrosis at advanced ages, thus mimicking DMD progression in humans (Stedman et al. 1991).

1.6 INFLAMMATION IN MUSCLE DAMAGE AND REPAIR

Inflammation is the complex biological response of the vascular tissues and immune system to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process for the tissue. In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism. However, uncontrolled inflammation can also lead to several diseases (i.e. asthma, rheumatoid arthritis). It is for this reason that inflammation needs to be tightly regulated by the body.

Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli. It is a short-term process characterized by the infiltration of the tissue by leukocytes and plasma proteins. Inflammatory mediators such as pro-inflammatory cytokines (i.e. IL-1β, IL-6, TNFα) act in parallel to propagate and mature the inflammatory response. Acute inflammatory reactions are characterized by rapidly resolving vascular changes, oedema and infiltration. The inflammatory process quickly ceases once the harmful stimulus has been removed and the tissue repaired. When the harmful stimuli are persistent, prolonged or even chronic inflammation can occur. Chronic inflammation is a pathological condition that leads to a progressive shift in the type of cells that are present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue for a long period of time.

Muscle damage produces a stereotypic classical inflammatory response in which neutrophils rapidly invade, followed by macrophages. This inflammatory response coincides with the initiation of muscle repair, which involves activation and proliferation of satellite cells, followed by their terminal differentiation. Recent investigations have begun to explore the relationship between inflammatory cell functions and skeletal muscle injury and repair.

Neutrophils are rapid invaders of muscle after damage. Morphological observations indicate that the invading neutrophils may be phagocytic, but they also have the ability to release proteases that can help degrade cellular debris that may be produced by muscle damage. However, neutrophils can also release high concentrations of cytolytic and cytotoxic molecules that can damage muscle or other healthy bystander tissues. The role of neutrophils in damaging healthy muscle tissue has been demonstrated in response to several perturbations. Current findings have shown that neutrophils are capable of direct lysis of muscle cell membranes through a superoxide-

dependent mechanism (Tidball 2005a). No direct evidence is yet available to show that neutrophils can also play a beneficial role in muscle repair or regeneration.

The role of **macrophages** in influencing the course of muscle injury or remodeling is more complex than the role of neutrophils, because macrophages are rich sources of diverse growth factors and cytokines, as well as free radicals. Macrophages are also professional antigen-presenting cells, so they can play important roles in regulating the cellular immune response to injured muscle.

On one hand, macrophages can injure muscle cells *in vitro* and *in vivo*. Cytotoxicity assays have shown that they can lyse target muscle cells and that their cytolytic capacity is increased by the presence of neutrophils (Nguyen and Tidball 2003). Macrophages also increase muscle membrane lysis *in vivo* in the *mdx* mouse. Depletion of macrophages from *mdx* mice by intraperitoneal injections of a macrophage-specific antibody resulted in an 80% reduction in muscle membrane lysis in vivo (Wehling et al. 2001). This finding shows that macrophages can play a major role in promoting mechanically-initiated muscle damage.

On the other hand, several observations indicate a potentially important role for macrophages in promoting muscle repair and remodeling after injury. For example, conditioned media from cultures of peritoneal macrophages or macrophage cell lines can increase the rate of proliferation of myoblasts *in vitro* as well as increasing the number of MyoD-expressing cells (Merly et al. 1999; Cantini et al. 2002). Macrophages have been also shown to rescue myogenic cells from apoptosis (Sonnet et al. 2006). Moreover, it has been recently described that depletion of macrophages is sufficient to inhibit experimentally-induced muscle regeneration (Arnold et al. 2007; Tidball and Wehling-Henricks 2007).

Evidence exists that muscle cells can release positive and negative regulators of inflammatory cell invasion, thereby playing an active role in modulating the inflammatory process. In particular, muscle-derived nitric oxide can inhibit inflammatory cell invasion of healthy muscle and protect it from lysis by inflammatory cells, *in vivo* and *in vitro*. On the other hand, muscle-derived cytokines can signal for inflammatory cell invasion, at least *in vitro* (Chazaud et al. 2003).

Whether the inflammatory process has an overall beneficial or detrimental effect on muscle function seems to depend on the magnitude and length (acute *versus* chronic) of the response, the previous history of muscle use, and possibly on injury-specific interactions between muscle and the invading inflammatory cells.

Depending on the cytokine environment, macrophages can differentiate into distinct subsets that play specific immunological roles. **Classical activation** is driven by the T Helper 1 (Th1) cytokine IFN-γ, which leads to the release of NO and pro-

inflammatory cytokines such as TNFα, IL-1β and IL-6. Classically activated macrophages are involved in the acute response to tissue damage and bacterial infection. By contrast, **alternative activation** is regulated by the Th2 cytokines IL-13 and IL-4 that induce the expression of molecules such as mannose receptor (CD206), and the enzyme arginase. These alternatively activated macrophages are more restricted to anti-parasitic responses and tissue repair (Gordon and Taylor 2005) (Fig. 5).

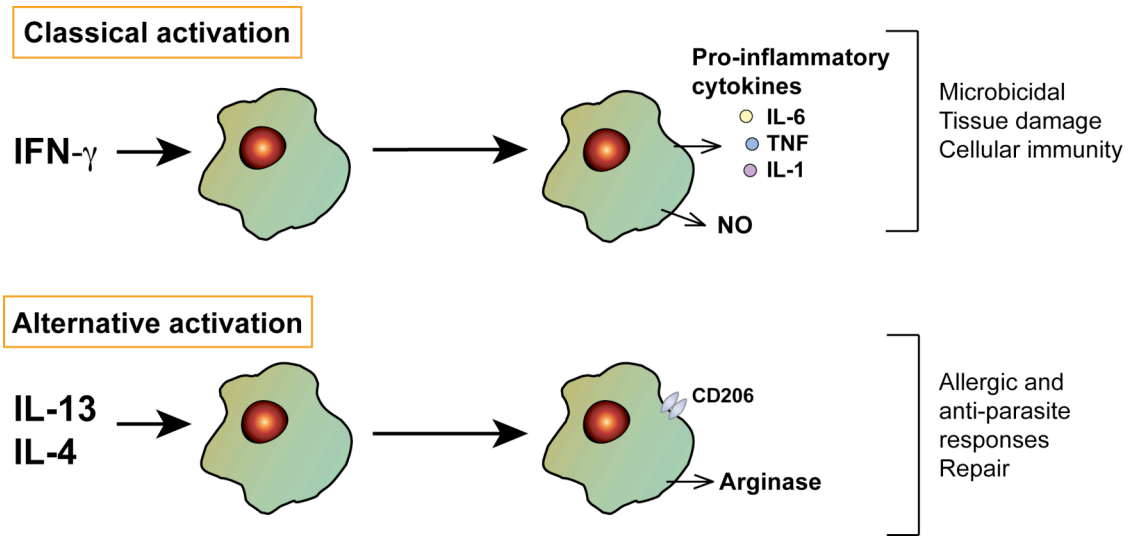

Figure 5. Differential activation of macrophages depending on the cytokine environment. Classically activated macrophages produce pro-inflammatory cytokines and NO whereas alternatively activated macrophages express CD206 and arginase. Adapted from (Gordon 2003).

The specific role of these different macrophage subtypes has never been studied in the context of muscle damage and repair.

1.7 MUSCLE SATELLITE CELLS IN MUSCLE REPAIR

Muscle satellite cells are a population of quiescent, undifferentiated mononuclear myogenic cells. Since their first description (Mauro 1961), satellite cells have been primarily identified in situ by their anatomical localization within the basal lamina surrounding individual myofibers, juxtaposed between the plasma membrane of the muscle fiber and the basement membrane, in a satellite position. Important morphological features of satellite cells are an increased nuclear-to-cytoplasmic ratio, reduced organelle content, and a smaller nucleus size displaying increased amounts of heterochromatin compared with fiber myonuclei. These characteristics reflect the finding that satellite cells

are mitotically quiescent and transcriptionally less active than myonuclei (Schultz et al. 1978). Different molecular markers specific to this cell population have been also identified (i.e. M-cadherin, Syndecan-3/4, Pax7, c-Met, CD34 etc) (Charge and Rudnicki 2004).

In the course of muscle regeneration, satellite cells first exit their normal quiescent state (activation) to start proliferating into myoblasts. After several rounds of proliferation, the majority of the satellite cells differentiates and fuse to form new myofibers or to repair a damaged one. Satellite cell activation is not restricted to the damaged site. Indeed, damage at one end of a muscle fiber will activate satellite cells all along this fiber leading to the proliferation and migration of the satellite cells to the regeneration site. However, recruitment of satellite cells from adjacent muscles is seldom observed and requires damage to the connective tissue separating the two muscles. After proliferation, quiescent satellite cells are restored underneath the basal lamina for subsequent rounds of regeneration (Schultz et al. 1985). The process of satellite cell activation and differentiation during muscle regeneration is reminiscent of embryonic muscle development. Muscle Regulatory Factors (MRFs) are transcription factors, which regulate muscle-specific gene expression during both processes. There are four MRFs: Myf5, MyoD, Myogenin and MRF4. Quiescent satellite cells do not express MRFs, while activation is characterized by the rapid upregulation of two MRFs, Myf5 and MyoD (Smith et al. 1994; Cornelison and Wold 1997; Cooper et al. 1999).

Upon satellite cell activation, MyoD up-regulation appears within 3-12 h and is detectable before any sign of cellular division. After proliferation, expression of Myogenin is upregulated in cells beginning their differentiation program (Smith et al. 1994; Cornelison and Wold 1997). This is followed by the activation of the cell cycle arrest protein p21 and permanent exit from the cell cycle. The differentiation program is then completed with the activation of MRF4 and of muscle-specific proteins, such as α-actin and MHC, and the fusion of myoblasts to repair damaged myofibers (Fig. 6).

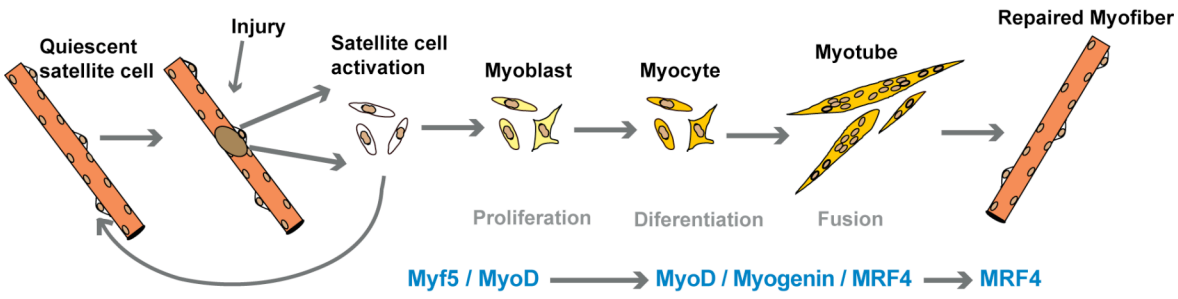

Figure 6. Representation of the MRFs expression during the myogenic process. At the end of the proliferation stage, myoblasts withdraw the cell cycle and differentiate to myocytes. Later, the myocytes align and start to fuse, forming multinucleated myotubes. The progression of the myogenic process is controlled by the sequential activation of the MRFs: Myf5, MyoD, Myogenin and MRF4. (Adapted from (Sartorelli and Caretti 2005)).

Despite its complexity, myogenesis can be partially recapitulated in cell culture *in vitro*; MPCs can be isolated from skeletal muscle and grown under permissive culture conditions to differentiate, fuse and form myotubes. These primary myoblast proliferate when cultured in medium with high content of serum and rich in growth factors (Growth Medium, GM). When the serum concentration in the medium is lowered and the growth factors removed (Differentiation Medium, DM), myoblasts withdraw from the cell cycle and cease proliferating, and then start differentiating toward myocytes, which will align and fuse to form multinucleated myotubes (Fig. 7).

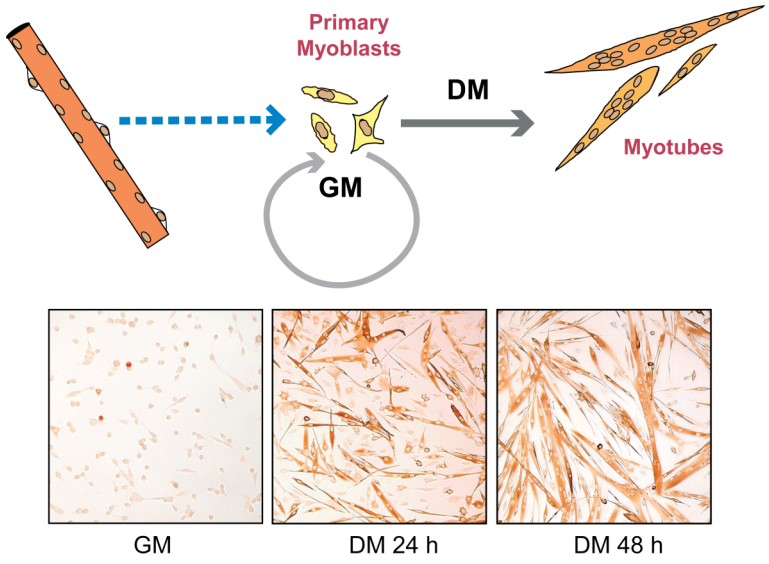

Figure 7. Primary myoblasts derived from satellite cells can be isolated from mouse skeletal muscle. Primary myoblasts can be cultured *in vitro* in growth medium (GM) or differentiation medium (DM).

1.8 FIBROSIS

Fibrosis is characterized by the excessive deposition of extracellular matrix components (mainly collagen) usually as a result of an injury, leading to thickening and scarring of connective tissue. Fibrotic changes can occur in various vascular disorders

and in all the main tissues and organs, including the skin, kidney, lung, liver and muscle. In some cases, it might ultimately lead to organ failure and death.

The key cellular mediator of fibrosis is the fibroblast, which when activated (myofibroblast) serves as the primary collagen-producing cell. Myofibroblasts are generated from a variety of sources including resident mesenchymal cells, epithelial and endothelial cells which undergo epithelial/endothelial-mesenchymal (EMT/EndMT) transition, as well as from circulating fibroblast-like cells called fibrocytes that are derived from bone-marrow stem cells. Myofibroblasts are activated by a variety of mechanisms, including paracrine signals derived from lymphocytes and macrophages, autocrine factors secreted by myofibroblasts, and pathogen-associated molecular patterns (PAMPS) produced by pathogenic organisms that interact with pattern recognition receptors (i.e. TLRs) on fibroblasts. Cytokines (IL-13, IL-21, TGFβ), chemokines (MCP-1, MIP-1β), angiogenic factors (VEGF), growth factors (PDGF), peroxisome proliferator-activated receptors (PPARs), acute phase proteins (SAP), caspases, and components of the renin-angiotensin-aldosterone system (ANG II) have been identified as regulators of fibrosis in different settings (reviewed in (Wynn 2008)).

Pathogenic fibrosis typically results from chronic inflammatory reactions that persist for several weeks or months and in which inflammation, tissue destruction and repair processes occur simultaneously. Most chronic fibrotic disorders have in common a persistent inflammatory stimulus that sustains the production of growth factors, proteolytic enzymes, angiogenic factors and fibrogenic cytokines, which together stimulate the deposition of connective tissue elements that progressively remodel and destroy normal tissue architecture (Wynn 2007). Although severe acute (non-repetitive) injuries can also cause marked tissue remodeling, fibrosis that is associated with chronic (repetitive) injury is unique in that the adaptive immune response is thought to have an important role.

Studies using various cytokine-deficient mice showed that fibrogenesis is strongly linked with the development of a Th2 CD4+ T-cell response, involving IL-4, IL-5 and IL-13. Although an equally potent inflammatory response develops when Th1 CD4+ T cells, which produce IFN-γ, dominate, under these circumstances, the development of tissue fibrosis is almost completely attenuated.

Numerous studies have indicated that macrophages and fibroblasts are the main effector cells involved in the pathogenesis of fibrosis. Although CD4+ T cells are clearly important, their main contribution could be to control the activation and recruitment of macrophages and fibroblasts. Macrophage activation was first described as a Th1-INF-γ-mediated process; however, as previously mentioned, it is now clear that macrophages differentiate into at least two functionally distinct populations depending on whether they are exposed to Th1 or Th2 cytokines. Th1 cytokines activate nitric-oxide synthase 2

(NOS2) expression in “classically activated” macrophages, whereas the Th2 cytokines IL-4 and IL-13 preferentially stimulate arginase-1 activity in “alternatively activated” macrophages (Gordon and Taylor 2005). L-Arginine is the substrate for both enzymes; however, NOS2 generates l-hydroxyarginine, l-citrulline and nitric oxide (NO), whereas arginase-1 promotes the production of l-ornithine. l-Ornithine is the substrate for two additional enzymes, ornithine decarboxylase (ODC) and ornithine amino transferase (OAT), which generate polyamines and l-proline, respectively. Because polyamines are crucial for cell growth and proline is a substrate for collagen synthesis, both the ODC and OAT pathways are thought to be important in repair processes. Collagen synthesis is strictly dependent on the availability of l-proline; therefore, the preferential activation of arginase-1 compared with NOS2 was recently proposed to be a possible explanation for the pro-fibrotic activity of IL-13 and the anti-fibrotic activity of IFN-γ (Wynn 2004) (See Fig. 8).

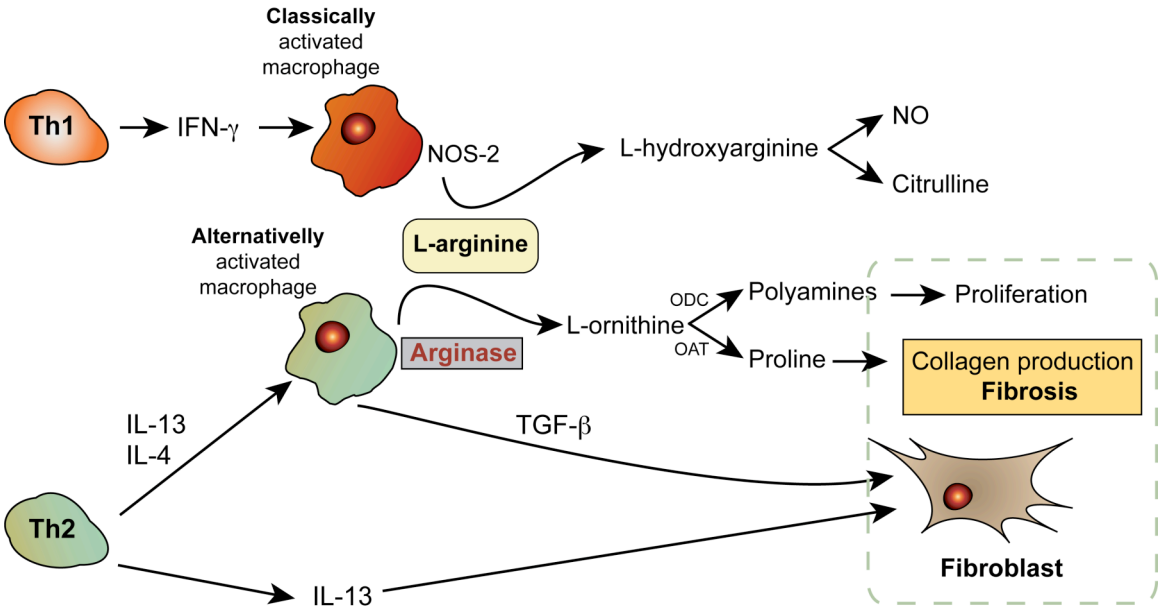

Figure 8. Type-1 cytokines (IFN-γ) stimulate NO production in classically activated macrophages, whereas IL-13 and IL-4 preferentially activate Arginase and production of L-ornithine in alternatively activated cells. L-ornithine serves as a substrate for ODC and OAT, which generate polyamines and proline, respectively. Proline is an essential amino acid used in collagen production. Fibroblasts are another significant target of IL-13 activity. IL-13 may act directly on fibroblasts or indirectly by stimulating and activating TGFβ production in macrophages. Adapted from (Wynn 2003).

Alternatively activated macrophages also express TGFβ, which has been the most intensively studied regulator of the ECM and has been linked with the development of fibrosis in a number of diseases (Sime et al. 1997; Sato et al. 2003). There are three isotypes of TGFβ in mammals, TGFβ1, -2 and -3, all exhibiting similar biological activity. Although a variety of cell types produce and respond to TGFβ, tissue fibrosis is primarily attributed to the TGFβ1 isoform, with circulating monocytes and tissue macrophages being the predominant cellular sources. Once activated, TGFβ signals through transmembrane receptors that trigger signaling intermediates known as Smad proteins, which modulate transcription of important target genes, including pro-collagen I and III (Roberts et al. 2003). Macrophage-derived TGFβ1 is thought to promote fibrosis by directly activating resident mesenchymal cells, which then differentiate into collagen-producing myofibroblasts.

TGFβ has also been related to muscle dystrophy-associated fibrosis in *mdx* mice (Gosselin et al. 2004; Andreetta et al. 2006); however, in comparison with other tissues, very little is known about the mechanisms/pathways driving fibrosis in dystrophic muscle.

2. THE PLASMINOGEN ACTIVATION SYSTEM

In multicellular organisms, extracellular proteolysis is important to many biological processes involving a dynamic rearrangement of cell-cell and cell-ECM interactions. Activation of the zymogen plasminogen into the active serine proteinase, plasmin, is a highly regulated and widely employed mechanism for the generation of extracellular proteolytic activity. Activation of plasminogen is exerted by two distinct plasminogen activators (PAs), tPA (tissue-type plasminogen activator) and uPA (urokinase-type plasminogen activator) (Collen and Lijnen 1991; Irigoyen et al. 1999). uPA binds to cells through the well-characterized uPA receptor (uPAR, CD87), while alpha-enolase and annexin-II among others, have been identified as cellular receptors for plasminogen and tPA, respectively (Lopez-Alemany et al. 1994; Hajjar and Acharya 2000; Blasi and Carmeliet 2002; Mondino and Blasi 2004), serving to localize PA and plasmin activities pericellularly. Plasmin is the major enzyme responsible for the dissolution of fibrin clots at both intravascular and extravascular sites; for that reason the PA system is classically known as the fibrinolytic system. uPA and plasmin are additionally implicated in numerous non-fibrinolytic processes leading to ECM degradation, either directly by proteolytic cleavage of ECM components such as fibronectin or laminin, or indirectly through the activation of latent matrix metalloproteinases (MMPs) (Lijnen et al. 1998; Blasi 1999). Furthermore, uPA and plasmin, as well as some MMPs, have been shown to activate several latent growth factors *in vitro*, including transforming growth factor β (TGFβ), hepatocyte growth factor/scatter factor (HGF/SF) and basic fibroblast growth factor (bFGF), whose activities are crucial for cell migration and tissue remodeling *in vivo* (Lyons et al. 1988; Naldini et al. 1992; Mars et al. 1993; Rifkin et al. 1999). Thus, several mechanisms account for the important implication of the plasminogen activation/plasmin system in different physiopathological processes involving ECM degradation, tissue remodeling and cell migration, including mammalian ovulation, trophoblast invasion, post-lactational mammary involution, neurite outgrowth, excitotoxic-induced neuronal death, nerve regeneration, skin wound healing, inflammation, glomerulonephritis, angiogenesis and tumor cell invasion (Plow et al. 1995; Blasi 1999; Mondino and Blasi 2004; Plow and Hoover-Plow 2004).

Because unrestrained generation of proteolytic activity may be hazardous to the cells, plasmin activity is tightly controlled at the level of PAs by plasminogen activator inhibitors (PAI-1 and PAI-2), and at the level of plasmin by α2-antiplasmin (Collen and Lijnen 1991; Irigoyen et al. 1999). PAI-1 is the primary physiological inhibitor of uPA. It regulates the proteolytic activity of uPA directly via its serine proteinase activity, and indirectly by regulating the levels uPA-uPAR complex through promotion of its endocytosis (Nykjaer et al. 1997; Degryse et al. 2001; Herz and Strickland 2001). uPAR and PAI-1

have also been implicated in non-proteolytic cellular processes. uPAR can directly promote integrin-mediated cell adhesion on a vitronectin (VN) substrate in the presence of uPA (Wei et al. 1996; Chapman and Wei 2001); through its avid interaction with VN, PAI-1 may inhibit VN-mediated cell adhesion and migration (Deng et al. 1996; Stefansson and Lawrence 1996). These interactions also induce cell signaling (Ossowski and Aguirre-Ghiso 2000; Blasi and Carmeliet 2002; Resnati et al. 2002). Thus, the plasminogen activation system exerts its biological functions not only through the catalytic activity of its components (leading to fibrinolysis and fibrin-independent proteolysis) but also through modulating the interaction between ECM-integrins, which does not necessarily involve catalytic activity of PAs (Fig. 9).

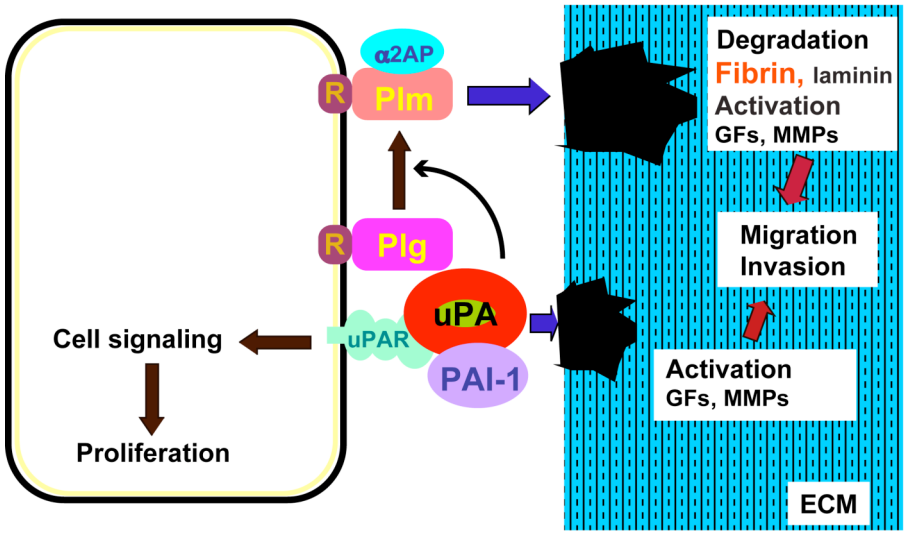

Figure 9. The Plasminogen Activation System. Active plasmin (Plm) is generated from its zymogen plasminogen (Plg) by urokinase-type plasminogen activator (uPA), which, in turn, can be inhibited by plasminogen activator inhibitor (PAI-1). uPA binds to its specific receptor uPAR while Plg can associate with alpha-enolase Plg receptor on the cell surface. Besides fibrinolysis, uPA and plasmin participate in both, growth factor and metalloproteinase activation as well as extracellular matrix (ECM) degradation, which together contribute to different cellular processes including cell proliferation and migration.

2.1 Components of the Plasminogen Activation System

2.1.1 Plasminogen (Plg)

Plasminogen is secreted, mainly by the liver, as a single chain 92-KDa glycoprotein. The molecule is composed of five kringle-like domains containing “lysine-

binding sites" and a carboxyl-terminal domain homologous to other trypsin-like proteases. The kringle domains mediate interactions of plasminogen and plasmin with substrates, inhibitors and cell receptors, which are crucial for the activation of plasminogen and localization of proteolytic activity of plasmin. The cleavage of the Arg561-Val562 peptide bond by plasminogen activators (PA) converts plasminogen to the active enzyme plasmin, consisting of two polypeptide chains liked by a disulfide bond. The amino-terminal heavy A-chain possesses all the kringle domains. The carboxyl-terminal light B-chain contains the catalytic site composed of His602, Asp645 and Ser740 (Castellino and Ploplis 2005).

Plasminogen receptors are abundant on cell surfaces and are not limited to a single class of molecules. In particular, free lysine binding sites within the kringle domains of plasminogen support its interaction with cell surfaces. The specific class of plasminogen receptor is cell-type specific and several receptors have been identified, among them α-enolase and annexin II (Miles et al. 1991; Hajjar et al. 1994). Plasminogen activation is enhanced as a consequence of cell surface binding and moreover, plasmin activity is enhanced and protected from its inhibitor α2-antiplasmin (Plow et al. 1995).

2.1.2 UROKINASE-TYPE PLASMINOGEN ACTIVATOR (UPA)

uPA is a 53-KDa serine protease produced as a single-chain protein (scuPA) (Gunzler et al. 1982). When secreted, scuPA (pro-uPA) is converted to the active two-chain form uPA (tcuPA) by cleavage of the peptide bond Lys158-Ile159 by plasmin. The two peptide chains of uPA are linked by disulfide bonds, and the molecule contains three functional domains. In the carboxyl-terminal region is a serine protease domain (SPD; also called the B chain) making up most of the low-molecular-weight (LMW) uPA with full specific activity (Steffens et al. 1982). The non-catalytic amino-terminal fragment (ATF), corresponding to the A chain, contains the kringle domain, and the epidermal growth factor (EGF)-like domain (GF). The two chains are linked by a connecting peptide. Thus, the uPA molecule has at least two completely independent parts: the catalytic carboxyl-terminal chain and the non-catalytic amino-terminal chain, of which the initial GF domain of 32 residues is responsible for the specific interaction with uPAR (Appella et al. 1987).

Besides converting scuPA to active tcuPA, plasmin can further cleave uPA to a 33-KDa form, which lacks the GF and the kringle domains but still retains full activity (Stump et al. 1986).

2.1.3 TISSUE-TYPE PLASMINOGEN ACTIVATOR (TPA)

tPA is a 70-KDa protein, which is secreted as a precursor in single-chain form. Plasmin converts the precursor by cleaving the peptide bond Arg275-Ile276 to give an active two-chain form held together by a single interchain disulfide bond as in uPA. Unlike uPA, however, single-chain tPA also has a significant activity (Nienaber et al. 1992). The tPA molecule is composed of four functionally distinct domains: (1) an amino-terminal region of 47 residues known as the fibronectin-like domain or finger domain, (2) an EGF-like domain (GF), (3) two kringle regions (K1 domain and K2 domain), and (4) a serine protease region with the active-site residues His322, Asp371 and Ser478 (Pennica et al. 1983). The first three domains are located in the amino-terminal chain and have modulatory activities for the enzyme.

2.1.4 PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 (PAI-1)

PAI-1 is a single-chain glycoprotein with a molecular weight of 50-KDa, and it belongs to the serpin gene family (Dellas and Loskutoff 2005). PAI-1 is synthesized as an active molecule that converts spontaneously to the latent conformation unable to form complexes with PAs. The half-life of this latent form is about 60 min at 37ºC. Active PAI-1 interacts with the adhesive glycoprotein VN in plasma and in the extracellular matrix. This interaction stabilizes PAI-1 and doubles its lifetime (Declerck et al. 1988). Thus, VN may be a cofactor for PAI-1. The high affinity binding site for PAI-1 in VN has been mapped to the somatomedin B domain (Seiffert et al. 1994). It is known that PAI-1 bound to VN prevents VN-integrin binding, independently from its proteinase-inhibitory functions and therefore down-regulates the VN-integrin mediated cell activities. The PAI-1- and the integrin-binding sites are localized in the same amino-terminal region of VN, in a close proximity excluding simultaneous binding of both ligands (Ngo et al. 2001).

An important property of PAI-1 is its ability to induce internalization and degradation of uPAR-bound uPA. The entire process requires the low-density lipoprotein (LDL)-receptor-related protein (LRP), which can interact with both PAI-1 and uPAR. The quaternary complex uPAR-uPA-PAI-1-LRP is internalized inside the cell. uPA and PAI-1 are then engulfed by lysosomes and digested, while uPAR is recycled to the cell surface (Nykjaer et al. 1997).

2.1.5 PLASMINOGEN ACTIVATOR INHIBITOR TYPE 2 (PAI-2)

PAI-2 is a single-chain protein of 47 KDa. Its inhibitory potency is 15 times less than that of PAI-1 (Ellis et al. 1990). PAI-2 is generally characterized as an inhibitor of the extracellular serine protease uPA. However, most newly synthesized PAI-2 remains intracellular, with only a fraction of PAI-2 secreted as a glycosylated product (Kruithof et al. 1995). Thus, PAI-2 has the unique feature of existing in both secreted and cytosolic forms. In its secreted form, PAI-2 participates in the control of tissue remodeling and fibrinolysis. In its cytosolic form, it plays an important role in intracellular proteolysis involved in processes such as apoptosis and inflammation (Dickinson et al. 1995; Antalis et al. 1998).

2.1.6 UPA RECEPTOR (UPAR)

uPAR is a heavily glycosylated protein of approximately 55-KDa. It is a glycosylphosphatydilinositol (GPI)-anchored protein composed of three domains (D1, D2 and D3). uPAR binds with high affinity to uPA, scuPA and ATF, as well as VN. It associates with the integrin family members (α3, α5) (Wei et al. 1996) and with the G-protein-coupled receptor FPRL1 (Resnati et al. 2002) to modulate cell adhesion and migration.

In addition to the membrane-anchored form, uPAR is released from the plasma membrane by cleavage of the GPI anchor and can be found as a soluble molecule (suPAR). Both uPAR and suPAR can be cleaved in the region that links domain D1 to domain D2 to yield a D1 fragment and a D2D3 fragment. The D2D3 fragment has direct chemotactic activity (Fazioli et al. 1997).

uPAR not only functions as a proteinase receptor, but also affects migration, adhesion, differentiation and proliferation through intracellular signaling. The active molecular complex seems to be a trimeric uPA-uPAR2 complex (Sidenius et al. 2002). Several of the signaling functions of uPA do not require its proteolytic activity, as catalytically inactive uPA derivatives (such as scuPA and ATF) are equally effective. Conversely, the expression of uPAR and its ability to bind uPA are required for signaling. uPAR lacks a cytosolic domain, but at least three types of transmembrane proteins have been identified as mediators of uPAR and they are involved in signaling in response to uPA. These mediators are integrins, G-protein-coupled receptors and caveolin. As a

result, uPAR activates intracellular signaling molecules such as tyrosine- and serine-protein kinases (PI3K, Rac, MEK, ERK…) (Ossowski and Aguirre-Ghiso 2000).

2.1.7 FIBRINOGEN

Fibrinogen is a circulating 340-KDa glycoprotein, primarily synthesized by hepatocytes. It is comprised of two symmetric half molecules, each consisting of one set of three different polypeptide chains termed Aα, Bβ and γ, that are linked together by disulfide bonds. The molecule is highly heterogeneous due to alternative splicing, extensive post-translational modification and proteolytic degradation. Each of the three polypeptide chains of the human fibrinogen molecule is encoded by a separate gene located on chromosome four (Herrick et al. 1999).

The predominant Aα chain of circulating fibrinogen contains around 610 amino acid residues (70 KDa), the Bβ chain of fibrinogen consists of 461 amino acids (56 KDa). The γ chain is heterogeneous with respect to both charge and size; however, the most abundant form, denoted as γ- or γ_A, consists of 411 residues (48 KDa).

The fibrinogen molecule has three distinct domains; two terminal D domains (67 KDa), each linked to a single central E domain (33 KDa) by a triple-stranded array of the polypeptide chains believed to exist in the form of a helical coiled coils. There is extensive post-translational modification of the protein including phosphorylation, sulphation, glycosylation and hydroxylation (Herrick et al. 1999).

Like other blood proteins, fibrinogen is secreted into the plasma and circulates throughout the body. Fibrinogen is most classically known for its role in blood coagulation. At sites of tissue injury, upon initiation of the coagulation cascade, the serine protease thrombin cleaves peptides from the amino-terminal regions of the Aα and Bβ chains. The cleavage of these peptides, termed fibrinopeptide A and fibrinopeptide B, exposes polymerization sites and initiates the formation of a polymer which form an insoluble fibrin clot (Sidelmann et al. 2000) (Fig. 10).

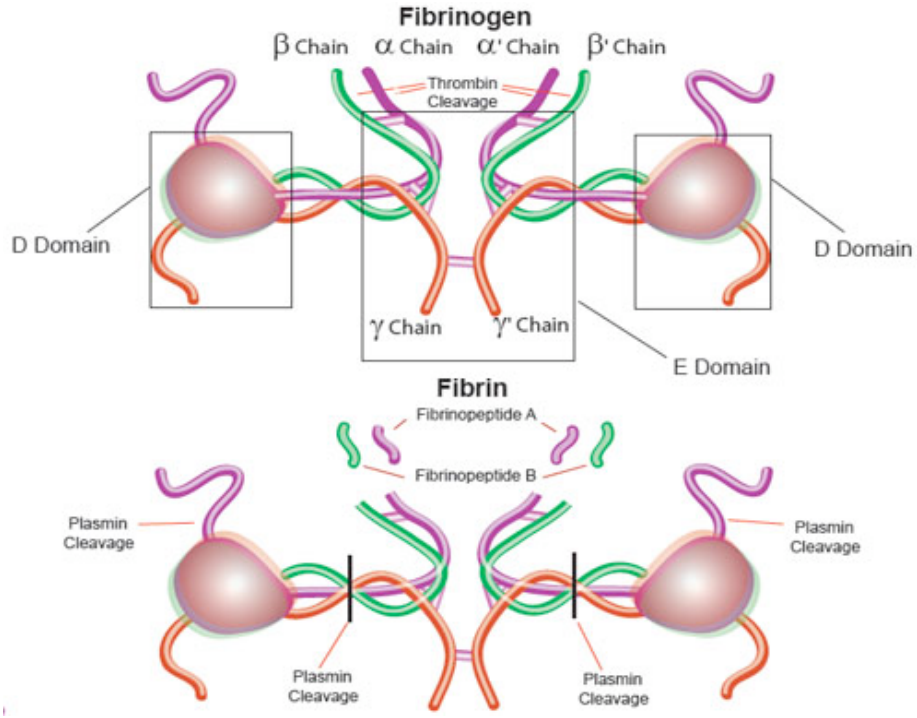

Figure 10. Schematic diagram of fibrinogen. There are three sets of nonidentical chains, which are bound by disulphide bonds at the amino-terminal end. Removal of fibrinopeptides A and B at this end via the action of thrombin, leads to cross-linking and the formation of the fibrin clot.

The role of fibrinogen in blood coagulation includes its capacity to aggregate platelets leading to thrombus formation. Mechanistically, fibrinogen binds to the αIIbβ3 receptor on platelets *via* the γ-chain of its C-terminus (Holmback et al. 1996). Fibrinogen engagement with the αIIbβ3 integrin induces platelet adhesion and aggregation thereby promoting blood coagulation (Phillips et al. 1988).

Fibrin is considered a provisional matrix because it is subjected to proteolytic degradation by plasmin. Plasmin digestion of fibrin results in the production of two soluble fibrin degradation products, namely D-dimmer and fragment E (Gaffney 2001). Although clearly important in haemostasis, the fibrin clot also provides a scaffold for cell adhesion, spreading, migration and proliferation.

Experiments from cases of human disease or from mice genetically lacking components of the coagulation and proteolytic pathways have shown that fibrin exacerbates some disease pathogenesis like skin wound healing, glomerulonephritis, multiple sclerosis and Alzheimer's disease (Romer et al. 1996; Kitching et al. 1997; Adams et al. 2007; Paul et al. 2007).

Notably, genetic depletion of fibrinogen rescues the effects of plasminogen deficiency in liver, lung and kidney damage, as well as in impaired wound healing, showing that fibrin is the major substrate for plasmin *in vivo* (Bugge et al. 1996).

2.1.7.1 FIBRINOGEN AS AN INFLAMMATORY MOLECULE

Recent evidence has expanded the role of fibrinogen beyond its function in blood clotting as an active participant orchestrating the extent of the immune system's inflammatory response. Fibrinogen can mediate diverse biological responses due to its ability to affect different cell types by binding to unique cellular receptors. As a result, fibrinogen is distinctive among blood proteins due to a molecular structure that contains binding sites for receptors expressed on cells that are central mediators of the inflammatory process.

Fibrinogen has been shown to play a central role in tissues with an inflammatory component. Often referred to as an *acute phase response* protein, fibrinogen is significantly upregulated upon inflammatory conditions (Schultz and Arnold 1990). In physiological conditions, fibrinogen circulates within the bloodstream at concentrations generally ranging from 2-4 mg/mL, but upon tissue damage or inflammation this concentration can increase several fold (Schultz and Arnold 1990). These changes in circulating fibrinogen levels have implicated fibrinogen as a risk factor for diverse pathological conditions including cardiovascular disease (Koenig 2003), stroke (Catto and Grant 1995) and Alzheimer's disease (AD) (van Oijen et al. 2005).

Fibrinogen, for example, has been demonstrated to promote inflammation at sites of implanted biomaterials. The molecular mechanism of this increased inflammatory response requires interaction with the CD11b/CD18 integrin (also known as Mac-1, CR3 or $\alpha M\beta 2$) (Tang et al. 1996). Biochemical studies have extensively characterized fibrinogen binding to CD11b/CD18 and demonstrated a requirement of specific sequences on the C-terminus of fibrinogen's γ-chain (Ugarova et al. 1998). Interaction studies using a diverse array of techniques, have elucidated the unique fibrinogen sequences that are critical for binding to the CD11b/CD18 receptor. Fibrinogen binds to the alpha subunit of CD11b upon a conformational alteration in the γC domain of fibrinogen, which unmasks a cryptic binding epitope (Lishko et al. 2002). While there are several regions involved in fibrinogen binding to CD11b/CD18, the critical site for this interaction is found on the C-terminus of fibrinogen's γ-chain (γ residues 377-395) (Ugarova et al. 2003). Fibrinogen signaling through CD11b/CD18 can elicit intracellular signaling. Fibrinogen *via* CD11b/CD18 can induce in monocytes production of tissue factor (Fan and Edgington

1991) and TNFα (Fan and Edgington 1993). Fibrinogen-CD11b/CD18 signaling can activate extracellular signal-regulated kinase 1/2 (ERK1/2) or the phosphoinositide-3 kinase (PI3K) pathway to elicit neutrophil survival (Whitlock et al. 2000), whereas stimulation of the nuclear factor kappa B (NF-κB) pathway causes increased IL-1β expression in monocytes (Perez et al. 1999). Although less well characterized, fibrinogen has also been found to bind to CD11c/CD18, an integrin critical for dendritic cell activation (Ugarova and Yakubenko 2001). The ability of fibrinogen to interact with distinct leukocyte receptors on several cell types might allow fibrinogen to promote diverse responses during different stages of inflammation.

The generation of a fibrinogen knock-in mice where the biochemically identified binding site of fibrinogen to CD11b was genetically mutated, further provided support for the fibrinogen/CD11b interaction *in vivo* (Flick et al. 2004b). By substituting alanine residues in fibrinogen's γ-chain, which are requisite for the binding to the CD11b/CD18 integrin, this mouse termed Fibγ$^{390-396A}$ is incapable of supporting fibrinogen signaling through CD11b/CD18 while still retaining all hematologic functionality. Fibγ$^{390-396A}$ mice failed to support CD11b/CD18-mediated adhesion of primary neutrophils, monocytes, and macrophages, and mice expressing this fibrinogen variant were found to exhibit a major defect in the host inflammatory response following acute challenges (Flick et al. 2004b). Fibrinogen interactions with CD11b/CD18 also play a major role in inflammation in the nervous system, as it has been demonstrated in animal models for multiple sclerosis (Adams et al. 2007).

In addition to CD11b/CD18, fibrinogen may contribute to the inflammatory response *via* the Toll-Like Receptor 4 (TLR4). Experiments using macrophage cells from mice deficient for TLR4 showed that fibrinogen/TLR4 interactions can induce chemokine expression (Smiley et al. 2001). In addition to monocytes/macrophages and neutrophils, fibrinogen induces the adhesion and activation of mast cells through its interaction *via* the C-terminus γ-chain (γ residues 408-411) with the αIIbβ3 integrin receptor (Oki et al. 2006).

The proinflammatory effects of fibrinogen have also been observed in the cardiovascular system. In myocardial infarction, fibrinogen interactions with the endothelial cell receptor vascular endothelial cadherin (VE-cadherin) promotes increased leukocyte infiltration and infarct size after reperfusion (Petzelbauer et al. 2005). Binding of fibrinogen *via* the N-terminus of the β chain (β residues 15-42) to VE-cadherin (Gorlatov and Medved 2002), also regulates other endothelial cell functions, such as migration and capillary tube formation (Martinez et al. 2001). Fibrinogen additionally binds *via* distinct RGD sequences in the C-terminus of the Aα chain (α residues 572-574) to the integrin αvβ3 (Smith et al. 2000) and *via* a sequence in the γ chain (γ residues 117-133) to the Intracellular Adhesion

Molecule-1 (ICAM-1) (Altieri et al. 1995) to mediate endothelial cell adhesion. Thus, fibrinogen, in addition to serve as a critical regulator of blood homeostasis, plays a major role in promoting inflammatory responses by utilizing multiple receptors expressed by cells that are key mediators of the onset and progression of inflammation, such as monocytes, macrophages, microglia, mast cells and endothelial cells.

2.2 CONSEQUENCES OF GENE INACTIVATION OF PLASMINOGEN ACTIVATION SYSTEM COMPONENTS *IN VIVO*.

Studies in mice using targeted inactivation of uPA, tPA, PAI-1, uPAR, plasminogen and fibrinogen genes have confirmed that disruption of the plasminogen activation system contributes not only to various vascular disorders but also to the perturbation of reproduction, wound healing, infection, cancer and brain function among others. The main phenotypes resulting from genetic inactivation of different components of the plasminogen activation system in mice are summarized in Table 2.

Deficiency	Phenotype	Reference
tPA	- increased thrombotic susceptibility	(Carmeliet and Collen 1995)
	- mild glomerulonephritis	(Kitching et al. 1997)
	- reduced neuronal degeneration	(Tsirka et al. 1995)
	- reduced renal fibrosis	(Hu et al. 2008)
	- impaired neuronal migration	(Seeds et al. 1999)
	- exacerbated cholestatic liver injury	(Wang et al. 2007)
	- increased E. coli-induced abdominal sepsis	(Renckens et al. 2006)
	- increased skin collagen deposition	(de Giorgio-Miller et al. 2005)
uPA	- increased thrombotic susceptibility	(Carmeliet and Collen 1995)
	- impaired neointima formation	(Carmeliet and Collen 1995)
	- impaired macrophage function	(Carmeliet and Collen 1995)
	- reduced decidual vascularization	(Irigoyen et al. 1999)
	- reduced trophoblast invasion	(Irigoyen et al. 1999)
	- reduced platelet activation	(Irigoyen et al. 1999)
	- exacerbated antigen-induced arthritis	(Busso et al. 1998)
	- reduced metastasis	(Almholt et al. 2005)
tPA:uPA	- severe spontaneous thrombosis	(Carmeliet and Collen 1995)
	- impaired neointima formation	(Carmeliet and Collen 1995)
	- reduced ovulation and fertility	(Carmeliet et al. 1994)
	- cachexia and shorter survival	(Carmeliet et al. 1994)
	- severe glomerulonephritis	(Kitching et al. 1997)
	- abnormal tissue remodeling	(Carmeliet and Collen 1995)
uPAR	- increased severity of renal fibrosis	(Zhang et al. 2003)
	- increased susceptibility to Streptococcus pneumoniae	(Rijneveld et al. 2002)
	- impaired neutrophil recruitment	(Gyetko et al. 2000)
	- reduced ischemic brain damage	(Nagai et al. 2008)
Plasminogen	- shortened life expectancy	
	- diminished growth rates	(Bugge et al. 1995)
	- spontaneous fibrin deposition	(Bugge et al. 1995)
	- spontaneous ligneous conjunctivitis	(Drew et al. 1998)

	- compromised clot lysis	(Ploplis et al. 1995)
	- attenuated inflammatory cell recruitment	(Ploplis et al. 1998)
	- enhanced resistance to pathogens	(Nordstrand et al. 2001)
	- severe glomerulonephritis	(Kitching et al. 1997)
	- exacerbated antigen-induced arthritis	(Busso et al. 1998)
	- enhanced lung collagen deposition	(Swaisgood et al. 2000)
	- impaired keratinocyte migration	(Lund et al. 1999)
	- impaired wound healing	(Lund et al. 1999)
	- reduced neuronal degeneration	(Tsirka 1997)
	- reduced morbidity and mortality of Lewis lung carcinoma	(Bugge et al. 1997)
	- reduced sarcoma growth	(Curino et al. 2002)
	- attenuated angiogenesis	(Oh et al. 2003)
	- accelerated atherosclerosis	(Xiao et al. 1997)
PAI-1	- bleeding tendency	(Carmeliet et al. 1993b)
	- mild hyperfibrinolytic state	(Carmeliet et al. 1993b)
	- reduced thrombotic incidence	(Carmeliet et al. 1993b)
	- reduced atherosclerosis	(Dellas and Loskutoff 2005)
	- reduced bleomycin-induced pulmonary fibrosis	(Eitzman et al. 1996)
	- attenuated obesity and insulin resistance	(Ma et al. 2004)
	- reduced tumor growth and angiogenesis	(McMahon et al. 2001)
	- attenuated antigen-induced arthritis	(Van Ness et al. 2002)
PAI-2	- reduced adipose tissue development	(Lijnen et al. 2007)
Fibrinogen	- spontaneous bleeding events	(Suh et al. 1995)
	- failure of pregnancy	(Suh et al. 1995)
	- wound healing defects	(Drew et al. 2001a)
	- diminished glomerulonephritis	(Drew et al. 2001b)
	- diminished lung metastasis	(Palumbo et al. 2000)
	- reduced collagen-induced arthritis	(Flick et al. 2007)

Table 2. Phenotypes resulting form targeted gene disruption of components of the plasminogen activation system in mice.

3. THE PLASMINOGEN ACTIVATION SYSTEM IN MUSCLE REGENERATION.

Extracellular proteolysis and tissue remodeling take place during skeletal muscle formation and pathological muscle regeneration. A number of proteolytic enzymes have been proposed to play a role during muscle regeneration, either in the inflammatory response, in the activation of satellite cells and/or in the migration of myoblasts across the basal lamina and in their further fusion to form the terminal muscle fiber (Couch and Strittmatter 1983; Hughes and Blau 1990). Metalloproteinases such as MMP-2 and MMP-9, meltrin-α and cathepsin B seem to be required for myotube formation *in vitro* (Gogos et al. 1996; Kherif et al. 1999). Moreover, the expression of MMP-2 and MMP-9 has been reported in the degeneration-regeneration process of myofibers *in vivo* (Kherif et al. 1999; Carmeli et al. 2004). The mechanism of MMP activation in most cell types involves a proteolytic activation cascade initiated by uPA/plasmin (Lijnen et al. 1998). Muscle injury induces the expression of uPA, Plg and PAI-1 during the initial regeneration phase (Festoff et al. 1994; Lluis et al. 2001; Chen et al. 2002; Suelves et al. 2002; Zhao et al. 2002). Alpha-enolase plasminogen receptor and uPAR expression are also upregulated in regenerating muscle (Lopez-Alemany et al. 2003; Lopez-Alemany et al. 2005; Suelves et al. 2007). Using a genetic approach, our group has demonstrated that uPA, but not tPA, activity is required for efficient skeletal muscle regeneration *in vivo* (Lluis et al. 2001). Similarly to wild-type mice, tPA-deficient mice completely repaired experimentally-injured skeletal muscle, while uPA-deficient mice were unable to repair the damage (Lluis et al. 2001), indicating that no redundancy exists between both PAs in muscle. Moreover, the muscle regeneration capacity of Plg-deficient mice was severely impeded, indicating that uPA-dependent plasmin activity is necessary for skeletal muscle regeneration *in vivo* (Ploplis et al. 1995; Suelves et al. 2002) (see Fig. 11). Most importantly, uPA- and Plg-deficient mice accumulated fibrin/ogen in the degenerating muscle after injury, while defibrinogenation of the mice rescued significantly the regeneration defect (Lluis et al. 2001; Suelves et al. 2002). These results strongly suggest that alterations in the homeostasis of the plasminogen activation system (i.e. fibrin/ogen accumulation) may have a pathogenic role in sustaining muscle degeneration.

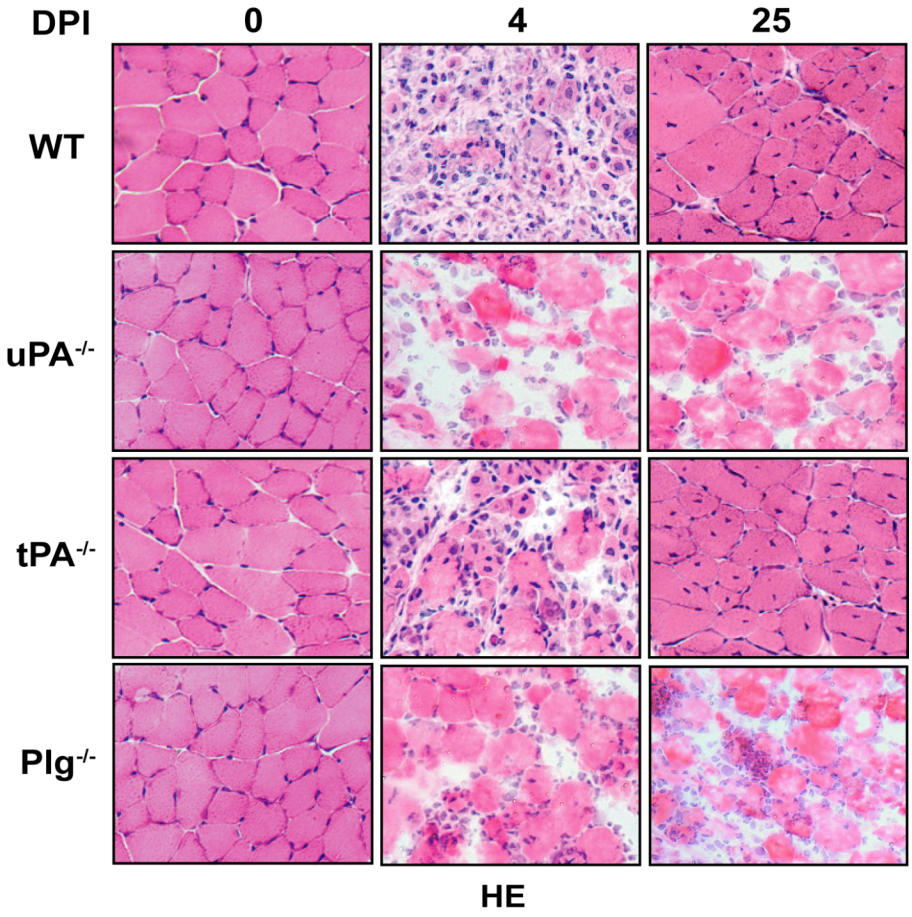

Figure 11. uPA- (but not tPA-) and Plg-deficient mice show a persistent regeneration defect following muscle injury. Frozen sections of CTX-injured muscles from WT, uPA-, tPA- and Plg-deficient mice, respectively, were stained with Hematoxilin/Eosin (HE) at 4 and 25 days post-injury (DPI). Contralateral control muscles were also stained with HE (0 DPI). In WT and tPA-deficient mice, regeneration is complete after 25 days, as evidenced by the presence of centrally nucleated fibers (CNF). In uPA- and Plg-deficient mice, however, a regeneration defect is still visible 25 DPI.

As previously described, inflammation is a process frequently associated to tissue repair, since degenerating tissues are invaded by inflammatory cells. Accordingly, in response to muscle injury, neutrophils, macrophages and T lymphocytes accumulate at the injury site in mice and rats during the inflammatory response (Orimo et al. 1991; Pimorady-Esfahani et al. 1997; Charge and Rudnicki 2004). Mice with a specific deficit in uPA or plasminogen show a reduced staining for Mac-1- and T11-positive cells two days after injury, indicating that the number of macrophages and T lymphocytes reaching the injury site is reduced in the absence of uPA or plasminogen (Lluis et al. 2001; Suelves et al. 2002). This suggests that uPA/plasmin activity may have a profound effect on inflammation and inflammation-related muscular disease. Previous studies with uPA-

deficient mice demonstrated that uPA is required for the pulmonary inflammatory response to *Cryptococcus neoformans* and *Pneumocystis carinii*, since a lack of uPA resulted in inadequate inflammatory cell recruitment, uncontrolled infection and death (Gyetko et al. 1996; Beck et al. 1999). Similarly, monocyte and lymphocyte recruitment was significantly diminished in plasminogen-deficient mice after thioglycollate-induced acute peritoneal inflammation (Ploplis et al. 1998). Additionally, components of the PA system may regulate the expression or/and activity of cytokines involved in inflammatory processes. Plasmin has been shown to release macrophage derived IL-1 and to activate TGFβ (Matsushima et al. 1986; Keski-Oja and Koli 1992), while uPA potentiates lipopolysaccharide-induced expression of IL-1β, MIP-2 and TNFα in neutrophils (Abraham et al. 2003). It has been shown that activated macrophages (equivalent to those which accumulate at the site of muscle damage) produce soluble factors (FGF and PDGF) which are highly chemoattractant and also mitogenic for muscle precursor cells (Robertson et al. 1993). Thus, the activated macrophages which accumulate in response to muscle damage will not only phagocytose necrotic tissue but also facilitate the repair of damaged myofibers.

Furthermore, uPA/plasmin can cleave and activate latent forms of growth/angiogenic factors such as bFGF, TGFβ and HGF/SF (Naldini et al. 1992; Odekon et al. 1994; Charge and Rudnicki 2004), which are expressed within injured muscle and are believed to promote the activation of quiescent satellite cells after injury *in vivo* (Tatsumi et al. 1998; Yablonka-Reuveni et al. 1999; Shefer et al. 2001). Other studies have shown that growth factor-dependent proliferation and migration of satellite cells require the cell-associated plasminogen activation system (Fibbi et al. 2002). There is also evidence for a direct role for uPAR/uPA/PAI-1 tripartite complex during human myogenic migration and cell fusion (Bonavaud et al. 1997; Chazaud et al. 2000). Therefore, modulation of plasminogen activation may directly or indirectly influence satellite cell recruitment and the growth and differentiation of cellular constituents in regenerating muscle.

OBJECTIVES

The plasminogen activation (fibrinolytic) system is well known to play a critical role in remodeling of the extracellular matrix. As described above, it also plays a role in experimentally-induced muscle regeneration; however, its role in muscle dystrophy remains unknown. Results from our laboratory show that different components of the system (uPA, uPAR, PAI-1 and plasmin) are highly expressed in *mdx* with respect to WT muscle. Importantly, we have observed that fibrin/ogen accumulates in *mdx*, but not in WT muscle, suggesting an imbalance of the system leading to fibrin/ogen deposition in *mdx* muscles. Thus, we hypothesized that the plasminogen activation system may be involved in the progression of muscle dystrophy. Since fibrosis is a major contributor to muscular dystrophy pathology, and the plasminogen activation system has been implicated in certain fibrotic conditions, a connection with dystrophic fibrosis was also hypothesized. Our aim in this PhD Thesis was to analyze the consequences of manipulating the plasminogen activation system on muscle regeneration and *mdx* distrophinopathy.

Specifically, the main objectives of my PhD project were:

- Study the consequences of uPA or PAI-1 deficiency in *mdx* dystrophy progression.
- Study the role of fibrin/ogen deposition in dystrophic muscle.
- Study the possible mechanisms by which fibrin/ogen can influence dystrophy progression.

MATERIALS AND METHODS

1. GENERATION OF DOUBLE-MUTANT MICE

uPA, uPAR, fibrinogen and PAI-1 knock-out male mice (Carmeliet et al. 1993a; Carmeliet et al. 1994; Suh et al. 1995; Dewerchin et al. 1996) were crossed with *mdx* female mice (Jackson Laboratories; USA). Male F1 mice were bred with *mdx* female mice, and their F2 heterozygous male and female offspring was intercrossed. The resulting F3 generation showed the expected Mendelian distribution of +/+, +/- and -/- genotypes, all of them in an *mdx* background. The uPA, uPAR, fibrinogen and PAI-1 genotypes were confirmed by polymerase chain reaction (PCR) of tail or ear biopsy genomic DNA, as previously described (Carmeliet et al. 1993a; Carmeliet et al. 1994; Suh et al. 1995; Dewerchin et al. 1996). The *mdx* genotype was confirmed by Western blotting of muscle biopsies, using an anti-dystrophin antibody (Novocastra, UK, 1:200). All animal experiments were approved by the Catalan Government Animal Care Committee.

2. MORPHOMETRIC ANALYSIS

At selected times, muscles of indicated mice were removed after cervical dislocation, frozen and stored at –80º C prior to analysis. 10 µm-thick cryostat sections were collected and stained with hematoxylin/eosin (HE). Muscle degeneration (%) was determined morphometricaly and expressed as a percentage of the total muscle cross-sectional area (CSA). Muscle fiber regeneration (%) was determined morphometricaly and expressed as the percentage of total muscle fibers containing central nuclei (CNF) present in the entire cross-section of the muscle. Pictures were obtained on a Leica DM6000B microscope equipped with a video camera (Leica DFC500). All parameters were measured using the ImageJ program (National Institute of Health, USA).

3. BIOCHEMICAL AND FUNCTIONAL ASSESSMENT OF MUSCLE

Serum creatine kinase (CK) was measured with the indirect CK colorimetric assay kit and standards (Thermo Electron, USA), according to manufacturer's instructions. **Grip strength assay**: forearm grip strength was measured as tension force using a computerized force transducer (Grip Strength Meter, Bioseb) to measure peak force exerted by a mouse's forearms as its grip was broken by the investigator pulling the mouse by the base of the tail away from the transducer of the grip strength meter. Three trials of 3 measurements per trial were performed for each animal with a few minutes

resting period between trials. The average tension force (Newton, N) was calculated for each group of mice. The 100% value was arbitrarily assigned to the recorded force of uPA$^{+/+}$*mdx* mice (Fig. 14) or saline-treated *mdx* mice (Fig. 30). **Treadmill assay**: the treadmill apparatus (Treadmill, Panlab) consisted of a motor-driven belt varying in terms of speed (5 to 150 rpm) and slope at 10º. 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 (FRS 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 the treadmill at constant speed (5 rpm), to obtain baseline values for locomotion in the intact state.

4. IMMUNOHISTOCHEMISTRY

The following primary antibodies were used for immunohistochemistry: rat anti-Mac-1 (M1/70 Hybridoma Bank), anti-T11 conjugated with fluorescein (Coulter Immunology, 1:50), rat anti-F4/80 (Serotec, 1:200), rat anti-Ly-6G (BD Pharmingen, 1:200), rat anti-CD2 (Caltag Laboratories, 1:200), goat anti-Fibrin/ogen (Nordic, 1:100), rabbit anti-MR (CD206) (kindly provided by Dr. Anne Regnier-Vigourox, 1:100), mouse anti-Arginase I (BD Biosciences, 1:200), mouse anti-TGFβ (Abcam, 1:1000), rabbit anti-p-Smad2 (Cell Signaling, 1:50), mouse anti-Smad2 (Cell Signaling, 1:20). Depending on the antibody, immunohistochemistry was performed with the TSA Cyanine 3 system (PerkinElmer Life Sciences) or as previously described (Suelves et al. 2002).

5. INDUCTION OF MUSCLE REGENERATION

Skeletal muscle regeneration was induced by intramuscular injection of 150 µl of 10^{-5} M cardiotoxin (CTX) (Latoxan, France) in the tibialis or 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 hind limb muscles, and contralateral intact muscles were used as control. Morphological examinations were performed at the indicated days after injury.

6. CHARACTERIZATION OF MUSCLE FIBROSIS

Quantification of collagen content in diaphragm muscle was performed according to (Lopez-De Leon and Rojkind 1985). Histological characterization of muscle fibrosis after Sirius Red or Masson's trichrome staining was also done by image analysis quantification.

7. SYSTEMIC DEFIBRINOGENATION

Twelve-day-old uPA$^{-/-}$*mdx* or *mdx* mice were daily injected intraperitoneally with ancrod (an anti-coagulating enzyme from *A. rhodostoma* (Bell et al. 1978); Sigma, 1-3 U of ancrod per day) or with a saline solution for 18, 60 or 180 days, and killed at 1 month, 2.5 months or 6.5 months of age, respectively. Muscles were dissected and frozen prior to analysis.

8. ZYMOGRAPHY

Three hundred μg of muscle extracts were size-fractionated on a 10% non-reducing SDS acrylamide gel, which was washed for 30 minutes in 2.5% Triton X-100–phosphate-buffered saline and for 30 minutes in distilled water. The SDS-PAGE gel was then laid onto a casein gel containing 2% nonfat dry milk, 0.25 mM Tris-HCl pH 7.6, 1% agarose, 0.25 X phosphate-buffered saline, and 15 μg/ml plasminogen (Chromogenix), and incubated in a humid chamber at 37°C until caseinolytic bands (corresponding to uPA or/and tPA) were visualized and photographed.

9. PREPARATION OF MUSCLE EXTRACTS AND WESTERN BLOT ANALYSIS

Muscle extracts were prepared 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 were resolved by SDS-PAGE and transferred to PVDF membranes. Antibody dilutions: anti-fibrin/ogen (kindly provided by Dr. K. Dano, Denmark; 1:3000); anti-alpha-Tubulin (Sigma; 1:4000).

10. BONE MARROW TRANSPLANTATION

Donor BM cells were obtained by flushing the femurs and tibiae of uPA$^{+/+}$*mdx* or uPA$^{-/-}$*mdx* mice with RPMI-1640 medium (Gibco BRL), and were transplanted into 2 months-old uPA$^{-/-}$*mdx* mice, after lethal irradiation (9 Gy). The reconstituting cells (5 x 10^6

cells) were injected intravenously into the tail of the recipient mice within 24 h after irradiation. The mice were placed in sterile cages, and fed with sterile chow until the reconstitution of BM was completed 8 weeks after the transplantation. No changes in general health status were noted in the recipient mice. Two months after transplantation animals were killed and muscles dissected and frozen prior to analysis.

11. DMD PATIENT STUDY

Specimen preparation for histopathological examination: Specimens were obtained by standard quadriceps muscle biopsy. Ten-µm-thick frozen transverse muscle sections were stained by Masson's trichrome. **Immunohistochemistry**: Fibrin/ogen deposits were detected with a monoclonal anti-human fibrin/ogen antibody (Accurate Chemical & Scientific Corporation, 1:200). Bound primary antibodies were detected by the SuperPicTure™ Polymer Detection Kit (Zymed) and diaminobenzidine (DAB) as the chromogen. **Image acquisition**: true colour (RGB) consecutive non-overlapping images (x 20) acquired on a Zeiss Axioplan 2 microscope so that > 90% of the tissue section surface was represented (20-80 images per specimen). **Morphometry**: quantification of fibrosis was carried out by colour image segmentation and automatic measurement using AxioVision 4.4 image analysis software. The ratio of the total area of fibrosis to the total area of all muscle fibers present in a fascicle (13-25 fascicles per biopsy) was used to estimate the extent of fibrosis (fibrosis index). Cases were classified according to the spread and intensity of fibrin/ogen immunostaining. In order to jointly represent all morphological parameters on the same graph, continuous variables were collapsed to four discrete classes using the 25^{th}, 50^{th} and 75^{th} percentiles. Curves were fitted after displaying the values of the 3 groups in function of age.

12. CELL CULTURE AND ISOLATION OF PRIMARY CELLS

Bone marrow-derived macrophages were obtained from *mdx* mice and cultured as previously described (Celada et al. 1984). When indicated, primary macrophages were stimulated with Fibrinogen (500 µg/ml; Sigma) + 50 µM $MnCl_2$, actinomycin D (2 µg/ml; Sigma), cycloheximide (10 µg/ml; Sigma), recombinant IL-1β (30 ng/ml; R&D Systems), recombinant IL-13 (5 ng/ml; Peprotech), recombinant TGFβ (5 ng/m; R&D Systems), BAY11-7085 (5µM; BIOMOL), a blocking antibody anti-CD11b (15 µg/ml; eBioscience) or a blocking antibody anti-IL-1β (30 ng/ml; R&D Systems).

Primary fibroblasts were isolated from *mdx* mice diaphragm. Briefly, after dissection from *mdx* mice, diaphragms were minced to $1mm^3$ pieces in a sterile Petri dish

and the small pieces distributed in a 12-well plate containing 0,5 ml DMEM-F12 50% FBS per well. The plates were then incubated at 37°C with 5% CO_2 for 10 days (changing the medium every 2 days) to allow fibroblasts to migrate out of the tissue explants. When cells reached 50% confluence, the minced tissue masses were removed and fibroblasts were trypsinized and subcultured in 60 mm∅ culture plates. When indicated, primary fibroblasts were treated with Fibrinogen (500 μg/ml; Sigma), a blocking antibody anti-αV (20 μg/ml; Chemicon), recombinant TGFβ (10 ng/ml; R&D Systems), or conditioned medium from cultured macrophages -stimulated or not with fibrin/ogen- in the absence or presence of a TGFβ neutralizing antibody (30 μg/ml; R&D Systems).

Satellite cell-derived primary myoblasts were obtained as described in (Mitchell and Pavlath 2004). Primary myoblast cultures were maintained on collagen-coated dishes in Ham's F10 medium supplemented with 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.001% Fungizone and 5 ng/ml bFGF.

13. PROLIFERATION ASSAY

WT, and uPAR$^{-/-}$ satellite cells were cultured in Ham's F-10 medium containing 20% FBS. 3.5 x 10^4 cells were plated in 12-well plates. After 18 hours in culture, proliferating cells were labeled with BrdU (1.5 μg/ml; Sigma) for 2 hours. BrdU-labeled cells were detected by immunostaining using rat anti-BrdU antibody (Oxford Biotechnology; 1:500) and a specific secondary biotinylated goat anti-rat antibody (Jackson Inmunoresearch; 1:250). Antibody binding was visualized using Vectastain Elite ABC reagent (Vector Laboratories) and DAB. BrdU positive cells were quantified as percentage of the total number of cells analyzed.

14. MIGRATION ASSAY

Macrophage migration was assayed on transwells (3-μm pore size, Beckton Dickinson, Bedford). 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 x 10^4 macrophages/transwell in RPMI-1640 containing 1% FBS were added to the upper chamber of the transwell and the conditioned medium of muscle satellite cells, previously concentrated 5-fold using Centrifugal Filter Device (Millipore, Bedford), was added to the lower chamber. Satellite cell migration was performed on 8-μm pore size transwells. Satellite cells from WT or uPAR$^{-/-}$ mice (5 x 10^4 cells/transwell) in Hams F-10 containing 1% FBS were added to the upper chamber of transwells. Transwells were coated with

matrigel, before addition of the cells. After 8 h incubation at 37ºC, cells on the upper side of the transwell membrane were removed using cotton swabs and cells on the underside were fixed with 70% ethanol and stained with 2% crystal violet. Cells on the underside surface were counted (12 fields per filter). Experiments were performed in triplicate.

15. RNA ISOLATION AND QUANTITATIVE RT-PCR

RNA was analyzed by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells or muscle tissue using Tripure reagent (Roche Diagnostic Corporation). DNase digestion of 10 µg of RNA was performed using 2 U DNase (Turbo DNA-free™, Ambion). Complementary DNA was synthesized from 2 µg of total RNA using the First-Strand cDNA Synthesis kit (Amersham Biosciences). PCRs were performed in a LightCycler® 480 System using a LightCycler® 480 SYBR Green I Master (Roche Diagnostic Corporation). Sequences of specific primers used are shown in Table 3. Thermocycling conditions were as follow: initial step of 10 min at 95ºC, then 45 cycles of 15s denaturation at 94ºC, 10s annealing at 60ºC and 15s extension at 72ºC. Reactions were run in triplicate, and automatically detected threshold cycle (Ct) values were compared between samples. Transcripts of the ribosomal protein L7 gene were used as endogenous normalization control.

Gene	Primer	Sequence
IL-6	Forward Reverse	5'-GAGGATACCACTCCCAACAGACC-3' 5'-AAGTGCATCATCGTTGTTCATACA-3'
TNFα	Forward Reverse	5'-CGCTCTTCTGTCTACTGAACTT-3' 5'-GATGAGAGGGAGGCCATT-3'
IL-1β	Forward Reverse	5'-CCAAAATACCTGTGGCCTTGG-3' 5'-GCTTGTGCTCTGCTTGTGAG-3'
MIP-2	Forward Reverse	5'-AAGTTTGCCTTGACCCTGAA-3' 5'-AGGCACATCAGGTACGATCC-3'
Ym1	Forward Reverse	5'-CCAGCATATGGGCATACCTT-3' 5'-CAGACCTCAGTGGCTCCTTC-3'
Ym2	Forward Reverse	5'-GCTGGACCACCAGGAAAGTA-3' 5'-CACGGCACCTCCTAAATTGT-3'
Arginase I	Forward Reverse	5'-AACACGGCAGTGGCTTTAACC-3' 5'-GGTTTTCATGTGGCGCATTC-3'
TIMP-1	Forward Reverse	5'-TTCCAGTAAGGCCTGTAGC-3' 5'-TTATGACCAGGTCCGAGTT-3'
TGFβ	Forward Reverse	5'-CTCCACCTGCAAGACCAT-3' 5'-CTTAGTTTGGACAGGATCTGG-3'
Collagen I	Forward Reverse	5'-GGTATGCTTGATCTGTATCTGC-3' 5'-AGTCCAGTTCTTCATTGCATT-3'
IL-13	Forward Reverse	5'-ATTGCATGGCCTCTGTAACC-3' 5'-CTTCCTCCTCAACCCTCCTC-3'

L7	Forward Reverse	5'-GAAGCTCATCTATGAGAAGGC-3' 5'-AAGACGAAGGAGCTGCAGAAC-3'

Table 3. Sequence of the murine primers used for qRT-PCR

16. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Macrophages were stimulated with fibrinogen or LPS (positive control). The NF-κB inhibitor BAY11-7085 was added when indicated. Nuclear extracts (NE) were obtained as described in (De Cesare et al. 1995). For electrophoretic mobility shift assays, 10 μg of nuclear extracts were incubated in 50 mM Tris-HCl pH 7.9, 12.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg of poly dI-dC for 10 min at room temperature to titrate out nonspecific binding before the addition of 15,000–20,000 cpm labeled oligonucleotide, and the reaction was further incubated for 20 min at 30°C. When unlabeled competing oligonucleotides were added, nuclear extracts were pre-incubated for 30 min at room temperature, before the addition of the labeled probe. Samples were loaded on a pre-run polyacrylamide gel (29:1 in 0.25x Tris borate-EDTA) and electrophoresed at 200 V. Gels were dried and autoradiographed at -80°C. The sequences of the sense strands of the oligonucleotides used in EMSAs are as follows: NF-κB, 5'-AGTTGAGGGGACTTTCCCAGGC-3' and p53 (as unspecific) 5'-ACACACATGCCTCAGCAAGTCCCAGA-3'.

17. STATISTICAL ANALYSIS

Quantitative data were analyzed by Student's *t*-test, unless otherwise specified. $P<0.05$ was considered statistically significant.

RESULTS

1. uPA DEFICIENCY EXACERBATES MUSCULAR DYSTROPHY IN *MDX* MICE

1.1 uPA ACTIVITY IS INDUCED IN *MDX* MUSCLE DYSTROPHY

It has been previously shown that uPA mediates the recovery of experimentally-injured muscle (Lluis et al. 2001), but its role in *mdx* dystrophy remains unknown. We therefore first analyzed, by zymography, uPA activity in *mdx* muscle extracts before and after onset of muscle degeneration. At 14 days of age (i.e. before disease onset), the activity levels of uPA were undetectable in WT mice and in *mdx* mice (not shown). In contrast, after disease onset (i.e. 30 days of age), the activity levels of uPA were increased in *mdx* muscle but not in WT muscle (Fig. 12). These changes were specific for uPA, as no lytic band corresponding to tPA (at 72 kDa) was detected by zymography (Fig. 12). Thus, uPA activity is specifically increased in *mdx* dystrophic muscle during disease.

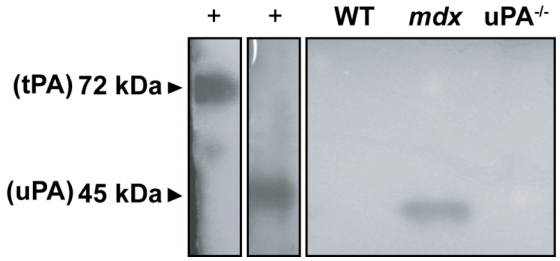

Figure 12. uPA activity in *mdx* dystrophic muscle. Zymographic analysis of WT, *mdx* and uPA$^{-/-}$ muscles. Purified murine uPA (45 kDa) and tPA (72 kDa) were used as a control for activity (+).

1.2 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* and uPA$^{-/-}$*mdx* littermates. Both genotypes were healthy at birth and did not show any signs of muscle injury or differences in muscle size prior to disease onset (Fig. 13a). Beyond 3 to 4 weeks of age, obvious signs of muscle dystrophy were detectable in uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* mice. However, compared to uPA$^{+/+}$*mdx* mice, uPA$^{-/-}$*mdx* mice suffered from a much more severe dystrophinopathy, at least up to 4 months of age, as characterized by more widespread and extensive myofiber degeneration and necrosis (Fig. 13a). Indeed, uPA$^{-/-}$ *mdx* muscles contained larger areas of muscle damage (Fig. 13b). Moreover, the number

of centrally nucleated fibers (CNFs) (indicator of muscle regeneration) was lower in uPA$^{-/-}$*mdx* than in uPA$^{+/+}$*mdx* muscle (Fig. 13c).

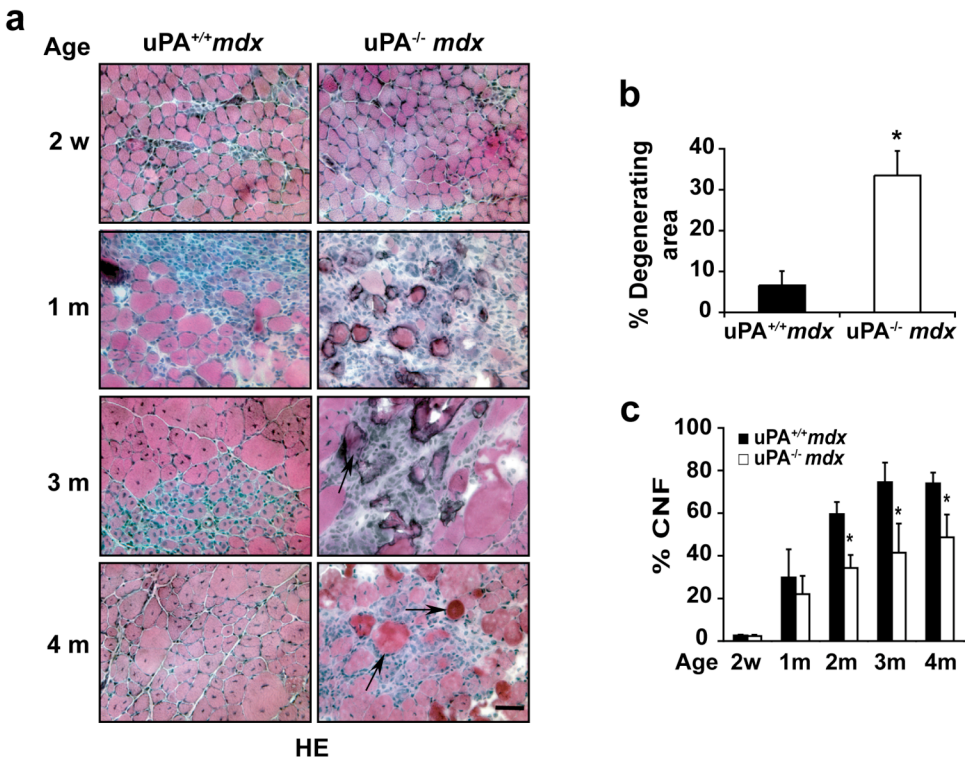

Figure 13. uPA deficiency exacerbates *mdx* muscle degeneration. a. Muscle sections of uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* mice of 2 weeks of age, and of 1, 3 and 4 months of age were stained with HE. Non-phagocytosed necrotic fibers in uPA$^{-/-}$*mdx* muscle sections are indicated with black arrows. Magnification bar: 50 µm. **b.** Percentage of total muscle degenerating area of 1 month-old mice. **c.** Reduced muscle regeneration in uPA$^{-/-}$*mdx* mice. Percentage of CNF at the indicated ages. Data are mean ± SEM; N=6 animals per group; *: $P<0.05$.

To ascertain worsening in the pathology of the whole skeletal musculature, we measured the serum levels of CK, a biomarker of sarcolemmal damage (Bulfield et al. 1984). Consistent with the more severe muscle degeneration, uPA$^{-/-}$*mdx* mice showed ~2-fold higher serum CK levels as compared to uPA$^{+/+}$*mdx* mice at 2.5 months of age (Fig. 14a). To determine the functional status of the diseased muscle, we used grip-strength and treadmill assays (performed by María Martínez de Lagrán and Mara Dierssen at the CRG). Compared to uPA$^{+/+}$*mdx* mice, muscle strength at 2.5 months of age was significantly decreased in uPA$^{-/-}$*mdx* mice in both assays (Fig. 14b, c). Altogether, these findings provide histological, biochemical and functional evidence that uPA deficiency aggravates muscle degeneration and attenuates regeneration in *mdx* muscle.

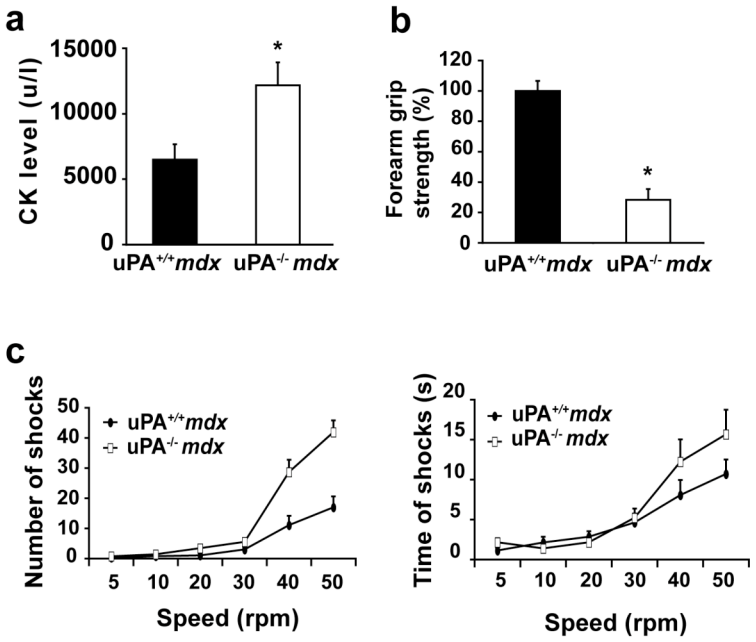

Figure 14. uPA$^{-/-}$*mdx* mice have increased muscle damage and reduced muscle strength. a. Serum CK levels in uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* mice at 2.5 months of age. **b, c.** Comparison of functional muscle strength between uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* mice at 2.5 months of age in grip strength (b) and treadmill (c) assays, as described in Materials and Methods. Data are mean ± SEM*;* N=10 animals per group; *: *P*<0.05.

1.3 BONE MARROW-DERIVED UPA PROMOTES THE INFILTRATION OF INFLAMMATORY CELLS INTO *MDX* DYSTROPHIC MUSCLE

In experimentally-injured muscle, uPA is produced by satellite cells and by inflammatory cells (Lluis et al. 2001). Though T-lymphocytes and neutrophils also infiltrate in dystrophic *mdx* muscles, infiltrated macrophages appear to be the major inflammatory cell type (Engel and Arahata 1986; Pimorady-Esfahani et al. 1997; Spencer et al. 2001; Tidball 2005a). We aimed to analyze the impact of uPA deficiency in the inflammatory response in *mdx* muscular dystrophy. Before disease onset (i.e. at 14 days of age), Mac-1$^+$ macrophages and T-11$^+$ T-lymphocytes were rarely detected in uPA$^{+/+}$*mdx* or uPA$^{-/-}$*mdx* muscles (Fig. 15a). After disease onset (i.e. at 30 days of age), these inflammatory cells had infiltrated the dystrophic muscle of uPA$^{+/+}$*mdx* mice (Fig. 15a). However, compared to uPA$^{+/+}$*mdx* mice, the number of infiltrated Mac-1$^+$ and T-11$^+$ cells in uPA$^{-/-}$*mdx* muscle was reduced up to ~50% (Fig. 15a). Consistent herewith, loss of uPA also reduced the number of infiltrated inflammatory cells in CTX-injured muscle (Fig. 15b). This was not due to a

genotypic difference in the number of circulating leukocytes in the peripheral blood (not shown).

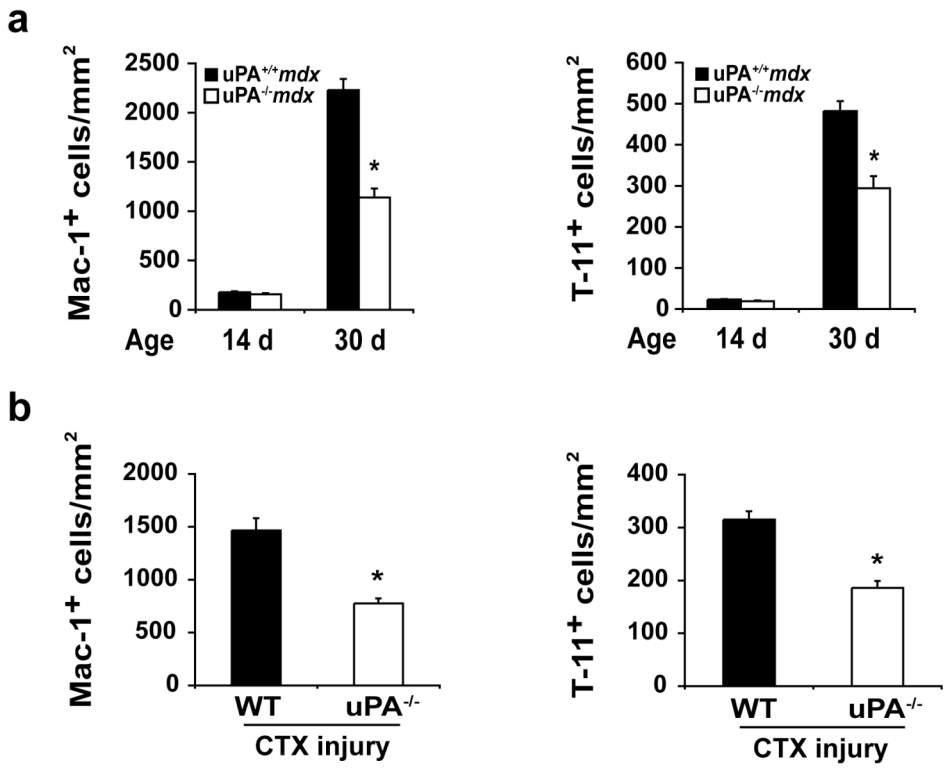

Figure 15. uPA promotes the infiltration of inflammatory cells into *mdx* dystrophic muscle and CTX-injured muscle. a. uPA deficiency reduces the inflammatory response in *mdx* dystrophic muscle. Number of Mac-1 and T-11 positive cells in muscle sections of uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* mice at 14 days of age (i.e. before onset of degeneration) and at 30 days of age (15 days after onset of degeneration). **b.** Number of Mac-1 and T-11-positive cells in muscle sections of WT and uPA$^{-/-}$ mice at 2 days after CTX injury. Data are mean ± SEM*;* N=5 animals per group; *: *P*<0.05.

This reduced infiltration and accumulation of inflammatory cells in uPA$^{-/-}$*mdx* dystrophic muscles was likely attributable to the fact that they lack uPA needed to invade injured tissues. Indeed, when performing *in vitro* migration experiments using Transwells, uPA$^{-/-}$*mdx* and uPA$^{-/-}$ macrophages were found to migrate less towards conditioned medium from satellite cells, compared with control cells (Fig. 16).

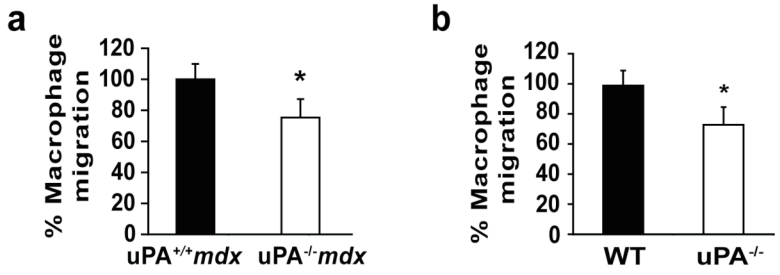

Figure 16. uPA deficiency reduces macrophage migration *in vitro*. a, b. Migration assays were carried out in Transwells. Conditioned medium from *mdx* (a) or WT (b) mouse primary satellite cell cultures was placed in the lower chamber of the Transwell. Macrophages obtained from uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* mice (a) or WT and uPA$^{-/-}$ mice (b) were placed in the upper chambers. The value 100% was arbitrarily given to the number of migrating uPA$^{+/+}$*mdx* (a) or WT (b) macrophages. Data are mean ± SEM; N=3 experiments performed in duplicate; *: P<0.05.

Therefore, we evaluated whether the conditional restoration of uPA expression in the BM of uPA$^{-/-}$*mdx* mice achieved via the transplantation of uPA$^{+/+}$*mdx* BM (termed uPA$^{-/-}$*mdx*$^{(\text{uPA+/+}mdx\text{-BM})}$ mice) could revert the deficient inflammatory response. As a negative control, we transplanted uPA$^{-/-}$*mdx* BM into uPA$^{-/-}$*mdx* mice (termed uPA$^{-/-}$*mdx*$^{(\text{uPA-/-}mdx\text{-BM})}$ mice). We found that the transplantation of uPA-expressing BM increased the infiltration of inflammatory cells into dystrophic muscle. Indeed, compared uPA$^{-/-}$*mdx*$^{(\text{uPA-/-}mdx\text{-BM})}$ mice, muscles in uPA$^{-/-}$*mdx*$^{(\text{uPA+/+}mdx\text{-BM})}$ mice became infiltrated with plenty of (uPA expressing) inflammatory cells (Fig. 17). Together, these data demonstrate that uPA is necessary for inflammatory cells to infiltrate the degenerating myofibers of *mdx* mice.

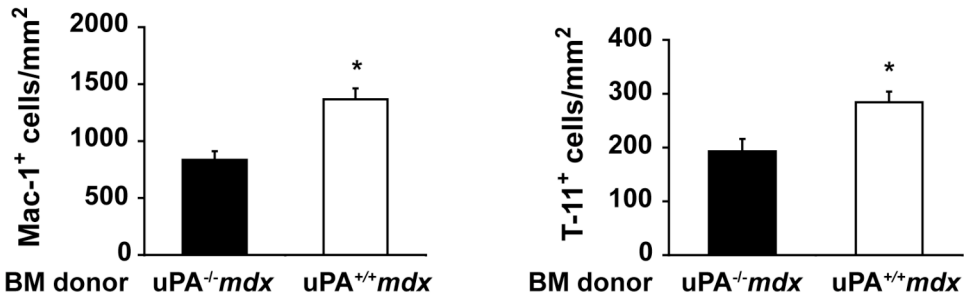

Figure 17. BM-derived uPA promotes the infiltration of inflammatory cells into *mdx* dystrophic muscle. Number of Mac-1 and T-11-positive cells in muscle sections of uPA$^{-/-}$*mdx* mice transplanted with BM from uPA$^{-/-}$*mdx* or uPA$^{+/+}$*mdx* donor mice, respectively. Data are mean ± SEM; N=4 animals per group; *: P<0.05.

1.4 REDUCTION OF FIBRIN/OGEN LEVELS BY BM-DERIVED UPA OR ANCROD TREATMENT REDUCES THE EXACERBATED DEGENERATION OF UPA$^{-/-}$*MDX* MICE

It has been previously shown that the persistent muscle degeneration in uPA$^{-/-}$ mice after injury was mediated, at least in part, by impaired dissolution of intramuscular fibrin or fibrinogen (fibrin/ogen) deposits (Lluis et al. 2001). We therefore analyzed in uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* muscle the extent of fibrin/ogen accumulation before and after disease onset. Prior to disease onset (14 days of age), fibrin/ogen was undetectable by immunostaining or Western blotting in uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* muscles (not shown). However, at the first disease peak (30 days of age), fibrin/ogen deposits were readily detectable in muscles of both genotypes (Fig. 18a). Importantly, however, compared to uPA$^{+/+}$*mdx* muscles, fibrin/ogen deposition was increased in uPA$^{-/-}$*mdx* muscles, up to 2.5-fold (Fig. 18a). Interestingly, prior transplantation of uPA-expressing BM cells attenuated this increased deposition of fibrin/ogen in uPA$^{-/-}$*mdx* mice (Fig. 18b).

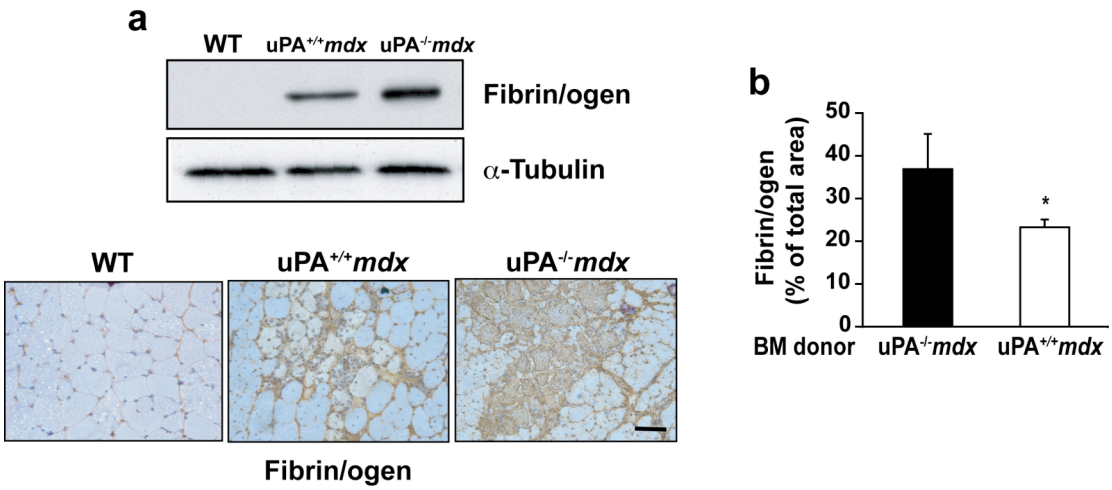

Figure 18. Fibrin/ogen deposition is increased in the muscle of uPA$^{-/-}$*mdx* mice. a. *Top*: Western blotting analysis of extracts of WT, uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* muscles at 30 days of age, using an anti-fibrin/ogen antibody. Alpha-tubulin was analyzed as a loading control. *Bottom*: Fibrin/ogen deposition in WT, uPA$^{+/+}$*mdx* and uPA$^{-/-}$*mdx* muscles at 30 days of age was analyzed by immunohistochemistry. Magnification bar: 50 µm. **b.** Fibrin/ogen levels were quantified in muscle sections of uPA$^{-/-}$*mdx* mice transplanted with BM from uPA$^{-/-}$*mdx* or uPA$^{+/+}$*mdx* donor mice. Fibrin/ogen was detected by immunohistochemistry, quantified and represented as the percentage of total muscle area. Data are mean ± SEM; N=4 animals per group; *: $P<0.05$.

To directly prove that the increased accumulation of fibrin/ogen mediated the exacerbated dystrophic disease in uPA$^{-/-}$*mdx* mice, we treated uPA$^{-/-}$*mdx* mice with ancrod, an established defibrinogenating agent known to drastically reduce plasma fibrinogen levels and fibrin/ogen deposition (Bell et al. 1978; Lluis et al. 2001; Akassoglou

et al. 2002). Daily delivery of ancrod (1 U per day), starting at 12 days after birth and continuing for 18 days thereafter, effectively reduced the accumulation of fibrin/ogen in uPA$^{-/-}$*mdx* muscles (Fig. 19a). Importantly, compared to saline, the area of degenerated muscle in uPA$^{-/-}$*mdx* mice was significantly reduced by ancrod treatment, indicating that the increased deposition of fibrin/ogen mediated the severe muscle degeneration in uPA$^{-/-}$*mdx* mice (Fig. 19b, c). Of note, muscles in uPA$^{-/-}$*mdx*$^{(uPA+/+mdx\text{-}BM)}$ mice, exhibited less severe signs of degeneration 2 months after transplantation, in comparison with uPA$^{-/-}$*mdx*$^{(uPA\text{-}/\text{-}mdx\text{-}BM)}$ mice (Fig. 20a). Consistent with this, serum CK levels were lower in uPA$^{-/-}$*mdx*$^{(uPA+/+mdx\text{-}BM)}$ than in uPA$^{-/-}$*mdx*$^{(uPA\text{-}/\text{-}mdx\text{-}BM)}$ mice (Fig. 20b). Thus, uPA-expressing BM-derived cells attenuate muscle degeneration in uPA$^{-/-}$*mdx* mice and are required for dissolving fibrin/ogen deposits in dystrophic *mdx* muscle.

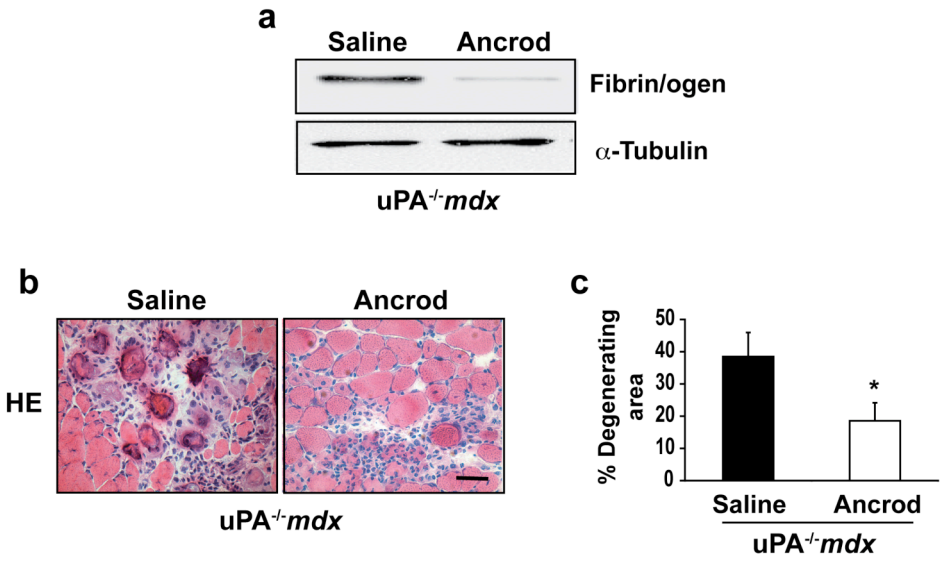

Figure 19. Reduction of fibrin/ogen levels by ancrod treatment reduces the exacerbated degeneration of uPA$^{-/-}$*mdx* mice. a. 12 day-old uPA$^{-/-}$*mdx* mice were daily injected intraperitoneally with ancrod or with saline solution for 18 days, up to 30 days of age. Comparison of fibrin/ogen levels in muscle of uPA$^{-/-}$*mdx* mice (at 30 days of age) after ancrod or saline treatment. **b.** HE staining of muscle sections. Magnification bar: 50 µm. **c.** Percentage of total degenerating muscle area of saline- or ancrod-treated uPA$^{-/-}$*mdx* mice. Data are mean ± SEM; N=5 animals per group; *: $P<0.05$.

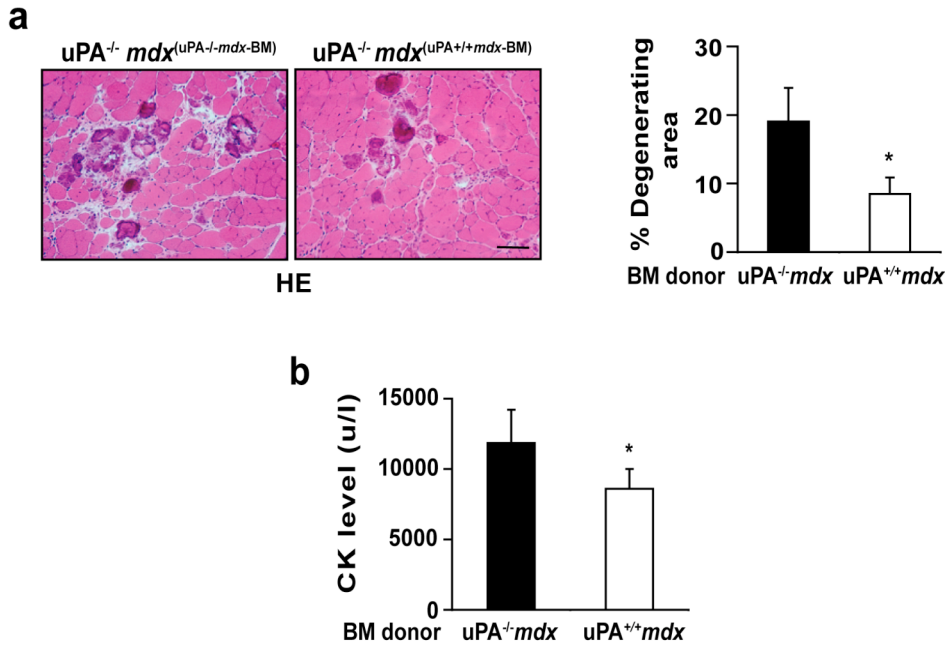

Figure 20. Transplantation of uPA-expressing BM ameliorates the severe uPA$^{-/-}$*mdx* muscular dystrophy. BM from uPA$^{+/+}$*mdx* or uPA$^{-/-}$*mdx* donor mice was transplanted into uPA$^{-/-}$*mdx* mice, and different muscle parameters were analyzed 2 months after transplantation. **a.** Left: HE staining of muscle sections. Magnification bar: 50 μm. Right: Percentage of total muscle degenerating area in uPA$^{-/-}$*mdx* mice transplanted with BM from uPA$^{-/-}$*mdx* or uPA$^{+/+}$*mdx* donor mice. **b.** Reduced muscle damage in uPA$^{-/-}$*mdx* mice transplanted with BM from uPA$^{+/+}$*mdx* donor mice as reflected by decreased serum CK levels. Data are mean ± SEM; N=4 animals per group; *: *P*<0.05.

1.5 DISPENSABILITY OF UPAR FOR MUSCLE REGENERATION AFTER INJURY AND IN *MDX* DYSTROPHINOPATHY

By binding to its receptor uPAR, uPA is capable of exerting its proteolytic effects at the pericellular level, but it also enables uPA to promote cell proliferation and migration via non-proteolytic 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. 21).

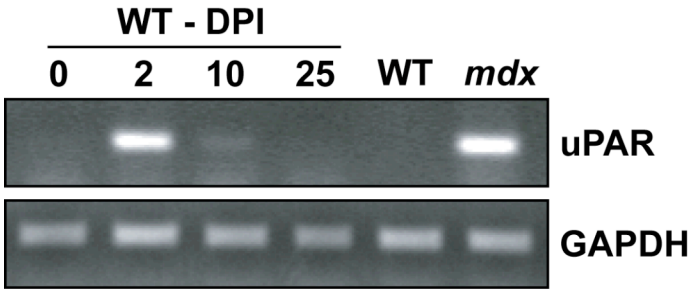

Figure 21. uPAR is expressed in CTX-injured WT muscle and *mdx* muscle. Induction of uPAR expression in CTX-injured WT muscles and *mdx* muscle as shown by RT-PCR.

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 days post-injury (Fig. 22a). Consistent herewith, the infiltration of inflammatory cells in injured muscle was not affected in the absence of uPAR (Fig. 22b).

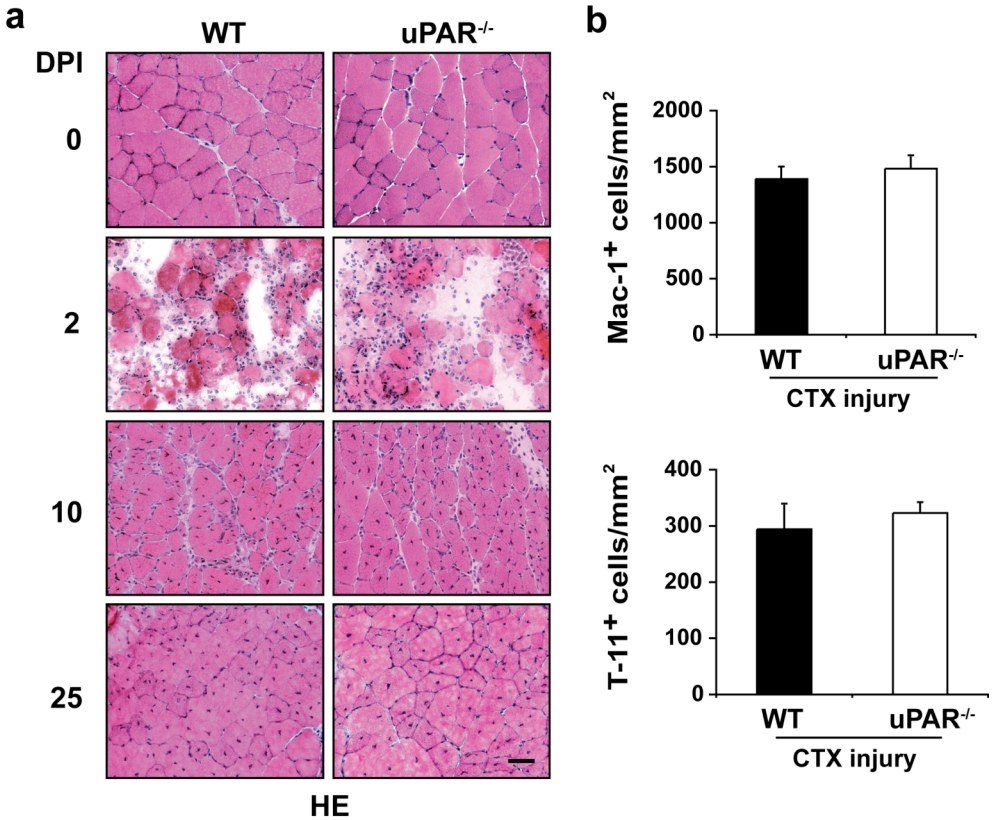

Figure 22. uPAR is dispensable for muscle regeneration after CTX injury. a. Muscle sections of control (non-injured) and CTX-injured WT and uPAR$^{-/-}$ mice stained with HE. Magnification bar: 50 μm. **b.** Number of Mac-1 and T-11 positive cells in muscle sections of WT and uPAR$^{-/-}$ mice at 2 days after CTX injury. Data are mean ± SEM; N=4 animals per group.

Most importantly, muscle CSA and the extent of muscular dystrophy was also similar in *mdx* and uPAR$^{-/-}$*mdx* mice (Fig. 23). Indeed, the percentage of muscle degeneration was not different between *mdx* and uPAR$^{-/-}$*mdx* mice (Fig. 23b). In addition, the number of infiltrated macrophages and T-cells did not differ between *mdx* and uPAR$^{-/-}$*mdx* mice (Fig. 23a). Interestingly, the percentage of CNF was slightly increased in uPAR$^{-/-}$*mdx* mice (Fig. 23b); however, satellite cell-derived primary myoblasts from uPAR$^{-/-}$ mice presented normal proliferation and migration rates *in vitro* (Fig. 24). 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.

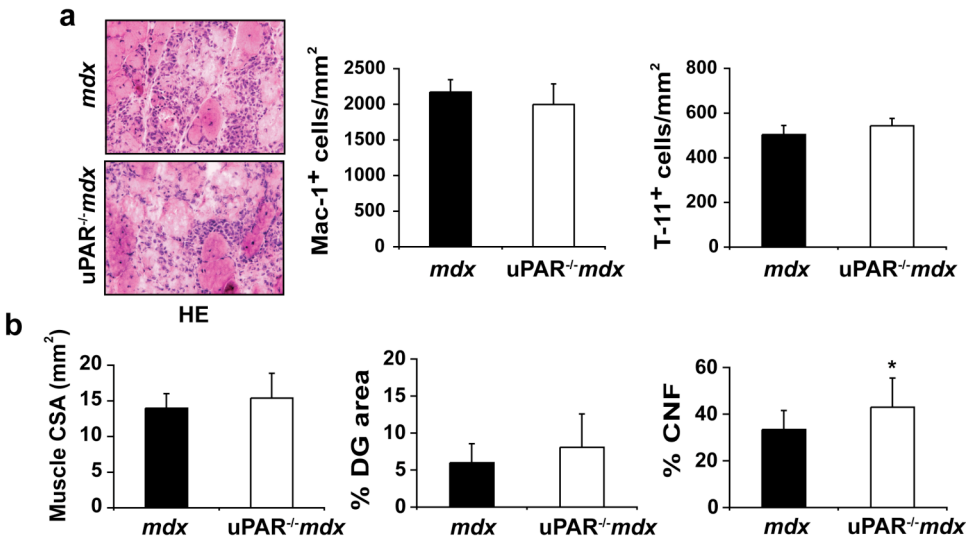

Figure 23. uPAR deficiency does not affect *mdx* muscle dystrophy progression. a. *Left*: HE staining of 1 month-old *mdx* and uPAR$^{-/-}$*mdx* mice muscle sections. Magnification bar: 50 μm. *Right*: Number of Mac-1 and T-11 positive cells in muscle sections of *mdx* and uPAR$^{-/-}$*mdx* mice at 30 days of age. **b.** *Left*: Muscle CSA of 1 month-old *mdx* and uPAR$^{-/-}$*mdx* mice. *Middle*: Percentage of total muscle degenerating area of 1 month-old *mdx* and uPAR$^{-/-}$*mdx* mice. *Right*: Percentage of CNFs in 1 month-old *mdx* and uPAR$^{-/-}$*mdx* mice. Data are mean ± SEM; N=6 animals per group; *: P<0.05.

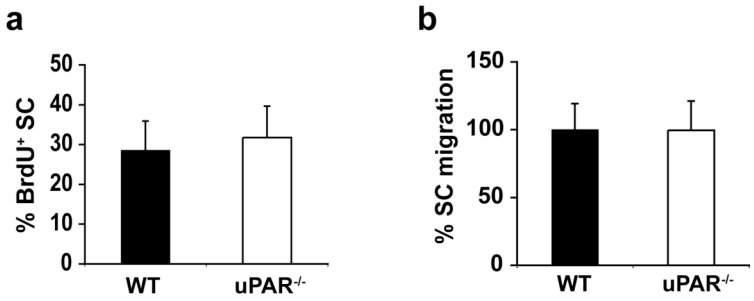

Figure 24. uPAR$^{-/-}$ SCs have normal proliferation and migration rates. a. SCs were cultured in growth medium in 12-well plates. Proliferation rates were expressed as percentages of BrdU-positive cells. **b.** Cell migration assay on Transwells. The value 100% was arbitrarily given to the number of WT SCs that had migrated. Data are mean ± SEM; N=3 experiments performed in triplicate.

2. FIBRINOGEN DEPOSITION DRIVES INFLAMMATION-MEDIATED FIBROSIS IN MUSCULAR DYSTROPHY IN *MDX* MICE

2.1 FIBRIN/OGEN ACCUMULATES IN DYSTROPHIC MUSCLE OF DMD PATIENTS AND *MDX* MICE

We have shown above in Section 1 that the excessive fibrin/ogen accumulation in uPA$^{-/-}$*mdx* mice exacerbates *mdx* muscle dystrophy. Thus, our next goal was to study in detail the involvement of fibrin/ogen deposition in muscle dystrophy progression and gain insights on its mechanism(s) of action.

We first analyzed fibrin/ogen deposition in muscles of DMD patients. Compared to muscles of healthy individuals or of fibromyalgia patients, DMD muscles showed significant fibrin/ogen accumulation (Fig. 25a). Similarly, in *mdx* mice muscles, fibrin/ogen deposits were readily detectable after disease onset, while absent before disease onset (Fig. 25b, c). Thus, fibrin/ogen deposition is associated with muscle dystrophinopathy.

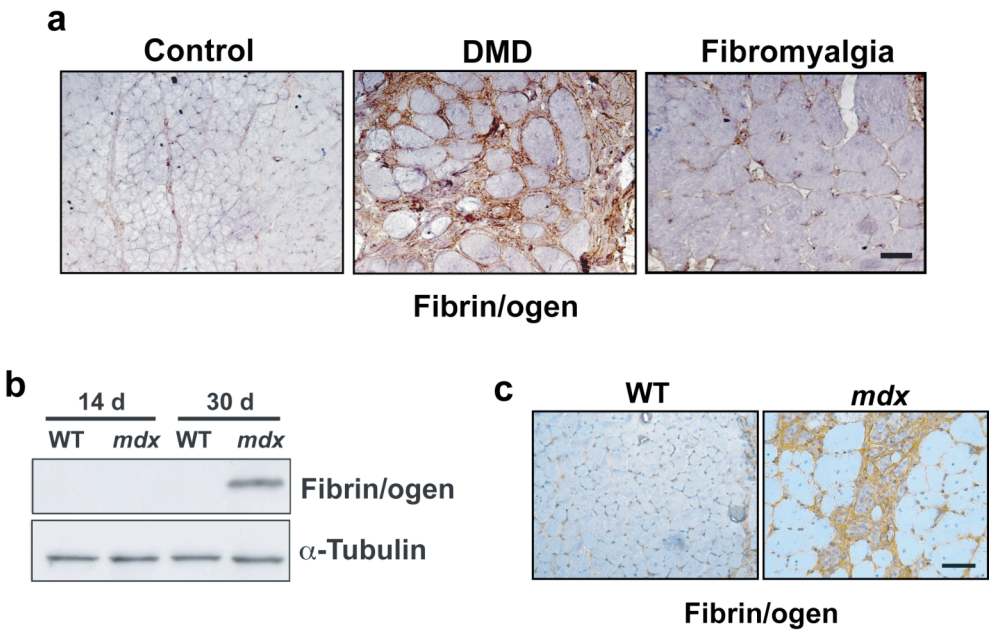

Figure 25. Fibrin/ogen accumulates in muscles of DMD patients and *mdx* mice. a. Compared to the strong staining for fibrin/ogen (brown) in a muscle biopsy of a DMD patient (*middle panel*), minimal or no fibrin/ogen accumulation was observed in muscle biopsies from healthy controls (*left panel*), or patients with fibromyalgia (*right panel*). **b.** Western blotting analysis of extracts of WT and *mdx* muscles before and after disease onset (14 and 30 days of age, respectively), using an anti-fibrin/ogen antibody. **c.** Fibrin/ogen immunostaining in muscles of WT and *mdx* mice of 30 days of age. Magnification bars in a and c: 50 μm.

2.2 GENETIC DEFICIENCY OF FIBRINOGEN ATTENUATES *MDX* MUSCLE DYSTROPHY

To further analyze the importance of fibrin/ogen deposition in *mdx* muscle, we bred the *mdx* mice into the fibrinogen-deficient background (*Fib*$^{-/-}$), and analyzed the consequences of fibrin/ogen deficiency on dystrophic disease in gastrocnemius muscle. No phenotypic differences between *Fib*$^{-/-}$*mdx* and *Fib*$^{+/+}$*mdx* mice were noted at 14 days of age (data not shown). Consistent with the absence of fibrin/ogen deposits in *mdx* muscle before disease onset, no signs of dystrophy were found in either genotype at this age (data not shown). As expected, at one month of age, *Fib*$^{+/+}$*mdx* but not *Fib*$^{-/-}$*mdx* mice exhibited fibrin/ogen deposition in damaged muscle areas (Fig. 26).

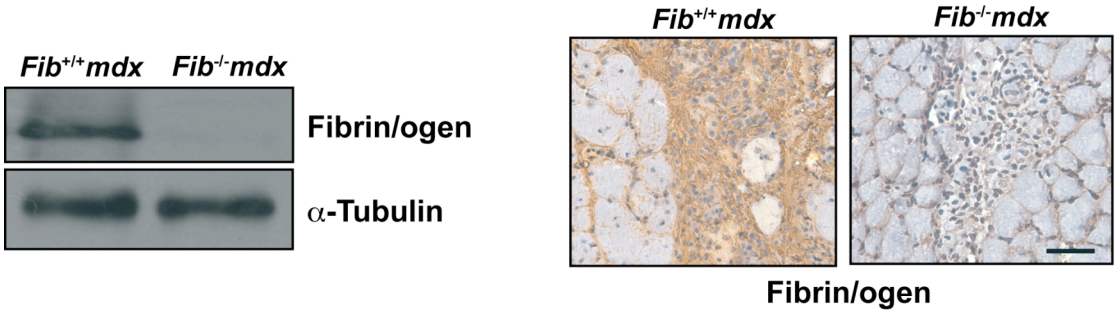

Figure 26. *Fib*$^{-/-}$*mdx* mice do not accumulate fibrin/ogen in their muscles. *Left*: Western blotting analysis of *Fib*$^{+/+}$*mdx* and *Fib*$^{-/-}$*mdx* muscle extracts of 1-month-old mice, using an anti-fibrin/ogen antibody. Alpha-tubulin was analyzed as a loading control. *Right*: Immunostaining of muscles of 1-month-old mice showing absence of fibrin/ogen deposition in *Fib*$^{-/-}$*mdx* mice as compared to *Fib*$^{+/+}$*mdx* mice. Magnification bar: 50 µm.

Compared to *Fib*$^{+/+}$*mdx* mice, we found less degeneration and larger centrally-nucleated regenerating myofibers in *Fib*$^{-/-}$*mdx* mice muscles (Fig. 27a, b). Inflammation was also attenuated in *Fib*$^{-/-}$*mdx* muscle, as evidenced by the reduced number of infiltrated macrophages (Fig. 27c). Notably, a similar attenuation of inflammation and larger size of regenerating fibers was observed in muscles of *Fib*$^{-/-}$ mice with respect to *Fib*$^{+/+}$ mice in a time-controlled regeneration model following CTX-induced muscle injury (Fig. 28). Thus, in the absence of fibrin/ogen, muscle inflammation and degeneration are reduced in *mdx* mice, while regeneration is enhanced.

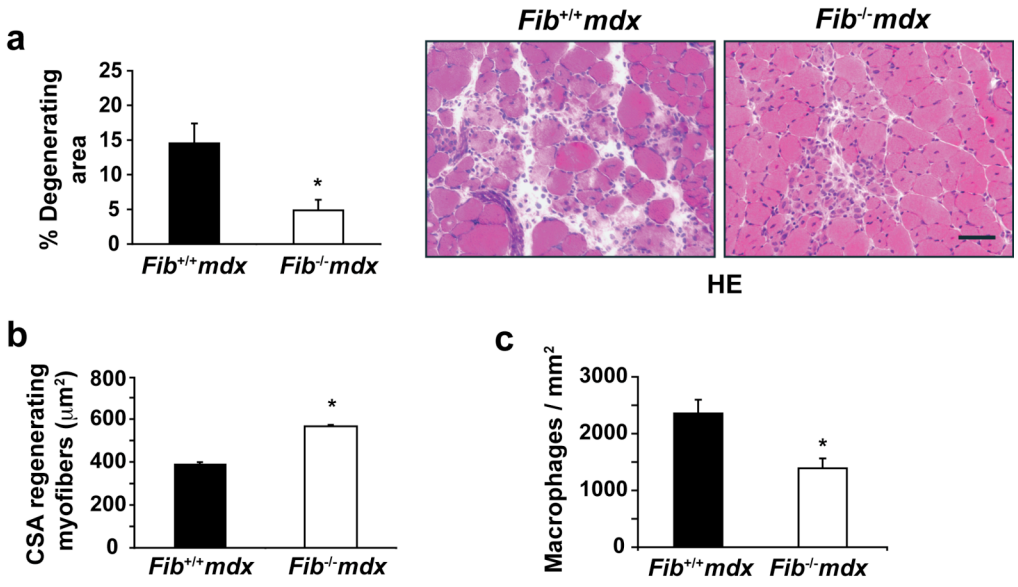

Figure 27. Fibrinogen deficiency attenuates *mdx* muscle dystrophy. a. Reduced percentage of total muscle degeneration area in *Fib*$^{-/-}$*mdx* versus *Fib*$^{+/+}$*mdx* mice at 1 month of age (*left*), as determined by morphometric analysis on HE stained muscle sections (representative sections are shown on the *right*). Magnification bar: 50 µm. **b.** Increased CSA of CNFs in gastrocnemius muscle of 1-month-old mice in *Fib*$^{-/-}$*mdx* mice as compared to *Fib*$^{+/+}$*mdx* mice. **c.** Reduced inflammatory infiltration in *Fib*$^{-/-}$*mdx* muscle. Macrophages (F4/80 immunostaining) were quantified in damaged areas of gastrocnemius muscle. Data are mean ± SEM; N=5 animals per group; *: $P<0.05$.

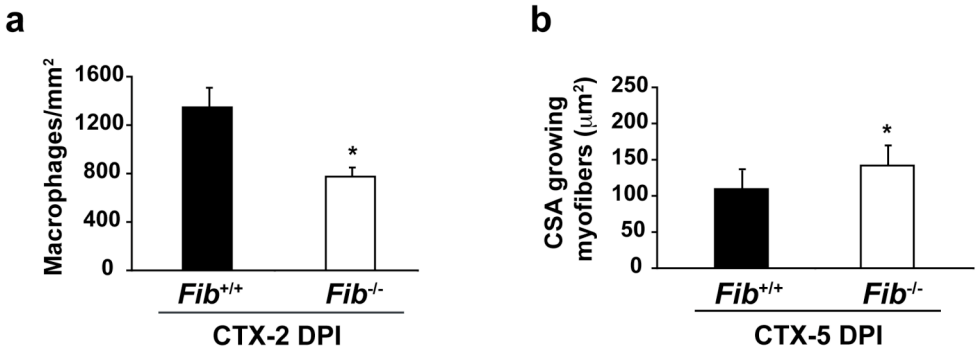

Figure 28. Reduced inflammation and enhanced growth of regenerating myofibers in fibrinogen-deficient mice after CTX injury. Muscle injury was performed by CTX injection in gastrocnemius muscles of *Fib*$^{+/+}$ and *Fib*$^{-/-}$ mice. At 2 DPI, quantification of the number of macrophages per mm^2 of degenerating area showed reduced infiltration in *Fib*$^{-/-}$ mice (**a**). At 5 DPI, the CSA of regenerating central-nucleated myofibers was higher in *Fib*$^{-/-}$ mice as compared to *Fib*$^{+/+}$ controls (**b**). Data are mean ± SEM; N=3 animals per group; *: $P<0.05$.

2.3 PHARMACOLOGICAL DEPLETION OF FIBRIN/OGEN REDUCES DEGENERATION AND ENHANCES REGENERATION IN *MDX* DYSTROPHIC MUSCLE

To confirm our genetic experiments and, more importantly, to evaluate whether compounds targeting fibrin/ogen might elicit beneficial effects in *mdx* mice, we examined the extent of muscle dystrophy after pharmacological depletion of fibrin/ogen. We therefore treated 12-day-old *mdx* mice with the defibrinogenating agent ancrod (Bell et al. 1978), or saline as a control, until the age of 2.5 months, and analyzed the severity of dystrophic disease in gastrocnemius muscle samples. As shown in Figure 29a, the administration of ancrod resulted in a significant reduction of fibrin/ogen accumulation in *mdx* muscles, as expected. Compared to saline, *mdx* mice were clearly protected against severe muscular dystrophy after ancrod treatment, based on quantitative morphological analyses of muscle sections (Fig. 29b, c). Ancrod treatment reduced the area of muscle degeneration by approximately 40% (Fig. 29c). In addition, ancrod-treated *mdx* mice -as *Fib*$^{-/-}$*mdx* mice - contained larger CNFs compared to saline-treated controls (Fig. 29d), suggesting that fibrin/ogen loss by genetic or pharmacological approaches also promoted myofiber regeneration.

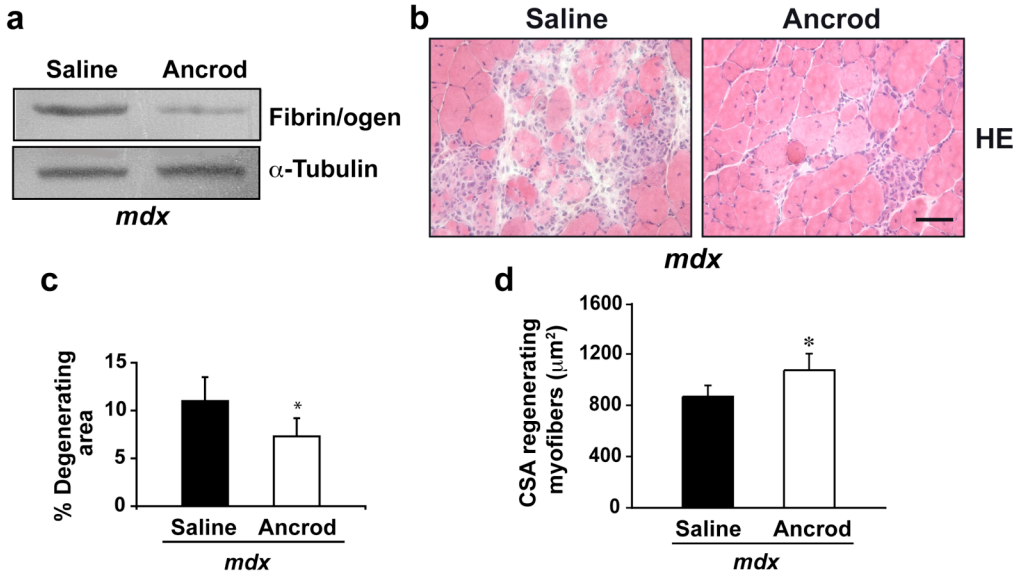

Figure 29. Fibrin/ogen depletion reduces degeneration and enhances regeneration in *mdx* muscle. 12 day-old *mdx* mice were daily injected intraperitoneally with ancrod or with saline solution, up to 2.5 months of age. **a.** Reduction of fibrin/ogen levels in *mdx* muscle by ancrod treatment, as shown by Western blotting with an anti-fibrin/ogen antibody. Alpha-tubulin was analyzed as a loading control. **b.** HE staining of muscle sections of saline- and ancrod-treated *mdx* mice. Magnification bar: 50 μm. **c.** Percentage of total muscle degeneration area in saline- versus ancrod- treated *mdx* mice. **d.** Increased CSA of CNFs in gastrocnemius muscle of 2.5-month-old mice in ancrod- as compared to saline-treated *mdx* mice. Data are mean ± SEM; c, d: N=6 animals per group. *: $P<0.05$.

2.4 FIBRIN/OGEN DEPLETION PRESERVES MUSCLE FUNCTION

To ascertain functional improvement by fibrin/ogen depletion, we analyzed the serum levels of CK and muscle strength. After 2 months of treatment *in vivo*, there was a marked decline in serum CK levels in ancrod-treated mice as compared to saline-treated controls (Fig. 30a). Consistent with this result, fibrin/ogen depletion improved the physical performance of *mdx* mice, as shown by preserved skeletal muscle strength and preserved capacity of *mdx* mice to run at high speed (forearm grip strength and treadmill assays; performed by María Martínez de Lagrán and Mara Dierssen at the CRG) (Fig. 30b, c). Thus, reduction of fibrin/ogen levels protects *mdx* muscle from functional deterioration.

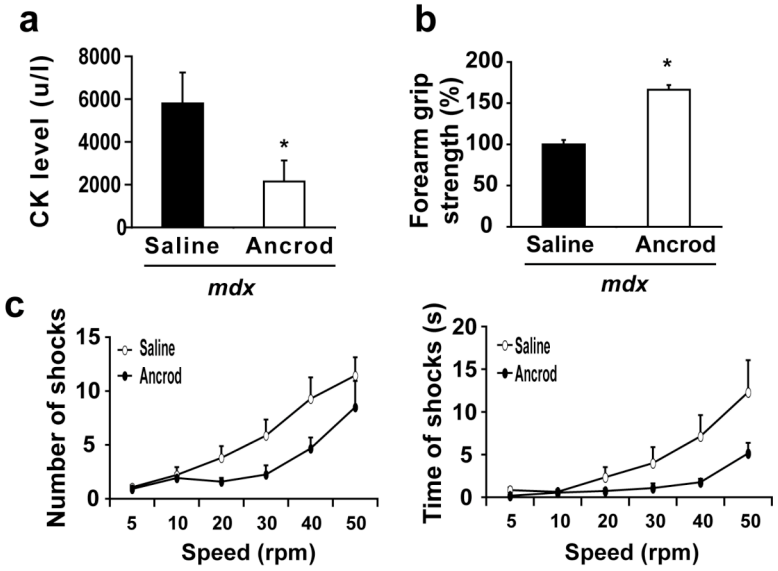

Figure 30. Ancrod treatment reduces muscle damage and increases muscle strength in *mdx* mice. a. Reduced muscle membrane damage in ancrod-treated *mdx* mice. Serum CK concentration was measured in *mdx* mice of 2.5 months of age treated with ancrod or saline for 2 months. **b, c.** Increased muscle strength in ancrod versus saline-treated *mdx* mice. Comparison of functional strength of 2.5 month-old *mdx* mice, treated with ancrod or saline for 2 months, was assessed by the performance in grip strength (b) and treadmill (c) assays, as described in Materials and Methods. Data are mean ± SEM; N=10 animals per group. *: *P*<0.05.

2.5 FIBRIN/OGEN DEPLETION REDUCES INFLAMMATION IN *MDX* MUSCLE

Damage in *mdx* dystrophic muscle is tightly associated with persistent inflammation (Tidball 1995; Pizza et al. 2005; Hodgetts et al. 2006). Classically-activated, pro-inflammatory macrophages are the major inflammatory cell type in degenerating muscle and are critically involved in promoting *mdx* muscle damage (Wehling et al. 2001; Tidball 2005a). In agreement with the reduced numbers of infiltrated macrophages in *Fib*$^{-/-}$*mdx* mice (see above), we found that the number of inflammatory cells (neutrophils, macrophages and T-cells) was also reduced in *mdx* muscles following ancrod treatment (Fig. 31).

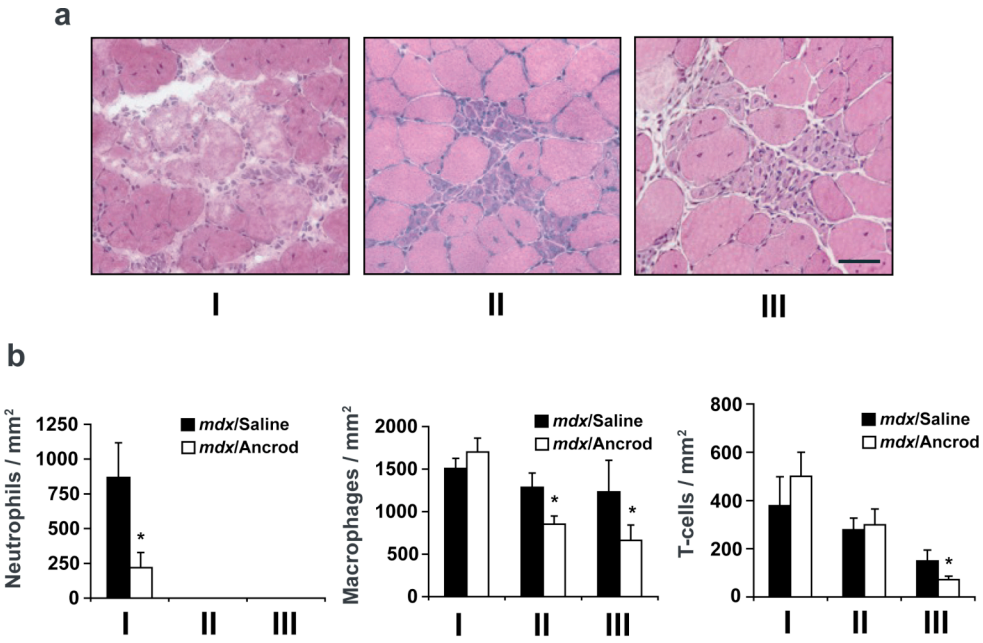

Figure 31. Fibrin/ogen promotes inflammation in *mdx* muscle. a. Representative pictures of the different areas analyzed in gastrocnemius muscles of saline- or ancrod- treated *mdx* mice of 2.5 months of age. Three distinct consecutive phases were analyzed: I. Areas with extensive degeneration, persistence of necrotic fibers and absence of regenerating myofibers. II. Areas of very small nascent myofibers and absence of necrotic fibers. III. Areas of early myofiber regeneration containing well-defined, small regenerating myofibers and bigger ones than in stage II. Magnification bar: 50 µm. **b.** Cell counts for neutrophils, macrophages and T-cells, detected by immunohistochemistry using anti-Ly-6G, anti-F4/80 and anti-CD2 antibodies, respectively, and quantified at different stages of the degeneration/regeneration cycle as detailed above. Data are mean ± SEM; N=5 animals per group; *: $P<0.05$ vs. *mdx*/saline.

Compared to WT controls, the levels of pro-inflammatory cytokines such as TNFα, IL-6, MIP-2 (the functional mouse homolog of human IL-8) and IL-1β were greatly increased in muscle extracts of *mdx* mice, and significantly reduced when *mdx* mice were treated with

ancrod (Fig. 32).

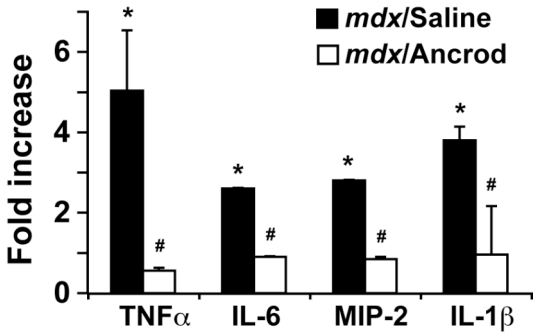

Figure 32. Ancrod-treated *mdx* muscles have reduced levels of pro-inflammatory cytokines. Gene expression analysis by qRT-PCR in muscle extracts of WT and saline- or ancrod-treated *mdx* mice of 2.5 months of age. Results are expressed as fold induction over expression level in WT muscle extract. Data are mean ± SEM; N=3 animals per group. *: $P<0.05$ vs WT mice; #: $p<0.05$ vs *mdx*/saline.

We next investigated whether fibrin/ogen had a direct effect on the cytokine production capacity of primary macrophages derived from *mdx* mice. Fibrinogen induced the rapid expression of TNFα, IL-6, MIP-2 and IL-1β in these cells, as in a classical pro-inflammatory macrophage response (Fig. 33b). In agreement with the capacity of fibrinogen to activate the NF-κB pathway via engaging its receptor Mac-1 ((Sitrin et al. 1998; Rubel et al. 2003) and Fig. 33a), treatment of primary macrophages with the NF-κB inhibitor BAY11-7085 abrogated fibrinogen-induced pro-inflammatory cytokine production (Fig. 33b). This induction was also impaired in the presence of a Mac-1 blocking antibody (Fig. 33b). These results indicate that, by engaging Mac-1 and activating NF-κB, fibrin/ogen upregulates the expression of pro-inflammatory cytokines including IL-1β .

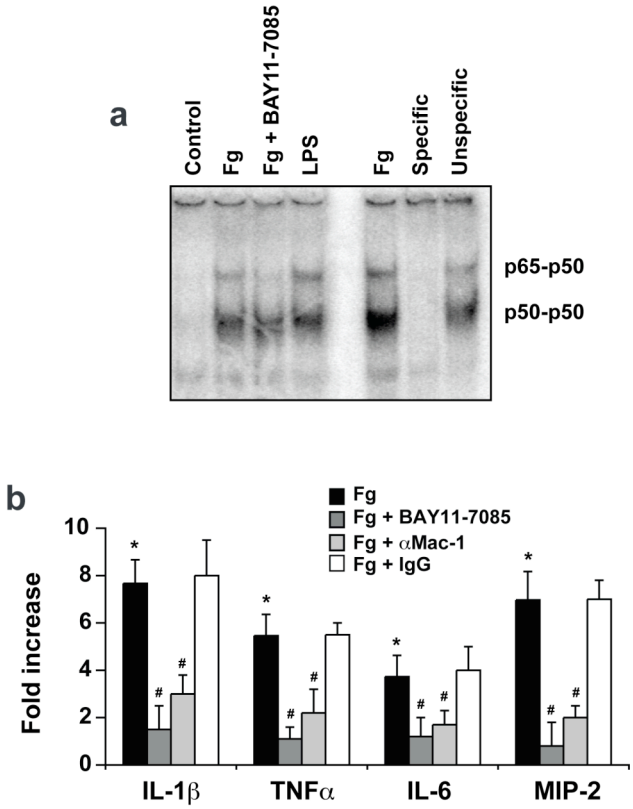

Figure 33. Fibrinogen induces pro-inflammatory cytokine expression in macrophages. a. Macrophages were stimulated with fibrinogen or LPS (positive control) for 1 h. The NF-κB inhibitor BAY11-7085 was added when indicated. Nuclear extracts were incubated with a radiolabeled double-stranded oligonucleotide containing the NF-κB binding site, and DNA-protein binding activity was analyzed by EMSA. When indicated, nuclear extracts were incubated with the labeled NF-κB oligonucleotide in the absence or presence of a 150-fold molar excess of unlabeled competitors: NF-κB (specific competitor) and p53 (unspecific competitor). **b.** Macrophages from *mdx* mice were treated with fibrinogen (6 h) ± BAY11-7085, a Mac-1 blocking antibody, or IgG control antibody as indicated. When indicated, gene expression was analyzed by qRT-PCR. Results are fold-induction values with respect to control conditions. N=3 experiments; *: *P*<0.05 vs control conditions; #: *P*<0.05 vs Fg alone.

2.6 FIBRIN/OGEN DEPLETION REDUCES FIBROSIS IN *MDX* DIAPHRAGM

Fibrosis is a hallmark of DMD, characterized by intramuscular deposition of fibrillar collagens, which increases with age, enhances disease severity and reduces life expectancy. In *mdx* mice, while degeneration is prominent in limb muscles at early disease stages, fibrosis is a characteristic feature of the diaphragm muscle, increasing with age, and thus mimicking DMD progression in humans (Stedman et al. 1991). Since fibrin/ogen deposition has been associated with a number of fibrotic conditions, particularly lung fibrosis (Olman et al. 1995; Swaisgood et al. 2000; Idell 2003), we studied

whether fibrin/ogen depletion might affect collagen accumulation in the diaphragm of *mdx* mice.

We therefore first analyzed collagen deposition in *mdx* diaphragm at different ages by Sirius red staining. The Sirius red positive area was consistently increased in 2.5, 4, 6 and 12 month-old *mdx* mice compared to *mdx* mice prior to disease onset (2 weeks of age) (Fig. 34a), indicating progressive deposition of collagen in *mdx* mice with age. Similar data were obtained with trichrome staining as well as with immunostainings for collagen I and VI (not shown).

We next studied whether the increased fibrosis in *mdx* diaphragm was associated with deposition of fibrin/ogen. Quantification of the area of positive fibrin/ogen staining at different ages revealed an increase of fibrin/ogen deposition with age, which started early after disease onset (Fig. 34a). However, the area of fibrin/ogen staining was already considerably increased at month 1 of age, while collagen deposition showed a clear increase only from 2.5 months onwards, suggesting that deposition of fibrin/ogen precedes fibrosis in *mdx* muscle (Fig. 34a). Importantly, pharmacological reduction of fibrin/ogen levels decreased fibrosis in *mdx* diaphragms of 2.5- and 6.5-month-old mice (Fig. 34c). Consistent herewith, less collagen was deposited in the diaphragm of *Fib*$^{-/-}$*mdx* mice as compared to *Fib*$^{+/+}$*mdx* mice (Fig. 34b). Thus, these data strongly suggest that fibrin/ogen precedes and promotes fibrosis in *mdx* muscle.

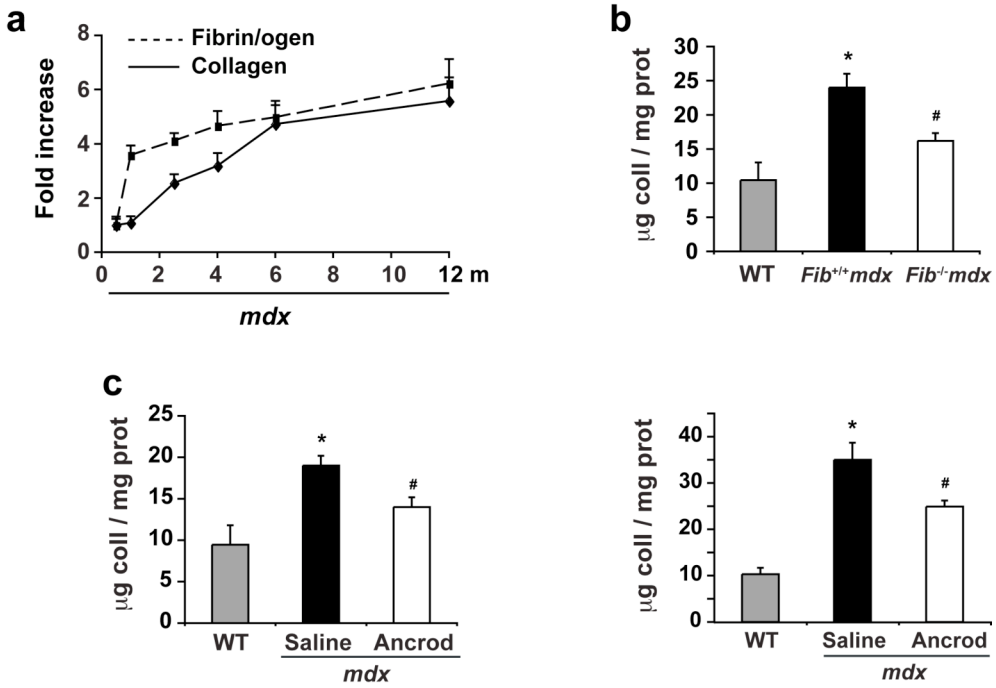

Figure 34. Fibrin/ogen promotes fibrosis in *mdx* diaphragm. a. Fibrin/ogen levels detected by immunostaining (*dashed line*) and fibrotic tissue identified by Sirius Red staining (*continuous line*) were analyzed in diaphragm sections of 0.5, 1, 2.5, 4, 6 and 12 month-old *mdx* mice and WT mice, and quantified as percentage of stained area. *Mdx* mice values were normalized with respect to aged-matched WT mice values. Data are presented as fold increase with respect to 0.5 month-old *mdx* mice. **b.** Fibrosis extent was quantified in diaphragm sections of $Fib^{+/+}mdx$ and $Fib^{-/-}mdx$ mice (3.5 months of age) using the micromethod described by (Lopez-De Leon and Rojkind 1985). Age-matched WT mice were included as a control. **c.** Fibrosis was quantified as in b in diaphragms of saline- or ancrod-treated mice of 2.5 (*left panel*) and 6.5 months of age (*right panel*). Age-matched WT mice were included as a control. Data are mean ± SEM; N=5 animals per group. *: $P<0.05$ vs WT mice; #: $P<0.05$ vs $Fib^{+/+}mdx$ or *mdx*/saline.

2.7 FIBRIN/OGEN DEPLETION REDUCES TGFβ EXPRESSION IN *MDX* DIAPHRAGM

Recent work showed that the pro-fibrotic cytokine TGFβ promotes fibrosis in *mdx* diaphragm (Hartel et al. 2001; Gosselin et al. 2004; Andreetta et al. 2006). We therefore analyzed the expression of TGFβ in *mdx* diaphragm and found that it progressively increased with age (up to 12 months), both at the mRNA (Table 4) and protein levels (mean±SD: 110±39, 210±50 and 745±183 pg/mg of protein at 2.5, 6 and 12 months, respectively). Notably, the increased expression of TGFβ correlated with the age-induced fibrosis in *mdx* diaphragm. Consistent herewith, also the expression of the TGFβ target gene products collagen I and tissue inhibitor of metalloproteinases-1 (TIMP-1) (Chen et al. 1999; Hall et al. 2003; Akool el et al. 2005) – known to be pro-fibrotic (McCrudden and Iredale 2000) – was upregulated in *mdx* diaphragm with aging (Table 4). Importantly, consistent with the observed attenuated fibrosis, fibrin/ogen depletion for 6 months in *mdx* mice reduced the expression of TGFβ and of the TGFβ-regulated genes collagen I and TIMP-1 (Table 5). Moreover, phospho-Smad2 staining was drastically reduced in diaphragms of ancrod-treated (Fig. 35) and fibrinogen-deficient *mdx* mice (not shown), indicating that interfering with fibrinogen deposition impaired functional TGFβ signaling. These results thus suggest that fibrin/ogen promotes fibrosis by increasing TGFβ expression and signaling in *mdx* diaphragm.

Genes	2,5 m	6 m	12 m
TGFβ	1.2±0.5	3.1±0.1*	4.5±1.5*
Collagen I	2.2±0.4*	3.3±1.1*	5.2±1.6*
TIMP-1	4.7±0.7*	14.0±4.0*	21.9±7.2*
Ym1	2.5±8.3	4.6±1.4*	5.0±2.1*
Ym2	1.5±0.4	7.2±1.0*	22.1±5.0*
IL-13	4.9±0.7*	11.8±3.3*	11.64±6.1*

Table 4. Expression of fibrosis related genes in diaphragms of *mdx* mice at different ages. RNA from diaphragms of *mdx* and WT mice at the indicated ages was analyzed by qRT-PCR. Results are expressed as fold induction over expression in WT mice; N=3 animals per group; *: *P*<0.05 vs WT.

Gene	*mdx*-saline	*mdx*-ancrod
TGFβ	3.2±1.4*	1.5±1.2#
Collagen I	3.5±0.7*	1.8±1.0#
TIMP-1	14.3±0.9*	7.9±0.2#
IL-1β	4.8±1.3*	3.1±0.4#
Ym1	5.6±1.7*	2.3±1.6#
Ym2	7.5±1.4*	2.6±0.7#
IL-13	10.7±0.3*	12±1.3*

Table 5. Expression of fibrosis related genes in diaphragms of saline- or ancrod-treated *mdx* mice. RNA from diaphragms of saline- or ancrod-treated *mdx* mice of 6.5 months of age and from age-matched WT mice was analyzed by qRT-PCR. Results are expressed as fold induction over expression in WT mice; N=3 animals per group; *: *P*<0.05 vs WT mice; #: P<0.05 vs *mdx*-saline.

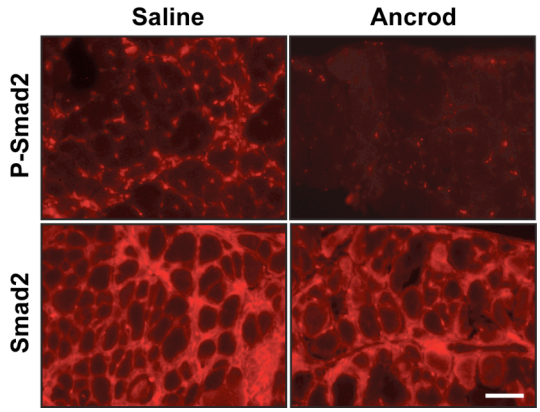

Figure 35. Ancrod treatment reduced TGFβ signaling in *mdx* diaphragm. Immunostaining of diaphragms of *mdx* mice, treated with ancrod or saline for 6 months, using anti-P-Smad2 and Smad2 antibodies. Magnification bar: 50 μm.

2.8 FIBRIN/OGEN INDUCES TGFβ SYNTHESIS BY *MDX* MACROPHAGES VIA INTERMEDIATE IL-1β EXPRESSION

Prompted by the finding that fibrin/ogen-driven fibrosis *in vivo* seemed to involve TGFβ, we investigated the potential link between fibrin/ogen and TGFβ. Since inflammatory cells are a critical source of TGFβ in *mdx* diaphragm (Gosselin et al. 2004;

Zhou et al. 2006), we analyzed TGFβ expression in primary *mdx* macrophages in response to fibrinogen. When macrophages were cultured in the absence of fibrinogen, TGFβ was not expressed, as analyzed by RT-PCR (data not shown). When these cells were stimulated with fibrin/ogen for 6 hours, no TGFβ expression was found either (data not shown). By contrast, after 48 hours, fibrin/ogen activated the expression of TGFβ in *mdx* macrophages (Fig. 36a). At this latter time point, ELISA on conditioned medium also showed that fibrin/ogen promoted the secretion of TGFβ by *mdx* macrophages (mean±SD, pg/ml: 1414±270 in control *versus* 3102±343 with fibrin/ogen; *N*=3; *P*<0.05). Consistent with this late induction pattern, fibrin/ogen-stimulated TGFβ mRNA expression in *mdx* macrophages was blocked not only by actinomycin D but also by cycloheximide (Fig. 36a), thus suggesting the involvement of a newly synthesized protein intermediate. Since the cytokine IL-1β has been shown to induce TGFβ gene transcription (Lee et al. 2006), and IL-1β is an early fibrin/ogen-inducible gene product (Fig. 33b, and (Perez et al. 1999)), we reasoned that IL-1β might be a mediator of the induction of TGFβ gene expression by fibrin/ogen. We first confirmed that, in *mdx* macrophages, recombinant IL-1β stimulated TGFβ expression at 48 hours (Fig. 36b). More importantly, we demonstrated that, at 48 hours, the fibrin/ogen-dependent TGFβ induction in these cells was significantly reduced when the function of IL-1β was blocked using an IL-1β neutralizing antibody (Fig. 36b), indicating that fibrin/ogen induced TGFβ through the intermediate action of IL-1β. In addition, and consistent with the expression of TGFβ, IL-1β expression was increased in 6.5-month *mdx* diaphragms compared to WT, while it was reduced in fibrin/ogen-depleted *mdx* mice (Table 5).

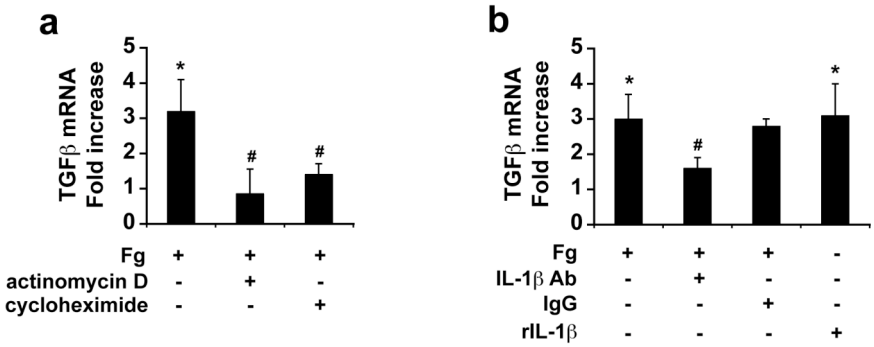

Figure 36. Fibrinogen regulates TGFβ expression in macrophages. a. *Mdx* macrophages were treated with fibrinogen (Fg) ± actinomycin D or cycloheximide for 48 h. TGFβ expression was analyzed by qRT-PCR. Values are fold-induction with respect to control conditions. **b.** Macrophages from *mdx* mice were treated with fibrinogen (Fg) ± an IL-1β neutralizing antibody, control IgG antibody or recombinant IL-1β for 48h, as

indicated. TGFβ expression was analyzed by qRT-PCR. Results are fold induction with respect to control conditions. N=3 experiments. *: *P*<0.05 vs control conditions; #: *P*<0.05 vs Fg alone.

Confirming the pro-fibrotic action of IL-1β via increasing the expression of TGFβ *in vivo* (Kolb et al. 2001; Bonniaud et al. 2005), treatment of *mdx* mice with an IL-1 receptor antagonist (IL-1ra) for 1 month reduced both TGFβ levels and the extent of collagen deposition in *mdx* diaphragm (Fig. 37). Taken together, our results suggest that fibrin/ogen induces an early pro-inflammatory macrophage response, which includes IL-1β synthesis; fibrinogen-induced IL-1β will in turn promote the synthesis of TGFβ.

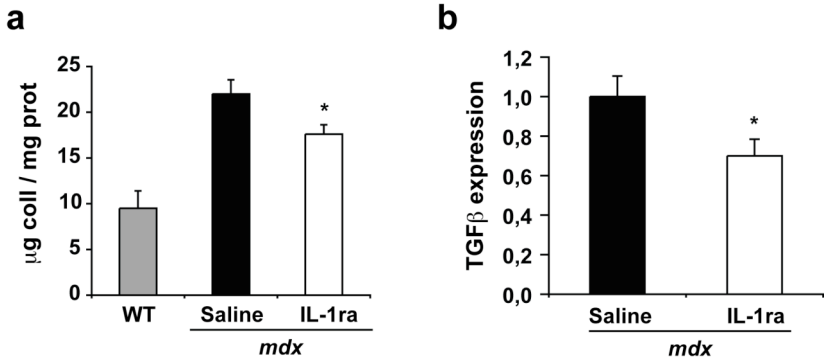

Figure 37. IL-1β inhibition reduces collagen deposition in *mdx* diaphragm. a. 2.5 months-old *mdx* mice were daily injected subcutaneously with IL-1ra or saline, during 5 weeks. Collagen content was analyzed in the diaphragm muscle of age-matched WT, saline- and IL-1ra treated *mdx* mice. **b.** RNA from diaphragms of saline- or IL-1ra-treated mice was analyzed by qPCR. Results are expressed in relative values. N=4 animals per group. *: *P*<0.05.

2.9 FIBRIN/OGEN-INDUCED TGFβ STIMULATES COLLAGEN PRODUCTION BY *MDX* FIBROBLASTS

Next, the functional relevance of the fibrin/ogen-induced TGFβ by *mdx* macrophages was studied. Because collagen-producing cells, fibroblasts, are indeed the key players in fibrosis development, and since TGFβ is known to promote fibrosis by increasing collagen production by fibroblasts (Chen et al. 1999; Verrecchia and Mauviel 2007), we tested the functionality of conditioned medium from fibrin/ogen-stimulated *mdx* macrophages by analyzing its capacity to induce collagen I expression in fibroblasts derived from *mdx* diaphragm. We first confirmed that recombinant TGFβ stimulated collagen I expression in *mdx* fibroblasts (positive control; Fig. 38a). Compared to control, conditioned medium from fibrin/ogen-stimulated *mdx* macrophages also induced collagen I expression in *mdx* fibroblasts (Fig. 38b). This effect was specific for TGFβ, since

collagen I induction was reduced in the presence of a neutralizing antibody against TGFβ (Fig. 38b). Similar data were obtained when analyzing the expression of the TGFβ target gene TIMP-1 in *mdx* fibroblasts (Fig. 38). Thus, by inducing TGFβ expression in macrophages, fibrin/ogen may stimulate the synthesis of collagen by *mdx* fibroblasts, and its further accumulation, thereby promoting fibrosis development.

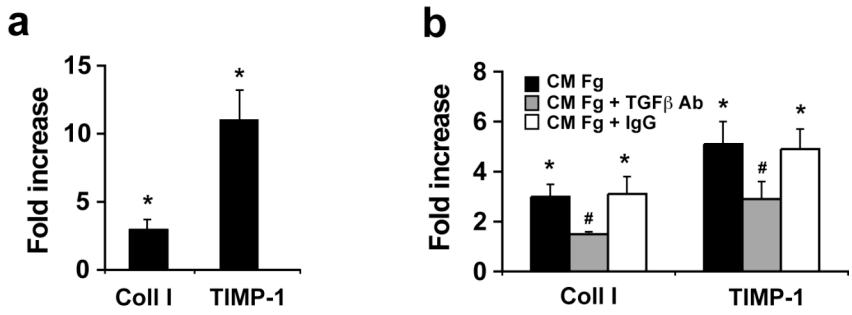

Figure 38. Fibrinogen-induced TGFβ stimulates collagen production by *mdx* fibroblasts. a. Primary fibroblasts derived from diaphragm of *mdx* mice were treated with recombinant TGFβ for 24 h. Gene expression was analyzed by qRT-PCR. Results are fold induction with respect to control conditions. *: *P*<0.05 vs control conditions. **b.** Primary fibroblasts derived from diaphragm of *mdx* mice were incubated with conditioned medium (CM) from *mdx* macrophages treated or not with fibrinogen for 24 hours. When indicated, a TGFβ neutralizing antibody or control IgG were added. Gene expression was analyzed by qRT-PCR. Results are fold induction with respect to control-conditioned medium. *: *P*<0.05 vs control conditioned medium; #: *P*<0.05 vs conditioned medium from fibrinogen-treated macrophages. Data are mean ± SEM; N=3 experiments performed in duplicate.

2.10 FIBRIN/OGEN-INDUCED TGFβ STEERS A PRO-FIBROTIC, ALTERNATIVE MACROPHAGE ACTIVATION RESPONSE

Apart from activating fibroblasts, TGFβ, in conjunction with Th2-derived cytokines such as IL-13, can amplify the expression of arginase I – a key enzyme in the initiation of collagen synthesis by fibroblasts (Wynn 2004; Bronte and Zanovello 2005). – in alternatively activated macrophages (Boutard et al. 1995; King et al. 1998; Munder et al. 1998; Takeuchi et al. 1998; Mills et al. 2000). Notably, alternative activation of macrophages has been associated to certain fibrotic pathologies (Gordon 2003; Wynn 2004). Typically, alternatively activated macrophages are identified by specific cell surface markers such as CD206 (also known as mannose receptor) (Stein et al. 1992) and express high levels of chitinase-like secretory lectins Ym1 and Ym2, and of TGFβ and

TIMP-1 (Wynn 2004; Mora et al. 2006). However, no link between alternatively activated macrophages and muscular dystrophy has ever been established.

We first quantified, by CD206 immunostaining, the number of alternatively activated macrophages in WT and *mdx* diaphragms. While unchanged in WT mice, the number of $CD206^+$ cells increased progressively with age in *mdx* diaphragms (Fig. 39a). In addition, qRT-PCR analysis showed an age-dependent increased expression of the macrophage alternative activation markers Ym1 and Ym2 in diaphragms of *mdx* mice (Table 4). Hence, these findings phenocopied the age-dependent deposition of fibrin/ogen and collagen in *mdx* diaphragm. Importantly, fibrin/ogen depletion in *mdx* mice reduced significantly the number of $CD206^+$ alternatively activated macrophages (Fig. 39b) and the expression of Ym1 and Ym2 (Table 5) in the diaphragm.

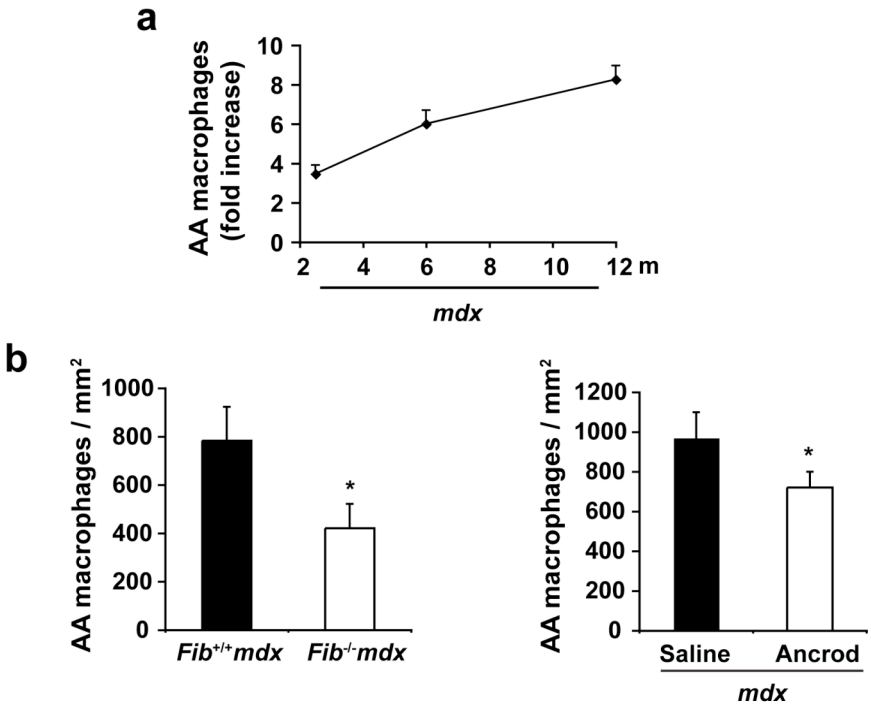

Figure 39. Fibrin/ogen enhances alternative macrophage activation in *mdx* diaphragm. a. Alternatively activated (AA) macrophages ($F4/80^+CD206^+$; detected by immunohistochemistry) were quantified in diaphragms of WT and *mdx* mice of different ages, and expressed as fold increase with respect to WT mice. **b.** AA macrophages were detected as in a and counted in diaphragm muscle sections of $Fib^{+/+}mdx$ and $Fib^{-/-}$ *mdx* mice *(left panel)* and saline- or ancrod-treated mice of 6.5 months of age *(right panel)*. Data are mean ± SEM; N=6 animals per group; *: $P<0.05$.

By double immunostaining, CD206$^+$ macrophages co-expressed both arginase I (Fig. 40) and TGFβ (Fig. 41), thus suggesting that fibrin/ogen regulates the presence of alternatively activated pro-fibrotic macrophages in *mdx* diaphragm.

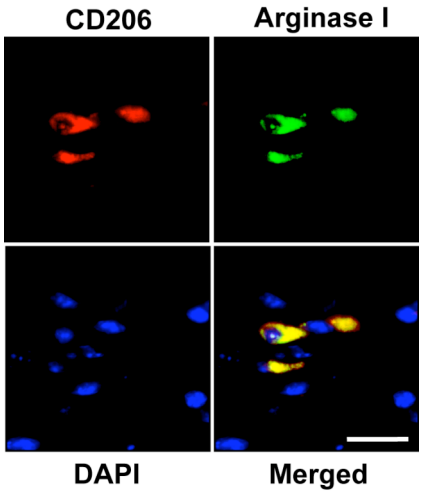

Figure 40. CD206$^+$ cells co-express Arginase I. Immunohistochemistry showing double positive cells for CD206 (red) and Arginase I (green) in a 8 month-old *mdx* diaphragm. Magnification bar: 50 µm.

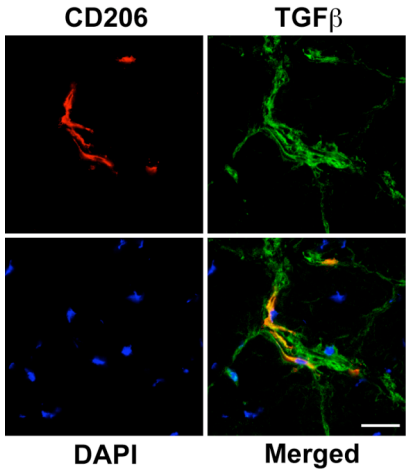

Figure 41. CD206$^+$ cells co-express TGFβ. Immunohistochemistry showing double positive cells for CD206 (red) and TGFβ (green) in an *mdx* diaphragm section. Magnification bar: 50 µm.

We next reasoned that fibrin/ogen-induced TGFβ might amplify the alternatively activated macrophage response and, hence, collagen synthesis. TGFβ alone induced the expression of arginase I in *mdx* macrophages, but not that of Ym1 and Ym2 (Fig. 42), suggesting that TGFβ is not sufficient to induce a full alternative macrophage activation

response. IL-13 induced, however, the expression of arginase I, Ym1 and Ym2 in *mdx* macrophages – as expected – while simultaneous treatment with TGFβ and IL-13 resulted in the synergistic induction of all three alternatively activated macrophage gene products, particularly of arginase I (Fig. 42). These data suggest that fibrin/ogen, by inducing TGFβ, can stimulate collagen synthesis through amplifying the activation of arginase-producing alternatively activated macrophages.

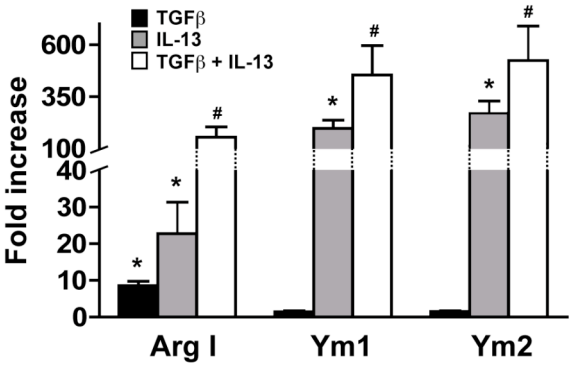

Figure 42. TGFβ and IL-13 synergistically induce alternative activation gene products. Macrophages from *mdx* mice were treated with recombinant TGFβ, IL-13 or both for 24 h. Gene expression was analyzed by qRT-PCR. Results are fold induction with respect to control conditions. *: *P*<0.05 vs control conditions; #: *P*<0.05 vs TGFβ and IL-13 alone. Data are mean ± SEM; N=3 experiments performed in triplicate.

Interestingly, although IL-13 expression increased age-dependently in *mdx* diaphragm (Table 4), it was not downregulated after fibrin/ogen depletion (Table 5), indicating that fibrin/ogen did not affect Th2 cytokine levels, but rather promoted the Th2 cytokine synergism with TGFβ for the alternative activation of macrophages. These data thus suggest that fibrin/ogen amplifies pro-fibrotic alternative macrophage activation through TGFβ induction.

2.11 FIBRIN/OGEN DIRECTLY INDUCES COLLAGEN SYNTHESIS IN *MDX* FIBROBLASTS

Based on the reported capacity of matrix-fibrin/ogen to enhance proliferation and migration of dermal fibroblasts (Rybarczyk et al. 2003), we considered the possibility that fibrin/ogen might also promote *mdx* fibrosis by directly regulating collagen production by *mdx* fibroblasts. Fibrin/ogen induced the expression of collagen in *mdx* fibroblasts (Fig. 43), and this induction was independent of new protein synthesis (not shown). Consistent with the reported binding of fibrin/ogen to fibroblasts via engaging αvβ3 integrins

(Rybarczyk et al. 2003; Orr et al. 2006), we found that incubation of *mdx* fibroblasts with an anti-αv antibody, abrogated the induced collagen expression by fibrin/ogen (Fig. 43). Similar effects were obtained with TIMP-1 (Fig. 43). Thus, these results further support the idea that fibrinogen can promote fibrosis in *mdx* diaphragm by direct and indirect fibroblast activating mechanisms, resulting in extracellular accumulation of collagen.

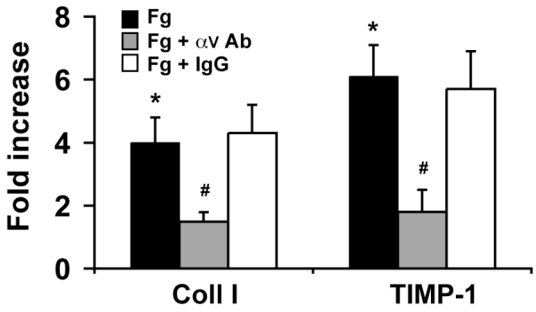

Figure 43. Fibrinogen directly induces collagen synthesis in *mdx* fibroblasts. Primary fibroblasts derived from diaphragm of *mdx* mice were treated with fibrinogen ± an αv neutralizing antibody or control IgG for 5 h. Gene expression as analyzed by qRT-PCR. *: *P*<0.05 vs control conditions; #: *P*<0.05 vs Fg alone. Data are mean ± SEM; N=3 experiments.

2.12 FIBRIN/OGEN DEPOSITION AND FIBROSIS IN DMD PATIENTS

We finally wanted to analyze the association between fibrin/ogen deposition and fibrosis in human DMD biopsies (this analysis was done in collaboration with Dr. R. Gherardi at INSERM). Collagen deposition (fibrosis) was prominent in DMD muscles and particularly found in the same areas occupied by fibrin/ogen (Fig. 44a). To investigate the relationship between the extent of fibrin/ogen deposition and fibrosis, a total of 39 DMD cases were collected, and the patients were divided in 3 non-overlapping groups according to their age: the *early* group (2-5 years; *N*=13), the *mid* group (6-7 years; *N*=14), and the *late* group (8-11 years; *N*=12). The extent of fibrin/ogen deposition and fibrosis was evaluated in all groups. Quantitative analysis revealed that fibrin/ogen accumulation was already significant at 6-7 years (mid group) remaining high thereafter. In contrast, collagen deposition increased only at older ages (8-11 years; late group) (Fig. 44b), suggesting that fibrin/ogen deposition preceded the development of fibrosis. Indeed, a strong correlation was found between fibrin/ogen accumulation and fibrosis in DMD muscle (*P*<0.0007; Fig. 44c), supporting the link between fibrin/ogen accumulation and deposition of collagen as suggested by our findings in *mdx* mice.

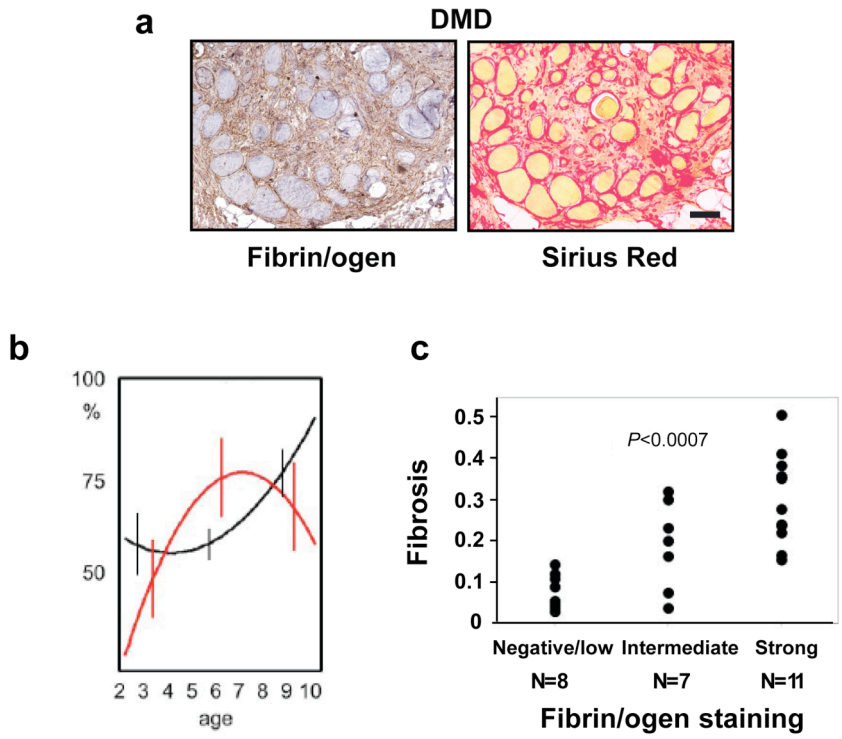

Figure 44. Correlation between fibrin/ogen accumulation and fibrosis in DMD patients. a. Representative example of fibrin/ogen immunostaining and Sirius red staining in muscle sections of DMD patients. Magnification bar: 50 μm. **b.** Integrated representation of fibrin/ogen accumulation (*red line*) and fibrosis (*black line*) in DMD patients, based on curve fitting of 3 data points, corresponding to quantitative morphometric results in the early, mid and late group (see Materials and Methods). **c.** Correlation between fibrin/ogen accumulation and fibrosis in DMD patients (multiple range tests after a significant Kruskal-Wallis test, Statgraphics Plus 5.0). Samples were divided in three groups: negative/low (N=8), intermediate (N=7), and strong (N=11) immunostaining for fibrin/ogen.

2.13 *Fib*γ$^{390\text{-}396A}$ MICE HAVE DIMINISHED INFLAMMATION AND REDUCED COLLAGEN DEPOSITION

Given the importance of preserving fibrinogen-clotting function, strategies targeting only fibrinogen signaling without affecting its pro-coagulant properties might be suitable for human therapeutic intervention. *Fib*γ$^{390\text{-}396A}$ knock-in mice, generated by Dr. J. Degen's group, produce a modified fibrinogen protein incapable of binding Mac-1, but preserving its clotting capacity (Flick et al. 2004b; Adams et al. 2007). These mice show a reduced inflammatory phenotype after bacterial infection and joint disease induction (Flick et al. 2004b; Flick et al. 2007). Based on these findings, we examined the phenotype of *Fib*γ$^{390\text{-}396A}$ mice, in parallel with the *Fib*$^{-/-}$ mice, after CTX-induced muscle injury – in an attempt to reproduce the sequential stages of inflammation, degeneration, fibrosis and ongoing regeneration characteristic of *mdx* muscle. Importantly, the number of macrophages was

reduced in the injured muscle of *Fib*γ$^{390\text{-}396A}$ mice compared to WT mice, to a similar extent to the reduction found in *Fib*$^{-/-}$ mice in parallel experiments (Fig. 45), reinforcing the relevance of fibrinogen, and specifically of fibrinogen/Mac-1 binding, in the inflammatory response in damaged muscle. Of note, collagen deposition was also decreased in damaged *Fib*γ$^{390\text{-}396A}$ muscle (similarly to the reduction found in *Fib*$^{-/-}$ muscle) (Fig. 45), supporting our conclusion that fibrinogen, via Mac-1 binding, promotes inflammation-mediated fibrosis; these results may also support the potential use of fibrinogen/Mac-1 interfering molecules to slow-down inflammation and fibrosis during DMD progression.

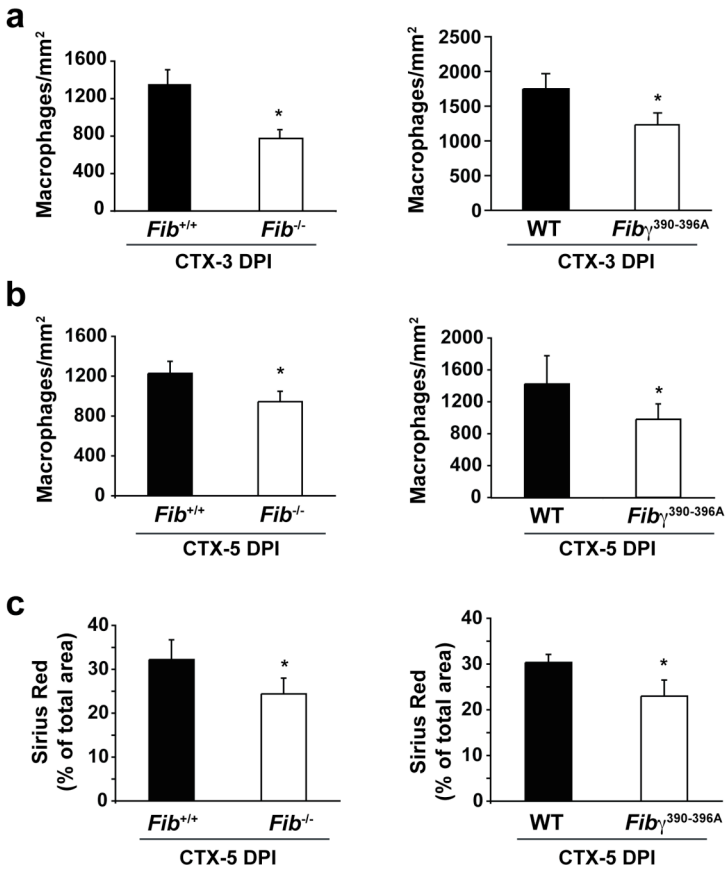

Figure 45. Cardiotoxin-injured *Fib*$^{-/-}$ mice or *Fib*γ$^{390\text{-}396A}$ mice have diminished inflammation and reduced collagen deposition. Muscle injury was performed by CTX injection in gastrocnemius muscles of *Fib*$^{-/-}$ mice, *Fib*γ$^{390\text{-}396A}$ and their respective WT controls. **a, b.** The number of macrophages per mm^2 of damaged area was quantified at 3 DPI (a) and 5 DPI (b) in *Fib*$^{+/+}$ and *Fib*$^{-/-}$ mice (*left panels*) or WT and *Fib*γ$^{390\text{-}396A}$ mice (*right panels*). **c.** The extent of collagen deposition was detected by Sirius Red staining and quantified in *Fib*$^{+/+}$ and *Fib*$^{-/-}$ mice (*left panel*) or WT and *Fib*γ$^{390\text{-}396A}$ mice (*right panel*). Data are mean ± SEM; N=4 animals per group; *: *P*<0.05.

3. PAI-1 DEFICIENCY ACCELERATES CTX-INDUCED MUSCLE REGENERATION BUT EXACERBATES *MDX* MUSCULAR DYSTROPHY

3.1 PAI-1 EXPRESSION IS INDUCED IN CTX-INJURED MUSCLE AND DYSTROPHIC MUSCLE

It has been shown that the absence of uPA exacerbates muscle degeneration in experimentally-injured muscle (Lluis et al. 2001) as in *mdx* dystrophic muscle (Section 1; (Suelves et al. 2007)). PAI-1 is the main inhibitor of uPA, and its expression is also induced in both experimentally-injured muscle and *mdx* muscle (Fig. 46a, b), suggesting a potential involvement of this molecule during the process of muscle degeneration/regeneration. Importantly, PAI-1 expression is also induced in muscles of DMD patients in comparison with healthy muscles (Fig. 46c).

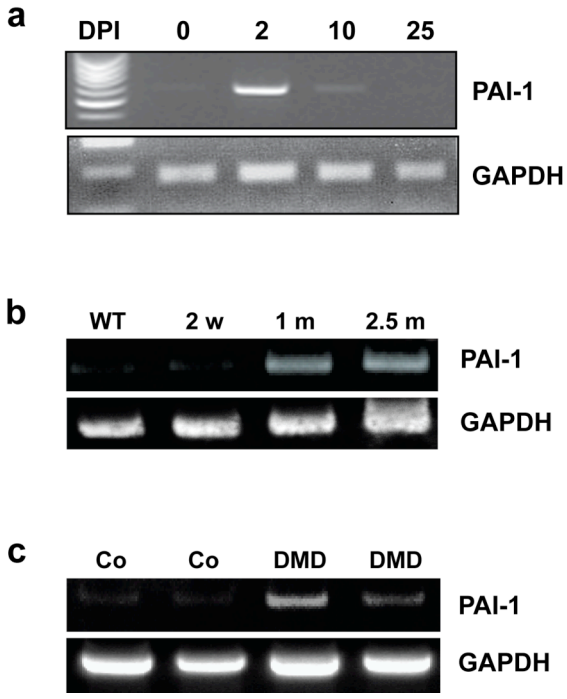

Figure 46. PAI-1 is induced during the process of skeletal muscle regeneration and in muscle dystrophy. **a.** RNA was isolated from non-injured (0 DPI) and cardiotoxin-injured (2, 10, 25 DPI) skeletal muscle. RT-PCR was performed to detect the expression of PAI-1 and GAPDH as a loading control. **b.** PAI-1 expression in WT and *mdx* muscles before (2 w) and after disease onset (1 and 2.5 m) was analyzed by RT-PCR. GAPDH was used as a loading control. **c.** PAI-1 expression in muscles of DMD patients and healthy individuals (Co) was analyzed as in b.

3.2 GENETIC LOSS OF PAI-1 ACCELERATES SKELETAL MUSCLE REGENERATION

To study the possible implications of PAI-1 in the process of experimentally-induced muscle regeneration, gastrocnemius muscles of WT and PAI-1-deficient (PAI-1$^{-/-}$)

mice were injured with CTX and muscles were collected at 0, 4 and 25 days post-injury. The morphometric analysis provided evidence that the muscle regeneration process was accelerated in PAI-1$^{-/-}$ mice, as evidenced by the reduced extent of degeneration and increased percentage of CNFs at early times after injury with respect to WT mice (Fig. 47). This accelerated regeneration in PAI-1$^{-/-}$ mice was accompanied by a reduced extent of fibrin/ogen deposition and a transiently increased inflammatory response (not shown).

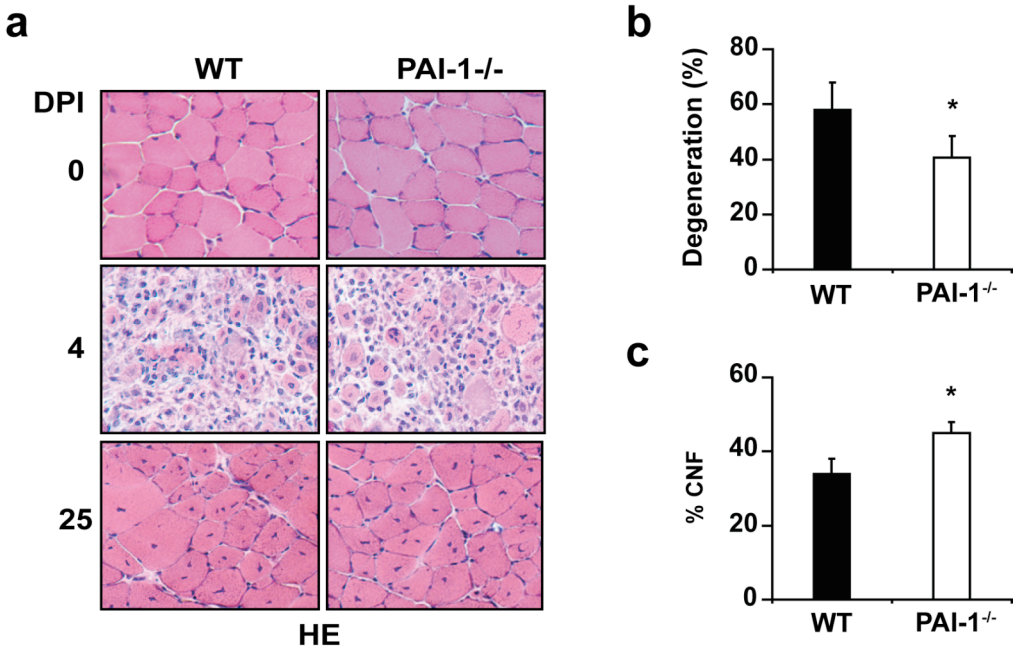

Figure 47. PAI-1-deficient mice show an accelerated regeneration. a. Frozen sections of CTX-injured muscles from WT and PAI-1 deficient mice were stained with HE at 4 and 25 DPI. Contralateral control muscles were also stained with HE (0 DPI). In WT and PAI-1 deficient mice, regeneration is complete after 25 days, as evidenced by the presence of CNFs. **b.** Analysis of muscle fiber degeneration (%) was determined microscopically and expressed as a percentage of the total muscle area. **c.** Muscle fiber regeneration was quantified on micrographs and expressed as the percentage of total muscle fibers containing central nuclei (CNF) present in the entire cross-section of the muscle. PAI-1-deficient mice show a reduced extent of degeneration and increased regeneration with respect to WT mice four days after injury. Data are mean ± SEM; N=4 animals per group; *: $P<0.05$.

3.3 PAI-1 DEFICIENCY AGGRAVATES *MDX* MUSCULAR DYSTROPHY

To evaluate the contribution of PAI-1 to muscle dystrophy, we intercrossed *mdx* mice with PAI-1$^{-/-}$ mice and analyzed PAI-1$^{+/+}$*mdx* and PAI-1$^{-/-}$*mdx* littermates before and after disease onset. Neither genotype showed any sign of muscle dystrophy at 2 weeks of age; however, after disease onset, PAI-1 deficiency severely exacerbated muscle dystrophy in *mdx* mice (Fig. 48a). Muscle breakdown was enhanced in PAI-1$^{-/-}$ *mdx* mice

compared to PAI-1$^{+/+}$ *mdx* mice at 2.5 and 3.5 months of age, as evidenced by the increase in muscle degenerating area and serum CK levels and in the former mice (Fig. 48b, c). This suggests that loss of PAI-1 is deleterious for *mdx* muscle dystrophy progression. To ascertain the influence of PAI-1 on the functional status of the *mdx* muscle, we performed a treadmill assay. Compared to PAI-1$^{+/+}$*mdx* mice, muscle strength was significantly decreased in PAI-1$^{-/-}$*mdx* mice at 2.5 months (Fig. 48d) and 3.5 months (not shown). Altogether, these findings provide histological, biochemical and functional evidence that the absence of PAI-1 leads to *mdx* dystrophy exacerbation.

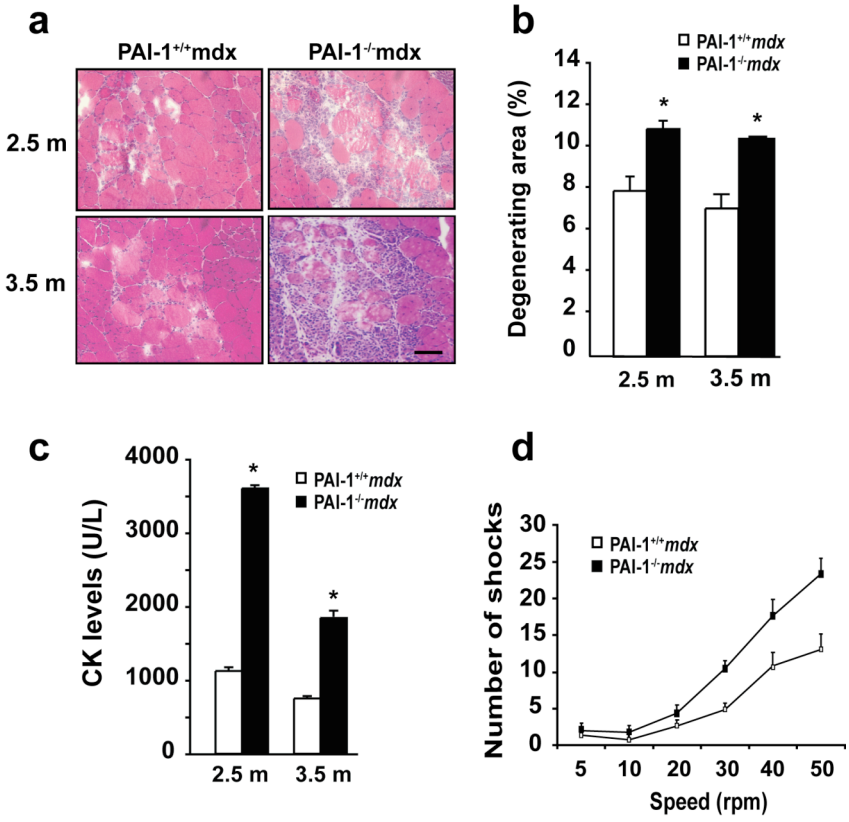

Figure 48. PAI-1 deficiency exacerbates *mdx* muscle dystrophy. a. Muscle sections of PAI-1$^{+/+}$*mdx* and PAI-1$^{-/-}$*mdx* mice of 2.5 and 3.5 months of age were stained with HE. Magnification bar: 50 µm. **b.** Percentage of total muscle degenerating area of 2.5 and 3.5 months-old mice. **c.** Serum CK levels in PAI-1$^{+/+}$*mdx* and PAI-1$^{-/-}$*mdx* mice at 2.5 and 3.5 months of age. **d.** Comparison of functional muscle strength between PAI-1$^{+/+}$*mdx* and PAI-1$^{-/-}$*mdx* mice at 2.5 months of age in treadmill assays performed as described in Materials and Methods. Data are mean ± SEM; N=7 animals per group; *: P<0.05.

DISCUSSION

The specific mechanisms and factors contributing to the progression of muscular dystrophy are largely unknown. One of the findings of the present Thesis work is that uPA plays an important reparative role in muscular dystrophy. Indeed, uPA expression and activity increases during dystrophic disease and genetic loss of uPA exacerbates muscle dystrophinopathy and worsens muscle performance in the *mdx* mouse model of Duchenne's disease. Importantly, these defects in the absence of uPA are significantly rescued by transplantation of uPA-expressing BM, thus highlighting the importance of uPA-secreting BM-derived cells in muscular dystrophy. The data also indicate an important deleterious role for fibrin/ogen deposits in dystrophic muscle and the requirement of uPA to dissolve them. Notably, muscle dystrophinopathy is unaffected in the absence of uPAR, suggesting that uPA exerts its effect independently of its receptor.

The exposed findings, not only show that uPA is produced by BM-derived cells, but also that these cells require uPA for their infiltration into dystrophic muscle. Accordingly, macrophages show 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; Pizza et al. 2005; Tidball 2005a; Hodgetts et al. 2006), although recently, evidence is also accumulating on a positive role for inflammatory cells during muscle regeneration (Tidball 2005a; Sonnet et al. 2006; Arnold et al. 2007; Pelosi et al. 2007; Tidball and Wehling-Henricks 2007). Indeed, we have found less inflammation but increased muscle degeneration in uPA$^{-/-}$*mdx* mice, while transplantation of uPA-expressing inflammatory cells rescues these degenerative defects. Thus, it is conceivable that inflammatory cells require uPA to infiltrate degenerating muscles of dystrophic mice, phagocytose cellular debris and initiate the repair process. Indeed, it has been demonstrated that the activation and the release of pro-recovery 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 uPA-mediated activation of leukocytes might involve mactinin, an alpha-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. Taken together, we propose that uPA drives the infiltration and function of inflammatory cells, required to create a beneficial environment for the repair of dystrophic muscle.

Abundant reports 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 upregulated 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, the results clearly show that loss of uPAR does not affect the degeneration/regeneration process, neither it impairs the inflammatory response in dystrophic muscle, indicating that uPAR is not required for either process. Consistent with this notion, no degeneration or inflammatory phenotype is 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; Gyetko et al. 2001; Rijneveld et al. 2002; Deindl et al. 2003), indicate that uPAR and uPA may operate at different steps and even independent of each other.

Another pre-requisite for efficient regeneration of dystrophic muscle appears to be the prevention of 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 are enhanced in *mdx* mice. Importantly, reduction of fibrinogen levels by ancrod treatment attenuates the severe muscle degeneration in uPA$^{-/-}$*mdx* mice. Thus, by preventing excessive fibrin/ogen accumulation, uPA produced by BM-derived inflammatory cells might attenuate muscle degeneration and persistent inflammation in *mdx* mice.

In this thesis, evidence is provided for a role of fibrin/ogen deposition in dystrophy progression. The fibrin/ogen accumulation in the dystrophic muscles of *mdx* mice promotes persistent inflammation, which in turn may lead to increased degeneration, and impaired regeneration. Genetic deficiency or pharmacological depletion of fibrin/ogen in *mdx* mice counteracts these processes thereby attenuating disease progression. Importantly, fibrin/ogen depletion reduces fibrosis in *mdx* diaphragm. Indeed, our data indicate that fibrin/ogen, through binding its receptor Mac-1, initiates the expression and release of TGFβ in *mdx* macrophages via the prior induction of IL-1β. Fibrinogen-induced TGFβ will, in turn, stimulate collagen expression in resident fibroblasts, and will further amplify its synthesis, by steering a pro-fibrotic, alternative macrophage activation

response. Notably, fibrin/ogen also induces directly collagen expression by *mdx* fibroblasts, via binding its receptor avb3 integrin. Fibrin/ogen accumulation also appears to be positively associated with fibrosis in DMD patients.

A critical question is how fibrin/ogen depletion attenuates the progression of muscle disease in *mdx* mice. We have found that fibrin/ogen affects several processes.

Intramuscular fibrin/ogen deposits can stimulate macrophages expressing Mac-1, an established fibrin/ogen receptor (Flick et al. 2004a). Following fibrin/ogen-Mac-1 engagement, macrophages express chemokines and cytotoxic cytokines including IL-1β, IL-6, MIP-2 and TNFα, as in a classical pro-inflammatory response. Prevention of this response via fibrin/ogen depletion may largely explain the reduced number of inflammatory cells observed in *mdx* muscle following fibrin/ogen depletion. Importantly, the presence of fibrin/ogen deposits critically regulates the expression of cytokines known to promote muscle degeneration such as TNFα (Grounds and Torrisi 2004; Hodgetts et al. 2006). The data do not exclude other fibrin/ogen-mediated effects on inflammatory cells such as Mac-1-mediated inhibition of inflammatory cell apoptosis (Sitrin et al. 1998; Rubel et al. 2003) and the increased release of free radicals (Pizza et al. 2001; Cheung and Tidball 2003; Nguyen and Tidball 2003).

Although inflammation is a necessary process after injury, allowing phagocytosis of tissue debris and promoting commencement of tissue repair, persistent inflammation is deleterious for most tissues, including muscle. It has been proposed that sarcolemmal damage resulting from absent dystrophin is exacerbated by the endogenous inflammatory response and that inflammatory cells and cytokines further damage the sarcolemma leading to myofiber necrosis (Spencer and Tidball 2001; Grounds and Torrisi 2004; Tidball 2005a). Neutrophils and macrophages can injure normal skeletal myotubes *in vitro* and *in vivo* (at least partly via inflammatory cell release of free radicals) (Pizza et al. 2001; Cheung and Tidball 2003; Nguyen and Tidball 2003), and reduction of T cells, neutrophils and macrophages from *mdx* mice *in vivo* reduced muscle degeneration and necrosis at early disease stages (Spencer et al. 2001; Wehling et al. 2001; Hodgetts et al. 2006). A similar protective effect in young dystrophic *mdx* muscle has been demonstrated by blocking TNFα-receptor interaction, supporting a key role for inflammation in the dystropathology of *mdx* mice (Grounds and Torrisi 2004; Hodgetts et al. 2006). Thus, the reduced overall inflammatory response, and of TNFα levels in particular, in fibrin/ogen-depleted *mdx* mice, may partly underlie the reduced degeneration and attenuated dystrophinopathy observed in these mice.

Our results also show that fibrin/ogen-depleted *mdx* mice have bigger regenerating

myofibers, indicating enhanced muscle regeneration – which is primarily mediated by increased recruitment of satellite cells, localized along the basal laminae. This effect might again be related to the inhibition of macrophage activation, since satellite cell differentiation and fusion might be blocked by fibrin/ogen-driven expression of TNFα and TGFβ in *mdx* macrophages (Szalay et al. 1997; Li et al. 1998; Argiles et al. 2000; Zhu et al. 2004). However, fibrin/ogen depletion might also directly affect satellite cell functions during regeneration, analogous to its effects on Schwann cells during nerve remyelination (Akassoglou et al. 2002; Adams et al. 2004). Fibrin/ogen is not a normal component of the ECM in healthy muscle, but becomes incorporated after muscle injury by extravasation. Importantly, we have observed that fibrin/ogen co-localizes extensively with fibronectin and partially with laminin in the *mdx* muscle ECM (not shown). Thus, fibrin/ogen may alter the pro-myogenic effect of these ECM components on satellite cells. Satellite cell functions on laminin and fibronectin are mediated by arg-gly-asp (RGD)-dependent integrins, α7β1 and α5β1, respectively. Importantly, mice deficient in α7 and α5 integrins (the receptors for laminin and fibronectin, respectively) develop muscle degenerative phenotypes (Mayer et al. 1997; Taverna et al. 1998; Disatnik and Rando 1999). Because fibrin/ogen also contains two RGD peptides and can bind to α5β1 integrin on other cell types, such as endothelial cells (Suehiro et al. 1997), we could hypothesize an inhibitory effect of fibrin/ogen on satellite cell functions on fibronectin due to a blockade of the fibronectin-mediated integrin signaling. Overall, fibrin/ogen deposition during muscle degeneration in *mdx* mice could contribute to destabilizing the ligand-receptor interactions of the extracellular matrix with the satellite cell integrins thus interfering with the regeneration process.

Surprisingly little is known about the regulatory pathways driving interstitial fibrosis in muscular dystrophy – a clinical hallmark and severity parameter determining life expectancy in DMD disease. In this thesis, a novel role of fibrin/ogen in the fibrotic transition of dystrophic muscle is unveiled and the underlying mechanisms by which fibrin/ogen accumulation might promote the persistent deposition of collagen in *mdx* diaphragm are investigated. Previous studies showed that neutralization of TGFβ by administration of an anti-TGFβ antibody or the proteoglycan inhibitor of TGFβ decorin reduced connective tissue proliferation and fibrosis in *mdx* mice diaphragm (Gosselin et al. 2004; Andreetta et al. 2006). However, what drives TGFβ production in dystrophic muscle is not known. Our results indicate a role of fibrin/ogen in the synthesis and release of TGFβ by *mdx* macrophages, via the induction of IL-1β, thereby allowing subsequent collagen synthesis by *mdx* fibroblasts. Amongst the different inflammatory cytokine genes that can be activated by fibrin/ogen (Fan and Edgington 1993; Perez et al. 1999; Smiley et

al. 2001), we focused on IL-1β in view of its known pro-fibrotic action *in vivo* (Kolb et al. 2001) and, in particular, of its capacity to stimulate TGFβ expression and fibrosis *in vitro* and *in vivo* (Kolb et al. 2001; Bonniaud et al. 2005; Lee et al. 2006). In agreement with this, we have shown that IL-1β blocking *in vivo*, reduces collagen deposition in *mdx* diaphragm. Our results indicate that fibrin/ogen can induce an early proinflammatory response, which, subsequently leads to the production of TGFβ, suggesting that fibrin/ogen can induce a macrophage activation switch over time, by modulating the macrophage microenvironment, thereby promoting fibrosis. Hence, the fibrin/ogen – IL-1β – TGFβ axis represents a novel pathway by which dystrophic muscle is transformed into fibrotic tissue. Our data do not exclude, however, the possible involvement of other cytokines or additional mechanisms in *mdx* fibrosis development.

We have further demonstrated that fibrin/ogen-induced TGFβ may potentiate the alternative activation of macrophages. Though they have been implicated in other fibrotic pathologies (Gordon 2003; Wynn 2004), this study is the first to implicate alternatively activated macrophages in fibrotic development during *mdx* muscle dystrophy. In particular, we have found that fibrin/ogen increases the number of alternatively activated macrophages in *mdx* diaphragm. Moreover, via increasing TGFβ secretion, fibrin/ogen amplifies arginase I expression in alternatively activated macrophages in response to IL-13, thereby indirectly promoting collagen synthesis by resident fibroblasts. Overall, apart from reinforcing the emerging link between tissue inflammation and fibrosis (Stramer et al. 2007), we provide evidence for a fibrin/ogen – IL-1β – TGFβ axis, which can drive multiple pathways during the fibrotic transition of dystrophic muscle.

Finally, our studies also show that fibrin/ogen directly stimulates the expression of collagen and TIMP-1 by *mdx* fibroblasts, via the specific engagement of the αvβ3 integrin, a known fibrin/ogen receptor (Rybarczyk et al. 2003; Adams et al. 2004). Taken together, these findings introduce the integrative concept that fibrin/ogen promotes tissue inflammation and fibrosis in *mdx* muscular dystrophy (see Fig. 49).

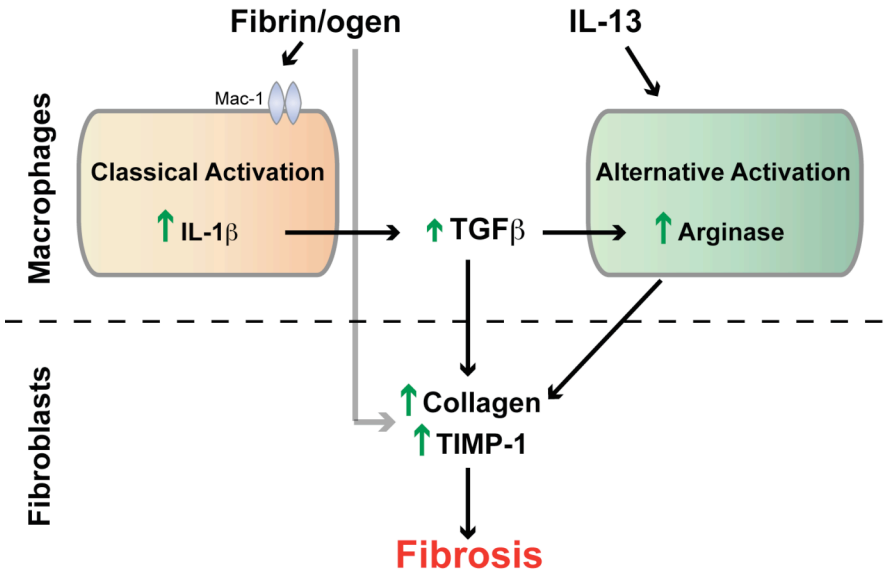

Figure 49. Proposed model for the role of fibrin/ogen in the muscle dystrophy-associated fibrosis. Fibrinogen promotes collagen production by three distinct but possibly overlapping mechanisms in *mdx* muscle. *i*. TGFβ is produced in response to fibrinogen stimulation of pro-inflammatory, classically activated *mdx* macrophages (through Mac-1 binding) and via the intermediate induction of IL-1β (a direct fibrinogen-inducible gene), as an anti-inflammatory counteracting mechanism involving a macrophage activation switch (Van Ginderachter et al. 2006). Macrophage-secreted TGFβ is free to bind and activate TGFβ receptors expressed by fibroblasts from *mdx* diaphragm, and directly induced collagen synthesis. *ii*. TGFβ can also promote the alternative activation of macrophages initiated by IL-13 Th2 cytokine. By upregulating arginase activity in these cells, TGFβ (in conjunction with IL-13) is known to promote fibroblast proliferation, collagen production and ultimately, fibrosis (see (Wynn 2004) for arginase-mediated collagen synthesis for details). *iii*. Because fibroblasts express αvβ3 fibrin/ogen receptors (Rybarczyk et al. 2003; Adams et al. 2004), fibrin/ogen might also directly activate the collagen-producing machinery in fibroblasts.

Despite intense research efforts, DMD is still an incurable and fatal disease. A potential advantage of a fibrin/ogen-based therapeutic approach over current anti-inflammatory/fibrotic strategies is that targeting fibrin/ogen interactions is likely to impose a block on the activation and recruitment of leukocytes only locally, i.e. within the dystrophic muscle where fibrin/ogen is deposited. Indeed, anti-TGFβ antibody therapies in muscle dystrophy reduce fibrosis but also increase inflammation (Andreetta et al. 2006). Hence, by targeting fibrinogen, one might prevent both inflammation and fibrosis in dystrophic muscle.

Importantly, muscle fibrosis not only accelerates the clinical decline in DMD young

patients but also represents a major obstacle for successful engraftment of stem cells in ongoing experimental cell-based therapies in DMD aimed at correcting the primary defect (i.e. dystrophin replacement or rescue). Thus, reducing inflammation-mediated fibrosis by targeting fibrin/ogen might also facilitate the homing of donor stem cells to muscle, and hence, the success of these primary therapies.

CONCLUSIONS

The following conclusions can be drawn from the results presented in this PhD Thesis:

1. BM-derived uPA promotes *mdx* muscle repair, by facilitating the infiltration of BM-derived inflammatory cells, which may help removing muscle debris, and by preventing the excessive deposition of fibrin/ogen.

2. uPAR deficiency does not affect experimentally-induced muscle regeneration or *mdx* muscular dystrophy, indicating that uPA exerts its effects independently of its receptor.

3. PAI-1 deficiency accelerates experimentally-induced muscle regeneration but exacerbates *mdx* muscular dystrophy.

4. Fibrin/ogen accumulates in dystrophic muscles of DMD patients and *mdx* mice; it promotes muscle degeneration and fibrosis in *mdx* mice and it is positively associated with fibrosis in DMD patients.

5. Fibrin/ogen promotes fibrosis at least via two mechanisms: driving the synthesis of TGFβ by macrophages, which can further steer an alternative activation response; and directly stimulating collagen synthesis in fibroblasts.

APPENDIX

The main objective of this PhD thesis has been to study the role of the fibrinolytic system in muscle regeneration and muscle dystrophy progression, and understanding the underlying mechanisms of action. This work has resulted in several publications, which are detailed below. At the very beginning of my PhD thesis I was involved in a project focused on the transcriptional regulation of PAI-1 by the alkylating agent MNNG.

The alkylating carcinogen N-methyl-N'-nitro-N-nitrosoguanidine activates the plasminogen activator inhibitor-1 gene through sequential phosphorylation of p53 by ATM and ATR kinases.

Vidal B, Parra M, Jardí M, Saito S, Appella E, Muñoz-Cánoves P.

Thromb Haemost. 2005 Mar;93(3):584-91.

Alpha-enolase plasminogen receptor in myogenesis.

Lopez-Alemany R, Suelves M, Diaz-Ramos A, Vidal B, Munoz-Canoves P.

Front Biosci. 2005 Jan 1;10:30-6. Review.

The plasminogen activation system in skeletal muscle regeneration: antagonistic roles of urokinase-type plasminogen activator (uPA) and its inhibitor (PAI-1).

Suelves M, Vidal B, Ruiz V, Baeza-Raja B, Diaz-Ramos A, Cuartas I, Lluis F, Parra M, Jardi M, Lopez-Alemany R, Serrano AL, Munoz-Canoves P.

Front Biosci. 2005 Sep 1;10:2978-85. Review.

uPA deficiency exacerbates muscular dystrophy in MDX mice.

Suelves M, Vidal B, Serrano AL, Tjwa M, Roma J, López-Alemany R, Luttun A, de Lagrán MM, Díaz-Ramos A, Jardí M, Roig M, Dierssen M, Dewerchin M, Carmeliet P, Muñoz-Cánoves P.

J Cell Biol. 2007 Sep 10;178(6):1039-51.

Fibrinogen drives dystrophic muscle fibrosis via a TGFβ/alternative macrophage activation pathway.

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The plasminogen activation system in skeletal muscle regeneration: antagonistic roles of urokinase-type plasminogen activator (uPA) and its inhibitor (PAI-1).
Front Biosci. 2005 Sep 1;10:2978-85.

Suelves M, Vidal B, Serrano AL, Tjwa M, Roma J, López-Alemany R, Luttun A, de Lagrán MM, Díaz-Ramos A, Jardí M, Roig M, Dierssen M, Dewerchin M, Carmeliet P, Muñoz-Cánoves P. *uPA deficiency exacerbates muscular dystrophy in MDX mice.* J Cell Biol. 2007 Sep 10;178(6):1039-51. Epub 2007 Sep 4.

Erratum in:
J Cell Biol. 2007 Oct 8;179(1):165. Díaz, Maria Angels [corrected to Díaz-Ramos, Angels].

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