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Review

The Role of Urokinase-type Plasminogen Activator (uPA) and Transforming Growth Factor Beta 1 (TGFβ1) in Muscle Regeneration

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Abstract. *Skeletal muscle regeneration is a highly synchronized process involving the activation of various cellular and molecular events, coordinating inflammation and regeneration processes which are crucial for the beneficial outcome of tissue remodeling. Fibrosis, a failure of tissue remodeling, is initiated with muscle regeneration; however, it is the result of an excessive inflammatory response, representing an imbalance between enhanced production and deposition and impaired degradation of extracellular matrix (ECM) components of the muscle. Therefore, factors influencing the relative degree of muscle fiber regeneration as compared to the amount of scar formation have a critical role in functional muscle remodeling. Herein we have focused on the role of urokinase-type plasminogen activator (uPA) and transforming growth factor beta 1 (TGFβ1) in ECM degradation and reconstitution in muscles.*

Skeletal muscle shows an enormous ability to adapt to mechanical loading conditions by changing its mass and phenotype *via* mechanisms that seem to be intrinsic to the muscle (1-4). Exercise is one of the most powerful stimuli for inducing structural, metabolic and functional re-organization of skeletal muscle cells, while muscle plasticity also occurs in a number of physiopathological processes, such as muscular dystrophies, inflammatory myopathies, or adult muscle aging (4-6). Furthermore, skeletal muscle has the remarkable ability to initiate a rapid and extensive repair process in response to metabolic or mechanical damage following unaccustomed or excessive exercise, preventing the loss of muscle mass (1, 6-8). As a postmitotic tissue, skeletal muscle lacks ongoing cell replacement and, therefore, there must be an effective local cellular repair system (9, 10). Although much has been learned about the events involved in skeletal muscle regeneration, the molecular mechanisms that regulate this process remain largely undefined (11).

Skeletal muscle repair is a highly synchronized process involving the activation of various cellular and molecular responses, where the coordination between inflammation and regeneration is crucial for the beneficial outcome of the repair process following muscle damage (6, 12). Cellular processes of myofiber regeneration are successfully completed when the activation, proliferation and subsequent differentiation and fusion of muscle satellite cells follow the infiltration of inflammatory cells, leading to new myofiber formation and enabling muscle repair and growth (12-14). However, a process of fibrosis is initiated concomitantly with muscle regeneration, as fibroblasts are activated and attracted into the sites of damage during muscle inflammation (12). Tissue regeneration depends on a balance between pro-inflammatory and anti-inflammatory factors that determine whether the damage will be resolved with muscle cell replacement or with scar formation (12, 15). During this process, the extracellular matrix (ECM) surrounding the skeletal muscle appears to play an important role in maintaining the structure of the muscle and a scaffold for myofiber regeneration (6, 16), while factors such urokinase-type plasminogen activator (uPA) and transforming growth factor beta 1 (TGFβ1) have been implicated as key modulators of skeletal muscle regeneration, since they

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contribute to ECM degradation and reconstitution, and, hence, to muscle tissue remodeling (11, 14, 17, 18). This review focuses on the molecular and cellular regulation of muscle regeneration, particularly in the context of the uPA/TGFβ1 bioregulation system and its interaction with the repair process, following exercise-induced muscle damage where extensive muscle tissue remodeling occurs.

Skeletal Muscle Damage and Repair: An Overview

Skeletal muscle fibers are repeatedly damaged and repaired throughout life and certain fundamental characteristics of the repair process following muscle damage have been recognized and described, although the complex regulatory pathways of muscle regeneration remain poorly understood (6, 7, 12, 13, 19). Muscle damage can be caused by events inside the muscle cells, such as metabolic deficits, ischemia or innate genetic defects and disease, or it can result from external events, such as injection of toxic agents, heat, cold, transplantation, mechanical stress, or various activity models, such as stretching or eccentric exercise (6, 7, 20-25). In most cases, the damage can involve muscle fibers, connective tissue and vascular and nerve supplies (22, 26-28). Regeneration of myofibers and reconstitution of the ECM are the main focus of this review, although the muscle regeneration process also involves the essential aspects of revascularization and reinnervation (6).

In order to study the muscle regeneration process in a controlled and reproducible way, animal experimental models of muscle damage have been developed in respect to the various causes of damage mentioned above, including laboratory animal models with abnormal degeneration and regeneration due to spontaneous or artificial deregulation of specific genes (6). In particular, special attention has been paid to the cellular responses activated and the mechanisms implicated in exercise-induced muscle damage and regeneration (6, 7, 22, 24, 29-31), with eccentric exercise (where the activated muscle is lengthened) used extensively as a model to study exercise-induced muscle damage, since it is particularly potent at inducing damage (20, 30, 32).

As a result of excessive physical activity, such as resistance training and mostly eccentric muscle contractions, the contractile system of muscle fibers sustains mechanical damage, characterized by disruption of the normal myofilament structures in sarcomeres, damage to sarcolemma, loss of fiber integrity and leakage of muscle proteins usually restricted to the cytoplasm of the muscle cell, such as creatine kinase, into the blood (20, 24, 29, 33-35). Eccentric exercise-induced mechanical stress has also been proposed to result in disruption of connective tissue or the ECM surrounding the myofibers (26-28, 36).

Muscle tissue repair following damage can be considered as a process consisting of four interdependent phases: degeneration, inflammation, regeneration and fibrosis (Figure 1), where, apart from the role of growth and differentiation factors, the degree of damage and the interactions between muscle and the infiltrating inflammatory cells appear to affect the successful outcome of the muscle repair process (12, 37). Although the phases of this process are similar after different causes of damage, the kinetics and amplitude of each phase may depend on the particular muscle damaged, the extent of damage, or the animal model used (6, 7, 38, 39). In the present review, regeneration of skeletal muscle following exercise-induced mechanical damage will be treated further.

Skeletal Muscle Degeneration and Inflammation

Necrosis of damaged muscle tissue and activation of an inflammatory response define the initial phases of muscle repair. Infiltrating inflammatory cells, such as neutrophils, macrophages and leukocytes, invade the muscle at the site of damage, potentially contributing to the damage, removing the necrotic tissue and promoting revascularization (6, 40-42). The initial events of muscle repair consist of an intrinsic degeneration within the fiber, since muscle fibers contain intrinsic proteolytic and degradative pathways that respond to the initiating mechanical lesion (7, 29). This phase occurs during the several hours prior to the arrival of phagocytic cells and continues during the phase of inflammation. It consists of autogenetic processes that begin the degradation of the lipid and protein structures within the damaged muscle cells (30). As early as 1-6 hours following exercise-induced muscle damage, neutrophils are the first inflammatory cells to begin accumulating at the site of injury, destroying necrotic tissue while working in conjunction with macrophages residing within the muscle (43, 44). Factors released by the damaged muscle activate these inflammatory cells which release chemotactic agents and provide the chemotactic signals to circulating inflammatory cells to invade the damaged muscle and begin a digesting process surrounding necrotic tissue (6, 24, 45). Because damaged muscle tissue does not appear to be chemoattractive for neutrophils or macrophages earlier than 24 hours following damage, there might be other mechanisms to provide the signals necessary to initiate the chemotaxis of inflammatory cells into the damaged muscle. Human muscle satellite cells, when they are activated to proliferate, and probably myoblasts, have been shown to immediately release factors that attract monocytes and macrophages to the site of damage (12).

A phagocytic process, characterized by a typical inflammatory response in the muscle tissue, is evident by 2 to 6 hours after the damage, with rapid invasion of the damage site by neutrophils and macrophages that potentially contribute to the muscle damage. A rapid breakdown of the damaged muscle fibres proceeds from lysosomal proteases, free radicals

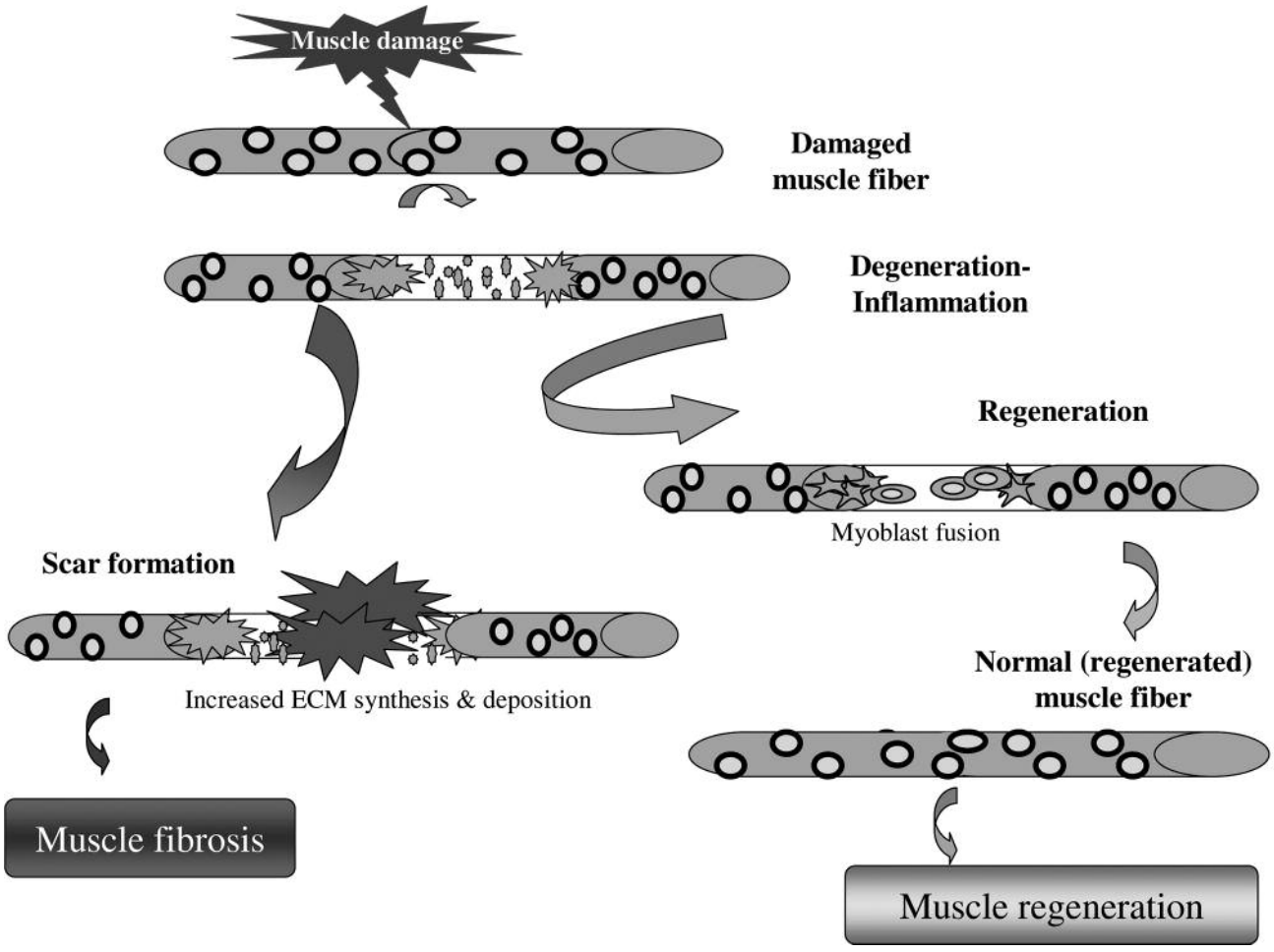

Figure 1. *Stages of muscle repair process following damage. Damaged muscle fibers undergo the degeneration and inflammation phases, which involve local myofiber necrosis and inflammatory cell infiltration. Fully functional recovery of muscle tissue depends on the precise coordination between inflammatory and regeneration processes following damage. An excessive inflammatory response with a subsequent increase in ECM production and deposition within the broken and between the regenerated muscle fibers would result in muscle fibrosis. Hence, myoblast migration and fusion to form the terminal (regenerated) muscle fiber should begin before scar tissue proliferates excessively and obstructs the regenerating muscle cells between the stumps of the disrupted myofibers. ECM: Extracellular matrix.*

or other oxidants released by macrophages *via* a superoxide-dependent mechanism which results in lysis of muscle fiber membranes (30, 42). This inflammatory process intensifies through 2 to 4 days following muscle damage and is important in the removal of the necrotic tissue and for the stimulation of muscle fiber regeneration (30). Macrophages become the predominant inflammatory cells of the phagocytic process 2 days post damage, infiltrating the injury site to phagocytose cellular debris, while they may also activate myogenic cells contributing to the muscle regeneration process (6, 46, 47). Muscle damage and healing should be considered as processes intimately related to inflammatory cell invasion and interaction with the damaged tissue, and the efficiency of muscle regeneration appears to be dependent on efficiency of the inflammatory cell invasion (12, 42). It seems that a continuous sequence of inflammation and repair, where immune cells interact with the damaged tissue, characterizes the process of tissue recovery, while coordination between inflammation and regeneration is crucial for muscle recovery following damage (12, 37, 42). It has been proposed that skeletal muscle interacts actively with the immune system by secreting various chemokines, cytokines and cell adhesion molecules of innate immunity (48).

Cytokines and growth factors released at the injury site by activated inflammatory cells and muscle cells act as mediators, by facilitating or retarding the influx of inflammatory cells into the site of damage thereby modulating the inflammatory process, while they also influence local blood flow and

vascular permeability (12, 24, 37, 49). TGFβ1 appears to be a chemotactic factor for macrophages and leukocytes (50), while activated macrophages at the site of muscle damage produce highly chemoattractive and mitogenic factors for muscle precursor cells, such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), facilitating the repair of damaged myofibers (14, 47). TGFβ1 is found in ECM in the form of latent TGFβ1 and its activation requires the proteolytic action of the uPA/plasmin system. The role of the TGFβ1/uPA bioregulation system has been implicated in several pathophysiological processes (51-62).

Moreover, a combination of cytokines produced by macrophages and other cellular sources activate and attract fibroblasts into the site of damage. The subsequent route of the inflammation process depends on the relative balance between pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, and anti-inflammatory cytokines, such as IL-4 and IL-10, leading to adequate repair of the damaged tissue without producing an excessive response (12, 24, 63, 64). This balance between pro-inflammatory and anti-inflammatory factors determines whether the damage will be resolved with muscle fiber replacement and reconstitution of a functional contractile apparatus, or with scar formation (12, 15). A limited inflammatory response could theoretically reduce excessive muscle degeneration and signals for scar formation, but it may also inhibit strong signals that promote the regenerative process, due to the reduced availability of growth factors and cytokines (37).

Muscle regeneration is completed successfully when the infiltration of inflammatory cells is followed by muscle repair and growth, processes which involve activation, proliferation and terminal differentiation of satellite cells (12, 13). Satellite cell activation should begin before scar tissue proliferates excessively and obstructs the regenerating muscle cells between the stumps of the disrupted myofibers (65-67) (Figure 1).

Skeletal Muscle Regeneration: Role of Satellite Cells

Muscle regeneration follows the degeneration and inflammation phases of the muscle repair process, beginning after the inflammatory cells have cleared necrotic tissue. Its primary cellular component has been established to be the activation of muscle satellite cells (6), which participate in the reconstitution of damaged tissue. The myofiber regeneration process is dependent on the proliferative activation of satellite cells and their subsequent myogenic differentiation into myoblast-like cells (68-70). The proliferation and myogenic differentiation of satellite cells enable the regeneration of existing myofibers or the generation of new myofibers (19, 71) (Figure 2).

Damaged muscle fibers constitute the site of mobilization of the small, mononucleated satellite cells, which are located between the basal lamina of the muscle and the sarcolemma of myofibers (72). They have little cytoplasm and express no muscle proteins (22). Satellite cells are the main, if not the only, cell type that contributes to muscle regeneration. It has been reported (73) that muscle regeneration could also occur by other cell types (*e.g.* bone marrow and hematopoietic stem cells), or by de-differentiation of mature muscle fibers in some amphibian species and *in vitro* de-differentiation of mammalian C_2C_{12} myotubes (74). However, it remains an open question if such de-differentiation could occur in damaged mammalian skeletal muscle *in vivo* (75, 76).

The molecular mechanisms that are involved in the regulation of satellite cell activation include the inflammatory response and the release of certain growth factors. The role of polymorphonuclear lymphocytes and macrophages, leukocytes, cytokine IL-6 and several growth factors has been implicated in satellite cell activation *in vivo*, although the actual stimulus for their activation has still to be defined (37, 73). In particular, an attractive aspect has been developed in the literature for the influential role of insulin-like growth factor-I (IGF-I)-induced actions, especially those of IGF-IEc (mechano-growth factor; MGF) on skeletal muscle satellite cells, as a potential mediator of muscle regeneration process (12, 77, 78).

Upon exposure to signals from the damaged environment, satellite cells near the injury site are induced to proliferate and migrate; there is also evidence that they approach the injury from other sites within the muscle, even from adjacent muscles (19, 73). It has been proposed that the disruption of the sarcolemma and basal lamina after muscle damage could release and activate quiescent satellite cells and muscle stem cells residing between these structures (37, 66), however, recruitment of satellite cells from contiguous muscles appears more seldom, requiring damage to the connective tissue that separates the two adjacent muscles (79). Nevertheless, it has been shown that myoblasts can migrate across basal lamina and contribute to the repair of damaged fibres (80).

In the course of muscle regeneration, satellite cells begin the repair process after their activation as early as 24 hours post-damage followed by a proliferative response in which some or all of the activated cells undergo multiple mitotic cycles. After this initial phase, the beginning of the regenerative phase is marked by the subsequent differentiation of the majority of the activated satellite cells or their progeny into myoblast-like cells. They then become incorporated into the damaged segments of myofibers, providing the extra set of genes for supporting the repair process, early enough for cell death to be avoided and thus preventing subsequent functional deficit of the muscle (81), and providing the extra nuclei required for increased protein synthesis during regeneration (2, 37, 47, 66, 68, 73, 76, 81-83). From animal experimental models of muscle damage, it

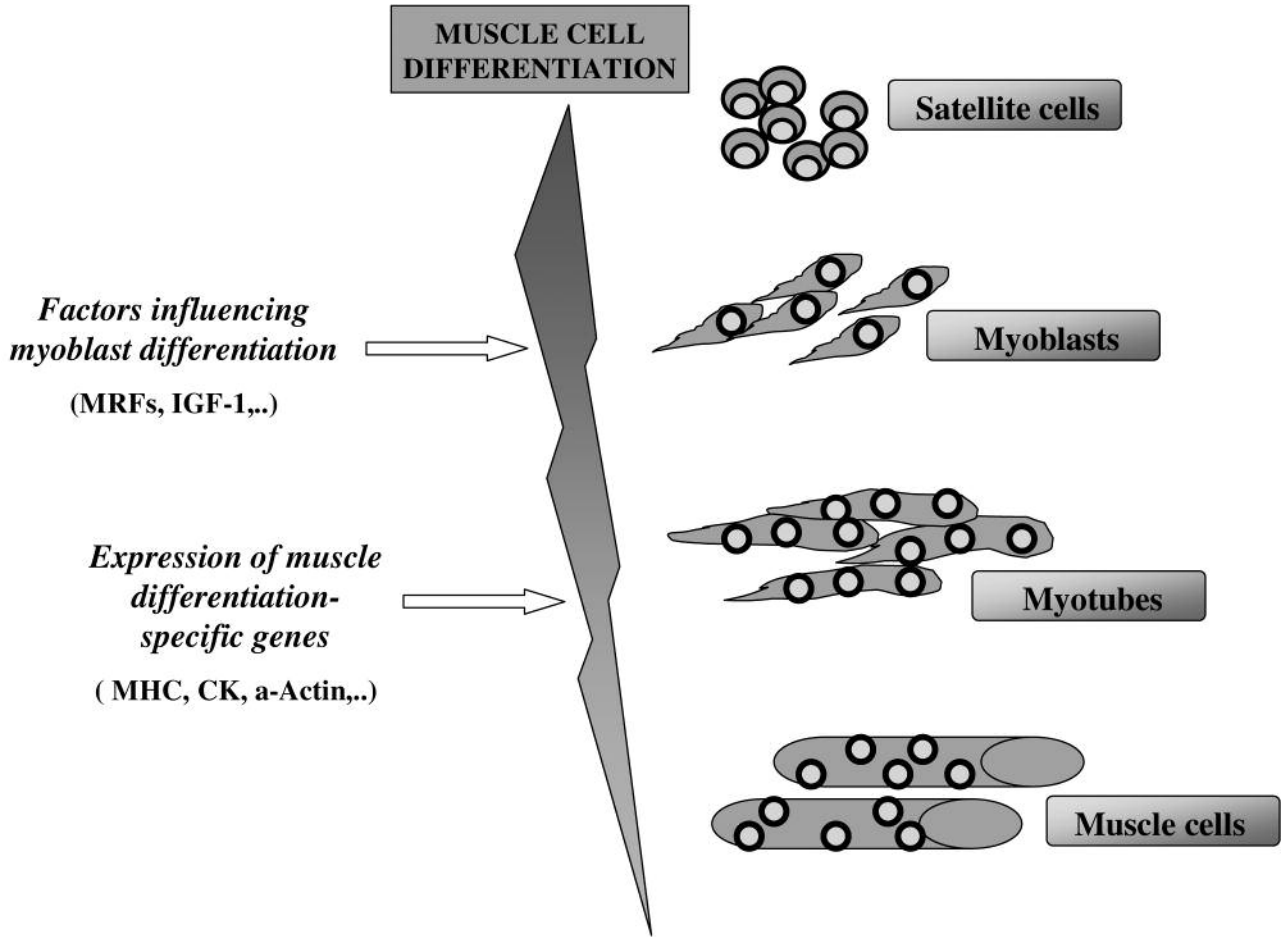

Figure 2. *Mononucleated quiescent satellite cells are activated to enter the cell cycle and proliferate. This phase is followed by their early differentiation into myoblasts, which is characterized by the up-regulation of the MRFs. Myoblasts subsequently start to fuse into multinucleated myotubes in a late differentiation phase, expressing muscle differentiation-specific proteins to form mature muscle fibers. CK: Creatine kinase; IGF-1: Insulin-like growth factor-1; MHC: Myosin heavy chain; MRFs: Myogenic regulatory factors.*

has been shown that central myonuclei are observed in discrete portions of the regenerating fibers, indicating that cell fusion is rather focal to the site of damage and not diffuse during regeneration (6). After myogenic cell fusion has been completed, myonuclei of the new myofibers move to the periphery of the fiber, which increases in size and eventually culminates in a mature myofiber.

Differentiation of satellite cells into myoblasts involves induction of embryonic forms of myosin heavy chain (MHC) and the regulation of other muscle-specific proteins belonging to the family of myogenic regulatory factors (MRFs) (6) (Figure 2). These are transcription factors that control the expression of several muscle genes and function as main activators of skeletal muscle differentiation by activating genes encoding structural and regulatory muscle proteins (13, 84-86). Newly formed myofibers appear to be basophilic, reflecting high protein synthesis, while their expression of embryonic/developmental forms of MHC reflects *de novo* fiber formation (6). Myofiber regeneration following local muscle damage involves dynamic restructuring of the muscle's intermediate filament system (3-7 days following damage) (87), the formation of new multinucleated myotubes, which begin to be formed 3-4 days after damage (88), coinstantaneously with the appearance of fibres expressing embryonic MHC (3-10 days following damage) (13), and consequently the replacement of the damaged muscle fibers (89).

Skeletal Muscle Regeneration *versus* Fibrosis

Eccentric exercise-induced muscle damage also results in alterations to the ECM and a very complex reaction exists between muscle cells, ECM and inflammation mediators following muscle damage (26, 27). ECM contributes to

resistance of the deformation of the muscle tissue and also serves as a scaffold for the reconstruction of the regenerating muscle fibers, while it is important in directing further differentiation of myoblasts into functional myofibers (16, 18, 90). However, uncontrolled collagen deposition over the post-damage period would restrict the regenerative potential of the myofibers and have serious implications for the functional capacity of the regenerating muscle due to the replacement of contractile tissue with non-contractile material (15, 65, 91). Thus, factors influencing the relative degree of muscle fiber regeneration compared to the amount of scar formation appear to be critical in functional muscle repair (Figure 3).

The growth factors released from macrophages, myogenic precursor cells and other cellular mediators of the damage process do not always enhance muscle regeneration. In fact, it has been shown that factors such TGFβ1 and myostatin (MSTN, alternatively named growth and differentiation factor-8, GDF-8) actually inhibit the skeletal muscle regenerative process (92-95). TGFβs, namely TGFβ1, -β2, -β3 and also MSTN identified as a new member of the TGFβ family (96), are important cytokines that regulate the homeostasis of numerous cellular functions and multiple biological processes including cell growth, proliferation and differentiation, apoptosis, ECM synthesis, cell motility and adhesion (97-101). TGFβ1 actions appear to occur predominately through the recently identified cytoplasmic proteins belonging to the Smad family, which are TGFβ receptor kinase substrates that translocate into the cell nucleus to act as transcription factors (99, 102). TGFβ1 plays an important role in regulating tissue repair and remodeling following damage, which, particularly during muscle regeneration, involves regulation of an immune response, myoblast fusion and inhibition of myoblast proliferation (1, 6). It controls proliferation of most cell types (101). Reduced proliferation and potent inhibition of cultured satellite cells by TGFβs were found *in vitro* (103-105), while IGF-I can override such effects of TGFβ1 (22, 106) and activate satellite cell proliferation (2, 81, 107). Moreover, TGFβ1 is associated with muscle fibrosis in various muscle diseases, such as Duchenne muscular dystrophy (37), and it was found that prevention of extreme fibrosis, *e.g. via* blocking of TGFβ1 with its antagonists (108), can improve muscle healing and increase the regenerative potential of muscle fibers (12, 37). TGFβ1 exerts its effects on cell proliferation, migration and differentiation in part through its capacity to modulate the deposition of ECM components (99). TGFβ1 acts during inflammation and fibrosis to stimulate the production of ECM proteins and, concurrently, to inhibit their enzymatic degradation by stimulating the production of proteinase inhibitors (37, 102, 109, 110) (Figure 3). It has been proposed that TGFβ1 overproduction and subsequent deregulation of its functions leads to progressive deposition of ECM and tissue fibrosis, and TGFβ1 antagonists may act as antifibrotic agents (108, 111).

A process of fibrosis is potentially initiated concomitantly with skeletal muscle regeneration, through the activation of TGFβ1, in order to quickly support the rejoining of the damaged myofibers (12). The complex biological process of fibrosis involves an acute inflammatory response and is predominantly characterized by a transient activation of fibroblasts to proliferate, producing an excessive and abnormal deposition of ECM components in the affected tissue (102, 111). It has been shown that TGFβ1 is a key regulator of ECM assembly and remodeling (111) and it appears to be one of the most potent profibrotic stimuli to fibroblasts (102). TGFβ1 attracts fibroblasts to the site of damage where they increase their synthesis of ECM proteins (1) (Figure 3). Fibroblasts also overproduce TGFβ1 and it is considered that they produce the majority of collagen and glycoproteins, which remodel the ECM, replacing connective tissue with scar tissue and reducing the degree of tissue vascularity (37). The net accumulation of collagen in tissue fibrosis is a result of an imbalance between enhanced production and deposition and impaired degradation of ECM components (111). Fibroblasts exhibit considerable phenotypic heterogeneity in conditions involving tissue remodeling and fibrosis. TGFβ1 has been shown to regulate collagen mRNA expression in rabbit cardiac fibroblasts (112). It also appears that the development of fibrosis is an eventual result of TGFβ1-induced differentiation of myoblasts and muscle-derived stem cells into myofibroblasts (95). Phenotypically fibroblast-like cells, myofibroblasts, have been hypothesized to play an integral role in fibrotic responses in the heart (113, 114) and TGFβ1 expression and protein production may act as an autocrine/paracrine stimulus for collagen formation in cardiac myofibroblasts (114). Activation of cardiac fibroblasts through uPA/plasmin-activation of TGFβ1 or matrix metalloproteinases (MMPs) has been proposed to cause cardiac fibrosis. MMPs facilitate myofibroblast migration and up-regulate the activity of potentially fibrogenic cytokines such as TNFα and TGFβ1, while increased MMP activity in the heart paradoxically appears to contribute to fibrosis (115-117). Myofibroblast activation by TGFβ1 increases production of ECM components such as collagen, laminin and fibronectin (118, 119). It should be mentioned, however, that synthesis of these connective tissue proteins after muscle damage appears to follow a certain sequence, reflecting a particular function that each carries out during muscle healing (91).

In addition, there is a growing body of evidence that the uPA/plasmin system has an important contribution in the homeostasis of muscle fibers and their ECM (14). Muscle damage induces the expression of plasminogen activation system components, which are involved in skeletal muscle regeneration and remodeling (17, 23, 120). There are studies demonstrating the recruitment of an extracellular proteolytic

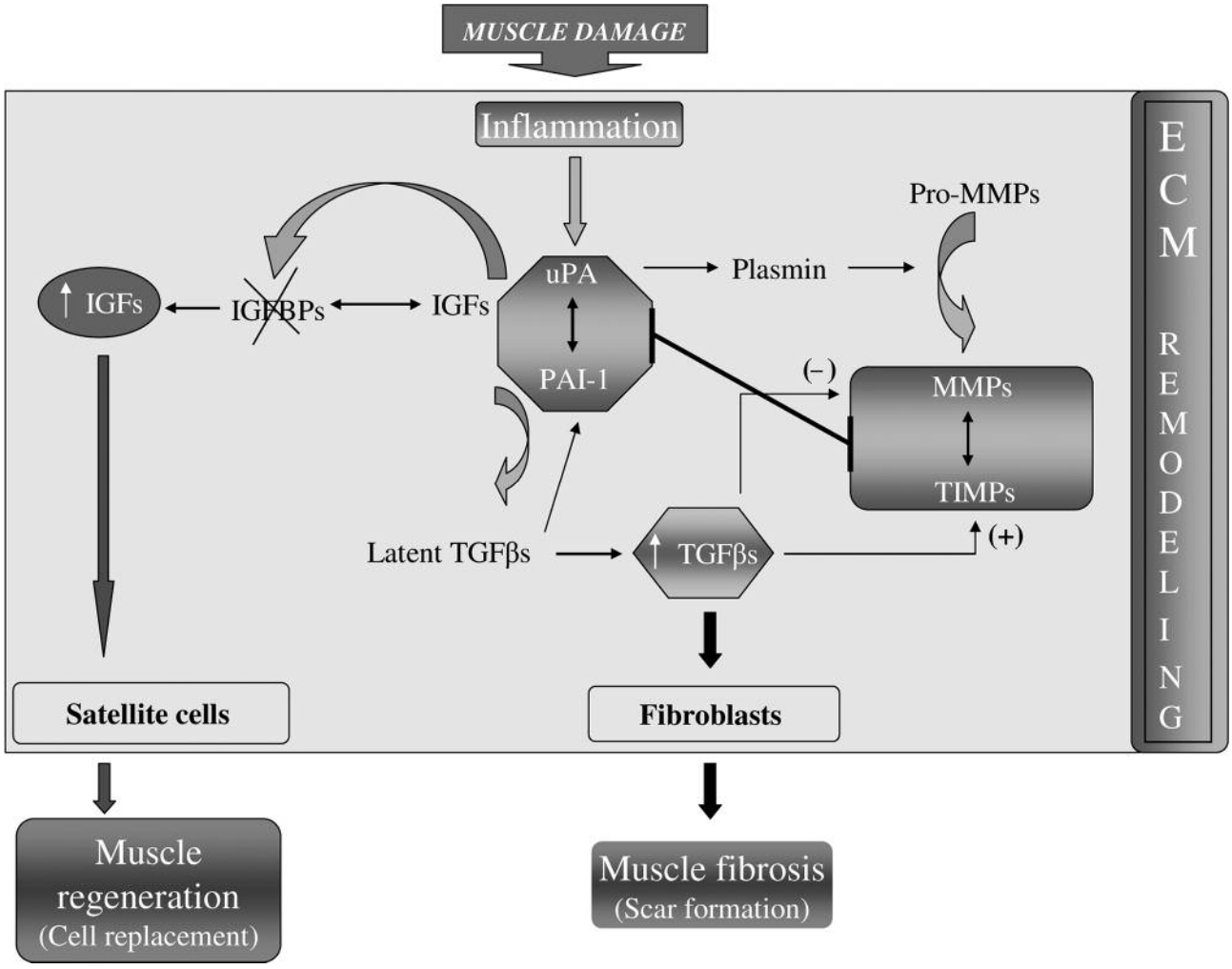

Figure 3. *Cellular and molecular events implicating a uPA/TGF β1 bioregulation system in ECM remodeling during the competitive processes of skeletal muscle regeneration and fibrosis following damage. ECM: Extracellular matrix; IGFs: insulin-like growth factors; IGFBPs: IGF binding proteins; MMPs: matrix metalloproteinases; PAI-1: plasminogen activator inhibitor-1; TGFβs: transforming growth factor β; TIMPs: tissue inhibitors of MMPs; uPA: urokinase-type plasminogen activator.*

cascade during these processes, where the plasminogen activation system constitutes an extensively used mechanism for the generation of the proteolytic activity in the ECM (14, 17, 23) (Figure 3). A number of extracellular proteolytic enzymes have been proposed to play an important role in the responses involved in muscle regeneration, *i.e.* in inflammation, the activation of satellite cells and the migration and further fusion of myoblasts to form terminal muscle fibers, while cell migration, ECM degradation and tissue remodeling are involved in processes regulated by the plasminogen activation/plasmin system (14). As discussed in detail below, components of the uPA/plasmin system probably regulate the expression and activity of cytokines involved in inflammatory process, since plasmin releases IL-1 derived from macrophages and also activates TGFβs (14). TGFβ-dependent and bFGF-dependent proliferation and invasion of satellite cells also require the cell-associated uPA/plasmin system (121).

uPA/plasmin System in Skeletal Muscle Regeneration

The main components in the plasminogen activation system include plasminogen (Plg), tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), urokinase-type plasminogen activator receptor (uPAR) and plasminogen activator inhibitors-1 and -2 (PAI-1, PAI-2) that control the activation of Plg (122). Most of these

components are expressed by human skeletal muscle (23, 123, 124), especially during the initial regeneration phase following *in vivo* muscle damage (17, 23, 120, 125, 126).

The end product of the plasminogen activation cascade is plasmin, as a result of the proteolytic conversion of the zymogen plasminogen (Plg) into this active serine proteinase (17). Plasmin is the major fibrinolytic enzyme responsible for the dissolution of fibrin at both intravascular and extravascular sites, and, together with other proteinases such as serine and metalloproteinases (MMPs), belongs to a group of carefully regulated and specialized matrix-degrading enzymes that appear to serve in matrix remodeling and cellular reorganization (17). Active plasmin is generated from the proteolytic, site-specific cleavage of its inactive precursor (Plg) by two distinct plasminogen activators, uPA and tPA (14). Although they have similar structure, as well as common physiological substrates and inhibitors, it appears that they have distinct physiological roles. uPA is selectively induced and, between the two pathways of Plg activation, the uPA-mediated pathway is the major one in skeletal muscle regeneration, while tPA activity is not required for efficient muscle regeneration *in vivo*, as demonstrated in uPA- and tPA-deficient mice following experimental damage of skeletal muscle (17, 23). Both uPA and plasmin activities are necessary, by contrast to the dispensable tPA activity, for the regeneration process following muscle damage (11, 14). Colocalizing uPA and plasminogen to the cell surface may optimize the rapid generation of plasmin (127). uPA is secreted as an inactive molecule, pro-enzyme uPA (pro-uPA), and only generates plasminogen activation after it has been converted to an active form (128). Since the result of plasminogen activation, plasmin, is the main factor involved in uPA activation and can efficiently activate pro-uPA, it leads to a phenomenon of reciprocal pro-enzyme activation, which is a central property of the plasminogen activation system (129, 130).

The formation of uPA-uPAR complex on the cell membrane localizes plasmin-mediated uPA activity in the pericellular space, which appears to have proteolytic, cell migratory, adhesive and chemotactic effects and plays a crucial role in cell migration and tissue remodeling (122, 131-133). However, the proteolytic conversion of the Plg into plasmin can occur without uPAR (134) and is a highly regulated and extensively used mechanism for the generation of extracellular proteolytic activity (17). Moreover, alpha-enolase constitutes a plasminogen receptor on several cell types that localizes and promotes plasminogen activation pericellularly, and appears to play a critical role in fibrinolysis and ECM remodeling (126). Unrestrained proteolytic activity would have deleterious effects on the cells, so plasmin activity is tightly regulated at the level of plasmin by alpha2-antiplasmin and at the level of PAs by PAI-1 and PAI-2, which regulate the proteolytic activity of plasminogen activators/plasmin. PAI-1 is the primary physiological inhibitor of uPA and regulates the levels of uPA-uPAR complex through promotion of its endocytosis (14, 124, 135, 136).

Accumulating evidence indicates that the different components of the plasminogen activation system play differential roles in muscle regeneration: the inhibition of uPA activity by PAI-1 appears to limit the efficiency of muscle regeneration, while PAI-1 deficiency reduces the extent of degeneration and accelerates skeletal muscle regeneration (11). The balance between PAI-1 and uPA may influence muscle regeneration through many pathways and previous *in vitro* studies have shown that PAI-1 and uPA can directly influence myogenesis (11, 121, 137-139), while there are also studies suggesting an integrated function rather than individual requirements of the different components of the plasminogen activation system in myogenesis (14, 140). Thus, plasmin activity is required for complete myoblast fusion and differentiation *in vitro*, as was shown after inhibition of its activity by α_2-antiplasmin, while uPA and Plg deficiency reduces the expression of muscle regeneration-specific genes, such as myogenin and MyoD (17, 23). PAI-1 deficiency demonstrated increased MyoD and developmental myosin expression after damage, as well as accelerated recovery of muscle morphology, protein levels and function compared with those in wild-type mice (11). uPA has been also suggested to be involved in regulation of migration and fusion of myoblasts (115, 123, 138, 141). It is able to induce proliferation, migration and fusion of satellite cells through both the proteolytic (*i.e.* plasmin formation, growth factor activation) and the non-proteolytic (*e.g.* uPAR-binding, matrix-binding) functions of uPA (121, 138, 139), while binding of uPA to its receptor seems to be necessary for human muscle satellite cell differentiation and may further concentrate and enhance uPA activity on the cell surface of migratory myoblasts (14, 123). Blocking uPA from binding to its receptor resulted in inhibition of myoblast migration and reduction of their differentiation into myotubes (124). Other studies have also shown that a decrease in uPA activity led to the inhibition of differentiation due to a lack of myoblast migration and fusion (142) and specific inhibition of uPA and plasmin proteolytic activity appears to abolish migration, fusion and differentiation of myoblasts *in vitro* (11, 138). Moreover, it has been proposed that the expression of alpha-enolase plasminogen receptor by migratory skeletal myoblasts could serve to enhance plasmin generation on their surface, facilitating efficient muscle regeneration (126).

Consistently with the biological context of skeletal muscle differentiation, it has been proposed that the function and cell localization of uPA are regulated to suit the changing needs of myoblasts (124). A regulated localization of uPA allows it to have a dual function: during differentiation, as a

predominantly cell-associated protease on undifferentiated myoblasts, providing the machinery that allows myoblast migration, which is necessary for their alignment and fusion; while as a soluble enzyme redistributed from the cell surface to the extracellular space, it regulates signals controlling myoblast differentiation (124).

Both uPA and PAI-1 may also contribute to tissue repair through pathways that do not involve plasminogen, including the regulation of growth factor activity and cell migration (11, 143, 144). It has also been proposed that they may modulate skeletal muscle regeneration through the regulation of ECM turnover. The removal of ECM barriers may be necessary for the efficient migration of satellite cells and macrophages during the repair process. More specifically, uPA and PAI-1 may modulate muscle regeneration by regulating the inflammatory response, since macrophage accumulation in the damaged muscle correlated with both the clearance of damaged tissue and the efficiency of regeneration (11). It was found that macrophages express uPA during the inflammatory response to skeletal muscle damage, as well as skeletal muscle stem cells later on during the regeneration process (17), although macrophages paradoxically appear to be important contributors to the development of cardiac fibrosis by their uPA overexpression (117). Regulation of uPA production is a critical event in inflammation and wound healing processes (128) and a reduced inflammatory response following muscle damage could be the cause of the reduced muscle regeneration in uPA-deficient mice, since the reduced accumulation of macrophages at the site of damage would alter the regeneration process (17, 23).

Besides its role as a protease, uPA may also stimulate chemotaxis of macrophages and neutrophils, as well as chemotaxis and proliferation of fibroblasts (133). In uPA- and Plg-deficient mouse models, it was shown that the absence of uPA and Plg resulted in less accumulation of macrophages and T lymphocytes at the site of muscle damage, suggesting a profound implication of uPA/plasmin activity in the inflammation process through its direction of cell migration (17, 23). The observed reduction of macrophage and T lymphocyte accumulation could be a result of either a reduced migratory capacity of these cells devoid of plasmin activity, or of their reduced capability of traversing fibrin-rich matrices, since fibrin could be a major matrix component impeding the migration of inflammatory cells in the absence of Plg (14, 23). Thus, it was suggested that plasmin plays a role in fibrin solubilization and in cellular reorganization of fibrin rich matrices, since a major role of plasmin is fibrinolysis, *i.e.* the degradation of fibrin deposits (23).

The importance of the uPA-mediated plasmin activity in clearance of extravascular muscle fibrin and in the normal regeneration process has been explored following muscle damage (17, 23), since the accumulation of fibrin in the extracellular basal membrane would impede inflammatory cell migration, obstruct the normal nutrition of the regenerating muscle and impair muscle regeneration (14). A pronounced regeneration defect, characterized by enhanced fibrosis and myotube degeneration, was shown following experimentally induced muscle damage in uPA- and Plg-deficient mouse models, which could indicate a protective role for Plg in skeletal muscle regeneration (17, 23). Increased fibrin deposition was detected in damaged skeletal muscle of uPA- and Plg-deficient mice, while systemic fibrinogen depletion appeared to restore muscle regeneration (17, 23). A pathogenic role in sustaining muscle regeneration was demonstrated for fibrin accumulation following muscle damage (14, 17, 23). It has been also proposed that plasmin deficiency may represent an impediment to cell migration because of the lack of its contribution to the degradation of matrix components other than fibrin (14). It appears that plasmin activity may be required to remove fibrin clots after damage and clear a path to allow the migration of different cells to the site of damage (11), while together with MMPs, it is needed to complete wound healing: uPA-uPAR complex participates in fibrinolysis, while MMPs have the capacity to split fibrin by acting as pericellular fibrinolysins (122, 145).

A proteolytic activation cascade initiated by uPA/plasmin is also involved in MMP activation during muscle regeneration (14, 146). Plasmin can directly activate several MMPs *in vivo* through proteolysis and it appears that the activation of MMP-2 and MMP-9 during skeletal muscle regeneration could be mediated by plasmin (14, 122, 146). These MMPs appear to be differentially expressed at different stages of degeneration and regeneration of experimentally damaged skeletal muscle (147). It has been proposed that MMP-9 expression is related to the inflammatory response and probably to the activation of satellite cells, since its expression is induced within 24 hours post damage and remains present for several days; while MMP-2 activation is concomitant with the regeneration of new myofibers (147). Meltrin-α and cathepsin B have been reported in myofiber degeneration-regeneration and appear to be required for myotube formation *in vitro* (147-150) since they can degrade other matrix components including collagen, elastin and laminin (151, 152). The degradation of collagen is catalysed by uPA through the formation of plasmin, which acts as an activator of the zymogen forms of collagenases (153).

Furthermore, increasing evidence supports the role of uPA in promoting invasiveness, fibrinolysis and matrix remodeling in various physiological and pathological processes other than muscle regeneration (154). uPA is expressed by many types of cancer cells (53, 155-157) and, based on its proteolytic capacity, it is thought to be important in tumor cell invasion (122, 158) and in optimizing the survival of metastatic cancer cells (61, 157, 159-163).

Finally, the uPA/plasmin system is implicated in several non-fibrinolytic processes, which lead to ECM degradation, either indirectly through the activation of latent MMPs, or directly by proteolytic cleavage of ECM components such as laminin, fibronectin and proteoglycans (14, 158, 164, 165). In many physiological and pathophysiological conditions, plasminogen activators and MMPs appear to act in concert (122). Thus, plasmin and uPA, together with some MMPs, can activate several latent growth factors and proteases, such as TGFβ1 and bFGF, whose activities have been proposed to be crucial for cell migration and tissue remodeling *in vivo* and *in vitro* (23, 146, 166-169).

uPA/Plasmin/TGFβ Bioregulation System in Skeletal Muscle Regeneration

Regulation of the bioactivity of various growth factors, such as TGFβ1 and bFGF, is a possible mechanism by which uPA and PAI-1 influence muscle regeneration (11). Plasmin-induced activation of latent TGFβ1 is facilitated by uPAR *via* bound uPA on the cell membrane (170). It is the secreted form that is ''latent'', *i.e.* the precursor molecule and a TGFβ binding protein (171); its activation requires extracellular proteolytic digestion by serine proteinases, resulting in the formation of active TGFβ1 dimer (166, 172). Activation of secreted latent-TGFβs through serine proteinases, such as plasmin, is a well regulated event that involves activation, localization and balance of different components of the system, representing a crucial event in regulating TGFβ activity (111, 142). The inhibition of activation of TGFβs by other serine proteinases, such as aprotinin, seems to stimulate skeletal muscle differentiation due to the reduced concentration of extracellularly active TGFβs (124). In fact, TGFβ1 inhibits myogenic differentiation (142) without inhibiting the expression or the binding activity of MRFs (173, 174). It was proposed that PAI-1 and a_2-antiplasmin could also stimulate differentiation by inhibiting plasmin-mediated activation of latent TGFβ1 (124). A decrease of uPA activity, together with an increase of PAI-1, may stimulate myogenic differentiation by inhibiting the formation of the uPA-mediated plasmin activity and hence the consequent activation of latent TGFβ1 (142).

Conversely, the regulation of uPA expression by TGFβ1, bFGF or MGF in various cell types (128, 175), as well as in myoblasts (141, 176), has also been documented. It was proposed that the regulated expression of uPA by these growth factors is involved in the responses of activated fibroblasts to tissue injury (128), or in the control of the proteolytic activity required during myoblast migration and fusion throughout the whole myogenic process (141), thus contributing to muscle regeneration (176). TGFβ1 induces a fibrinolytic pattern characterized by an up-regulation of PAI-1 and uPA receptor and a down-regulation of uPA in muscle satellite cells and it has been proposed that this pattern is exploited for satellite cell proliferation and invasion (121). The use of antibodies that blocked the interaction of PAI-1 with uPA was found to impair satellite cells migration and fusion *in vitro* (137, 140). In human keratinocytes, it was found that TGFβ1 enhanced uPA activity, resulting in the removal of PAI-1 from the ECM (177), while it stimulated the production of uPA and PAI-1 in rat osteoblast-like cells (178). It also appeared to stimulate uPA expression in myofibroblast-enriched cell cultures (128). In gingival fibroblasts, TGFβ1 inhibited uPA production, while it stimulated uPA and PAI-1 production in granulation-tissue fibroblasts, adding a crucial element to the cellular proteolytic balance where plasminogen activation may be inhibited due to PAI-1 synthesis (128). Taken together, these results could reinforce the proposed distinction in cell behavior among fibroblasts derived under different physiological conditions (128). Moreover, the concept of a molecule having opposite, biological context-specific effects is well established, hence a molecule may have opposite consequences in uninjured *versus* injured tissues, as has been proposed for PAI-1 (117). Thus, it was suggested that preservation of fibrous scar tissue may be the primary role of PAI-1 in wound healing, while in uninjured heart, its role may be the inhibition of uPA and the prevention of uPA-induced myocardiac collagen accumulation and fibrosis (117).

Another important activity of TGFβ1 is the transcriptional activation of genes that code for ECM proteins and their regulatory proteins, such as fibronectin, collagen and PAI-1 (101, 179, 180). Hence, TGFβ1 has a major influence on the reorganization of the ECM through the period of muscle regeneration and appears to be responsible for the remodeling of the ECM and basal membrane surrounding damaged myofibers and the activated satellite cells (1). As a result of repeated damage, TGFβ1 production increases, leading, in turn, to the progressive deposition of ECM; it has been proposed that such ECM deposition at the site of damage would lead to tissue fibrosis (181, 182).

Conclusion

The data discussed in this review suggest that plasminogen activation system components coordinate a regenerative response to muscle damage *via* proteolytic and non-proteolytic functions, compensatively to TGFβ-induced fibrotic responses following tissue damage. A regulated proteolytic cleavage *versus* enhanced deposition and impaired degradation of ECM components surrounding the damaged muscle appear to account for the contributory role of uPA and TGFβ1 to the balance between the competitive processes of muscle regeneration and fibrosis. This balance determines whether the damage will be resolved with muscle fiber replacement and reconstruction of a functional

contractile apparatus, or with scar formation. In the context of the beneficial outcome of the regeneration process following muscle damage, appropriate ECM degradation/reconstitution is crucial and the uPA/TGFβ1 bioregulation system appears to have a key role in this process as a main regulator of ECM remodeling.

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