DEVELOPING TISSUE ENGINEERING AND GENE THERAPY APPROACHES INVOLVING THE USE OF NERVE GROWTH FACTOR AND MUSCLE-DERIVED STEM CELLS TO IMPROVE THE REGENERATION OF DYSTROPHIC MUSCLE

by

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DEVELOPING TISSUE ENGINEERING AND GENE THERAPY APPROACHES INVOLVING THE USE OF NERVE GROWTH FACTOR AND MUSCLE-DERIVED STEM CELLS TO IMPROVE THE REGENERATION OF DYSTROPHIC MUSCLE

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In recent years, researchers have attempted to use gene- and cell-based therapies to restore dystrophin and alleviate the muscle weakness that results from Duchenne muscular dystrophy (DMD). Our research group has isolated a population of muscle-derived stem cells (MDSCs) from the postnatal skeletal muscle of mice. In comparison with satellite cells, MDSCs display an improved transplantation capacity in dystrophic *mdx* muscle that can be attributed to their ability to undergo long-term proliferation, self-renewal, and multipotent differentiation, including differentiation toward endothelial and neuronal lineages. The overall goal of this study was to investigate whether the use of nerve growth factor (NGF) improves the transplantation efficiency of MDSCs. Two methods of *in vitro* NGF stimulation were used: retroviral transduction of MDSCs with a CLNGF vector to constitutively express NGF and direct stimulation of MDSCs with NGF protein. Neither method of NGF treatment changed the marker profile or proliferation behavior of the MDSCs, but direct stimulation with NGF protein significantly delayed cells' *in vitro* differentiation ability. Stimulation with NGF also significantly enhanced the engraftment efficiency of MDSCs transplanted within the dystrophic muscle of *mdx* mice, resulting in better muscle regeneration. These findings highlight the importance of NGF as a modulatory molecule, the study of which will broaden our understanding of its biological role in the regeneration and repair of skeletal muscle by muscle-derived cells.

DESCRIPTORS

Cell Transplantation

Gene Therapy

Muscle Derived Stem Cells

Muscular Dystrophy

Muscle Regeneration

Nerve Growth Factor

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NOMENCLATURE

ACRONYMS, ABBREVIATIONS, AND SYMBOL DEFINITIONS

ACCW	Automated Cell's CytoWorks™
Bcl-2	A protein associated closely with progenitor cells
b-FGF	Basic fibroblast growth factor
bp	Base pair
BrdU	Bromodeoxyuridine
CD34	Cluster differentiation 34: A transmembrane cell surface glycoprotein restricted to the capillary endothelium and hematopoietic progenitor cells
CD45	Cluster differentiation 45: A transmembrane cell surface glycoprotein used as stem cell and hematopoietic cell marker
c-kit	Bone marrow-derived hematopoietic stem cell marker
CNPase	2', 3'-Cyclic-nucleotide 3'-phosphodiesterase
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle's Medium
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
EP cells	Early preplate cells
FACS	Fluorescence Activated cell Sorting

FGF-2	Fibroblast growth factor-2
Flk-1	A mouse homologue of KDR receptor, a VEGF receptor expressed on the common precursors of endothelial and hematopoietic stem cells
HBSS	Hanks' Balanced Salt Solution
H&E	Hematoxylin and eosin
HSC	Hematopoietic stem cells
IGF-1	Insulin-like growth factor type 1
LIF	Leukemia inhibitory factor
LP cells	Late preplate cells
LTP cells	Long-term proliferating cells
M-Cadherin	A transmembrane protein highly expressed in developing skeletal muscle
MDSCs	Unique population of muscle-derived stem cells isolated by researchers in our laboratory
MyHC	Myosin heavy chain
MyoD	A myogenic regulatory factor that is expressed at earlier stages of myogenic progression
NF	Neurofilament
NGF	Nerve growth factor
Pax7	A transcription factor required for satellite cell development
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PDGF	Platelet-derived growth factor
PM	Proliferation medium

Sca-1	Stem cell antigen-1
SCF	Stem cell factor
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
vWF	von Willebrand factor

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1.0 BACKGROUND

1.1 ADULT SKELETAL MUSCLE CHARACTERISTICS

The muscle fibers are the basic contractile units of skeletal muscles, individually surrounded by a connective tissue layer and grouped into bundles called perimysium to form a skeletal muscle (Figure 1.1A). Myofibers are multinucleated syncytia with their postmitotic myonuclei located at the periphery, as seen in the muscle cross-section stained with hematoxylin and eosin (H&E) (Figure 1.1B, arrow). As well as being rich in connective tissue, skeletal muscles are highly vascularized to provide essential nutrients for muscle function (Figure 1.1B, black arrowhead). As the myofiber matures, it is contacted by a single motor neuron that branches throughout the muscle (Figure 1.1B, white arrowhead). The functional properties of skeletal muscle including its contractile ability depend on the maintenance of a complex framework of myofibers, motor neurons, blood vessels, and extracellular connective tissue matrix. Therefore, revascularization, reinnervation, and reconstitution of the extracellular matrix are all essential aspects of the muscle regeneration process.

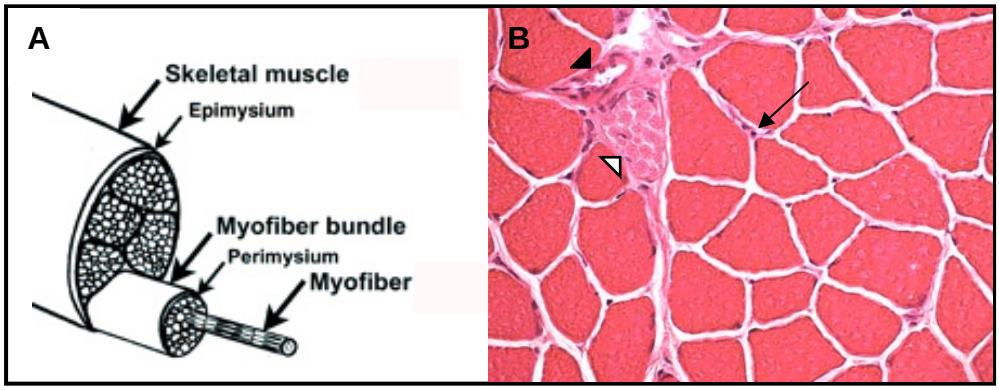

Figure 1.1 Morphological characteristics of adult mammalian skeletal muscle

1.2 MORPHOLOGICAL CHARACTERISTICS OF SKELETAL MUSCLE REGENERATION

Skeletal muscle is a heterogeneous tissue, containing vascular and neural cells in addition to the contractile myofibers. Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei [1, 2]. Minor lesions inflicted by day-to-day wear and tear elicit only a slow turnover of its constituent multinucleated muscle fibers. It is estimated that in a normal adult rat muscle, no more than 1–2% of myonuclei are replaced every week [2]. Nonetheless, mammalian skeletal muscle has the ability to complete a rapid and extensive regeneration in response to severe damage. Whether the muscle injury is inflicted by a direct trauma (i.e., extensive physical activity and especially resistance training) or innate genetic defects (i.e., DMD), muscle regeneration is characterized by two phases: a degenerative phase and a regenerative phase (Figure 1.2A).

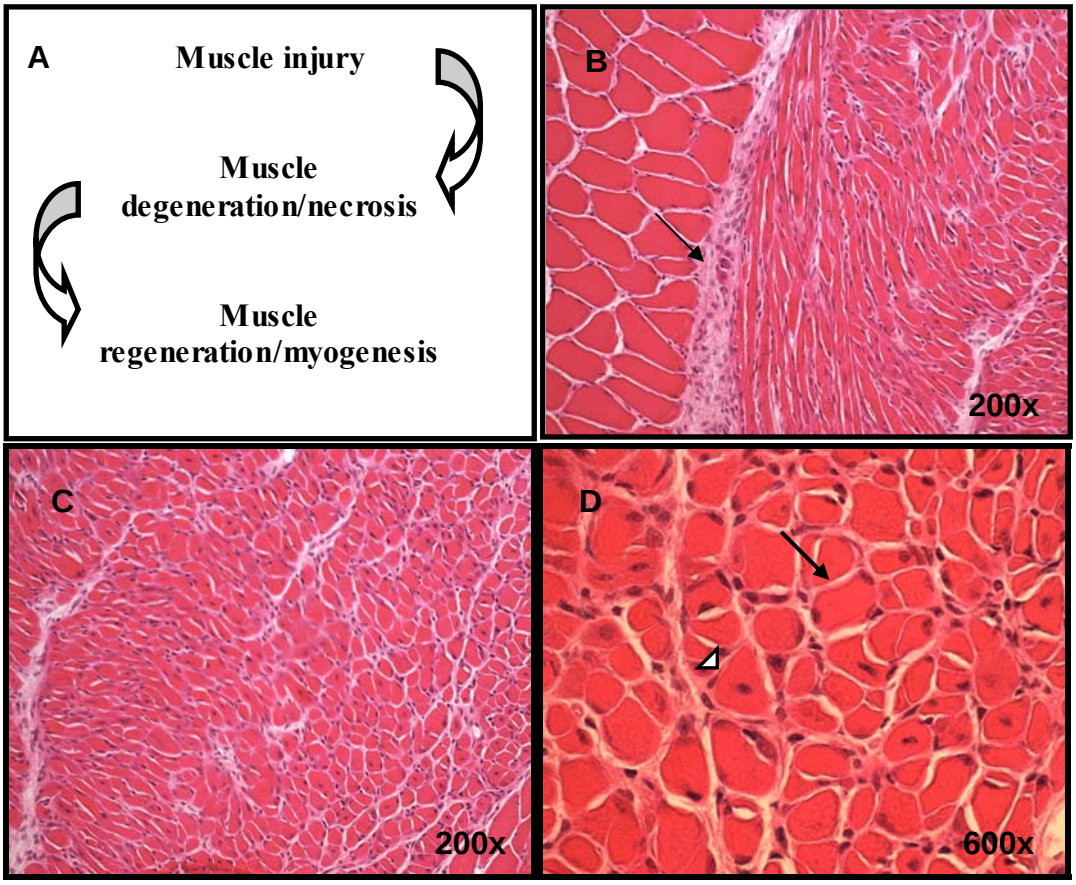

Figure 1.2 Skeletal muscle repair process

The initial event of muscle degeneration is necrosis of the muscle fibers. Figure 1.2B shows 10 μm cross sections of mice gastrocnemius muscle stained with H&E. Injury by cardiotoxin (CTX) injection in the muscle results in a rapid necrosis of myofibers and the activation of an inflammatory response leading to the loss of muscle architecture (compare Figure 1.2B with Figure 1.1B) including the formation of fibrosis (Figure 1.2B, arrow). This event is generally triggered by disruption of the myofiber sarcolemma resulting in increased myofiber permeability and disruption of the myofiber integrity. The early phase of muscle injury

is usually accompanied by the activation of mononucleated cells, principally inflammatory cells and myogenic cells. Present 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 (reviewed in Refs. [3, 4]). Neutrophils are the first inflammatory cells to invade the injured muscle, with a significant increase in their number being observed as early as 1–6 hours after myotoxin or exercise-induced muscle damage [5, 6]. After neutrophil infiltration and ~ 48 hours post-injury, macrophages become the predominant inflammatory cell type within the site of injury [4, 6]. Macrophages infiltrate the injured site to phagocytose cellular debris and may affect other aspects of muscle regeneration by activating myogenic cells [7-10]. Thus muscle fiber necrosis and increased number of nonmuscle mononucleate cells within the damaged site are the main histopathological characteristics of the early event following muscle injury (Figure 1.2C).

Myofiber regeneration is characterized by the activation of myogenic cells to proliferate, differentiate, and fuse to necrotic fibers for repair or to each other for new fiber formation. Notably, the expansion of myogenic cells provides a sufficient source of new myonuclei for muscle repair (reviewed in Refs. [11-13]). Numerous nuclear radiolabeling experiments have demonstrated the contribution of dividing myogenic cells to regenerate myofibers, by proliferation phase to form new muscle fibers followed by myogenic cells differentiation and fusion into mature muscle fibers [14-16]. Long-standing histological characteristics are still used to identify the mammalian skeletal muscle regeneration process. On muscle cross-sections, regenerating fibers are characterized by their small caliber and their centrally located myonuclei (Figure 1.2D, white arrowhead). Once fusion of myogenic cells is complete, newly formed myofibers increase in size, and myonuclei move to the periphery of the muscle fiber (Figure

1.2D, black arrow). Moreover, on muscle longitudinal sections and in isolated single muscle fibers, central myonuclei are observed in discrete portions of regenerating fibers or along the entire new fiber, suggesting that cell fusion is not diffuse during regeneration but rather focal to the site of injury [17]. In a time dependent manner after injury, the regenerated muscle fibers will become almost morphologically and functionally indistinguishable from undamaged muscle.

1.3 DUCHENNE MUSCULAR DYSTROPHY: A SKELETAL MUSCLE DISORDER

Duchenne muscular dystrophy (DMD) is a devastating muscle disease affecting about 1 in 3500 boys in all populations. It is an X-linked recessive disorder [18] where a mutation in the 2.5 million bp gene results in a failure to produce the 427 kDa protein called dystrophin at the sarcolemma of the muscle fibers [19-21]. Dystrophin and dystrophin associated protein complex (DAPC) form a link between the intracellular actin-based cytoskeleton and the extracellualr matrix (ECM) which plays a major role in maintaining plasma membrane integrity and stability [22-24] (Figure 1.3). Disruption of this complex leads to increased susceptibility to contraction-induced injury and sarcolemmal damage leading to myofiber necrosis [(Figure 1.4, compare normal human skeletal muscle (A) with dystrophic muscle (B)]. Indeed, upon muscle injury, a finely orchestrated set of cellular responses is activated, resulting in the regeneration of a well-innervated, fully vascularized, and contractile muscle apparatus. This repair process is present in DMD, but is not efficient enough to compensate for the necrotic process and fibrosis. Thus, in DMD patients, repeated cycles of degeneration-regeneration would exhaust the regenerating potential of myogenic precursor cells leading to massive activation of connective tissue that

results in muscle fibrosis [18] causing the muscle to undergo progressive weakness and wasting [25] which eventually leads to congestive cardiac and respiratory failure before adulthood. Despite extensive research in developing an effective approach of dystrophin delivery in dystrophic muscle (e.g., cell and gene therapy), there is no therapy capable of substantially slowing the course of the disorder and rescuing the diseased muscle tissue.

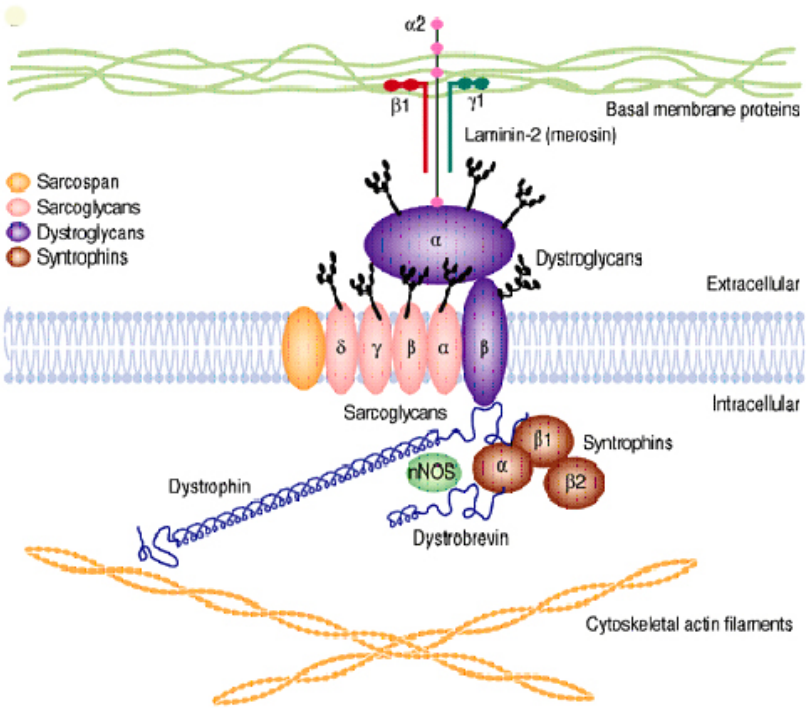

Figure 1.3 Membrane stabilization by dystrophin protein interaction with intracellular cytoskeleton, actin filaments, and the extracellular matrix (Adapted from Expert Reviews in Molecular Medicine 2002, Cambridge University Press)

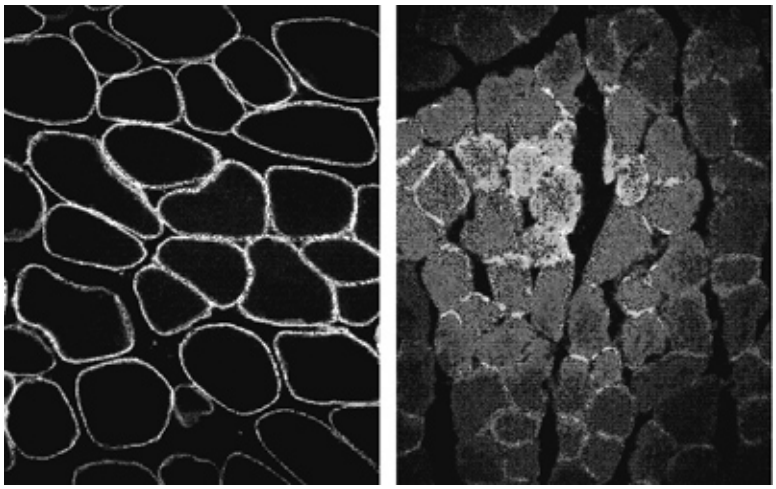

Figure 1.4 Immunohistochemical dystrophin labeling of skeletal muscle biopsies, taken from a normal individual versus a patient with Duchenne's muscular dystrophy (Courtesy of Johnny Huard, PhD, Pittsburgh, PA)

1.3.1 Animal Model of DMD

The biochemical and genetic animal homologue to human DMD is the *mdx* mouse. It is a spontaneously occurring mouse line deficient for dystrophin due to a point mutation in exon 23 of the dystrophin gene, which forms a premature stop codon [26]. The *mdx* mouse with less that 10% of the normal amount of dystrophin, and less than 0.1-0.01% of muscle fibers staining positively for dystrophin, is considered a true genetic homologue of DMD [27]. Although *mdx* mice are normal at birth, skeletal muscles show extensive signs of muscle degeneration by 3–5wk of age [28-30]. This acute muscle degeneration phase is accompanied by an effective regeneration process leading to a transient muscle hypertrophy [28, 29]. After this period, the degeneration/regeneration activity continues at lower and relatively constant levels throughout the life span of the animal. In fact, muscle of *mdx* mice differs from DMD patients in that it exhibits a greater degree of compensatory muscle regeneration and a scar fibrotic replacement

[28]. However, for reasons that remain unclear, in the older animals (~15 months), the muscle regeneration process is defective and the mice become extremely weak and die before wild-type littermates [31-33]. Such a milder histopathological modification is reflected by a slower progression of the disease.

1.4 CURRENT TREATMENTS AND THEIR LIMITATIONS

Two different therapeutic approaches have been explored in an effort to deliver normal dystrophin to murine and human dystrophic muscle: cell therapy based on myoblast transplantation (MT) and *ex vivo* gene therapy based on viral and non-viral vectors.

MT involves transplantation of primary myoblasts into defected muscle which contribute to the formation of new muscle fibers during repair and regeneration, and help in delivery of dystrophin [34-42]. The initial animal experiments and clinical trials, however, have suggested that although myoblast transplantation is feasible and introduced donor cells have fused with host myofibers around the site of injection and produced normal dystrophin [43-45], the amount of muscle fiber expressing dystrophin was not therapeutically significant and rather inefficient [39, 41, 42, 45, 46]. Donor cells may have also suffered from poor spread from the injection site, a low survival rate, and immune rejection by the host system [47-54]. In animal experiments, immunodeficient animals and/or immune-suppressive regimens [40, 44, 48, 55, 56], preirradiation of the injected muscle [44], and myonecrotic agents [35, 48] have been used extensively to improve the success of this technique. Although these approaches may be used to improve the restoration of dystrophin in *mdx* mice, the success of this technique remains rather

limited and for the most part, clinically impractical. To minimize the problem of immune rejection in human, autologous myoblast transfer has been employed, where primary myoblasts are removed from a patient via biopsy, expanded in cell culture, genetically manipulated with therapeutic genes, and re-introduced to the same patient. This technique therefore permits the introduction of myoblasts capable of expressing the transgene into defected muscle.

Virus-mediated delivery of the dystrophin gene to alleviate the biochemical deficiency in skeletal muscle became more of the research focus as a novel and attractive alternative. Viral and non-viral vectors have evolved rapidly. Plasmid, DNA, liposomes, viral vectors (e.g., adenovirus, adeno-associated virus, retrovirus, and herpes simplex virus) have already been used in the approach for gene delivery to muscles [57-64]. Two basic approaches for local gene therapy in the musculoskeletal system have been extensively investigated. Either the vectors are injected directly into the host tissue (*in vivo*) or the cells harvested via biopsy from different tissues (e.g., mesenchymal stem cells, muscle-derived cells, or dermal fibroblasts) are expanded *in vitro*, genetically engineered (transduced/transfected) *in vitro*, and re-introduced to the same patient (autologous) where they either replace degenerated fibers (as in the case of DMD) or form additional fibers expressing the desired gene (*ex vivo*) [65-67].

The advantage of direct (non-cell-based) approaches are low toxicity and immunogenicity [68], but the inability of most viral vectors to efficiently transduce or infect non-dividing muscle fibers is one of the major limitations [61, 69, 70]. In addition, the choice of the target cell is limited by the location of the defect, and insertion of genetic material into a specific type of cell is difficult to control. One way to overcome the difficulties of the direct approach and to help maximize the gene transfer efficiency and stabilize the expression was to develop an *ex vivo* method of gene transfer [71-80]. The *ex vivo* method has been

successfully used to deliver dystrophin in dystrophic muscle of *mdx* mice [81]. In humans, this method was used in the clinical setting to deliver and express factor IX for hemophilia B [80], interleukin 1 receptor antagonist protein for arthritis [82], human pro-insulin for diabetes [83], tyrosine hydroxylase for Parkinson's disease [84], and human growth factor for growth retardation [77]. The advantage of this method is multifaceted. First, the gene manipulation takes place outside the body (*in vitro*), thereby bypassing the need to inject massive amounts of virus into the patient. Also, we can select cells after transduction by special markers (e.g., neomycin) to increase the transduction efficiency and expression of the desired protein. In addition, this method give us enormous flexibility because we can choose the ideal cells for specific deficiency, For example, in case the of muscular dystrophies, muscle-derived stem cells would be an ideal choice for *ex vivo* gene therapy, with the reasons for this being discussed in detail in next the section.

It is important to realize that *ex vivo* gene therapy is not without limitations. It is clear that immunological problems associated with virally transduced cells still limit this technique. The efficiency of retroviral vector-mediated gene transfer is highly variable depending upon the vector design, the titer of the package virus, the type and species of the target cell, and is strictly dependent upon cell replication [85]. The integration of viral vectors into the genome of cells bears the risk of mutagenesis and the development of a potential malignancy. Consequently, all gene therapy techniques should be regarded with extreme caution. However, with viral vectors continuously being engineered to be less immunogenic, major advances can be expected in the near future.

1.5 MUSCLE-DERIVED STEM CELLS: POTENTIAL FOR MUSCLE REGENERATION

Stem cells are undifferentiated cells with unique features including i) appearance in early development and persistence throughout life; ii) self-renewal ability resulting in a large number of progeny; iii) long term proliferation potential while maintaining transient quiescent state; and iv) multilineage potential to enhance the new cell's incorporation into injured or diseased tissues. The stem cells' definition primarily emerged through extensive research on marker profiles, self-renewal, and the multi-potential behavior of hematopoietic stem cells (HSCs). On that note, the different populations of muscle-derived progenitor cells also appear to exhibit varied degrees of pluripotency. The most well-characterized muscle progenitor cells are satellite cells [86], usually referred to as "muscle stem cells." These unique undifferentiated myogenic cells have a committed fate and can regenerate injured skeletal muscle very efficiently [87, 88]. In addition to participating in the formation of myofibers, satellite cells can also differentiate into other lineages, such as adipocytic, osteoblastic, and chondrogenic [89, 90]. Satellite cells are integral to the development of skeletal muscle during embryogenesis and the regeneration of muscle fibers during postnatal life. During postnatal life, these cells are mitotically quiescent and reside between the basal lamina and the sarcolemma of myofibers. During the need for perceived growth or during post-natal reparative responses to stress or damage, satellite cells become activated, migrate, re-enter the cell cycle, differentiate, and fuse to form new regenerating myofibers [87, 88, 91]. Researchers have investigated the injection of satellite cells/myoblasts as a means to promote muscle repair in both animals and humans [88, 89]. The results suggest that, although the injected cells can improve muscle regeneration, various limitations such as poor

survival, limited dissemination of the injected cells, and immune rejection limit the success of this technique [47-54]. The development of using stem cells for transplantation may enable scientists to overcome these limitations because stem cells in theory are capable of long term proliferation, efficient self-renewal, and multilineage differentiation, all of which can improve the long-term survival of the cells post-transplantation [37, 92-94]. Investigators in our lab have obtained early myogenic progenitor cells highly proliferative, late-adhering, and Sca-1[+]/CD34 [+]/CD45[–]/c-Kit[–] called muscle-derived stem cells (MDSCs) using a preplating enrichment technique [95] (for details refer to Appendix A). This technique separates myogenic cells based on their adhesion to collagen-coated flasks. The fraction of more committed myogenic cells that were attached to bottom of flask at early time points [early preplate (EP)] exhibit in vitro marker profiles, as well as proliferation and fusion behavior comparable to that of satellite cells (Table 1.1). The cell population from the late preplate (LP) were called long-term proliferation (LTP) or muscle-derived stem cells (MDSCs).

Table 1.1 The Marker profile comparison between myogenic, stem, and blood cells, adapted from [96, 97]

Cell Types	Cell Markers *	EP	LTP (MDSCs)
Myogenic cells	Desmin	+	-/+
	M-cad	+	-
	Pax7	+	-
Stem cells	CD34	-/+	+(+)
	Sca-1	-(N)	+(+)
	Bcl-2	-(N)	+(+)
	Flk-1	N	+(N)
Blood Cells	c-Kit	-	-
	CD45	-	-

+: >90%, -: <5%, -/+: 5-30%, +/-: 40-80%, N: Not determined

*(refer to nomenclature for marker profiles name and description)

MDSCs have unique characteristics usually associated with non-committed progenitor cells such as i) long term proliferation ability *in vitro* and *in vivo*, ii) high self-renewal, iii) multipotent differentiation capability (particularly into blood vessel and nerve); and iv) immune-privileged behavior [96, 98]. In addition, MDSC are c-Kit[–]/CD45[–], eliminating their potential

hematopoietic origin. Moreover, they spontaneously express myogenic markers, MyoD and desmin (Table 1). Finally, the MDSCs have a high potential for myogenic differentiation *in vitro* and *in vivo*, when compared with satellite cells, and they display a significant improved transplantation capacity (higher number of dystrophin (+) myofibers) starting at 10 days up to 30 and 90 days post-transplantation in gastrocnemius of *mdx* mice [96].

Until recently, the satellite cells were presumed to be the sole source of myonuclei in muscle repair. However, recent findings have demonstrated the presence of multi-potential stem cells in various adult tissues, thereby challenging the widely held view that tissue-specific stem cells are predetermined to a specific tissue lineage. In fact, adult stem cells isolated from various tissues appear to differentiate *in vitro* and *in vivo* into multiple lineages depending on environmental cues. Progenitor cells isolated from bone marrow (BM) [37, 99-101], the adult musculature [37, 96, 102-104], the neuronal compartment [105, 106], and various mesenchymal tissues [107, 108] can differentiate into the myogenic lineage. In particular, BM and muscle adult stem cells have been shown to differentiate into muscle cells in vitro and to contribute to muscle regeneration in vivo (for review, see Refs. [12, 109, 110]). Although these various types of cells appear to be able to differentiate toward myogenic lineage their regeneration capacity in skeletal muscle is limited. Therefore, MDSCs compared to many other cell types are better candidates for skeletal muscle transplantations, particularly because these cells can highly regenerate skeletal muscle, be obtained easily from a superficial muscle biopsy (non-invasive manner) from patients, be expanded to the desire number, and most importantly, through multi-potential differentiation into endothelial and neural lineages, they may enhance the neural and vascular supply during muscle regeneration [96].

1.6 ROLE OF GROWTH FACTORS IN MUSCLE REGENERATION

General terms such as hormone, cytokine, and growth factor are principally of historical interest. Specific terms such as nerve growth factor were derived form early descriptions of a factor's action or source, consequently, such terms do not necessarily provide meaningful descriptions of their function but rather they exist as identifiers accepted by tradition. There are small proteins that serve as signaling agents for cells. Despite being present in plasma or tissue at concentrations that are generally measured in picomolar (ng/ml) range, growth factors are the principal effectors of such critical functions as cell division, matrix synthesis, and tissue differentiation in virtually every organ system [111].

Figure 1.5 shows a schematic of the mechanism by which growth factors regulate cell behavior in general. Growth factors elicit their cellular actions by binding to specific transmembrane receptor molecules (Receptor-binding domain) on their target cells membrane. These receptors serve as information transducers, converting information carried by a growth factor into a form that is usable by the cell. This ligand-receptor interaction activates the intracellular domain of the receptor (kinase domain) which possesses the enzymatic ability to transfer phosphate groups to proteins (kinase activity). This acts as an intracellular communication step. The presence or absence of the receptor defines whether or not a cell can respond to information in its external environment. Growth factor receptors are linked by a cascade of chemical reactions in the cytoplasm to various genes in the nucleus with the binding of transcription factors (proteins that bind to specific regulatory sequences of DNA) to activate gene transcription into messenger RNA (mRNA). The mRNA is then transcribed into protein to

be used within the cell. Often this cascade activates several genes at once. As a result, when use of a growth factor is considered to treat a specific cell defect, one must be aware that the factor may generate multiple effectors, even within a single cell type. While these results may be advantageous (as when both cell division matrix synthesis are desired for a repair response), it is a theoretical disadvantage if so-called mismatched effects (for example, cell division and matrix degradation) are stimulated simultaneously. Each family of growth factors has its own corresponding family of receptors. Despite marked differences in structure among receptor families, many of the key links in the gene-activating chain of reactions are shared by these families. Thus, binding of different growth factors to their respective receptors may lead to the same cellular response (such as cell division). Much more impressive than the similarities among post-receptor pathways, and much less well understood, are the differences. Many growth factors display pleiotrophic activity, eliciting a variety of effects in different stages of development. Although it is not yet clear how this remarkable versatility is achieved, these specific mechanisms probably will be important in the design of growth factor therapies that will be capable of activating only certain genes and not others. Knowledge about receptors is crucial to the successful application of growth factors as therapeutic agents. Clearly, treatment with growth factors will not help a problem caused by abnormalities in the receptor for that factor. In addition, the growth factors must be regulated, so as not extend treatment beyond the therapeutic level and to prevent overgrowth of various tissues in the target area.

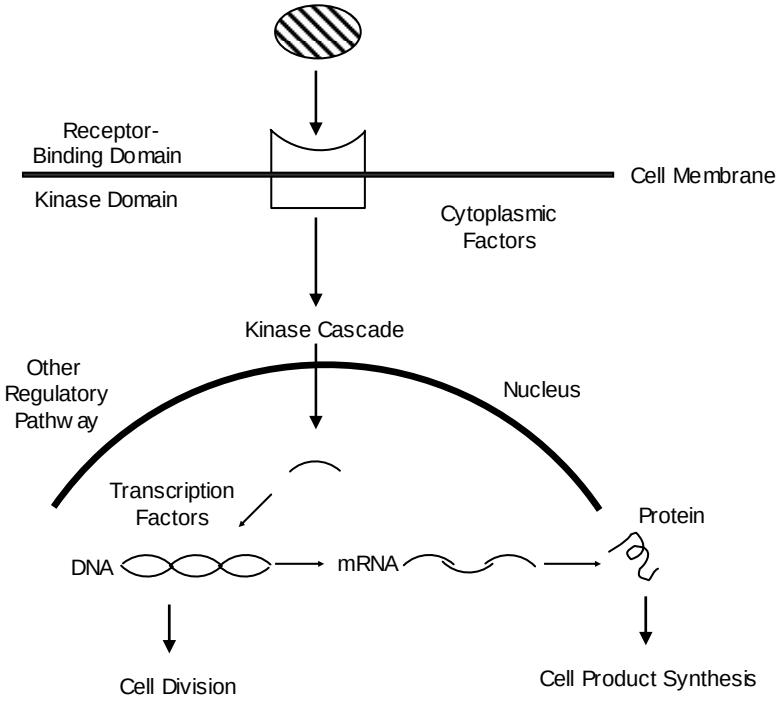

Figure 1.5 Schematic of the mechanism by which growth factors regulate cell behavior, adapted from [111]

It is well documented that growth factors can regulate skeletal myoblast proliferation and differentiation *in vitro* [112, 113] and act as stimulators or inhibitors (Table 1.2). Various growth factors are thought to play a role in different stages of muscle generation [114, 115] by stimulating satellite cells to release, proliferate, and terminally differentiate [116-119]. These factors that regulate muscle regeneration *in vivo* must act to maintain a balance between growth and differentiation in order for restoration of normal tissue architecture to occur. It is likely that a combination of many growth factors is involved in the regulation of myogenesis during muscle development and regeneration. The insulin-line growth factor (IGFs), basic fibroblast growth

factor (bFGF), platelet-derived growth factor (PDGF), leukemia inhibitory factor (LIF), and transforming growth factor beta (TGF-β) have been localized to muscle cells or other cell types present in muscle tissue [113, 120-123]. Expression of bFGF and the IGFs have been examined in regenerating skeletal muscles by immunocytochemistry and in situ hybridization, and they has been found to be up-regulated compared to non-injured muscles [121, 124, 125]. The pre-treatment of myogenic cells in culture with bFGF has shown to promote cell proliferation, resulting in an up to four-fold increase in myofiber regeneration [126]. In the mouse model, the IGF-1, bFGF, and to a lesser extent, nerve growth factor (NGF), directly injected post-injury have enhanced muscle regeneration in lacerated, contused, and strain-injured muscle [127-138].

Table 1.2 Effect of growth factors on the proliferation and fusion of myoblasts *in vitro*, adapted from [130]

Growth Factor*	**Proliferation**	**Fusion**
bFGF	Stimulates	Stimulates
IGF-1	Stimulates	Stimulates
NGF	Stimulates	Stimulates
α-FGF	Inhibits	Inhibits
PDGF	Inhibits	Inhibits
EGF	Inhibits	Inhibits
TGF-α	Inhibits	Inhibits
TGF-β	Inhibits	Inhibits

*(refer to nomenclature for the list of growth factors name)

While, in the past few years, much has been learned about the effects of these factors on musculoskeletal tissues, and a few notable therapeutic successes have been achieved, the understanding of their role in muscle diseases remains rudimentary. With continued progress in the basic science and clinical investigation of these factors, it is probable that they will become the method of choice for the prevention and treatment of a variety of current unsolved problems.

1.6.1 Nerve Growth Factor— NGF

The term "NGF" was introduced 50 years ago as a target-derived neutrophic factor that is essential for the development, survival, and differentiation of developing neurons in the peripheral sympathetic and sensory neurons [139, 140]. NGF belongs to the neurotrophin family of growth factors that are synthesized as precursors (pro-neurotrophins) that are proteolytically cleaved to mature and biologically active form [141]. Because neurotrophins are normally expressed at low levels, little is known about their processing and secretion by neurons and non-neuronal cells in vivo.

The ideas about the biological role of NGF have been dominated by concepts that arose from studies on the differentiation and survival of young neurons. Until recently, the expectation was that the biology of NGF would center on the classical target-derived neurotrophic factor paradigm in which NGF released by postsynaptic targets acts on presynaptic neurons to build or maintain functional contacts and enhance the function of well-defined neural circuits. Although this paradigm undoubtedly plays a critical role in both the peripheral nervous system (PNS) and central nervous system (CNS), it does not appear to be the sole role for NGF actions suggesting this molecule may have broader physiological effects. For example, NGF has been reported to be

expressed by the luminal epithelium of the epididymis and the germ cells of the rat and mouse testes [142], and the circulation levels of NGF change not only with age, but also during neuroendocrine disregulation, after neurological insults, and during autoimmune and allergic diseases [143-149]. More relevant to the work being presented here, NGF has been shown to promote the differentiation of muscle cells in culture [150]. Furthermore, Rende at al. in 2000 showed that NGF expression in skeletal muscle is not only associated with a classical target-derived neurotrophic function for peripheral nervous system neurons, but also with an autocrine action (locally binding to cell-surface receptors on the same cells that produced it) which affects the proliferation, fusion into myotubes, and cell morphology of developing myoblasts, thereby suggesting that among other roles, endogenous NGF signaling through both neurons and non-neuronal cells subserves neuroprotective functions and facilitates muscle repair. The regulated expression of NGF throughout adult life suggests multiple functions for NGF signaling, many of which are poorly understood.

1.6.2 NGF Receptors: TrkA and $p75^{NTR}$

The NGF functions as a dimmer of identical subunits linked together by noncovalent bonds and with molecular mass of about 26 kDa [151]. The functional activity of NGF is mediated by two classes of receptors: high-affinity receptor, TrkA (kd =10-11 M), and low-affinity receptor, $p75^{NTR}$ (Kd=10-9 M) [152-156].

A schematic drawing of the structural features of Trk and $p75^{NTR}$ is displayed in Figure 1.6. TrkA is a 140 kDs single-pass transmembrane protein with a single transmembrane domain and a single cytoplasmic tyrosine kinase domain that serves as a receptor tyrosin kinase (RTK)

for NGF. Neurotrophin-mediated activation of TrkA receptor leads to a variety of biological responses and elicits many of the classical neurotrophic actions ascribed to NGF, including proliferation and survival, axonal growth and remodeling, assembly and remodeling of cytoskeleton [157-159]. $p75^{NTR}$ is a 75-kDs transmembrane glycoprotein that belongs to a superfamily of cytokine receptors which includes TNF receptors (TNFR), Fas, CD27, CD40, and CD30. $p75^{NTR}$ binds all members of the neurotrophin family with approximately equal nanomolar affinity, and is therefore referred to as a neurotrophin receptor, and not as an NGF receptor. $p75^{NTR}$ has a distinctive extracellular-domain sequence that differs with TrkA, with four distinct cytosine-rich domains that are responsible for ligand-binding. The precise role of $p75^{NTR}$ in NGF signal transduction has not been fully elucidated. Several studies have indicated that stimulated of TrkA is necessary and sufficient to elicit a full biologic response and is required for cell survival, while other reports have highlighted the crucial role of the association of TrkA and $p75^{NTR}$ in regulating NGF biological activities on NGF-responsive cells [155]. These studies shed light on the often conflicting roles for $p75^{NTR}$ in mediating apoptosis and in augmentation Trk-induced survival and differentiation. The selectivity of proNGF for $p75^{NTR}$ suggests that its local secretion may determine whether apoptotic or survival actions predominate.

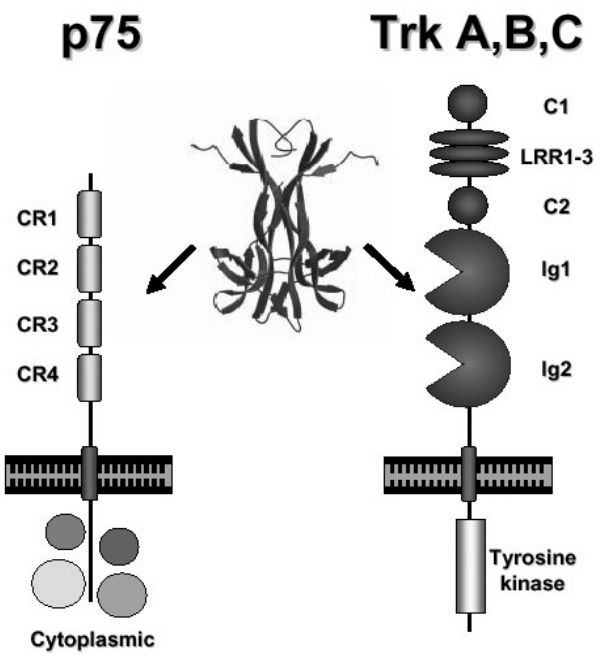

Figure 1.6 A schematic drawing of the structural features of Trk and p75 receptor, adapted from www.izn.uni-heidelberg.edu.de/download/LFB2004/Tucker.pdf

1.7 MDSCs & NGF AS A REMEDY FOR TISSUE ENGINEERING

Tissue Engineering has been defined as the application of the principles and methods of engineering and life sciences towards the development of biological substitutes to restore, maintain, or improve functions. It is our expectation that a cell-based therapy can help to provide a solution to the growing problem of tissue and organ failure. Therefore, there has been growing enthusiasm for a tissue-engineering approach that aims at utilizing stem cells to deliver genes of interest to improve healing of the musculoskeletal system. The feasibility of direct injection of

human recombinant growth factors for treatment of muscle injuries due to its safety and ease is practically hindered because high concentrations of the growth factor are often required to produce the beneficial effect. Indeed, studies have shown that growth factors exhibit a dose-dependent effect on myoblasts proliferation and differentiation *in vitro*, while *in vivo*, three consecutive injections of high concentration (100 ng) NGF, IGF-1, and bFGF are required to improve muscle healing in the mice model [127-138]. The relatively short biological half-life, the bloodstream's rapid clearance, and the limited adequate duration of growth factor delivery are the main reasons why large concentrations of growth factors are typically required.

In this regard, isolated muscle-derived stem cells obtained through the preplate technique would be the perfect candidate for cell-mediated therapy and the perfect choice for *ex vivo* gene delivery since these cells show i) long-term proliferation and self-renewal capacity ii) multilineage differentiation ability (e.g., myogenic, neurogenic, osteogenic, adipogenic, hematopoietic, and chondrogenic), and iii) potential immune-privileged behavior (i.e., the failure to trigger the immune response). While the direct *in vivo* injection of growth factors or stem cells is technically less complex, the indirect, *ex vivo*, gene delivery technique is safer because the gene manipulation (i.e. genetically engineering using viral vectors) takes place under controlled conditions outside the body. With the *ex vivo* approach, growth factors can be delivered using endogenous cells. These cells are capable of responding to stimuli created by injured tissue and can participate in healing process more effectively by delaying, ameliorating, or arresting the further degeneration.

2.0 PROJECT OBJECTIVES

The overall goal of this project is to evaluate a novel tissue engineering method for skeletal muscle repair. Both muscle-derived stem cell transplantation and *ex vivo* gene therapy are excellent candidates for growth factor delivery. We propose that direct stimulation of MDSCs with nerve growth factor (NGF) protein and genetically engineering MDSCs with retroviral transduction used for sustained delivery of NGF can hold great promise as the basis for tissue engineering and gene therapy applications to acquire muscle healing. The development of such a novel therapeutic strategy hold tremendous potential for the treatment of pathological conditions associated with poor muscle regenerative capacity, such as those observed during injuries and muscular dystrophies.

2.1 OBJECTIVE 1: EXAMINE THE PHENOTYPIC EFFECT OF NGF STIMULATION ON MDSCS *IN VITRO*.

A variety of growth factors epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1), and stem cell factor (SCF) have been shown to be potent stimulators of the proliferation and myogenic differentiation of MDSCs in vitro [160]. The current study aims to address the phenotypic behavior (proliferation and fusion) of MDSCs

under the influence of NGF. Two stem cell markers: Sca-1 and CD34 and two myogenic markers: desmin and Pax7 will be examined before and after NGF stimulation. The proliferation kinetics and myogenic cell behavior of the control and stimulated MDSCs will be monitored using a novel bioinformatic cell culture imaging system, allowing time-lapse image analysis, including cell division time and fusion behavior. The myogenic differentiation capacity of these cells will be investigated by the ability of cells to differentiate in vitro and to fuse and form myotubes. We hypothesize that MDSCs' marker profiles will remain the same while the myogenic marker expression, proliferation kinetics, and myogenic differentiation will change following NGF stimulation.

2.2 OBJECTIVE 2: EVALUATE THE EFFECT OF NGF STIMULATION ON MDSCS' REGENERATION CAPACITY.

Our preliminary studies indicate that growth factors promote the multipotent differentiation of MDSCs into muscle fibers, blood vessels, and peripheral nerve. They may also contribute to the formation of functional skeletal muscle tissue with adequate vascular and neural supplies. We hypothesize that NGF stimulation will promote multilineage differentiation, which in turn enhances engraftment efficiency (higher number of dystrophin-positive myofibers) and improves the regeneration capacity of MDSCs in *mdx* skeletal muscle.

3.0 INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive muscle disorder characterized by dystrophin deficiency that results in initial necrosis of muscle fibers, which in turn leads to progressive muscle weakness and, ultimately, death before or shortly after patients reach the second decade of life [19, 161]. Dystrophic muscle has a heightened susceptibility to structural damage and a decreased capacity to undergo self-repair.

Researchers have localized dystrophin in the sarcolemma of myofiber [162-164], where it is thought to play a role in maintaining plasma membrane integrity and stability [22-24]. Like the muscles of humans with DMD, the muscles of *mdx* mice are dystrophin deficient [165], which makes the *mdx* mouse an excellent genetic and biochemical model for DMD. Unlike the muscles of humans with DMD, however, the muscles of *mdx* mice show no progressive weakness or progressive fibrosis; instead, they exhibit muscle hypertrophy and maintain their regeneration capacity [166].

Although lack of dystrophin leads to progressive muscle degeneration, the evolution of DMD is likely to be dependent upon other factors, such as insufficient expression of growth-associated proteins. After skeletal muscle damage, quiescent myogenic stem cells, which are normally embedded in the basal lamina of the muscle fibers, are activated and migrate toward the damaged area, where they undergo a cycle of proliferation, fusion, and differentiation that culminates in the generation of myofibers that replace the damaged ones [167]. In most cases,

myogenic differentiation is measured as increased expression of muscle cells functions such as creatine kinase activity, fusion of single cells to form myotubes, or elevation of myosin heavy or light chain expression, or other proteins associated with the contractile apparatus. Various growth factors can regulate skeletal myoblast proliferation and differentiation and are known to play a role in different stages of new muscle regeneration, therefore enhancing the healing process [111, 114, 115]. In addition to stimulating cell proliferation, growth factors can maintain cell survival and regulate critical intracellular signal transduction pathways [168] under conditions that otherwise lead to apoptotic death.

To date, the list of growth factors known to affect the behavior of skeletal muscle cells or to be expressed in skeletal muscle tissue is extensive. However, few studies have investigated the role of NGF during skeletal muscle regeneration, and its exact mechanism of activity is poorly understood. In addition to acting as a target-derived factor for developing neurons, NGF has an autocrine effect on myoblast proliferation and fusion [169-171]. Moreover, adult knockout mice expressing a neutralizing antibody against NGF display a severe dystrophy and reduced muscle mass [172, 173]. Recent evidence suggests that NGF acts by binding to the high-affinity tyrosine-kinase receptor (TrkA) and the low-affinity p75-neurotrophin receptor ($p75^{NTR}$). TrkA is found in developing adult rat myoblasts [174] and during differentiation of muscle cells [170]. NGF and $p75^{NTR}$ are widely expressed in myoblasts, human myocyte cultures, and regenerating myofibers in the muscle of DMD patients [175, 176].

Previous studies in our laboratory have revealed that the delivery of human recombinant NGF protein via direct intramuscular injection improves both muscle recovery [130] and muscle force (fast-twitch strength) after strain injury [129]. However, the efficiency of direct intramuscular injection of growth factors varies according to the type of injury encountered and

is limited by the need to maintain high enough concentrations to achieve a therapeutic effect. In addition, the use of growth factor proteins to promote healing is severely hindered by the difficulty of ensuring their delivery to the injured site [177], their short biological half-lives [66, 177], and the bloodstream's rapid clearance of these molecules. For the study reported here, we used a combination of MDSC-based gene therapy and direct stimulation with NGF protein to examine the effects of NGF on the proliferation and differentiation capacity of MDSCs *in vitro* and their regeneration efficiency in *mdx* muscle *in vivo*.

4.0 MATERIALS AND METHODS

4.1 ANIMALS

Mdx mice (C57BL/10SCsn DMD^{mdx}/J) were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal protocols used for these experiments were approved by the Children's Hospital of Pittsburgh's IACUC committee (protocol # 3/02).

4.2 CELL ISOLATION AND CULTURING

A previously described modified preplate technique [96] was used to obtain MDSCs from normal (C57BL/6J) 3-week-old female mice. Cells were cultured at an initial density of 450 cells/cm^2 in flasks coated with collagen type I (Sigma-Aldrich Corp., St. Louis, MO) and maintained in proliferation medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin; all reagents from Gibco, Inc., Carlsbad, CA) containing 0.5% chick embryo extract (Accurate Chemical, Westbury, NY). After 2 days of growth (confluency < 50%), the

cells were trypsinized, counted, and replated to generate the quantity of cells needed for each experiment. The preplating technique was used to purify slowly adhering MDSCs if necessary.

4.3 GENERATION OF RETROVIRAL VECTOR EXPRESSING NGF

NGF cDNA was amplified from plasmid pSP72NGFpA (provided by Dr. Paul Robbins) using primers NGF1 (agg cgg ccg ccc acc atg ctg tgc ctc aag cca gtg aaa) and NGF2 (tca aga tct tca gcc tct tct tgt agc ctt cct) and Pfu DNA polymerase (Stratagene, CA). The PCR product was cut with restriction enzymes Not I and Bg III and cloned into the same 2 sites of retroviral vector pCLX [178]. The vector DNA was converted into a replication-defective retrovirus by co-transfection (with calcium-phosphate precipitation) into packaging cell line GP-293 (Clontech, Palo Alto, CA) with a plasmid, pVSVG, which expressed vascular stomatitis virus glycoprotein as the viral envelope. Conditioned medium containing retroviral vector was stored at -80 ^{o}C until use.

4.4 STIMULATION OR RETROVIRAL TRANSDUCTION OF MDSCS WITH NGF

MDSCs were plated at 20%–30% initial confluency and either stimulated with 100 ng/ml of NGF (Sigma-Aldrich) for 7 days (S-MDSCs) or retrovirally transduced with the CLNGF vector to express NGF (E-MDSCs) at a multiplicity of infection of 5 in the presence of polybrene (8 μg/ml). Normal MDSCs neither stimulated nor transduced served as the control group (C-MDSCs). E-, S-, and C-MDSCs were expanded for one week in proliferation medium (20%

serum) at an initial density of $8x10^4$cells/well on collagen type I–coated 6-well plates, where they remained for 48 hours; proliferation medium was then replaced with low-serum medium (2% FBS) in which cells were cultured for an additional 24 hours. Tissue culture supernatant was collected and spun at 1200 rpm for 5 minutes at 4 °C, and the level of functional NGF secreted by the cells in the tissue culture supernatant was measured by enzyme-linked immunosorbant assay (ELISA) (NGF Emax® Immunoassay System kit, Promega, WI) performed as detailed in the manufacturer's instructions.

4.5 CELL CHARACTERIZATION BY FLOW CYTOMETRY

Flow cytometry was used to analyze the expression of the cell surface markers cluster differentiation (CD34) and stem cell antigen-1 (Sca-1). Cultured cells were trypsinized, spun, washed in a buffer made of phosphate buffered saline (PBS) (Dulbecco phosphate-buffer salt solution 1X; Mediatech, Inc., Herndon, VA) containing 0.5% bovine serum albumin (BSA) (ICN Biomedicals) and 0.1% sodium azide (Sigma-Aldrich), and then counted. After trypsinization, the cells were maintained on ice for the remainder of the procedure. The cells were then divided into equal aliquots and spun into a pellet. A 1:10 mouse serum (Sigma-Aldrich) was used to resuspend each pellet, and the suspensions were incubated for 10 minutes on ice. Predetermined, optimal amounts of both direct and biotin-conjugated rat anti-mouse monoclonal antibodies (CD34 and Sca-1) were placed in each tube for 30 minutes. Each experimental tube received FITC-conjugate for CD34 and biotin-conjugated Sca-1. A separate cell portion received equivalent amounts of isotype control antibodies. After several rinses, all fractions (including the

controls) were labeled with streptavidin-allophycocyanine (APC) for 20 minutes. Just before the analysis, 7-amino-actinomycin D (7-AAD) was added to each tube to exclude non-viable cells from the analysis. All antibodies, including APC and 7-AAD, were purchased from BD PharMingen (San Diego, CA). At least 10,000 live cell events were analyzed via flow cytometry (FACS Aria cytometer using FACS Diva software, Becton Dickinson, San Diego, CA).

4.6 MYOGENIC MARKER EXPRESSION BY IMMUNOCYTOCHEMISTRY

A fraction of each group was evaluated by immunofluorescent staining for expression of the myogenic proteins desmin and Pax-7. Analysis was performed on methanol-fixed cells that were blocked with 5% goat serum in PBS for 1 hour. The cells were incubated for 1 hour with the following primary antibodies: mouse IgG anti-desmin (1:250; Sigma-Aldrich) and mouse anti-Pax-7 (1:50; R&D Systems, Minneapolis, MN). After being rinsed thoroughly with PBS, the cells were incubated for 30 minutes with the secondary antibody biotinylated goat anti-mouse IgG (1:250; Vector, Burlingame, CA). To fluorescently label the antigenic binding, the cells were washed and incubated with Streptavidin-Cy3 (1:500; Sigma-Aldrich) for 10 minutes; nuclei were then counterstained with DAPI (1:100; Sigma-Aldrich) in PBS. All dilutions were in 5% goat serum in PBS at room temperature. Negative control staining was performed using an identical procedure, with omission of the primary antibody. Northern Eclipse software (v.6.0, Empix Imaging, Mississauga, ON, Canada) was used to quantify the percentage of myogenic cells as the ratio of cells that strongly expressed desmin or Pax-7 to the total number of nuclei in 10 randomly chosen fields at 200x magnification.

4.7 CELL DIVISION ANALYSIS

The time lapse between cytokinesis events were recorded as the length of the cell division cycle from the time-lapsed video images from a novel microscope imaging system described below. For each population, 100 cells were selected and tracked. The division time (DT) of each cell was determined by direct observation of the cells from the time-lapsed video record. The initiation of cell division was marked at the time when two daughter cells were formed, and these cells were followed until their respective division. The lapse time between those two division events was recorded as the length of the cell division cycle. The average population doubling time (PDT) was calculated by fitting an exponential trend line to several measured data points. PDT was estimated by using the software package SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) to perform nonlinear regression in order to generate the best fit to the curve. The fraction of daughter cells that were actively entering the mitotic cell cycle (α) was calculated from experimental data using PDT and DT and solving the re-arranged Sherley model to obtain the correlation coefficient (R^2) for the nonlinear regression [179, 180], a value that indicates how well the data actually fit the model (such that $0 < R^2 < 1.0$).

4.7.1 Experimental Settings

Various cell culture and imaging settings such as cell plating densities, image acquisition intervals, duration of cell growth, viewfield limits, and optimized phase contrast have already

been tested [181]. These settings are important in determining the best densities at which the cells are able to interact with each other, while remaining visible. This way, the events can be captured accurately without the cells being lost to follow-up.

MDSCs from each group were plated at an initial density of 450-500 cells/well in a collagen type I–coated 12-well plate in 20% serum medium, as described in section 4.2. Cells were allowed to adhere for 6-12 hours. Using the microscopic imaging system, time-lapsed visible imaging was obtained for individual cells and subsequently, for growing colonies [182]. In these experiments, groups of 4 to 6 cells were selected for imaging. Coordinate positions of these view fields were recorded by the CytoWorks software program that subsequently controls the time and position of stage movement. Images of each view field were acquired at 10-minute intervals for 4 days. For each cell type and treatment condition, 15 view fields were selected from 6 wells. Cell population growth was monitored by counting the total number of cells, N, in the view field at 12-hour intervals.

4.7.2 Imaging System

Our imaging system showing in Figure 4.1 consists of a customized mechanical stage containing a cell culture system and microscopy (Nikon Eclipse TE-2000-U microscope) specifically designed for time-lapsed imaging over long periods of time. In the system, an environmentally-controlled biobox incubator is mounted to the stage of the microscope, which is in turn linked to a CCD camera (Automated Cell Technologies, Inc., Pittsburgh, PA). The x-y position of the stage is under the control of the user such that any position on a culture plate can be selected for viewing. The system accommodates any sized multi-well plates from 6-well to 384-well.

Multiple viewfields can be selected in each well. Each view field or x-y position that is selected becomes the location for time-lapsed imaging. Individual images are directly recorded in jpeg from the continuous images. Automated measures of the total numbers of cells can made at any time point and the division times can accurately determined by direct observation of cytokinesis.

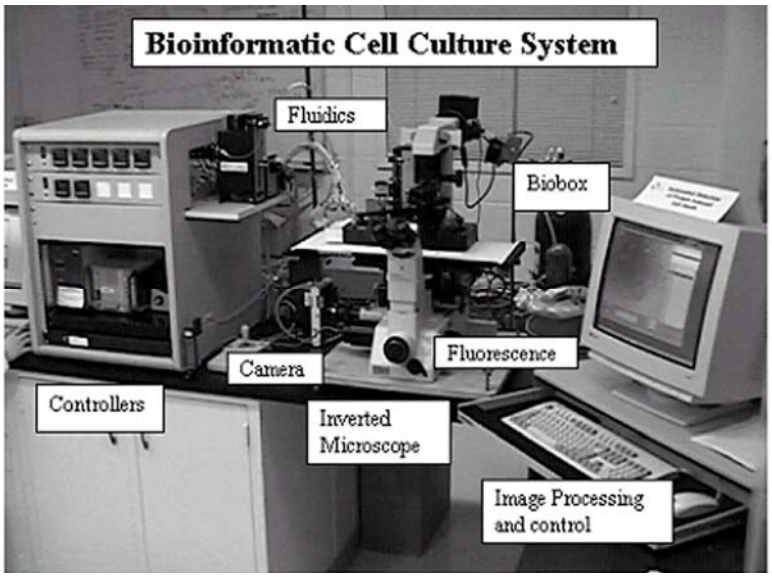

Figure 4.1 Bioinformatic Cell Culture and Imaging System (Courtesy of Bridget Deasy, PhD, Pittsburgh, PA)

4.7.3 Non-exponential Growth Model

This model was proposed by Sherley [180] and is particularly well-suited for studying the expansion of all cell populations and estimation of division time, mitotic fraction or population doubling time. Consequently, this model enables researchers to assess the behavior of a particular cell population under various culture conditions. The model is based on the Sherley

equation that is easy to use and offers a simple way of modeling cell growth for stem cell biologists based on the fraction of daughter cells that are dividing, α, while accounting for the presence of non-dividing cells:

$$N = N_0\left[0.5 + \frac{1-(2\alpha)^{(t/DT)+1}}{2(1-2\alpha)}\right]$$

where N is the number of cells at any time t, N_0 is the initial number of cells, and both DT and cell number at each time point are determined by image analysis directly through individual observations of cytokinesis. A brief derivation of this model, including assumptions inherent to its use, is provided in Appendix B.

4.8 MYOGENIC DIFFERENTIATION

Myogenic differentiation was evaluated by immunocytochemical staining for fast myosin heavy chain (MHC) expression. E-, S-, and C-MDSCs were plated at an initial density of 1000 cells/cm^2 in multi-well collagen type I–coated 12-well plates in high-serum DMEM (details above) for 2 days. To induce fusion, proliferation medium was replaced with differentiation medium (low serum: DMEM supplemented with 2% FBS and 1% penicillin-streptomycin) for an additional 3 to 4 days. Immunocytochemistry staining was performed as described above with the monoclonal mouse anti-MHC (1:250; Sigma-Aldrich) as the primary antibody to reveal fast MHC expression. Nuclei were visualized by DAPI (1:100; Sigma-Aldrich). Representative fields

were evaluated to determine the degree of differentiation (percent ratio of MHC-expressing nuclei to total number of nuclei), an indicator of differentiation efficiency.

4.9 MYOFIBER REGENERATION *IN VIVO*

A total of 2–3x10^5 C-, E-, or S-MDSCs were injected into the gastrocnemius muscle of 6–8 week-old male *mdx* mice. Mice in the E-MDSC group and their controls were immunosuppressed by subcutaneous injection of FK506 (2.5 mg/kg mouse body weight/day) beginning on the day of cell transplantation and continuing until the day of sacrifice [183]. Ten to fourteen days after transplantation, the gastrocnemius muscles were harvested, flash frozen in liquid nitrogen–cooled 2-methylbutane, and serially sectioned (10 μm). Dystrophin staining of cryopreserved tissue was performed on acetone-fixed, horse serum–blocked sections using a rabbit anti-dystrophin antibody (1:1000; provided by Dr. Terry Partridge) for 3 hours. Sections were then washed in PBS and incubated with biotinylated anti-rabbit IgG antibody for 1 hour. Next the sections were washed again and incubated with Streptavidin-Cy3 (1:300; Sigma-Aldrich) for 20 minutes. All incubations were at room temperature. Fluorescence microscopy was performed and digital images were acquired. Muscle regeneration was assessed by counting the number of dystrophin-positive myofibers in an area containing the largest graft and calculating the regeneration efficiency index (RI: the number of dystrophin-positive fibers in the host muscle per 10^5 donor cells) for ease of comparison and graphical display [103].

4.10 QUANTIFICATION OF DYSTROPHIN-POSITIVE MYOFIBERS USING NORTHERN ECLIPSE

The digital images of regenerated dystrophin-positive myofibers from gastrocnemius muscle sections immunostained against dystrophin were acquired using a Nikon Eclipse E800 microscope equipped with a Spot digital camera (Figure 4.2A). Northern Eclipse software package (v6.0, Epix Imaging, Inc.) was used to perform dimensional analysis of dystrophin-positive myofibers both manually and automatically. For manual count, each myofiber was numbered and counted using the manual counter provided by the software. For the automatic count, the images were converted into binary, black and white (8-bit grayscale) (Figure 4.2B). Using a manually-set threshold to delineate the immunofluoresence signal from the background, the Northern Eclipse software identifies myofibers that meet the chosen criteria by the user and places a circle or a number inside each individual myofiber. The pixel intensities were chosen as a set point so that any pixel darker than the set point is turned red/white while pixels lighter than the set point are turned cyan/black. The correct set point was determined manually by the user. This procedure is called thresholding (Figure 4.2C). During the thresholding, the user should maximize the number of connected components so that the myofibers remain distinct enough so that the software will be able to count each myofiber separately (Figure 4.3A). If the threshold point is at low pixel intensity, the red/white will flood the myofibers (Figure 4.3B), while with high pixel intensity, only a few fibers will be distinguished (Figure 4.3C), thereby resulting in a low count of myofibers and an under estimation of the data. Increasing the threshold will maximize the number of connected components (boundaries around each fiber), and the correct number of myofibers will be measured. The software determines the actual myofiber cross-sectional area of each fiber and provides quantitative measurements of the number of pixels

occupied by each individual fiber. The fiber area distribution (FAD) of 4000 individual myofibers per group was measured by determining the total number of pixels occupied by each fiber— a number that was easy to convert to μm^2 with analysis software. H&E staining of non-injected regions of the same grafts was used to detect the boundaries of the host *mdx* myofibers. The parameters such as cross-sectional area, diameter (maximal myofiber length in microns), minor axis diameter (the longest line through myofiber that is perpendicular to its orientation) and myofiber elongation (ratio of major axis to minor axis) were also calculated as median, 10^{th}, 25^{th}, 75^{th}, and 90^{th} percentiles (for detailed descriptions of each parameter refer to Appendix C). This data was compared with control or between groups, using non-parametric one-way ANOVA on ranks with a Dunn's method for multiple comparisons with an unequal sample size.

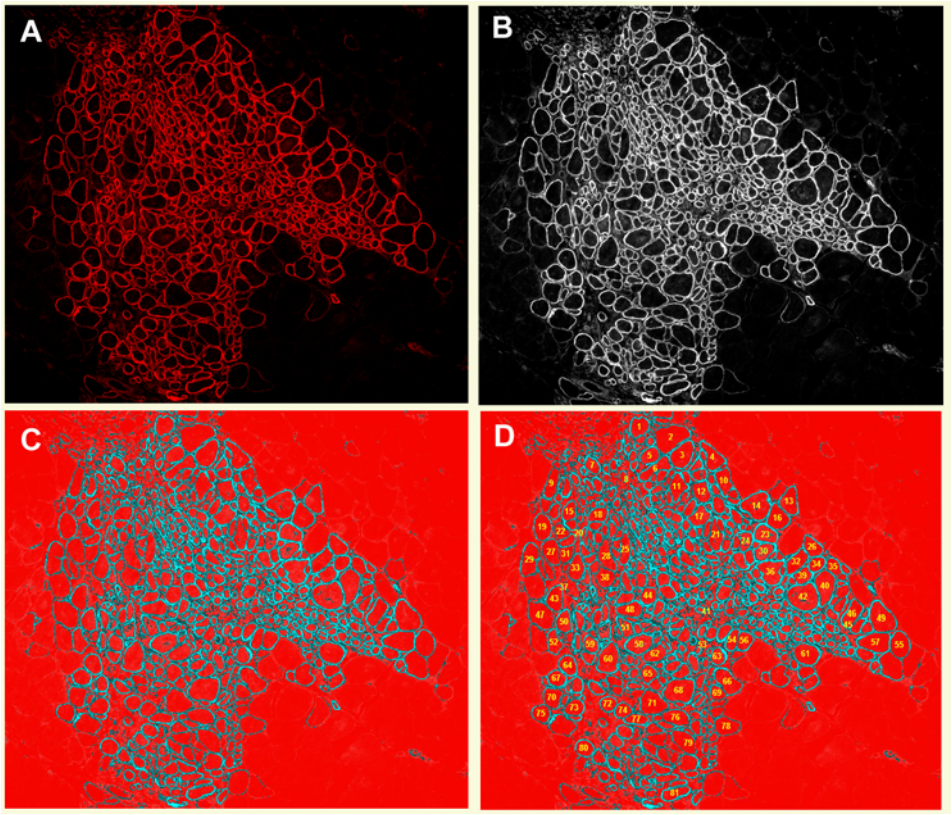

Figure 4.2 Dimensional analyses of immunohistochemically-labeled dystrophin-positive myofibers

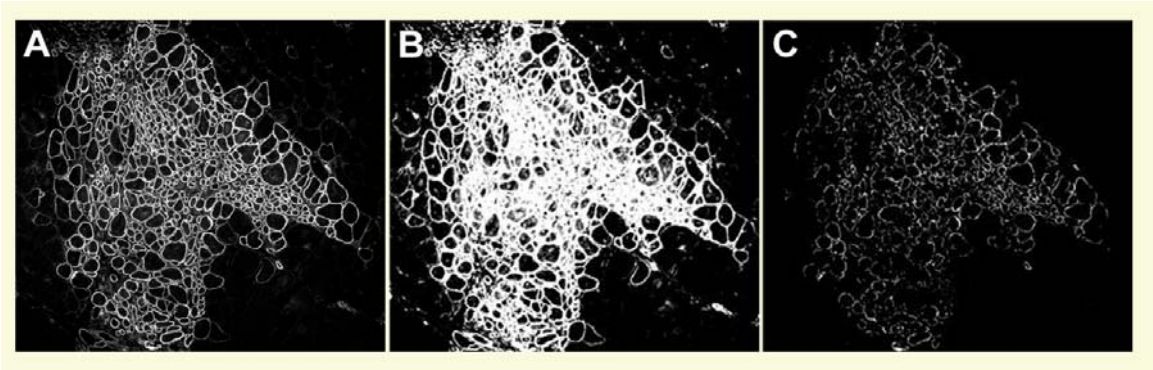

Figure 4.3 Challenges encountered during thresholding an image

4.11 STATISTICAL ANALYSIS

Differences with $p < 0.05$ were considered statistically significant. All values are given as the mean ± standard deviation of the mean (SD). Direct comparisons between treatment and control groups were made by using Student's *t* test or the Mann-Whitney Rank Sum test (where appropriate). Multiple group comparisons were made by using one-way analysis of variance (ANOVA). In cases where the data failed this test and indicated that the data varied significantly from a population with a normal distribution, nonparametric tests, the Kruskal-Wallis One Way Analysis of Variance on Ranks, were used ($p<0.05$ significance level). Nonparametric distributions were also detected and comparisons were made using Kruskal-Wallis one-way ANOVA on ranks with Dunnett's test for comparing treatment groups with a single control group or comparing between groups with an equal number of sample size; or Dunn's test for comparing treatments groups with unequal number of sample size. All statistical testing and regression analyses were performed using SigmaStat for Windows Version 2.0 (Copyright 1992-1995 Jandel Corporation).

5.0 RESULTS

5.1 QUANTITATIVE DETECTION OF NGF

Two different methods of NGF stimulation were used in this study: retroviral transduction of MDSCs with the CL-NGF vector to induce expression of human NGF (E-MDSCs) and direct stimulation of MDSCs with NGF protein (100 ng/ml) for 7 days (S-MDSCs). For both groups, normal MDSCs that were neither stimulated nor transduced served as controls (C-MDSCs). We used ELISA to measure the levels of NGF secreted by MDSCs *in vitro*. After transduction with CL-NGF that carries a 3' long-terminal repeat (LTR), virus packing signal (ψ), and human NGF cDNA driven by the cytomegalovirus promoter (CMV-P), genetically engineered MDSCs (E-MDSCs) were able to synthesize, process, and secrete active human NGF (Figure 5.1).

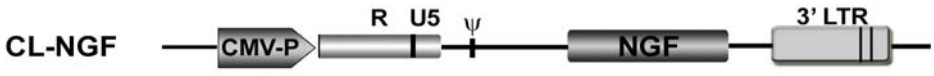

Figure 5.1 Schematic representation of the retroviral vector expressing NGF

Quantitative detection by ELISA of NGF protein within the cell supernatant revealed that the level of NGF secreted by E-MDSCs reached 307.4 ± 42.5 ng/10^6cells/24 hours 3 days after transduction and continued to be high 6 and 9 days after transduction (173.2 ± 11.1 ng/10^6cells/24 hours and 164.9 ± 28.2 ng/10^6cells/24 hours, respectively, *$p < 0.05$). In contrast, NGF-stimulated MDSCs (S-MDSCs) and control (non-treated) MDSCs (C-MDSCs) on average secreted NGF at barely detectable levels at the three time points (0.09 ± 0.17 ng/10^6cells/24 hours and 0.02 ± 0.37 ng/10^6cells/24 hours, averages for S-MDSCs and C-MDSCs, respectively, Figure 15).

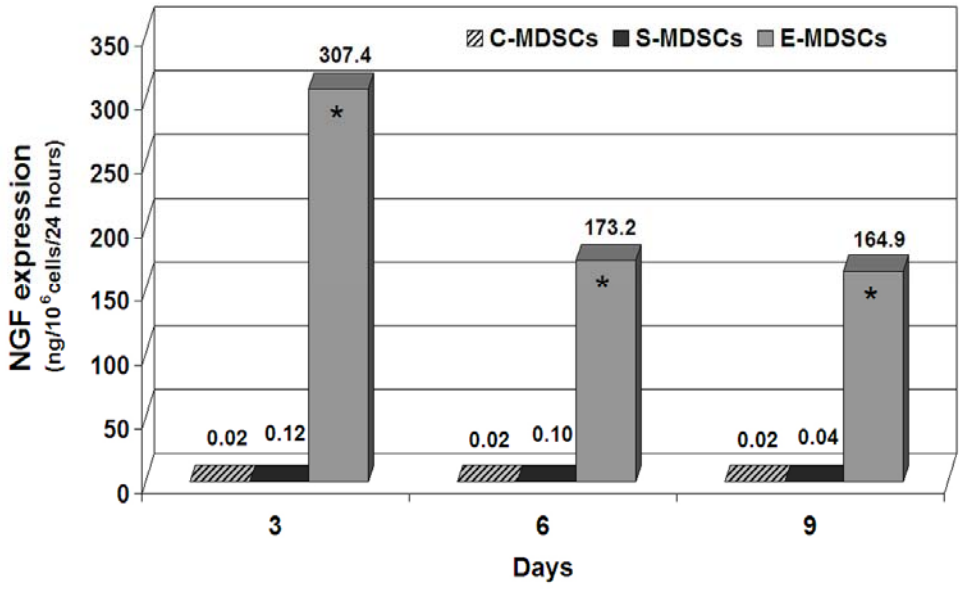

Figure 5.2 Quantitative detection by ELISA of the NGF protein in the cell supernatant

5.2 PROLIFERATION KINETICS

Growth factors stimulate the proliferation of myogenic precursor cells. To investigate the cellular response of MDSCs to NGF, we examined cellular division time (DT), and population doubling time (PDT). We fit experimental data sets for C-, E-, and S-MDSCs to the Sherley model equations by using nonlinear regression with the correlation coefficient $R^2 > 0.90$ to estimate mitotic fraction (α) (i.e., the fraction of daughter cells that are actively dividing). Our data suggest that the average DTs (C-MDSCs = 11.9 hours, E-MDSCs = 11.5 hours, and S-MDSCs = 12.1 hours) were not significantly different in the various groups ($p = 0.053$, Kruskal-Wallis analysis on ranks) and that the PDTs of the different cell groups were also quite similar (11–13 hours). Moreover, we observed a strong association (as indicated by the high correlation coefficient) for all of the groups ($R^2 = 0.99$), but no difference in the estimated α for the 3 groups (C-MDSCs = 0.94, E-MDSCs = 0.92, and S-MDSCs = 0.94). The mean mitotic fraction remained relatively constant among the groups (~0.94) (Figure 5.3). These results indicate that neither NGF transduction nor stimulation significantly alters the proliferation kinetics of MDSCs.

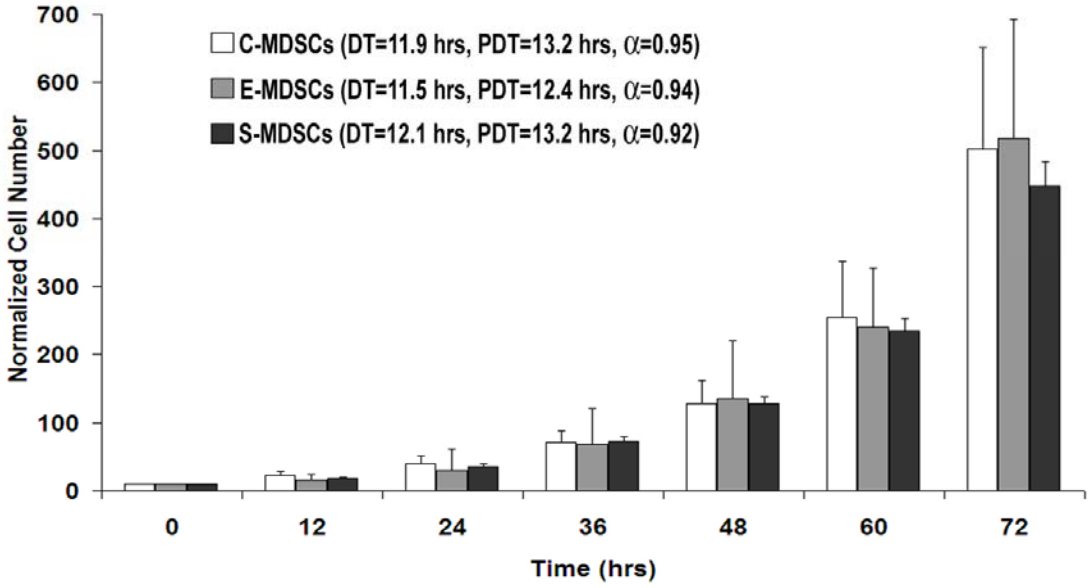

Figure 5.3 Proliferation kinetics of MDSCs

5.3 IN VITRO STEM CELL AND MYOGENIC MARKER PROFILES

We investigated the expression levels of the stem cell markers CD34 and Sca-1 by flow cytometry. Data were collected by performing logarithmic amplification on 5000 cells, excluding cell debris by combining forward and side scatters. This data is presented as dot plots in Figure 5.4A (the percentage of cells in each quadrant is indicated in the upper right-hand corner). Our results indicate high expression levels of the stem cell markers CD34 and Sca-1 (> 70%) by the

control group (C-MDSCs), 7 days after either retroviral transduction of the cells with the CL-NGF vector (E-MDSCs) or stimulation with 100 ng/ml NGF (S-MDSCs). We observed no significant difference between the groups (in terms of stem cell marker expression) after 2 weeks of *in vitro* expansion ($p = 0.655$). This marker stability suggests that NGF does not affect the stem cell marker expression of MDSCs *in vitro*.

We also used immunofluorescent staining to assess the cells' expression of two myogenic proteins: Pax-7 and desmin. Myogenic differentiation assay revealed low levels of Pax-7 and desmin expression. We quantified the expression of Pax-7 and desmin as the ratio of nuclei positive for Pax-7 or desmin to the total nuclei in 10 randomly chosen fields. As shown in Figure 5.4B, there were no significant differences in Pax-7 expression by the different groups of treated and untreated MDSCs ($p = 0.148$, n=6). Whereas E- and C-MDSCs expressed similar levels of desmin, we observed significantly more desmin-expressing cells in the S-MDSC group ($p < 0.05$, n=6).

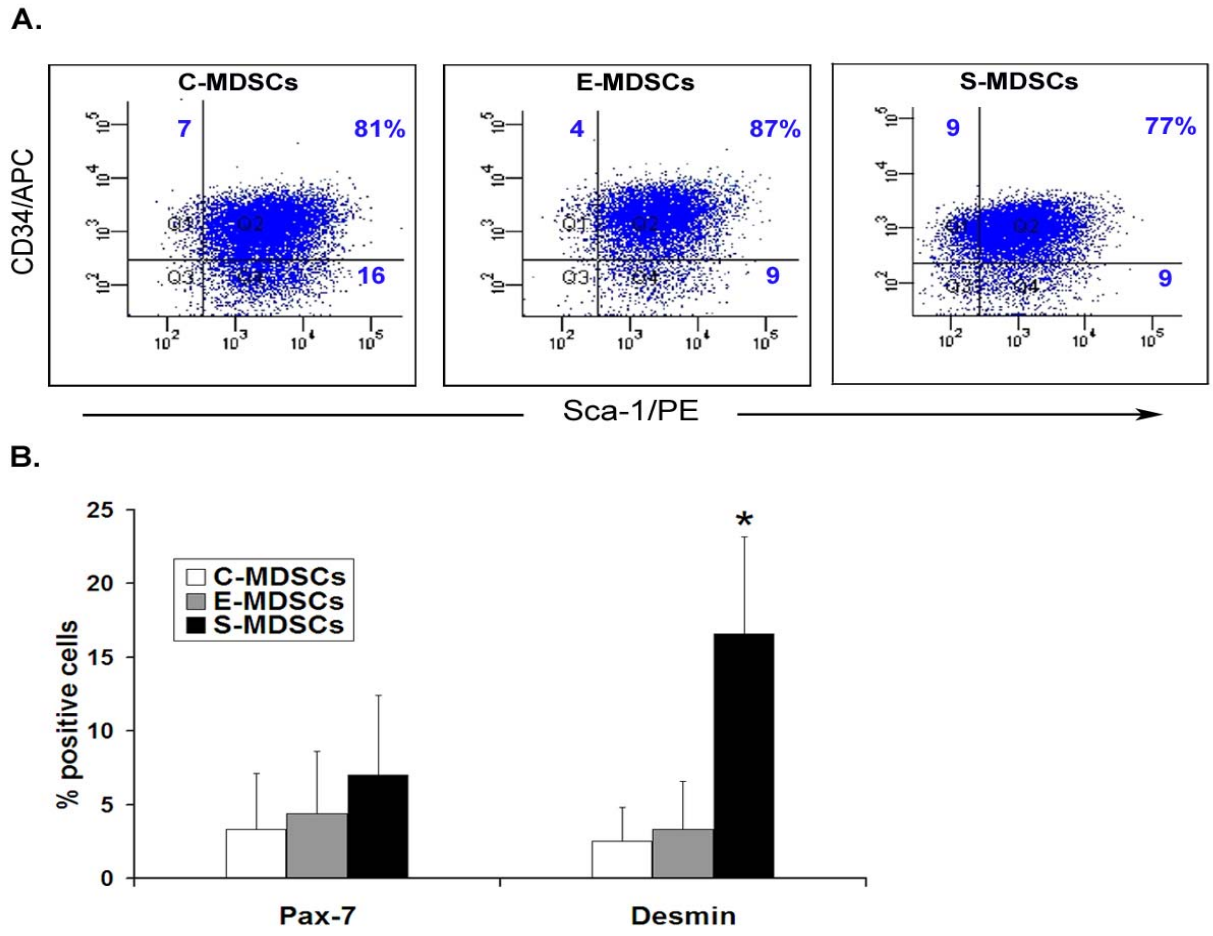

Figure 5.4 Marker profile analysis of MDSCs

5.4 IN VITRO MYOGENIC DIFFERENTIATION

After cultivating the cells under low-serum and high density conditions, we assessed myogenic differentiation in the 3 groups by performing immunohistochemical staining for fast myosin

heavy chain (MHC) expression to analyze myotube formation Representative images of MHC-positive C-, E-, and S-MDSCs (Cy3; red) overlaid on nuclear counterstain (DAPI; blue) are shown in Figures 5.5A–C. We defined differentiation efficiency as the percentage ratio of MHC-expressing nuclei to total number of nuclei. C-MDSCs consistently showed a differentiation efficiency of 35%, and E-MDSCs showed 30% differentiation efficiency. In contrast, the differentiation efficiency of S-MDSCs, 26%, was significantly lower than that of C-MDSCs, which indicates that S-MDSCs have a decreased ability to fuse and form multinucleated myotubes (*$p < 0.05$, n=3, Figure 5.5D). Scale bar represents 100 μm.

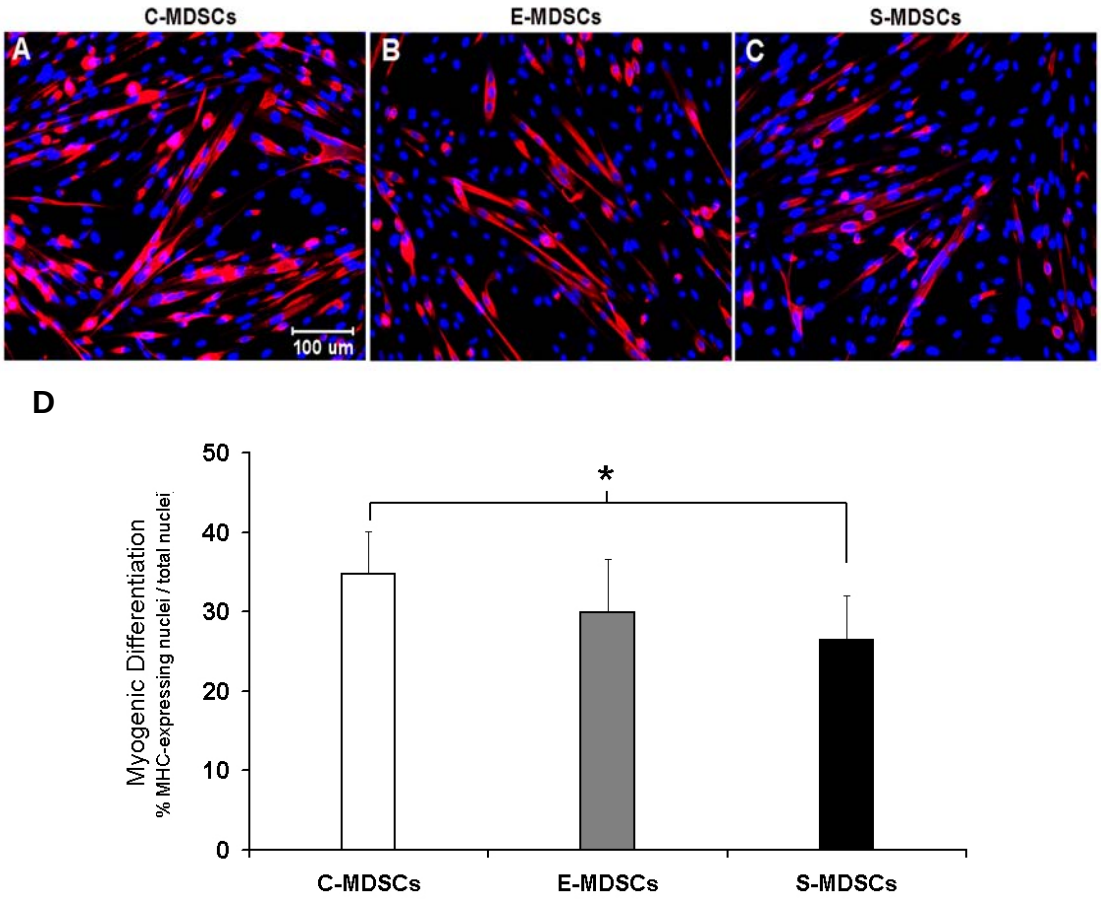

Figure 5.5 Myogenic differentiation *in vitro*

5.5 MUSCLE REGENERATION

We evaluated the ability of the 3 groups of cells (E-, S-, and C-MDSCs) to regenerate dystrophic skeletal muscle by transplanting $3x10^5$ cells from each cell group into the gastrocnemius muscles of 8-week-old male *mdx* mice. Fourteen days after transplantation, we sacrificed the animals; we harvested the injected gastrocnemius muscles, snap froze them, and sectioned them using a cryostat. Using immunohistochemical staining, we assessed the number of dystrophin-positive myofibers with manual count and quantitated muscle regeneration in terms of the regeneration index (RI: the number of dystrophin-positive fibers in the host muscle per 10^5 donor cells). The dystrophin-positive grafts of E- and C-MDSCs are shown in Figures 5.6A and 5.6B. The average RI of E-MDSCs was significantly larger than that of C-MDSC's (435.6 ± 85.5 vs. 197.5 ± 53.8, $*p < 0.001$, n=8, Figure 5.6C). Scale bar represents 250 μm.