Mesenchymal stem cells in skeletal muscle regeneration

Garza-Rodea, A.S. de la

Chapter 1

General introduction

Table of contents

1. Preface
2. Skeletal muscle tissue
   - 2.1 Structure of skeletal muscle tissue
   - 2.2 Sarcolemma and sarcoplasmic protein components
   - 2.3 Satellite cells
3. Myogenesis
   - 3.1 Skeletal muscle development during embryogenesis
     - 3.1.1 Paired box transcription factors
     - 3.1.2 Myogenic regulatory factors
   - 3.2 Skeletal muscle regeneration: homeostatic turnover and postinjury repair
4. Skeletal muscle pathologies
   - 4.1 Central and peripheral nervous system diseases
     - 4.1.1 Toxic or drug-related myopathies
     - 4.1.2 Autoinmmune reaction myopathies
     - 4.1.3 Traumatic myopathies
   - 4.2 Hereditary skeletal muscle diseases
     - 4.2.1 Metabolic myopathies
     - 4.2.2 Mitochondrial myopathies
     - 4.2.3 Skeletal muscle channelopathies
     - 4.2.4 Congenital and distal myopathies
     - 4.2.5 Congenital muscular dystrophies
5. Animal models of skeletal muscle regeneration
   - 5.1 Genetic models
   - 5.2 Induced skeletal muscle damage
     - 5.2.1 Chemical injury
     - 5.2.2 Physical injury
     - 5.2.3 Biological injury
6. Cell therapy for diseased and injured skeletal muscle
   - 6.1 Muscle-derived stem cells
   - 6.2 Multipotent stem cells
7. Outline of the thesis

1. Preface

A major challenge in regenerative medicine is to understand the process of tissue reconstruction after injury or disease and in particular the role of resident or transplanted stem cells during this process.
One of the most frequently studied tissues in regenerative biology is skeletal muscle. Gaining insight into the molecular, cellular and environmental factors governing skeletal muscle repair might help in the development of effective cell therapies for a variety of skeletal muscle diseases and injuries.
The word muscle is derived from the Latin *mus* (=mouse) and refers to the resemblance in shape of some muscles (notably the *musculus biceps*) to mice. Muscles of the body vary in size, shape and composition, according to their function. Muscle tissue is commonly divided into two categories: smooth and striated. The striated muscles are further subdivided into cardiac and skeletal muscles, which posses a similarly structured contractile system but differ from each other in their developmental and regenerative histogenesis. In the developing myocardium the progeny of each myogenic cell differentiates into a mononucleated cell displaying extensive proliferative activity followed by differentiation into rhythmically contracting cardiomyocytes. As described below, skeletal myogenesis proceeds in a very different manner involving the formation of multinuclear syncytia by fusion of mononucleated muscle precursor cells. The adult myocardium does not contain a reserve of myogenic stem cells and therefore possesses limited regeneration potential. In contrast, adult skeletal muscle tissue shows high regenerative capacity due to the presence of resident satellite cells, which represent a local reservoir of myogenic stem cells. In view of the aforementioned and other fundamental differences (e.g. in arrangement, innervation and metabolism) between cardiac and skeletal muscle tissue the two types of striated muscle are considered separate entities. The remainder of this thesis will deal with skeletal muscle tissue only.
Stem cell-based therapies represent promising approaches for the treatment of incurable diseases and tissue injury due to the capacity of these cells to self-renew and differentiate into specialized mature cells. Understanding the interaction of stem cells with the diseased/injured environment and their contribution to the repair process by release of regeneration-promoting signals or by differentiation into the lost cell types is crucial for the advancement of their clinical application.
This thesis focuses on one stem cell population, the mesenchymal stem cells (MSCs) and in particular on their role and utility in skeletal muscle regeneration.

2. Skeletal muscle tissue

2.1. Structure of skeletal muscle tissue

The multinucleated (syncytia) skeletal muscle cells - also known as muscle fibers or myofibers - are generally arranged in parallel to one another in the muscle belly. The two ends of the muscle belly are commonly attached to bone or terminated intrafascicularly by a muscle tendon called myotendinous junction. This is a fragile area where the myofibers often break. Several sheets of connective tissue are wrapping the muscle fibers (Figure 1.1): the epimysium is a deep fascia consisting of dense and irregular connective tissue that surrounds the entire muscle and extends into the muscle tendons; the perimysium is the layer that surrounds groups or fascicles of myofibers within a muscle; the endomysium, also referred to as the basal lamina or basement membrane is the connective tissue that surrounds each individual myofiber and lies outside the cell membrane or sarcolemma. The connective tissue of skeletal muscles hosts neurons and blood vessels of the myofibers and it is important for force transduction to the skeleton.

The nuclei of mature myofibers are normally located directly underneath the sarcolemma. In the cytoplasm or sarcoplasm of the myofibers hundreds of myofibrils are packed in a parallel arrangement. The “striated” appearance of cardiac and skeletal muscle tissues is largely due to the banding pattern formed by the precise alignment of thin (actin) and thick (myosin) filaments that together with their associated proteins, comprise the highly organized sarcomeres. The sarcomere is the functional unit of skeletal muscle cells that provides the mechanical force for contraction[1,2](Figure 1.1).

Myofibers can be classified in terms of contractile and metabolic properties. Type 1 or slow-twitch myofibers are red, produce relatively little force, are highly resistant to fatigue, contain many mitochondria and large amounts of myoglobin and oxidative enzymes, and derive their contractile energy (ATP) mainly from aerobic metabolism. Type 2 or fast-twitch muscle fibers have a pink to white appearance depending on their myoglobin content, utilize creatine phosphate and glycogen as main energy stores and rely for a large part on glycolysis to generate the energy needed for contraction. In humans, there are two types of fast-twitch myofibers (i.e. 2a and 2X). Type 2a muscle fibers contain many mitochondria and large amounts of myoglobin, have relatively high oxidative and glycolytic capacities and are moderately fatigue-resistant. Type 2X –for the human orthologue of rat myosin heavy chain 2X (MYH-2X) - myofibers have few mitochondria and low myoglobin content, generate ATP almost exclusively by anaerobic metabolism and fatigue easily. Human muscles contain myofibers type 1, mixed 1-2a, 2a, mixed 2a-2X and 2X. However, most human skeletal muscles contain type 1 and 2a[3]. The proportion of the three myofiber types within skeletal muscles may differ between

individuals and may change throughout life as a result of ageing and in response to exercise, nutrition and disease[4,5]. Rodent muscles contain myofibers type 1, mixed 1-2a, 2a, mixed 2a-2X, 2X, mixed 2X-2b and 2b[3]. Mouse muscles are predominantly composed of type 2b and 2X fibers, with 2A fibers representing a minor component and type 1 being rare and mostly confined to some muscles, such as the soleus[6].

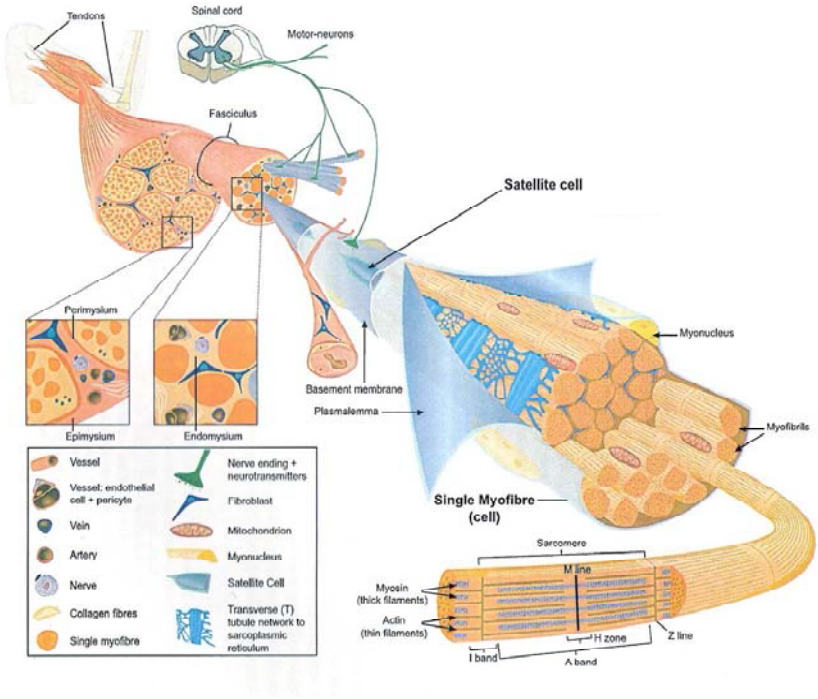


Figure 1.1 **Schematic representation of the structure of skeletal muscle.**
See description in the text. Insert: microphotograph of immunohistochemistry of human satellite cell detected by Pax7 antibody in healthy human muscle. Drawing adapted from Tajbakhsh S.[26].

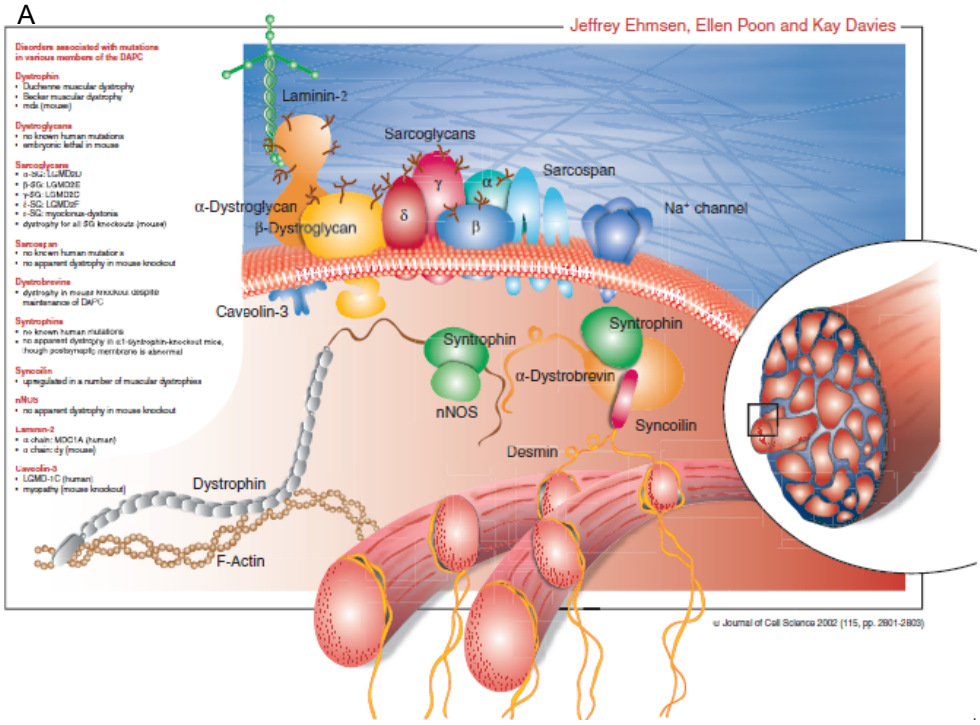
The activity of skeletal muscles is controlled by somatic motor and sensory neurons. The axons of α-motoneurons originate in the spinal cord (anterior horn) and end as neuromuscular junctions (NMJs) on individual myofibers after splitting into many terminal unmyelinated branches. Each muscle fiber is innervated by a single motor axon, which may also innervate other myofibers.
The NMJ is a specialized synapse formed by the swelling of the axon terminal of an α-motoneuron into a synaptic end bulb, filled with vesicles containing the

neurotransmittor acetylcholine. This synaptic end bulb is separated by the so-called synaptic cleft from the motor end plate, a highly folded, trough-like depression of the sarcolemma containing large numbers of muscle-type nicotinic acetylcholine receptors (nAChRs)[5].

2.2. Sarcolemma and sarcoplasmic protein components

A critical component of the sarcolemma of both skeletal and cardiac muscle is the dystrophin-glycoprotein complex (DGC) discovered in the 1990´s. This complex includes dystrophin, dystroglycans (α,β), syntrophins (α,β), α-dystrobrevin, sarcoglycans (α,β,γ,δ) and sarcospan[7] (Figure 1.2). The DGC complex is highly structured and links the extracellular matrix (ECM) via laminin-2 (α2, β1 and γ1 chains) to the subsarcolemmal γ-actin filaments. The DGC protects striated muscle cells from contraction-induced damage[8,9]. The DGC also participates in regulating proteins involved in cell signaling, such as neuronal nitric oxide synthetase (nNOS) and neurexins[10,11]. The DGC complex establishes, via α-dystroglycan, also a link between the intracellular compartment and extracellular proteins such as agrin, biglycan and perlecan[12].

A



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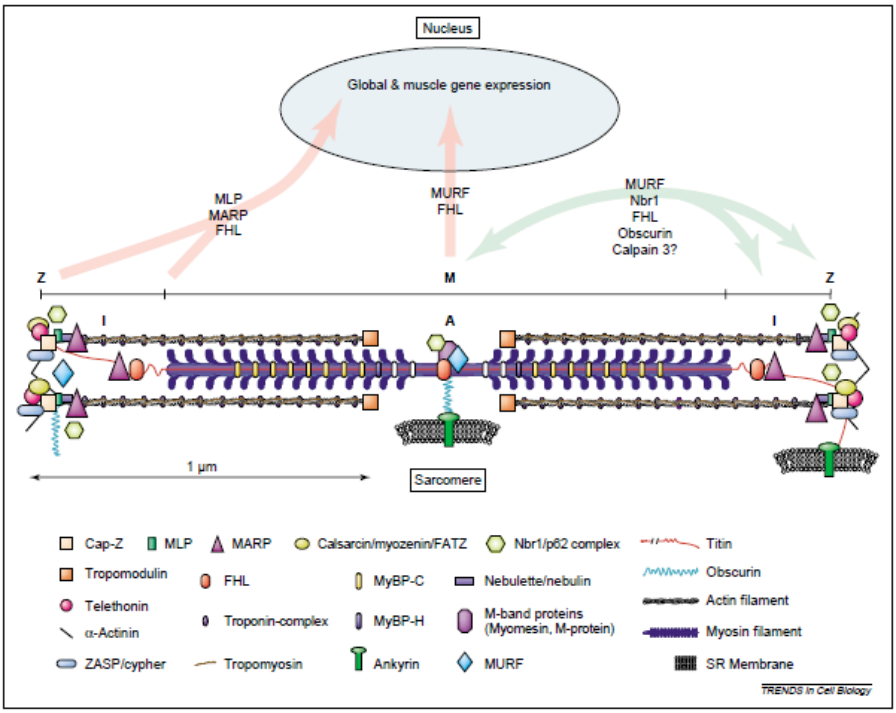

Figure 1.2 **Schematic organization of myofiber proteins.**
A: dystrophin that is localized at the sarcolemma and member of the dystrophin-glycoprotein complex (DGC), interacts with cytoplasmic, transmembrane and extracellular proteins in skeletal muscle. Through its C-terminus, dystrophin binds to the DGC. The DGC is comprised of sarcoplasmic proteins (dystrobrevin, syntrophins and neuronal nitric oxide synthase [nNOS]), transmembrane proteins (β-dystroglycan, the sarcoglycans, caveolin-3 and sarcospan) and extracellular proteins (α-dystroglycan and laminin-2). The N-terminus of dystrophin binds to the cytoskeleton through actin filaments where is called Z disc (costamers). Desmin links sarcoplasmic proteins with myonuclei proteins. Cartoon adapted from[292].
B: simplified scheme of the sarcomere which is a highly regular array of filaments of the contractile proteins actin and myosin that are crosslinked in the Z-disk (actin) and M-band (myosin). Actin and myosin form the major constituents of the I-bands and A-bands, respectively. Actin filaments are regulated by the tropomyosin-troponin complex, and thick filaments by the regulatory myosin-binding proteins-C and –H. the sarcomeres also contains numerous proteins with multiple localizations and with the potential to exchange between Z-disks, I-bands and M-bands (green arrows between compartments), as well as to translocate to the nucleus (orange arrows between compartments). Both titin and obscuring form links to the sarcoplasmic reticulum (SR) membrane via ankyrin 1.5 P62 can also translocate to the intercalated disk. Z disk, anchoring plane of actin filaments; I-band, region of variable length containing actin filaments and the elastic segment of titin; A-band, region of the myosin filament; M-band, anchoring plane of myosin filaments. Taken from Lange et al.[293].

Disruption of the connection between the cytoskeleton and ECM renders the sarcolemma fragile, which explains why mutations in genes encoding DGC proteins underly muscular dystrophies like Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) and the limb-girdle muscular dystrophies (LGMD) 2C through 2F with a characteristic pathology of contraction-induced sarcolemmal tearing followed by muscle degeneration.
The myonuclear envelop is connected to the subsarcolemmal filamentous actin cytoskeleton via a complex called linker of nucleoskeleton and cytoskeleton (LINC)[13,14]. The LINC is not only important for the migration and positioning of myonuclei but also for nuclear function including the regulation of gene expression and nuclear transport and may help myonuclei/fibers to withstand mechanical stress. Mutations in genes encoding various proteins associated with the nuclear envelope (e.g. emerin and lamin A/C) cause muscular dystrophies such as Emery-Dreifuss muscular dystrophy, *LMNA*-related congenital muscular dystrophy and LGMD1B most likely by disregulation of skeletal muscle gene expression and/or by disorganization of the internal structure of myofibers[15,16].

2.3 Satellite cells

Satellite cells are skeletal muscle-specific stem cells. Satellite cells were discovered at the beginning of 60's by Mauro[17] and are characterized as mononucleated, small, spindle-shaped cells with a thin rim of cytoplasm surrounding a heterochromatic nucleus. Satellite cells reside along the long axis of the myofiber in a shallow depression between the basal lamina and the sarcolemma referred to as the "satellite cell niche" (Figure 1.3).
Typically, satellite cells express *PAX7*[18]. Expression of other genes may depend on the activation state of the satellite cells[19] (Table 1.1). The frequency of satellite cells in the muscle tissue differs between species, with host age and with myofiber type. In neonatal mice satellite cells constitute ~30% of the nuclei in skeletal muscle. Their number decreases with age to ~4% in the adult and to ~2% in the aged (29- to 30- month-old) mouse[20,21]; in adult humans they comprise 3.8% of all skeletal muscle nuclei[22]. In general, slow-twitch myofibers contain approximately three times more satellite cells than fast-twitch myofibers[23].

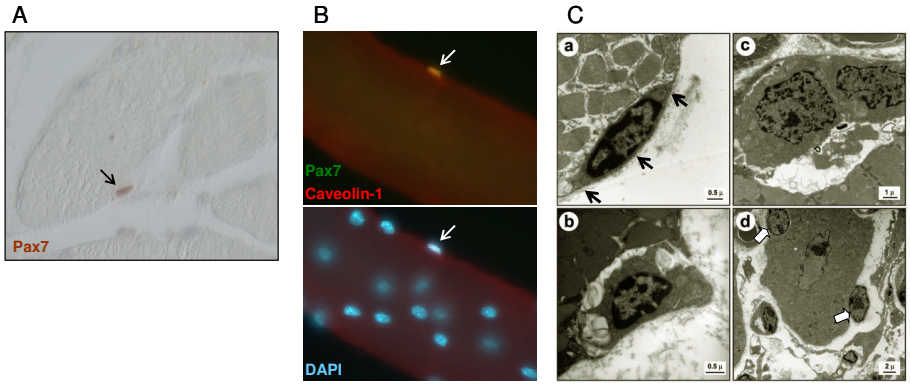

Figure 1.3 **Satellite cell location and activity.**
A: human satellite cell stained with Pax7 antibody in paraffin cross-section of healthy human muscle. B: upper, inmunostaining of satellite cells with Pax7 and Caveolin-1 antibodies in isolated fibers of adult mouse muscle; lower, DAPi counter stain for nuclei. C: Transmission electron microscopal picture of (a) mouse quiescent satellite cell, showing characteristically condensed heterochromatin in the nucleus and little cytoplasm. The cell lies in between the sarcolemma of mature myofibers and the continuous basal lamina (indicated by arrows); (b) an activated satellite cell with pseudopodia, less condensed heterochromatin and more cytoplasma near a degenerating myofiber 6 hours after cardiotoxin (CTX) injection in tibilis anterior muscle (TAM). Note the space between the satellite cell and the myofibers; (c) two newly divided progeny cells in a regenerating myofiber at day 2 after CTX; (d) a myotube with a central nucleus and two satellite cells (indicated by arrows) at day 5 after CTX damage. Taken from Yan Z et al.[294].

Table 1.1 Human and mouse satellite cell markers.

Marker	Q	A	D	Function
c-met	+	+	+	HGF receptor
CXCR4/SDF-1	+	+	+	Migration
Integrin$_{\alpha 7}$	+	+	+	ECM signaling, fusion
Integrin$_{\beta 1}$	+	+	+	ECM signaling, fusion
M-cadherin	+	+	+	Anchoring
NCAM (CD56)	+	+	+	Adhesion
Myf5	+	+	+	Myogenic commitment and transient amplification
MyoD	-	+	+	Activation and myogenic differentiation
Pax7	+	+	-	Multiple roles (see text)
Desmin	+/-	+	+	Cytoskeleton
Nestin	+	-	-	Cytoskeleton, nuclear organization?

A, activated (cycling) satellite cell; CXCR4/SDF-1, stromal derived factor 1 (SDF-1) is a ligand for CXCR4 receptor; D, differentiation myoblast; ECM, extracellular matrix; HGF, hepatocyte growth factor; NCAM, neural cell adhesion molecule-1; Q, quiescent satellite cell. Table adapted from Kuang S et al.[19]

Under normal physiological conditions, satellite cells of the adult muscle are quiescent. Upon exercise or injury satellite cells are activated through locally released signal molecules that play a key role in the regeneration process (Figure 1.4C). A more detail discussion of satellite cells and myoregeneration will be presented in the following sections.

3 Myogenesis

3.1. Skeletal muscle development during embryogenesis

In the embryo, multiple genes are involved in the specification, proliferation and differentiation of muscle stem cells. The products of many of these genes are redeployed during fetal and postnatal skeletal muscle growth as well as during skeletal muscle regeneration. For a more detailed description of embryonic myogenesis, several recent reviews can be consulted[24-26]. Here a brief description of embryonic myogenesis is presented with a summary of the genes involved at each stage (Table 1.2).

Table 1.2 Cell markers in skeletal muscle development in the limb of mouse embryos.

Stages	Associated genetic factors
Delamination	Pax3; c-met
Migration	c-met; Lbx1; CXCR
Proliferation	Pax3; c-met; Mox2; Msx1; (Myf5; MyoD)
Determination	Myf5; MyoD
Differentiation	Myogenin; Mcf2; (MyoD; Myf6)
Specific muscle formation	Lbx1; Mox2
Satellite cells	Pax7

Lbx1, Lady bird homolog; Mox, homeobox containing transcriptor factor 2; Msx1, Muscle segment homeobox. Table adapted from Buckingham M et al.[31]

Embryonic myogenesis begins in newly formed somites that occurs rostrocaudal. The somites are transitory epithelial structures formed in pairs by segmentation of paraxial mesoderm on either side of the neural tube that run along the back of the embryo. In response to instructive signals emanating from the surrounding tissues, the somites form two distinct compartments, the dermomyotome and the sclerotome. The sclerotome is located in the ventral part of the somites and gives rise to the cartilage and bone of the vertebral column, ribs and most of the skull. The dermomyotome, which comprises the dorsal part of the somites originates the dermatome and myotome. The myotome represnts the first skeletal muscle mass in the embryo and is divided in epaxial and hypaxial domains. The epaxial myotome, adjacent to the neural tube and notochord will give rise to the deep back muscles whereas the

musculature of the rest of the trunk, tongue and limbs derives from the hypaxial dermomyotome. The cells derived from the myotome are called muscle progenitor cells or muscle founder (stem) cells. A fraction of these cells delaminates from the epithelium of the hypaxial dermomyotome and migrates into the limb buds to eventually produce the arm and leg muscles[27-29] (Figure 1.4A).

The migration of the myogenic progenitor cells to the limb buds is guided by the cell surface receptors c-Met and CXCR4 and their corresponding ligands hepatocyte growth factor (HGF) and stromal cell-derived factor 1 (SDF1)[30]. The homeodomain- containing transcription factor *Lbx1* has also been shown to be implicated in the migration process[31,32].

Muscle progenitor cells can be identified by the presence of *PAX3* transcripts. Further, these cells respond to signals such as Wnts, hedgehog proteins, fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs) and antagonist of these transforming growth factor (TGF) superfamily members like noggin emanating from adjacent neural tube, notochord and ectoderm[33,34]. During their migration to the limb buds, the muscle progenitor cells have not yet activated the myogenic determination genes and it is only when they reach their destination that they begin to express the basic helix-loop-helix transcription factor genes *MYOD1* and *MYF5*[35]. Activation of these genes is assumed to depend on signaling molecules such as the Wnt7a and sonic hedgehog homologue (Shh).

Before skeletal muscle in the limb forms, the muscle progenitor cells undergo extensive proliferation. The activation of the myogenic differentiation program in these cells depends on the presence of MyoD1 and other transcription-regulating proteins such as the myocyte enhancer factors (i.e. *Mef2)*. Following their conversion into embryonic myoblasts, the cells fuse with each other to form primary or embryonic myofibers. A fraction of muscle progenitor cells continues to proliferate and, after their differentiation into fetal myoblasts, will give rise to secondary or fetal myofibers. These secondary myofibers use the embryonic myofibers as scaffold for their formation and are initially smaller in size than their predecessors. At this stage a basal lamina starts to enfold each myofiber and innervation is initiated as well. It is well known that the Pax3-positive cells that express now also *PAX7* begin to migrate and settle under the developing basal lamina into the characteristic satellite cell niche[27]. There are recent indications that Pax7-positive cells at mouse embryonic day 11.5 can give rise to satellite cells[36]. Pax3-positive somitic cells also give rise to embryonic myofibers and endothelium[37]. The primary myofibers are initially slow-twitch, but some rapidly convert to a fast phenotype and the secondary myofibers acquire the characteristics of fast-twitch[31,38]. During late prenatal and early postnatal development, satellite cells continue to divide at a slow rate and part of their progeny fuses with adjacent myofibers to bring about skeletal

muscle growth. It has been estimated that in adults >90% of the myonuclei are derived from the progeny of satellite cells[39]. At the end of the postnatal growth, satellite cells enter a quiescence state but can be reactivated upon skeletal muscle damage or an increase in skeletal muscle use (physiological adaptation). Following activation, satellite cells start to replicate to generate daughter cells which either differentiate and subsequently fuse among themselves or with damaged myofibers to restore skeletal muscle integrity (see below) (Figure 1.4C) or return to a quiescent state to maintain the stem cell pool.

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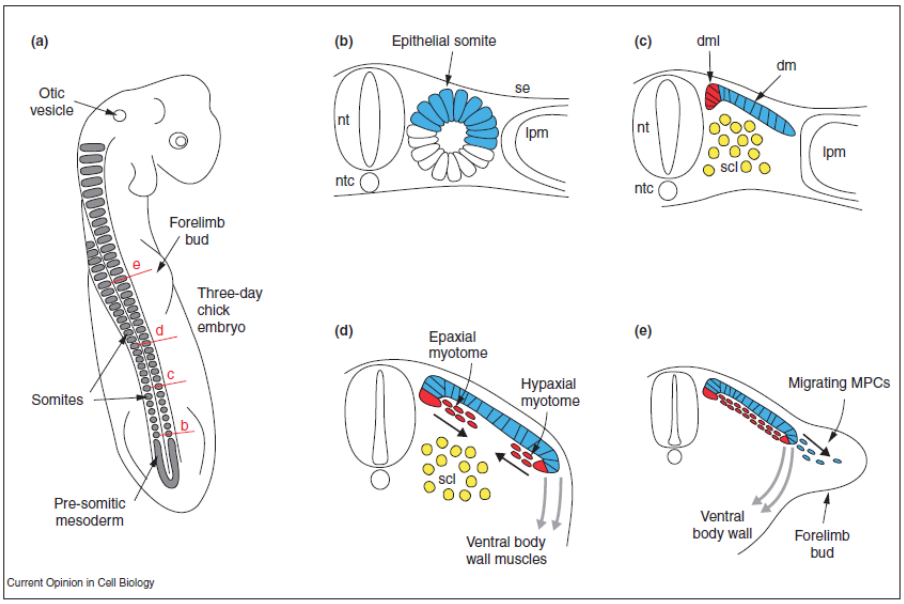

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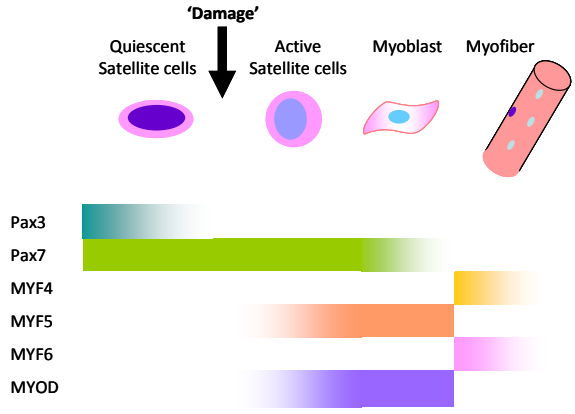

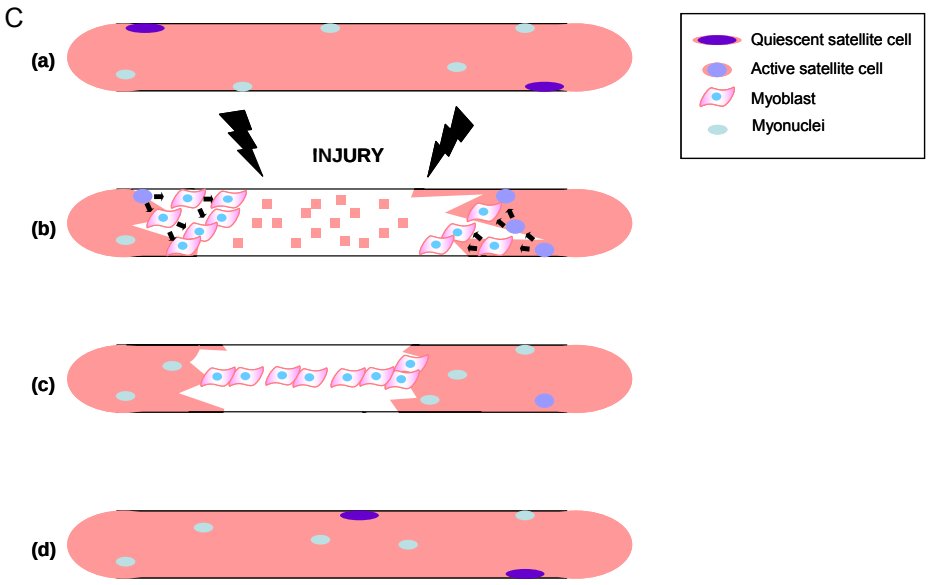

Figure 1.4 Myogenesis and myoregeneration.
A: domains of somatic myogenesis in the early chick embryo. (a) Schematic view of a three-day chick embryo with somites (gray) on either side of the neural tube. (b-e) Cross sections of somites and axial tissues on the right-hand side of the embryo at levels indicated in (a). (b) epithelial somite (level I). Newly formed somite receives signals from the neural tube (nt), notochord (ntc), surface ectoderm (se), and lateral plate mesoderm (lpm). Lineage tracing experiments have shown that the dorsal part of the somite is fated to form the dermomyotome (blue). (c) Somite at level VII. Dermomyotome (dm, blue) has differentiated from the dorsal region of the somite. The dorsomedial lip (dml) of the dermomyotome forms next to the neural tube. Ventral region of the somite undergoes an epithelium to mesenchyme conversion and forms the sclerotome (scl) that will later give rise to the vertebrae and ribs. (d) Thoracic/interlimb level somite. Cells from the dermomyotome translocate through the medial and lateral lips to form the epaxial and hypaxial myotome (both shown in red). The lateral lip of the dermomyotome grows ventro-laterally and will later comprise the ventral body wall muscles (gray arrows). (e) Forelimbs level somite. Myotome (red) continues to form subjacent to the dermomyotome (blue). Long-range migrating muscle precursor cells (MPCs) delaminate from the hypaxial dermomyotome and invade the limb bud. Taken from Bailey et al.[295].
B: expression of Pax genes and myogenic transcriptor factors during ontogenesis of adult skeletal muscle upon injury.
C: simplifies presentation of myoregeneration in adult skeletal muscle. (a) uninjured myofibers; (b) damage to the myofibers activates satellite cells into proliferation and migration; (c) myoblast fuse to replace lost fibers; (d) remodeling is completed and myonuclei and satellite cells are at their normal position.

With the exception of musculature of the tongue, which is derived from muscle progenitor cells in the four occipital somites, embryogenesis of the striated muscles of the neck and head progresses differently than that of the skeletal muscles of the trunk and limbs. The majority of neck/head muscles (including the facial, mastication, pharyngeal and laryngeal muscles) are derived from cranial paraxial mesoderm of the branchial arches while the extraocular muscles originate from anterior prechordal mesoderm[40-42]. The transcription factors orchestrating the early stages of craniofacial myogenesis also differ from those involved in the early phases of skeletal muscle formation elsewhere in the body. Instead of Pax3 and Pax7, several other transcription factors act upstream of the myogenic regulatory factors (see below) to initiate skeletal muscle formation in the neck and head. Some of these transcription factors (Isl1, Nkx2.5, Pitx2 and Tbx1) also play a role in cardiomyogenesis. It has been postulated that postnatal satellite cells in the head, that are of different origin from limbs and trunk, may also arise from a distinct cell populations[43] and therefore regulate myogenesis in this anatomical area differently than that of limbs and trunk[29].

3.1.1 Paired box transcription factors

"Paired box" transcription factors (Pax proteins) play essential roles in tissue specification and organogenesis. There are nine known *PAX* genes (*PAX1-9*) in mammals that are divided into four subgroups based on genomic structure, sequence similarity and function[44]. *PAX3* and *PAX7* are homologous genes associated, albeit not exclusively, with myogenesis[45,46]. *PAX3* and *PAX7* are, for example, also expressed in the (developing) central nervous system where they play a role in the commitment of precursor cells to different neuronal cell fates and in the maintenance of specific brain cell subtypes[47]. In the absence of both Pax3 and Pax7, somitic skeletal muscle development is arrested and muscle precursor cells fail to leave the myotome[48].

PAX3 is mainly expressed during embryogenesis and is involved in the delamination and migration of embryonic myoblast towards developing limb buds. Pax3 acts in a synergistic myogenic gene regulatory network together with members of the Dach, Eya, and Six families of transcription factors. Pax3 can directly activate *MYF5* and *MYOD1* expresion, thereby initiating myogenic differentiation. It has been suggested that Pax3 regulates the activation and early proliferation of satellite cells and that its downregulation is necessary for myoblast differentiation. In adults mice, Pax3 appears in a rare satellite cells subsets which declines with aging[49].

Pax7 is expressed in the mature satellite cells and its function is maintenance, proliferation and self-renewal in these cells. Skeletal muscles of *PAX7*$^{-/-}$ mice lack satellite cells, even though at birth their skeletal musculature is comparable to that of wild-type siblings. During postnatal development, *PAX7*$^{-/-}$

mice grow at a much slower rate than their wild-type siblings and they do not survive beyond 2 to 3 weeks of age[50]. Skeletal muscles of *PAX7*$^{-/-}$ mice are atrophic, with 30% reduction in fiber diameter as compared to that of wild-type mice. Furthermore, Pax7-deficient skeletal muscles fail to regenerate upon injury, which is consistent with the observation that Pax7 is required for the myogenic progression of satellite cells[51,52].
Taken together, these data indicate an important role of Pax3 in prenatal development, specification, and location of muscle precursor cells while Pax7 is especially important in determining and maintaining the adult population of satellite cells in the mature skeletal muscle.

3.1.2 Myogenic regulatory factors

The basic-helix-loop-helix domain-containing family of myogenic regulatory factors (MRFs) is involved in muscle cell determination and differentiation both during development and during postnatal skeletal muscle regeneration[24]. Members of this family of transcription factors in birds and mammals are: MyoD1 (also designated Myf3), MyoG (also known as Myf4 or myogenin), Myf5 and Myf6 (also known as herculin or MRF4). Phylogenetic analysis suggests that the four *MRF* genes arose through two consecutive gene duplication events causing *MYOD1* and *MYOG* to be most closely related to *MYF5* and *MYF6*, respectively. This may explain the partial redundancy in function observed between the MRFs in mouse gene knock-out studies[53]. Also, each of the MRFs can transactivate the genes encoding the other MRFs. Nonetheless, each of the MRFs displays a unique spatiotemporal expression pattern during skeletal muscle development and has a distinct role in the specification and differentiation of skeletal muscle progenitor cells that may differ depending on the muscle (group) involved[54] (Table 1.3). Primarily through studies in transgenic mouse models, a general picture of skeletal muscle development has emerged in which MyoD1 and Myf5 have overlapping roles in progenitor cell specification while MyoG and Myf6 are mainly involved in the terminal stages of skeletal myogenesis[53]. The recent finding of myotome formation and embryonic myogenesis in *MYOD1*$^{-/-}$*MYF5*$^{-/-}$ mice that have the *MYF5* gene inactivated without seriously disrupting the expression of the neighbouring *MYF6* gene, indicates that Myf6 can also act as a myogenic determination factor[55].
During the early embryonic phase of somatic myogenesis, Myf5, MyoD1 and Myf6 are responsible for myogenic cell determination with a more prominent role of Myf5 in the specification of the epaxial skeletal musculature and of MyoD1 in that of the hypaxial skeletal muscles. In the late embryonic phase of somatic myogenesis MyoG, Myf6 and MyoD1 cooperate in inducing myogenic cell differentiation and maturation through the activation of downstream skeletal muscle genes. In the early myotome, differentiated cells are present as

myocytes and muscle cell fusion only occurs in the mature myotome after expression of *MYOD1* and accumulation of M-cadherin[56]. The main factor responsible for the onset of differentiation in the myotome is Myf6[57]. After the embryonic phase of somatic myogenesis, *MYF6* expression is transiently downregulated with the gene being transcribed again at a high levels late during fetal skeletal muscle development[58]. *MYOG* is present in the differentiating skeletal muscle cells from the late stage of embryonic skeletal muscle development onward.

Table 1.3 Myogenic transcriptor factors in embryo and postnatal skeletal muscle.

Name	Embryonic action	Post natal action
MYOD1 (MYF3)	Induction of cell-cycle arrest and differentiation Myoblast proliferation and determination	Expressed in activated/proliferating satellite cells
MYOG (MYF4 OR MYOGENIN)	Associated with terminal differentiation and fusion of myogenic precursor cells to new or existing fibers	Expressed at the beginning of differentiation of satellite cells
MYF5	Myoblast proliferation and determination	Expressed in activated/proliferating satellite cells
MYF6 (HERCULIN or MRF4)	Possibly involved in differentiation of myotubes Associated with terminal differentiation and fusion of myogenic precursor cells to new or existing fibers	Present in newly formed myotubes
Mef2 (myocyte-specific enhancer factor; 2A,2B and 2C)	Induction and maintenance of muscle differentiation. Activation of muscle-specific gene expression: developing carding, skeletal and smooth muscle.	

During fetal and adult skeletal myogenesis the role of the individual MRFs is more defined although some actions still are overlapped (Figure 1.4B). *MYOG* is expressed when the progeny of satellite cells begins to differentiate. It is of interest that in knockout mice lacking *MYOG* satellite cell differentiation and skeletal muscle differentiation postnatal appears to proceed normally although these animals show a ± 30% reduction in body size as compared to the control mice[59]. This observation gave rise to the proposition that expression of other myogenic transcription factors, with overlapping activities, may compensate for the lack of *MYOG*[35].
MYF6 is not expressed in adult satellite cells but is co-expressed with *MYOD1* and *MYOG* in newly formed myotubes during skeletal muscle regeneration[60,61].

MYOD1 is not expressed in quiescent satellite cells, but is activated when these cells leave the basal lamina of the myofiber and begin to proliferate. Up-regulation of *MYOD1* is required for satellite cells to enter the proliferative phase that precedes terminal differentiation. In the absence of MyoD1, satellite cells have an increased ability to self-renew and a decreased differentiation potential. *MYOD1*$^{-/-}$ display high prenatal mortality. Although the frequency of satellite cells in these mice is 13-fold higher than that in wild-type siblings, their regeneration capacity is severely reduced[62]. The *MYOD1*$^{-/-}$ mice exhibit pronounced muscle atrophy[63], an incorrect maturation of nAChRs (i.e. delayed the transition from the fetal [alpha, beta, gamma, delta] to adult-type [alpha, beta, delta, epsilon] AChRs) and a reduction in NMJ fold density[64]. The latter two findings suggest an important role for MyoD1 plays in motor end plate formation.
MYF5 expression is largely restricted to replicating satellite cells and its expression is downregulated during differentiation[61]. However, there are indications of residual transcription of this gene in a subpopulation of quiescent satellite cells[65]. This may reflect the existence of heterogeneity in the satellite cell pool including both cells that are committed to undergo myogenic differentiation and cells that maintain “stemness” or return to quiescence[66-68].

3.2 Skeletal muscle regeneration: homeostatic turnover and postinjury repair

The regeneration of mature skeletal muscle tissue is a complex process involving satellite cells, myoblasts, immune cells and remodeling of the connective tissue. Furthermore, recent findings have raised the possibility that dedifferentiation of myofibers may yield an additional cell source to aid in the healing process of injured skeletal muscle[69]. The intensity of the regenerative response is proportional to the extent of tissue injury and the final outcome is influenced by innervation, vascularization, hormonal and nutritional status[70].
Mature skeletal muscle is a terminally differentiated tissue with myofibers containing postmitotic nuclei that do not divide. In normal muscle, contraction-induced sarcolemmal injury is common. Since skeletal muscle fibers have an efficient sarcolemmal repair system small tears in their plasma membrane normally are sealed within seconds[71] by a membrane sealing mechanism in which dysferlin acts as a Ca^{2+}-regulated fusogen[72,73]. In response to very mild injury (e.g. single eccentric stretch-induced injury) satellite cells immediately start to proliferate, but because of the rapid “intrinsic” recovery of the damaged myofibers, the satellite cell activation halts before the myoblasts have been formed[74]. Moderate to severe muscle damage induces satellite cells to proliferate and differentiate into myoblasts that fuse with pre-existing myofibers and with one another to replace part or all of the damaged myofibers (Figure 1.4C). After the initial wave of fusion, hypertrophy of regenerated skeletal

muscle fibers occurs by accretion of additional myoblasts. A normally functioning satellite cell repair system is able to completely regenerate a damaged skeletal muscle within 9 to 10 days.

Skeletal muscle injury occurs through a variety of mechanisms. These mechanisms can be divided in direct trauma (e.g. lacerations, contusions, and strains) or indirect causes (e.g. ischemia and neurological dysfunction). The different phases of healing of the damaged skeletal muscle are similar for all injury types. However, the functional recovery of the muscle differs considerably depending, amongst others, on severity of the initial damage and the age/condition of the affected individual[75-78] but this is not discus in this thesis.

Skeletal muscle regeneration proceeds in three distinct phases[79] in analogy to the healing of skin wounds. The first phase is *inflammation/degeneration*, characterized by accumulations of leucocytes (mainly neutrophils), myofiber necrosis of damaged segments and phagocytosis by macrophages of the necrotic debris. The infiltrating cells and the damaged myofibers release large amounts of growth factors, including HGF[80] and fibroblast growth factor (FGF) that activate satellite cells[21,81]. Nitric oxide (NO) production by increased NO synthase (NOS) activity is also important for satellite cell activation, possibly through activation of matrix metalloproteinases, which induce the release of HGF from the ECM[82,83].The second phase, *tissue repair or regeneration,* begins when satellite cells start to proliferate, migrate to the necrotic zone, differentiate into myoblasts and fuse to form multinucleated myotubes. Interestingly, during this phase embryonic and neonatal *myosin heavy chain genes* are temporarily re-expressed in the myofibers. After the initial wave of fusion, hypertrophy of regenerated fibers occurs by accretion of additional myoblasts. The last phase called *muscle healing, maturation or remodelling,* is characterized by a gradual recovery of the functional properties of the muscle, including the recovery of the tensile strength of its connective tissue component. Re-establishment of vascular and neural connections within the regenerating skeletal muscle is essential for its normal growth and function[84]. Reinnervation of the muscle tissue is essential for the full maturation and differentiation of the myofibers. Without innervation, myofibers become atrophic and eventually substituted by fibrofatty tissue[85,86].

The activation of satellite cells and the promotion of myoblast fusion heavily depends on growth factors (Table 1.4), including HGF[80], FGFs (e.g. FGF-1 and FGF-2), insulin-like growth factors (i.e. IGF-1 and IGF-2), epidermal growth factor (EGF), platelet-derived growth factors (i.e. PDGF-A and PDGF-B), tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6)[87-90]. The expression of the muscle-specific transcription factors including *MYOD1* and *MYOD* (Figure 1.4B) mark the differentiation of muscle precursor cells into myoblast[91]. Notch signaling appears to be necessary for the expansion of the myoblast pool and

Wnt signaling has been described to promote myogenic differentiation[92,93]. Detailed overviews of the function of these and other factors are provided elsewhere[21,87,89,90].

Table 1.4 Effect of growth factors on myoblasts *in vitro* and *in vivo.*

Growth factor	Cell proliferation	Cell differentiation
HGF	stimulates	stimulates
FGF-1	inhibits	stimulates
FGF-2	stimulates	inhibits
IGF-1	stimulates	stimulates
EGF	inhibits	inhibits
PDGF-A	inhibits	stimulates
PDGF-B	stimulates	inhibits
TNF-α	inhibits	inhibits
IL-6	stimulates	stimulates

HGF, hepatocyte growth factor; FGF, fibroblast growth factor; IGF-1, insulin-like growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TNF- α, tumor necrosis factor alpha. Table adapted from Filippin et al.[88] and Huard et al.[89]

Newly regenerated myofibers contain centrally localized nuclei. In humans, these nuclei move to a subsarcolemmal position (Figure 1.5). In contrast, in rodents regenerated myofibers remain centronucleated indefinitely[94]. Immature and regenerating myofibers show low expression of the genes encoding β-spectrin, dystrophin, nNOS, laminin β2 and integrin α7, but highly express the genes for utrophin, laminin α5, neural cell adhesion molecule (N-CAM), vimentin, desmin and major histocompatibility complex class I (MHC-I) molecules[23].

Most satellite cells in the regenerating muscle proliferate within the empty sleeves on the basal lamina of the degenerating myofibers. This microenvironment supports similarly the generation of new formed myofiber(s). The basal lamina is also of crucial importance during myoregeneration in serving as a mechanical scaffold for the orientation of the regenerating myofibers. This microenvironment supports equally well the generation of new skeletal muscle fibers. Muscle regeneration can often lead to significant remodeling of the muscle tissue with a variety of patterns. Regenerating (nascent) myotubes within the same basal lamina my not fuse, leading to the formation of clusters of smaller fibers, or may fuse only at one extremity, causing the formation of forked fibers (previously called “fiber splitting”). After segmental necrosis, regenerative processes are concentrated at the level of the damaged stump, giving rise to appearances called “budding”, and the reconstitution of myofiber integrity may be prevented by scar tissue that separates the two stumps that will result in the formation of new MTJ. Finally,

regenerating myofibers may occasionally form outside the basal lamina due to migration of satellite cells or possible contribution of non muscle stem cells, and remain as small fibers embedded in the interstitial tissue[95]. Further, the basal lamina it also acts as a reservoir of growth factors and a substratum of macromolecules favorable for skeletal myogenesis[84].

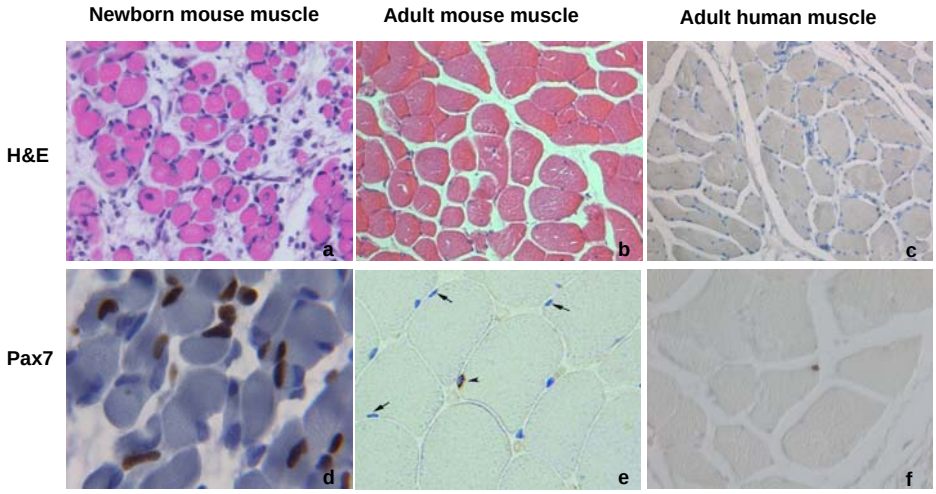

Figure 1.5 **Immuno histochemistry of skeletal muscle.**
Microphotographs of skeletal muscle of newborn mouse (a,d), adult mouse (b,e) and adult human (c,f). Staining of hematoxylin and eosin (H&E) in the upper panels and staining with Pax7 antibody in the lower panels. Notice the differences in morphology of fibers, myonuclei and frequency of Pax7 cells between newborn and adult muscle. Magnification 40x a,b and c; 100x d,e, and f.

Tissue reconstitution after injury involves not only the replenishment or replacement of parenchymal cells but also that of the supporting structures including blood vessels, nerves, connective tissue and stromal cells. Little is known about how this network of associated cells coordinates myofiber growth, homeostasis and repair in skeletal muscles. It has been recently shown that different stem cell types (e.g. bone marrow [BM]- and blood vessel wall-derived stem cells) besides to satellite cells have the potential to participate in skeletal muscle regeneration, though their contribution in the healthy tissue seems to be negligible[96,97].

The regenerative capacity of skeletal muscle in response to injury is significantly declines with age[98]. This is apparently not attributable to a decrease in the number or activity of the satellite cells but rather to an overall decline in the regenerative capacity of the aged muscle, as each phase of the

repair process seems to slow down and deteriorate with age[98,99]. There are also considerable differences in the kinetics of myoregeneration between different mammalian species. These differences seem to relate to muscle body size. A general rule is that “what takes a day in the rat takes a week in the cat”[100].

4 Skeletal muscle pathologies

A gross classification of the types of skeletal muscle diseases based on the underlying mechanism is presented in Table 1.5. Skeletal muscle tissue may also become indirectly affected in many acute or chronic diseases, but these secondary lesions will not be included in this overview.

4.1 Central and peripheral nervous system diseases

Many central and peripheral nervous system diseases lead to degeneration and atrophy of myofibers. Denervated myofibers shrink in size and may undergo changes in their internal architecture including a relative increase in slower myosin isoforms, sarcomeric disorganization and myofibrillar disruption, a decrease in the number of ribosomes and in the number and size of mitochondria and a relative increase in the terminal cisternae of the saroplasmic reticulum[101]. The basal lamina around individual myofibers often remains intact as they shrinks and becomes folded. Since one motor nerve supplies many myofibers, denervation will result in atrophic myofibers scattered at random in a skeletal muscle biopsy. The atrophic myofibers in the muscle is pathognomonic of skeletal muscle denervation. The effect of denervation on the satellite cells depends on the duration of the injury. Shortly after denervation satellite cell numbers increase whereas long-term denervation depletes the satellite cell pool[102-105].

The non-neurogenic acquired muscular diseases or myopathies have multiple origins. Accordingly, these myopathies can be divided in degenerative, caused by toxic agents, autoimmune reactions and traumatic.

Table 1.5 Types of human muscular diseases.

Disease	Histological features
Central and peripheral nervous system diseases "neurogenic"	
Spinal muscular atrophy (SMA I, II, III) Motor neurone disease Chronic neuropathy Simple neuropaty	Atrophy of small or large myofiber group(s) Grouping myofiber type (I or II) Myofibers with angulated shape
Acquired "myopathies"	
Toxic myopathies: alcohol/drug abuse, statins, diuretics, steroids, some antimalaria treatments	Rhabdomyolysis or massive necrosis of myofibers.
Inflammatory myopathies: - infectious agent (e.g. virus, bacteria, or parasite) - autoinmmune disease (e.g. dermatomyositis, polymyositis, inclusion body myositis, sarcoidosis, nodular focal myositis, vasculitis, myositis associated with fasciitis, myasthenic syndromes)	Hypertrophied fibers rare Necrosis Phagocytosis Inflammatory cells Lymphorrhages (lymphocyc infiltration) Perifascicular atrophy and reduced number of capillaries (dermatomyositis) Rimmed vacuoles (inclusion body myositis)
Trauma: lacerations, contusions, strains, high dose ionizing radiation, heat, ischemia	Inflammatory cells Necrosis Internal/central nuclei Variation in fiber size
Genetically based muscular diseases	
Metabolic myopathies: mitochondrial myopathies, lipid myopathies, glycogenoses, abnormal transport of ion channels, endocrine myopathies. Congenital myopathies: MDC1, Ullrich congenital muscular dystrophy, Integrin α7 deficiency, Fukuyama congenital muscular dystrophy, Muscle-eye-brain disease, Walker-Warburg syndrome	The muscles are replaced with fatty deposits over time. Focal muscle degeneration (weak and wasted) Wide variation in fiber size Necrotic fibers Phagocytosis Endomyseal fibrosis Internal nuclei increased Basophilic fibers Inflammatory response
Muscular dystrophies: - Autosomal dystrophies (e.g. LGMD, fascio-scapulo-humeral myopathy, oculo-pharingeal dystrophy) - X-linked muscular dystrophies (e.g. Becker and DMD)	

4.1.1 Toxic or drug-related myopathies

Common causes for toxic or drug-related myopathies[106] are the overuse of (cortico)steroids and alcohol[107]. The widespread use of statins has resulted that these drugs are presently the commonest cause of myalgia and increase of creatine kinase (hyperCKaemia) in peripheral blood. Other important causes of iatrogenic myopathies include the chronic use of immunosuppressive drugs (e.g. cyclosporine) and nucleoside analogue reverse transcriptase inhibitors. To understand human muscle pathology, laboratory animals are often applied. Studying the effect of drugs and other myotoxic agents in animal models has provided useful information on the pathogenesis of skeletal muscle disease and has helped to elucidate the cellular mechanisms involved. For example, zidovudine (also known as azidothymidine [AZT]), used in the treatment of acquiered immunodeficiency syndrome (AIDS), greatly increased our understanding of mitochondrial DNA turnover in skeletal muscle[108,109].
Drugs/toxins differ in the myopathology they induce. Focal mild myopathy results from intramuscular injection of opiates, lidocaine and benzodiazepines. Necrosis and rhabdomyolysis is the result of intoxication by alcohol, opiates (heroin and cocaine), anaesthetic agents, snake venoms and statins. Drug-induced skeletal muscle inflammation a myopathy resembling polymiosistis is caused by D-penicillamine, levodopa (also known as L-3,4-di-hydroxy-phenylalanine or L-DOPA), phenytoin, procainamide, leuprolide, propylthiouracil, cimetidine (Tagamet), α-interferon, L-tryptophan and aluminium hydroxide (used as adjuvant in various vaccine preparations). Mitochondrial damage/depletion is caused by AZT and cyclosporine. Myosin heavy chain loss is caused by high doses of intravenous corticosteroids given during mechanical ventilation under neuromuscular blockade with pancuronium (Pavulon). Type 2 myofiber atrophy may develop as a result of chronic alcohol abuse and high doses of corticosteroids. Vacuolar myopathy is a side effect of treatment with anti-malarial drugs (e.g. quinine and related agents and [hydroxyl] chloroquine). Lysosomal myopathy is side effect of the antiarrhytmic agent amiodarone and the prophylactic antianginal agent perhexiline maleate. Antimicrotubular myopathy is a side effect of colchicine and the cytostatic drug vincristine. The clinical presentation resulting from intoxications may be acute, subacute or chronic[110]. However, some drugs may cause a variety of pathological manifestations and clinical presentations[111]. Nonetheless, in all these cases, skeletal muscle regeneration follows the normal process through activation of satellite cells as described earlier.

4.1.2 Autoinmmune reaction myopathies

Inflammatory myopathies are a heterogeneous group of skeletal muscle diseases with diverse onset, clinicopathological features and etiologies. There are two main categories of inflammatory myopathies: the infectious

inflammatory myopathies caused by viruses, bacteria, fungi, protozoa or helminthes and the idiopathic (autoimmune) inflammatory myopathies which are further subdivided in focal and generalized forms[112]. Examples of generalized idiopathic inflammatory myopathies are polymyositis, adult and juvenile dermatomyositis, inclusion body myositis and necrotizing myopathy, which may also have a paraneoplastic etiology[113]. Common to the inflammatory myopathies is the presence of inflammatory cell infiltrates of variable composition and distribution and with contributions of $CD8^+$ T lymphocytes, B lymphocytes, plasma cells, histiocytes, dendritic cells and macrophages. Furthermore, these diseases are associated with various degenerative changes in the skeletal muscle fibers such as expression of MHC-I molecules on the sarcolemma, myofiber necrosis, perifascicular complement deposition (i. e. C5b-9), changes in blood vessels and loss of capillaries[113]. The most prominent clinical symptoms of inflammatory myopathies are skeletal muscle weakness and wasting[91,111,114].

The myasthenic syndromes are due to damage of the NMJs resulting in characteristic fatigable skeletal muscle weakness[115]. Myasthenia gravis is an acquired autoimmune disorder caused by antibodies to the different subunits of the nAChR (majority of cases) or to a muscle-specific receptor tyrosine kinase (MUSK), which are both concentrated in the postsynaptic membrane of the NMJ. Other examples of acquired myopathies with an involvement of the NMJ are Lambert-Eaton myasthenic syndrome and Isaac-Mertens syndrome, which are caused by antibodies directed against voltage-gated calcium and potassium channels, respectively. The congenital/inherited myasthenias are caused by mutations in genes encoding various key players in neuromuscular transmission like nAChR subunits, choline acetyltransferase and rapsyn, which is involved in nAChR clustering[111].

4.1.3 Traumatic myopathies

The traumatic skeletal muscle injuries are the most frequent. The damage can result from crush, contusion, laceration or freezing. Healing of these skeletal muscle injuries occurs via the normal regeneration process as discussed previously. Satellite cell activation is limited to areas where there is necrosis of myofibers. Regeneration might be completed in 9 to 10 days, depending on the severity of the injury. Satellite cell activation after trauma has been proposed to be triggered by the disruption of the integrity of the sarcolemma and basal lamina[116] or/and by cytokines release by infiltrating inflammatory cells[89].

Ischemic damage is an aggravating factor of many traumatic lesions. Regeneration in those cases depends on the severity of the damage. Usually it presents a normal process of muscle regeneration starting from the closest non-injured areas. In some cases the healing process presents certain

complications i.e. incomplete functional recover, recurrent injuries and/or scar tissue formation.

4.2 Hereditary skeletal muscle diseases

Hereditary skeletal muscle diseases include the metabolic myopathies, mitochondrial myopathies, skeletal muscle channelopathies, congenital myopathies, distal myopathies and (congenital) muscular dystrophies. For a comprehensive overview of the inherited myogenic disorders and the genes involved see www.musclegenetable.org.

4.2.1 Metabolic myopathies

Disorders that affect the metabolism of the muscle called metabolic myopathies, may cause exercise intolerance/muscle fatigue, cramps/myalgia, rhabdomyolysis/myoglobinuria and progressive skeletal muscle weakness[117]. These diseases, which all affect the energy supply of skeletal muscle tissue, can be subdivided in three different groups with regard to their biochemistry. Besides glycogen storage diseases, which includes Pompe's, Cori-Forbes, McArdle's and Tarui's disease and phosphorylase b kinase deficiency, the metabolic myopathies comprises fatty acid oxidation defects, and mitochondrial myopathies. Of these diseases, the mitochondrial myopathies are by far the most prevalent. Whether myoadenylate deaminase deficiency also belongs to the metabolic myopathies is still a matter of debate given that fact that 2% of the general population is homozygous for mutant *AMPD1* alleles and not all of them display clinical symptoms.

4.2.2 Mitochondrial myopathies

The mitochondrial myopathies are a heterogeneous group of neuromuscular disorders linked to mutations in the mitochondrial DNA or in nuclear genes encoding mitochondrial proteins[118]. The mutations in the mitochondrial DNA either affect mitochondrial protein synthesis *in toto* or components of the mitochondrial respiratory chain. The mutations in the nuclear DNA are associated with components of the mitochondrial respiratory chain or with proteins that are directly or indirectly involved in its proper assembly and function or in factors controlling the abundance and quality of the mitochondrial DNA. Some important mitochondrial myopathies include mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes, chronic progressive external ophthalmoplegia and myoclonic epilepsy with ragged-red fibers, which are linked to defects in the mitochondrial DNA and Kearns–Sayre syndrome, myoclonic epilepsy myopathy sensory ataxia, Alpers-Huttenlocher syndrome, coenzyme Q10 deficiency, Leigh syndrome, and Barth syndrome whose origin lies in nuclear DNA mutations[111].

4.2.3 Skeletal muscle channelopathies

Another group of inherited myogenic disorders are the skeletal muscle channelopathies[119,120]. These diseases are caused by defects in genes encoding ion channel proteins of the sarcolemma, T tubules and sarcoplasmic reticulum representing all major classes of voltage-gated ion channels (Na+, K+, Ca2+, Cl−), ligand-gated ion channels (e.g. nAChR, see above) and intracellular channels (ryanodine receptor 1 [RyR-1]). Clinically the skeletal muscle channelopathies fall into three main groups: those with myotonia only, those with myotonia and periodic paralysis and those solely with periodic paralysis. Inherited myogenic disorders that belong to this group are Becker's and Thomsen's myotonia congenital (CLCN1 gene), potassium-aggravated myotonia, paramyotonia congenital or Eulenburg's disease and hyperkalaemic periodic paralysis (SCN4A gene), hypokalaemic periodic paralysis (SCN4A or CACNA1S gene), Andersen-Tawil syndrome (KCNJ2 gene) as well as malignant hyperthermia and central core disease (RYR1 gene). A disease related to the skeletal muscle channelopathies is Brody's disease, which is caused by mutations in the ATP2A1 gene encoding the fast-twitch skeletal muscle sarcoplasmic reticulum Ca2+ ATPase[121].

4.2.4 Congenital and distal myopathies

Congenital myopathies are a molecularly, pathologically and clinically heterogenous group of hereditary diseases defined by hypotonia and skeletal muscle weakness, that usually present at birth or early childhood, and generally have slow progression. These disorders are associated with characteristic morphological defects in the structure of the myofibers as evident by histochemical staining or ultrastructural examination. Most but not all congenital myopathies originate from mutations in genes encoding sarcomeric components. On the basis of their morphological features, these diseases have been subdivided in four main groups characterized by the presence of (rod-shaped) protein accumulates, myofiber cores devoid of mitochondria, centrally localized myonuclei and disproportionate myofiber size variations, respectively. The best known congenital myopathies are the nemaline rod myopathies, which can be caused by mutations in at least six different genes[122].
Another group of hereditary muscle diseases dominated by mutations in genes encoding sarcomeric proteins are the distal myopathies. These diseases are generally characterized by progressive skeletal muscle weakness and atrophy beginning in the distal muscles of the upper and lower limbs especially those around the wrists and ankles[123].

4.2.5 Congenital muscular dystrophies

Congenital muscular dystrophies are a clinically and genetically heterogenous collection of inherited skeletal muscle diseases characterized by congenital hypotonia, delayed motor development and early onset of progressive muscle weakness accompanied by dystrophic alterations in skeletal muscle structure. Most of these diseases are either associated with defects in sarcolemmal/ECM proteins (e.g. merosin-deficient congenital muscular dystrophy/MDC1A [*LAMA2* gene], Ullrich syndrome and Bethlem myopathy [*COL6A* genes], congenital muscular dystrophy with integrin α7 deficiency [*ITGA7* gene]) or with defects in proteins that affect the glycosylation of α-dystroglycan and lead to a secondary reduction in laminin α2 (e.g. MDC1C, MDC1D, Fukuyama congenital muscular dystrophy, muscle-eye-brain disease and Walker-Warburg syndrome)[111,124,125]. Some of the congenital muscular dystrophies are caused by severe allelic variants of gene defects that more commonly produce mild forms of LGMD with onset in adolescence or adult life (see below).

The muscular dystrophies are a group of hereditary disorders, clinically characterized by skeletal muscle wasting and weakness. The clinical and genetic heterogeneity of these conditions is well recognized: some have prenatal onset, while others affect only adults, some are rapidly progressive, while others are associated with long periods of stability; some involve multiple organs including the heart and central nervous system, while others are largely restricted to skeletal muscles. In general, muscular dystrophies compromise patient mobility and quality of life, and in the most severe cases lead to extreme skeletal muscle weakness and premature death. They share histological features e.g. variation in myofiber diameter, necrosis and centronucleation of myofibers as signs of degeneration and regeneration, respectively, split myofibers, inflammatory infiltrates and, at later stages of disease, replacement of myofibers by fibroblasts and adipocytes[91,126,127].

The first identified gene associated with muscular dystrophy was the *DMD* gene located on the short (p) arm of the X chromosome at position 21.2 (Xp21.2), which encodes the protein dystrophin. This protein acts as a “molecular shock absorber” that dissipates part of the forces generated during sarcomere contraction to the extracellular matrix (see above). Lack of functional dystrophin leads to striated muscle fragility, contraction-induced sarcolemmal damage, myofiber necrosis and inflammation[128]. DMD is the most common and severe disease among similar dystrophic diseases that affects approximately 1 in 3,500 boys and is caused by deletions, duplications, or point mutations in the *DMD* gene that in most cases disrupt its open reading frame. A less severe phenotype is observed in BMD, in which mutations in the *DMD* gene yield a protein that retains part of its functionality. Other diseases belonging to the muscular dystrophies are the six types of Emery-Dreyfuss muscular dystrophy, the two forms of fasioscapulohumeral dystrophy, and the

more than twenty types of LGMD. Like DMD/BMD, several of the most severe LGMDs have been linked to mutations in genes encoding the DGC proteins (i.e. LGMD2C, LGMD2D, LGMD2E and LGMD2F)(reviewed in[91]).
In muscular dystrophy repeated episodes of skeletal muscle regeneration, brought about by recurring loss of muscular parenchyma, may lead to an early exhaustion of the proliferative potential of satellite cells, and to failure of maintaining muscle homeostasis. Once the myoregenerative capacity of the satellite cells has been exhausted, the skeletal muscle tissue is gradually replaced by fibrous or fatty connective tissue. Satellite-cell exhaustion may relate, at least in part, to shortening of telomere ends after repeated rounds of DNA replication[129], to recurrent exposure to inflammatory conditions and/or oxidative stress[130], to an accumulation of mutations in key satellite-cell regulatory genes introduced during repeated rounds of proliferation, or to a combination of these factors.
In other myopathies the exhaustion of satellite cells is not the predominant feature. For instance satellite cells from biopsies of patients with myotonic dystrophy type II, which is caused by the extension of the CCTG repeat in intron 1 of the *CNBP* gene, do proliferate but fail to differentiate[131]. Furthermore, it has been shown that the activation of satellite cells in human skeletal muscle diseases is more likely to occur in fibers of larger diameter, regardless of whether the predominant pathological pattern is neurogenic or myopathic and whether the satellite cell number of the biopsy is elevated[132].
It would be desirable to gain more insight in the precise role of satellite cells in these various skeletal muscle diseases for the design of more effective cell-based therapies. However, to study skeletal human muscle has several practical limitations such as the collection of muscle biopsies and the isolation, identification and expansion of satellite cells/muscle precursor cells. Therefore most of the work on skeletal muscle regeneration is performed in laboratory animals, particularly rodents.

5 Animal models of skeletal muscle regeneration

Skeletal muscle has a remarkable ability to regenerate. A variety of conditions in many different animal species have been used to investigate the regeneration process. These include invertebrates, amphibians, fishes, birds and mammalians. Among the latter are large animals like dogs, pigs and horses as well as many rodents of which the mouse is the most popular/represented. Since our focus of interest is the capacity and role of human MSCs in myoregeneration our choices were limited to immunodeficient animals. As in our laboratory, we had good experience with the engraftment of human cells in non-obese diabetic severe combined immunodeficient

(NOD/SCID) mice, it became the mainstay of the research described in this thesis. Therefore this review deals mainly with mouse models of skeletal muscle damage and repair. Regeneration of skeletal muscle has been studied in muscle of mice with different hereditary myopathies[133] as well as in animals (both wild-type and mutant) following various kinds of induced injuries.

5.1 Genetic models

Over the past few decades, a large number of genes involved in skeletal muscle diseased have been identified. At present, a large variety of hereditary animal models are available for studying skeletal muscle degeneration and regeneration. These include spontaneous mutants (as in the C57BL/10ScSn-*Dmd*^{mdx}/J or *mdx* mouse, a model for DMD), chemical- or irradiation-induced mutants (such as the *mdx*^{2Cv}, *mdx3*^{Cv}, *mdx4*^{Cv} *and mdx5*^{Cv} mouse) and animal engineered using recombinant DNA technology (e.g. the *mdx52* mouse, which has exon 52 of the *DMD* gene disrupted)[134]. The mouse models are the most used for practical reasons as logistics, the limited scale-up of reagents, the relatively low costs of housing, the availability of many research tools for mouse studies, the great knowledge of mouse biology and because of the many genetic defects available. Most of the genetically modified mice mimic certain specific aspects of the human disease, while others provide evidence for roles of genes as secondary modifiers of disease progression or as inhibitors of disease phenotypes[133]. Listed in Table 1.6 are engineered mice with (conditional) defects in genes encoding transcription factors, membrane receptors or cell signaling molecules, structural proteins and ECM components[87,100]. An inconvenience of these models is that their genetic background might differ substantially and consequently the progression of muscle regeneration. The latter is based on observations where muscle regeneration after crush-injury is greater in Swiss and SJL/J mice than in Balb/c mice[135,136] and on the findings that in Swiss mice show impaired phagocytosis of necrotic skeletal muscle isografts[137]. Further, comparing skeletal muscle damage and repair upon voluntarily running of mice from different strains including CBA/J, NMRI, C57BL, NIH, Swiss and Balb/c revealed marked differences between individuals and strains[138]. Accordingly, one has to take into account the genetically background of genetically modified mice in the experimental design.

Table 1.6 Targeted germline mutations affecting muscle degeneration/regeneration in mice.

Target mutation	Main observations	Ref.
Transcriptor Factors		
Myf5$^{-/-}$	Normal skeletal muscle Severe rib abnormalities Premature death	250,251
MyoD$^{-/-}$	Minor alterations Satellite cells with increased proliferation but delayed differentiation	252,253,62
Myf4$^{-/-}$	Severe muscle deficiency Secondary myogenesis altered Perinatal death	254,255,256
Myf6$^{-/-}$	Mild alteration of skeletal muscle	257,258
Myf5$^{-/-}$ / MyoD$^{-/-}$	No myoblast, no skeletal muscles Perinatal death	259
Myf5$^{-/-}$ / Myf6$^{-/-}$	Normal skeletal muscle Rib and axial muscle malformations Perinatal death	260
Pax7$^{-/-}$	Growth deficit, satellite cells absent	18
Slug$^{-/-}$	Fairly normal	261
MNF$^{-/-}$	Growth deficit Decreased proliferation but normal differentiation of satellite cells	163
DCG components		
MCK (Cre)-dystroglycan (LoxP)	Increased degeneration without fibrosis or fat replacement, muscle mass increase	262
Dystrophin$^{-/-}$ (*mdx*)	Increased degeneration without fibrosis or fat replacement, transient muscle hypertrophy Satellite cells proliferate normally	262
δ-Sarcoglycan$^{-/-}$	Increased degeneration without fibrosis or fat replacement, muscle mass increase	262
Growth factors		
FGF-6$^{-/-}$	Fairly normal muscle function	263,264
MSTN$^{-/-}$	Muscle mass increase (hyperplasia and hypertrophy)	265,147
Others		
Laminin β2$^{-/-}$	Abnormal differentiation of neuromuscular junctions	266
Desmin$^{-/-}$	Cardiac, skeletal and smooth muscle degeneration	267,268,269

FGF, fibroblast growth factor; LIF, leukemia inhibitory factor; MCK, muscle creatine kinase; MNF, myocyte nuclear factor; MSTN, myostatin; ND, not determined. Table adapted from Pastoret C et al.[23]and Charge et al.[87].

To date there are various mouse models in which the DGC has been disrupted by knocking out genes encoding dystrophin or other components of this complex such as dystroglycan and sarcoglycans. These knockout (KO) mice have provided useful insight into the regulatory pathways involved in skeletal muscle degeneration and regeneration. The *mdx* mouse, which is the most widely used model for DMD, has a spontaneous stop codon mutation in exon 23 of the *DMD* gene on a C57BL/10 background. These mice show massive skeletal muscle degeneration during the first 6 weeks of their life but in contrast to DMD patients at 1 year of age these mice have only mild fibrosis and limited

replacement of myofibers by fat cells[128,139]. In *mdx* mice skeletal muscle regeneration is going on throughout life by continuous recruitment of satellite cells. The mdx mutation does not seriously impair skeletal muscle function and the mice possess a nearly normal lifespan. This mild muscle damage is mainly due to the presence of relatively large numbers of dystrophin-positive myofibers called 'revertant fibers' (1-3%)[140] and up-regulation of utrophin that may partially compensate for the loss of functional dystrophin[141,142].
It is noteworthy to mention that currently the animal that best mimic DMD are the dystrophin-deficient dogs (i.e. golden retriever, beagle) which display a similar pathogenesis as human DMD patients characterized by progressive skeletal muscle adipofibrosis and weakness as well as premature death[23,143]. Nonetheless, *mdx* mice serve usefully for proof of principle purposes of therapeutic approaches, including gene replacement therapy[144,145], surrogate gene therapy[146-148], exon skipping[149-152], stop codon read through[153,154], myoblast transfer[155,156], stem cell therapy[157], and various drug or nutritional therapies[158,159].
Having a mouse model that can better mimic the more severe aspects of DMD, including increased mortality, increased cardiac and respiratory muscle weakness, and increased deficits in ambulation, would advance translational work. Recently, *mdx* mice were introduced that have a human-specific deletion in the mouse *cytidine monophospho-N-acetylneuraminic acid hydroxylase (Cmah)* gene that increases disease severity of the *mdx* model of DMD[160]. Sacco et al.[161] reported *mdx* mice lacking the RNA component of telomerase (*mdx/mTR*) have shortened telomeres in muscle cells and severe muscular dystrophy that progressively worsens with age. However, *mdx* mice carrying mutations in genes that perturb satellite cell function display a more severe dystrophic phenotype and eventually die prematurely[162-164].

5.2 Induced skeletal muscle damage

Whereas the muscular dystrophies present continuing simultaneous combinations of skeletal muscle degeneration and regeneration that can not be clearly separated, induced injury allows analysis of skeletal muscle regeneration with a sharply defined beginning and ending. For the study of interventions, as in our case with MSC implants, injury models -if properly utilized- have the advantage that the interpretation of the results is not hampered by modifying effects of the MSCs on the degeneration process.
Another attractive feature of induced injuries is that they can be applied locally to a group of muscles or a single muscle or even to part of a skeletal muscle. This limits possible adverse effects of skeletal muscle damage on the whole organism and facilitates tissue sampling for analysis. Further, myopathy-causing gene defects may affect other tissues/organs besides cardiac and skeletal muscle.

To study myoregeneration, a diversity of methods for destroying skeletal muscle tissue have been employed, which fall into three categories, namely chemical, physical and biological injury (Table 1.7).

5.2.1 Chemical injury

A variety of chemical compounds causes severe skeletal muscle damage following intramuscular administration.
Most employed are barium chloride ($BaCl_2$)[165]; bupivacaine[166] and two snake toxins (i.e. cardiotoxin and notexin)[167]. Other chemicals such as aldehydes, chloroquine (specific to type I myofibers), glycerol (promotes the replacement of myofibers by adipocytes)[168,169], vincristine and hypertonic solutions as well as solutions of varied temperature (hot or cold) and pH (acid or alkaline)[23] have been employed less frequently.
The snake toxins are the most widely used and the lesions caused by these agents are reproducible. Upon local injection, these toxins induce acute rhabdomyolysis. Satellite and stromal cells are not affected so that regeneration of the treated muscle(s) proceeds as described above. Accordingly, in most experiments described in this thesis skeletal muscle damage was induced by injection of cardiotoxin into the readily accessible tibialis anterior muscle (TAM).

5.2.2 Physical injury

Among the physical injuries used to study skeletal muscle regeneration are cold injury, crushing, surgical wounding, mincing and free grafts.
In clinical practice, skeletal muscle damage caused by exposure to environmental low temperatures or cryodamage is complex affecting not only the skeletal muscle parenchyma but also the intramuscular connective tissue, blood vessels and neurons as well as neighboring tissues/organs. This type of injury does therefore not allow the investigation of skeletal muscle regeneration *per se*. In contrast, localized exposure to a low temperature (e.g. by using liquid nitrogen) of a well-defined area of skeletal muscle has been used experimentally to induce a limited area of necrosis. In this model, regeneration of the necrotic muscle (with no viable cells remaining) is totally dependent on precursor cells from the surrounding viable skeletal muscle tissue[170].
Crush injury of skeletal muscle tissue causes necrosis resulting in a focal reduction of the satellite cells. Macrophages invade the area of injury and phagocytose the necrotic tissue. These cells also produce several growth factors that are mitogenic for muscle precursor cells. Mitotic activity of satellite cells has been observed at or near the site of injury but many if not all of the dividing satellite cells have migrated from undamaged areas surrounding the lesion[171].

Table 1.7 Induction of muscle damage.

Agent	Muscular damage characteristics	Ref.
Chemical:		
$BaCl_2$	Destruction of the architecture of muscle Persisting satellite cells and parenchyma cells	165
Bupivacaine (Mercaine)	Selective degeneration[#] of myofibers lasting 1-2 days Immature myofibres appear within 2-3 days Selectively kill myofibers No affection of satellite cells proliferation	270,271 272
Myotoxins		
Cardiotoxin (Protein kinase C activity that is a pore-forming agent)	Does not affect satellite cells or blood vessels and muscle innervation Degeneration[#] lasts 1-2 days Maximum satellite cell proliferation ~2-3 days Immature myofibres appear within 2-3 days Reinnervation begins ~3 days Extended inflammation	272 273
Notexin (Phospholipase A_2 neurotoxin peptide that block neuromuscular transmission)	Degeneration[#] lasts 1-2 days Maximum satellite cell proliferation ~2-3 days Immature myofibres appear within 2-3 days Reinnervation begins ~3 days Extended inflammation	274
Physical:		
Cryodamage	Complete absence of myogenesis only depending on time of exposure No viable cells remain only depending on time of exposure	170
Crushing	Focal reduction of satellite cells.	171
Surgical wounding	Transected myofibers often undergo abortive attempts of regeneration The regenerated ends of transected myofibers commonly become embedded in connective tissue	94 275 172
Irradiation	Depends on dose (mild or severe damage) Cell replication is blocked and apoptosis ensues Reduced phagocytic activity Dose dependant decrease in numbers of muscle precursor cells Dose dependant decrease in myotubes in the regenerating muscle	276 277 278 279
Mincing	Degeneration[#] lasts 7 days Maximum satellite cell proliferation reached ~7-9 days Absence of surviving fibers Regeneration occurs in a centripetal fashion following in growth of capillaries	23
Free muscle grafts	Degeneration[#] lasts 3-5days Reinnervation starts in weeks	174.173 183
Biological:		
Forced exercise	Increased skeletal mass, focal damage Focal regeneration of myofibers	181
Devascularization - Denervation	No survival of satellite cells Regeneration occurs in a centripetal fashion originating from healthy surroundings Immature muscle fibers appear within 5-7 days Damage to the basement membranes	182 174
Ischemia-reperfusion	Depending on time and pressure no survival of satellite cells can be found Regeneration occurs in a centripetal fashion Immature muscle fibers appear within 5-7 days depends on dose	184 100

[#]Degeneration is considered as inflammatory changes in myofibers and infiltration of inflammatory cells

Surgical wounding (i.e. transection) of muscle tissue produces a clean lesion, which in humans is usually followed by scarring at the site of injury. On either side of the incision, the transected myofibers often undergo abortive attempts at regeneration, and the regenerated ends commonly become embedded in connective tissue[94,172].

For studying regeneration of minced skeletal muscle tissue, a skeletal muscle piece is chopped into small fragments so that all myofibers are destroyed and all neurovascular connections are disrupted and subsequently transplanted subcutaneously or intramuscularly. This minced muscle retains a surprising capacity for regeneration of up to ~30% of its original mass, but the regeneration process is often accompanied by excessive scar formation[23]. Minced muscle grafts offer the unique opportunity to eliminate the problem of inhomogeneous distribution when studying the participation of specific cells like stem cells in the regeneration process. Also, many more of such cells may be mixed with the injured skeletal muscle tissue than can be accomplished by intramuscular injection.

Free transplantation consists of removing a skeletal muscle completely from its bed and replacing it orthotopically. In the standard graft, the tendons are surgically restored, but revascularization and reinnervation are allowed to occur spontaneously. This is basically an ischemia model. The center of the graft falls into a state of ischemic necrosis, and over a few days regeneration occurs along a centripetal gradient. Free grafting is size-limited, and the recovery course of the grafted muscle differs among species. In adult rats, the rectus femoris muscle regenerates fully after free grafting. It takes 6-7 weeks before the center of the graft has become revascularized[173,174]. In monkeys (palmaris longus muscles), the center of a free graft becomes filled with a dense core of collagenous connective tissue surrounded by a concentric rim of regenerated myofibers[175].

Cross-transplantation of either whole or minced skeletal muscles is a most valuable to distinguish between intrinsic properties of the graft and environmental influences on the regeneration process. Cross-transplantation was first carried out between fast- and slow-twitch muscles to investigate the trophic effect of the nerves on the functional differentiation of regenerating skeletal muscle[176]. Minced skeletal muscle tissue has also been transplanted between normal and dystrophic mice to establish whether duchenne muscular dystrophy is caused by a myogenic or neurogenic defect[177,178]. The same approach has also been used to study the effect of a diabetic environment on skeletal muscle regeneration[179]. A cross-age transplantation model showed that old skeletal muscle tissue grafted into young rats regenerated as well as recipient age-matched young skeletal muscles, whereas young skeletal muscle tissue grafted into old hosts regenerated as poorly as old skeletal muscles[180].

5.2.3 Biological injury

Examples of biological injury are forced exercise, denervation-devascularization and ischemia-reperfusion.
Forced or eccentric physical exercise induces focal damage of the myofibers and subsequent activation of satellite cells[181]. Devascularization-denervation represents a mild form of skeletal muscle injury that has been used mainly to study myofiber type specification[182,183]. Delay on innervation to skeletal muscle decreases the number of satellite cells and causes a severe reduction of number of myofibers[105].
Ischemia-reperfusion injury is brought about by temporary oxygen deprivation of an organ or part of an organ. Transient ischemia by vascular clamping[100,184] leads to anoxia of the deprived tissue and ultimate death of its cells. Upon release of the clamp, reperfusion is rapidly accomplished and followed within hours by infiltration of inflammatory cells in the damaged area. Skeletal muscle regeneration originates from precursor cells surrounding the damage tissue and from surviving precursors in the ischemic area itself, if any. A similar regeneration process is observed as with free transplants of whole or minced muscle[100]. In the latter cases, a proportion of the precursor cells in the graft may survive on the low supply of oxygen and nutrients due to diffusion from the surrounding tissues and reperfusion is accomplished by neovascularization.

6 Cell therapy for diseased and injured skeletal muscle

Cell therapy has as main goal to repair damage and replenish lost myofibers through systemic or local injection of cells with myoregenerative properties, thereby restoring skeletal muscle function. To be successful, the transplant should not only contain adequate numbers of cells capable of contributing to or, even better, differentiation into myofibers under the adverse conditions that prevail in a pathological microenvironment, but these cells also need to be delivered and distributed properly and should not be subject to immunological rejection. The problems associated with the latter requirements have not been solved adequately yet. Currently, they present by far the most urgent challenge in the field, in particular for the treatment of generalized skeletal muscle diseases.
Many different cell types have been investigated as potential therapeutics, mainly for muscular dystrophies but also for other neuromuscular disorders, urinary incontinence and local traumatic injury of skeletal muscle. In the next section, a concise description is given of the main cell types that are currently being evaluated for their myoregenerative ability.

Until now, research has been focused on myogenically committed precursor cells, muscle-specific stem cells and multipotent non-embryonic stem cells. Table 1.8 provides an summary of the main findings on the myogenic/regenerative properties of these cells with an emphasis on their clinical usefulness. The majority of the investigations on the use of cells to repair damage of diseased skeletal muscle tissue are directly or indirectly (e.g. improving quality of life) dealing with the treatment of DMD or similar degenerative myopathies and age-related skeletal muscle wasting (also known as sarcopenia). As mentioned above, the major obstacles are the procurement of sufficient cell numbers, how to achieve an adequate distribution of the cells over the whole body musculature and the prevention of rejection of the therapeutic cells. However, it should be kept in mind that several of these hurdles are of less significance in the treatment of isolated skeletal muscles or localized skeletal muscle injuries and defects.

6.1 Muscle-derived stem cells

The terms “muscle precursor cell” or “muscle progenitor cell“ (MPC) are used to describe all types of myogenic cells, irrespective of their origin, capable of giving rise to myofibers *in vivo*. They include quiescent and activated satellite cells, presumptive myoblasts (i.e. myoblast precursors) and myoblasts (postmitotic cells able to fuse to form myotubes). Pioneering experiments with mouse models of DMD demonstrated that MPCs of non-dystrophic donors can be transplanted into dystrophic muscle and give rise to dystrophin-positive myofibers[185]. This treatment has thus been extensively explored both in laboratory animals and in patients. Although clinical studies in which allogenic MPCs were injected into skeletal muscles of DMD patients was found to be safe, the treatment yielded only few dystrophin-positive myofibers (less than 1.5% at 6 months after transplantation) and did not result in functional benefit[186,187]. Major problems encountered during these studies include the poor dissemination of the donor cells after injection[188,189], the intense host immune response to the transplanted cells[190,191] and their limited survival[192,193]. Many attempts have been made (i.e. variation in cell numbers injected, modification of the cell delivery method, strategies to control acute rejection and donor cell conditioning [for example heat shock or Tubulyzine] prior to transplantation[194-196]) to overcome these limitations but none have been particularly successful.

Table 1.8A Therapeutic cells for skeletal muscle disease: muscle-derived cells.

Name	Anatomic origin and common markers	In vivo delivery of the cells	Contribute to:		Advantages	Disadvantages	Ref.
			myofiber formation	precursor population			
Satellite cells	Attached to the muscle fibers under the basal laminae Pax7, CD34, caveolin, calcitonin receptor, β1-integrin, M-caherin, a7-integrin, nestin, c-Met, syndecan-4, NCAM	i.m.	Yes	Yes	Myogenic stem cells Efficiently differentiation to myoblasts	Low frequency in muscle Cannot be expanded in culture Not systemic deliverable Poor migration Human satellite cells have not been tested in vivo yet	280,67,19 68,281
Myoblasts	Proliferation after activation of satellite cells MYF5, MYOD1, CD56, Desmin	i.m.	Yes	Yes	Myogenic precursors Easy to isolate and expand in vitro Used in clinical trials	Less efficient in contributing to muscle regeneration than satellite cells Not systemic deliverable Poor migration	282,283, 284 185 189
MDSCs	Myofiber periphery CD34, Bcl2, MNF, MyoD, M-cadherin	i.m. i.v.	Yes	ND	Easy to expand in vitro Multipotent	Human MDSCs have not been tested *in vivo* in patients	199,98, 197,285
CD133+	Myofiber periphery close to blood vessels CD133, CD34, Thy-1, CD45, Sca-1, CD146, CD90, CD44, LFA-1, L-selectin	i.a. i.m.	Yes	Yes	Systemic deliverable (cross blood vessel wall)	Difficult to prepare pure cell populations Difficult to expand *in vitro*	200 201
$PW1^{+}/Pax7^{-}$	Interstitial space of skeletal muscle	i.m.	Yes	Yes	Defined anatomical location	Human cells have not been isolated yet	202
$CD34^{-}$	Skeletal muscle CD56	i.m.	Yes	Yes	Myogenic cells without adipogenic potential Characterized from human skeletal muscle	Difficult to prepare pure cell populations	203

MDSCs, muscle-derived stem cells; i.a., intra arterial; i.m., intra muscular; i.v., intravenous; ND, not done.

Table 1.8B Therapeutic cells for skeletal muscle disease: multipotential stem cells.

Name	Anatomic origin and markers	In vivo delivery of the cells	Contribute to:		Advantages	Disadvantages	Ref.
			a) myofiber formation	b) precursor population			
SP	BM and skeletal muscle (interstitial) Sca-1, c-Kit, CD43, CD34, CD31, CD133	i.m. i.a. i.v.	Yes	Yes	Exhibit both hematopoietic and myogenic potential *in vivo* Cultured muscle SP can more efficiently engraft in dystrophic muscle after delivery via the femoral artery	Difficult to prepare pure cell population	(286) (287)
Myoendo-thelial cells	Myofiber periphery close to blood vessels CD34, CD144, CD56, CD31, CD133, Flk1, VE-Cadherin	i.m.	Yes	ND	Easy to expand *in vitro*	Difficult to prepare pure cell population Not systemic deliverable	(210)
Pericytes	Periphery of capillaries and microvessels in all tissues α-SMA, ALP, CD56, CD146, PDGFR-β1, CD10, CD13, CD44, CD73, CD90	i.a.	Yes	Yes	Tested in dystrophic mouse and dog models	Differences in regenerative ability between different cell preparations	(213) (214)
Mesoangio-blasts	Associated with the microvessels wall CD34, c-kit, Flk1, CD31,	i.a.	Yes	ND	Easy to expand *in vitro* Tested in mdx and golden retriever	Difficult to prepare pure cell population	(215) (216)
HSCs	BM and peripheral blood c-kit, Sca1, CD45, CD34, CD11b, CD14	i.v. i.m.	Yes	ND	Easy to isolate Well-characterized Systemically-deliverable	Very limited contribution to skeletal muscle	(288) (221)
MSCs	BM, AT, SM CD73,CD90, CD105, CD29, CD44, CD51, CD106, CD166, Stro-1	i.m.	Yes	Yes	Can be isolated from multiple organs Easy to expand *in vitro* Multipotent Easy acquisition harvesting from AT SM derived MSC contribute also to extracellular matrix components	Very limited contribution to skeletal muscle Poor distribution following systemic administration	(243) (244) (289) (290) (291) this thesis

HSC , hematopoietic stem cell; SP, side population; MSC, mesenchymal stem cells; BM, bone marrow; AT, adipose tissue; SM, synovial membrane; i.a., intra arterial; i.m., intra muscular; i.v., intravenous; ND, not done.

There are myogenic cells other than MPCs present in skeletal muscle tissue such as the so-called muscle-derived stem cells (MDSCs)[197-199], muscle-derived CD133^{+} [200,201], PW1^{+}/Pax7^{-} [202] and CD34^{-} cells[203] that can all contribute to skeletal muscle regeneration. Thus far, the precise origin of these cells and the extent to which they act as muscle stem cells during homeostasis are unclear.

Mouse MDSCs are distinct from satellite cells with regard to surface marker profile, proliferation capacity and myogenic fusion properties[197-199]. MDSCs also promote angiogenesis, probably through vascular entothelial growth factor (VEGF) secretion[197,204,205]. By repeatedly selecting slow-adhering cells in long-term cultures of mouse skeletal muscle, MDSCs were isolated that regenerate both skeletal and cardiac muscle better than myoblasts[204,205]. The higher myoregenerative capacity of MSDCs as compare to MPCs have been attributed to their sustained proliferation, self renewal capacity and resistance to oxidative/hypoxic stress[197,204,206].

Muscle-derived CD133^{+} cells, that can also be isolated from peripheral blood, represent one of the most promising stem cell types for treating muscular dystrophies. These cells when freshly isolated from adult human normal blood samples express on their surface a pattern of adhesion molecules such as CD44, lymphocyte function-associated antigen-1 (LFA-1), P-selectin glycoprotein ligand-1 (PSGL-1), very late antigen-4 (VLA-4), L-selectin and the chemokine receptor CCR7 that renders them to be able to migrate through blood vessel walls[207]. Following intramuscular or intra-arterial injection into *SCID/mdx* mice, human blood-derived CD133^{+} cells contribute efficiently to skeletal muscle regeneration and contribute to the replenishment of the satellite cell pool[201]. Genetically “cured” DMD muscle-derived CD133^{+} cells that were injected intra-arterially or intramuscularly into *SCID/mdx* mice showed a better skeletal muscle regeneration in terms of distribution and number of dystrophin-positive myofibers as compared to animals that were given unmodified DMD blood-derived CD133^{+} [208]. Intramuscular transplantation of freshly isolated autologous muscle-derived CD133^{+} cells has been tested also in DMD patients[209]. Four or five treated patients developed an increased in capillary density and two of them displayed a change in the ratio of slow- to fast-twitch myofibers in the treated muscle[209]. Since CD133^{+} cells represent only a very small fraction of the mononucleated cells in the skeletal muscle tissue, the application of freshly isolated, unmanipulated cells is technically very difficult.

Recently, a cell population called PW1^{+}/Pax7^{-} interstitial cells (PICs) was identified[202] within the interstitial space of mouse skeletal muscle which could differentiate into both skeletal and smooth muscle lineages *in vitro* and contribute to skeletal muscle regeneration as well as give rise to both satellite cell and new PICs after intramuscular transplantation. However, their systemic deliverability and isolation from human have not yet been tested.

$CD34^-$ cells isolated from human skeletal muscle[203] were shown to be highly myogenic with no adipogenic potential when injected in cryodamaged skeletal muscle of immunodeficient mice. These cells have been demonstrated further to be able to occupy a satellite cell position consistent with their myoregenerative potential[203].
Skeletal muscle therefore seems to contain many different cell types that fulfill the definition of a muscle stem cell, but, as the immunophenotype of cells may not be constant and it is extremely difficult to prepare pure/homogeneous cell populations, one cannot rule out the possibility that a single rare cell type is responsible for the myoregenerative capacity residing in each of the aforementioned muscle stem cell populations.

6.2 Multipotent stem cells

Multipotent myogenic stem cells with myoregenerative capacity have also been found in the skeletal muscle interstitium and in many other organs/tissues. These cells include myoendothelial cells[210], side population cells[211], blood-derived $CD133^+$ cells[201], multipotent adult progenitor cells (MAPCs)[212], pericytes[213,214], mesoangioblasts[215-218], hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).
The pericytes and mesoangioblasts are very promising cells for clinical application because a significant proportion reaches skeletal muscles following systemic administration. Both cell types, mesoangioblasts and pericytes, are blood-vessel associated stem cells originate from embryonic[218] and postnatal[214] tissues, respectively. The ability of these cells to contribute to skeletal muscle regeneration has been demonstrated in both dystrophic (i.e. α-sarcoglycan null mice and *SCID*/*mdx*) mice and dystrophin-deficient golden retrievers[214-216,219]. These encouraging findings however, have still to be confirmed by other investigators. The results of a planned phase I clinical trial of allogenic human mesoangioblast transplantation in DMD patients are eagerly awaited[220].
Ferrari and collegues[221] in 1998 were the first to demonstrate *in vivo* the generation of skeletal muscle from BM cells. They used transgenic donor mice expressing a nuclear *LacZ* gene encoding nuclearly localized. After transplantation of unfractionated BM from these mice into immunodeficient *scid/bg* hosts and subsequently induction with cardiotoxin of muscle injury, β-galactosidase positive nuclei were unequivocally detected in the regenerated myofibers. This was regarded as evidence that murine BM contains transplantable progenitors that can be recruited to an injured skeletal muscle through the circulation, where they participate in muscle repair. The next year, Gussoni et al.[157] reported reconstitution of the hematopoietic compartment and incorporation of donor-derived nuclei into myofibers after intravenous transplantation of BM from non dystrophic mice into irradiated *mdx* mice. In a similar study, irradiated female *mdx* mice received BM from non-dystrophic

congenic male donors[222]. Y chromosome-containing myonuclei were detected in skeletal muscles and in the heart of the recipient animals albeit in very small numbers[222].
Allogeneic BM transplantation in dystrophic-deficient dogs did not result in a significant contribution of donor cells to the diseased skeletal muscle, i.e. no significant increases in the percentages of dystrophin-positive myofibers or the amount of wild-type dystrophin mRNA were observed even not following the surgical disruption of the blood/muscle barrier and after the mobilization of HSC from the BM with the aid of granulocyte colony-stimulating factor[223].
In a DMD patient who received allogeneic BM transplantation 12 years before being diagnosed with DMD, donor nuclei were identified in a small fraction of muscle myofibers (0.5-0.9%)[224]. This clinical observation emphasizes the ability of human BM cells to contribute to myofibers and to persist long-term in skeletal muscle[186,224,225].
MSCs isolated from BM are distinguishable from HSC by their repertoire of cell surface antigens (CD29, CD44, CD49A-F, CD51, CD73, CD90, CD105, CD106, CD166 and Stro1) and lack expression of specific hematopoietic lineage markers (CD11b, CD14 and CD45). Also, MSCs adhere to tissue culture plastics and thus can be easily separated from the non-adherent HSCs. In contrast to HSCs, MSCs are capable of extensive proliferation *ex vivo* without loss of their normal karyotype[226,227]. Furthermore, MSCs have the ability to give rise to adipocytes, osteoblasts and chondroblasts *in vitro*[228-230]. Cells with properties of MSCs have been isolated from many different tissues (e.g. skin, skeletal muscle[231], adipose tissue[232], synovial membrane[233], umbilical cord[234], the circulatory system[235], dental pulp[236], amniotic fluid and membrane[237] as well as from fetal blood, liver, BM and lung[238-242]. MSCs derived from BM as well as from some other sources have been shown to posses myogenic differentiation capacity *in vitro* and *in vivo*[243].
The ability to generate large numbers of MSCs from a small sample together with their multipotency and their capacity to fuse with myoblasts *in vitro* and to contribute to skeletal muscle regeneration in dystrophic and experimentally injured muscles, make these cells attractive candidates for cell-based therapies of degenerative myopathies.

7 Outline of the thesis

The aim of this thesis was to explore the role of human MSCs from different tissue sources in the regeneration/repair of skeletal muscle. For this purpose, several *in vivo* tissue damage models were employed. Most of the experiments were performed in NOD/SCID mice to avoid immunological rejection of the human cells.

Since there was little information regarding the myogenic properties of BM-MSCs *in vivo* we started investigating the participation of these cells in skeletal muscle regeneration following cardiotoxin-induced injury. In **chapter 2**, the histological features of the damage induced by cardiotoxin injection in TAMs is described. Then, the fate of locally injected BM-MSCs was studied up to 120 days after injury. The BM-MSCs were tagged by transduction with a lentivirus vector encoding cytoplasmically localized β-galactosidase to permit their tracking. The contribution of BM-MSCs to skeletal muscle regeneration was quantified by a precise method developed during the study. Hybrid myofibers were further analyzed with human muscle-specific markers (e.g. β-spectrin) to study nuclear reprogramming of donor cells.

Apart from the BM, adipose tissue (AT) and synovial membrane (SM) have been reported as useful sources of human MSCs. The literature contains several descriptions of their capacity to contribute to skeletal muscle regeneration[243,244]. The relative ability of human MSCs of different origin to participate in skeletal muscle repair is, however, difficult to determine as each cell source was investigated in isolation by different research groups. In addition, for clinical applications of human MSCs, the acquisition, availability and amount of the tissue source are issues to be consider as limitation for cell therapy. In **chapter 3**, we compared in a side-by-side study the myogenic properties of BM, SM and AT-MSCs derived from the same donors. In this way, inter individual and inter-laboratory variations were avoided and a fair comparison of the myoregenerative ability of human MSCs from different sources could be achieved both *in vitro* and *in vivo*. This study provided evidence that AT-MSCs can be consider as the preferential source for clinical applications in myopathies.

Chapter 4 described the results of a study on the regeneration of the panniculus carnosus muscle in mice following ischemia-reperfusion injury. For this purpose, pressure ulcers (PU) were induced in the dorsal skin of hairy, hairless, and diabetic mice by applying magnetic disks for different periods of time. PU were also induced in previously irradiated skin folds of mice in an attempt to achieve tissue damage resembling that of PU (decubitus) in human patients. The healing process of all layers of the skin (epidermis, dermis, subcutis) is described for each induction protocol. The ischemia-reperfusion model was used further to study the contribution of human BM-MSCs to the regeneration of the panniculus carnosus muscle as well as to other resident cell types of the skin.

While standardizing conditions for PU induction and trying to set up models of chronic wound healing (e.g. diabetes, irradiation) we encountered an undesirably large variation in the effects of streptozotocin (STZ), the agent employed to induce diabetes was encountered. **Chapter 5** reports on the cause of this problem and its elimination.

An intriguing question that arose in the course of our study was whether the finding with human cells in the regenerating murine muscle represents their therapeutic effect in patients. To circumvent the necessity of studies in vital human muscle we resorted to a surrogate condition with human muscle tissue implanted subcutaneously in mice. Such a model should allow the comparison of syngeneic with xenogeneic combinations of therapeutic cells (e.g. MSCs) and damaged tissue. In **Chapter 6**, we described attempt to establish subcutaneous implants of minced muscle tissue in immunodeficient mice. To address the proposition that interaction between human and murine cells may be suboptimal due to differences in the repertoire and structure of cell adhesion molecules and growth factor receptors we tested cross mixes of mouse or human skeletal muscle implants with murine or human mesenchymal stem cells. Unfortunately, we were not able to fully exploit this system due to the very limited supply of healthy human skeletal muscle tissue. Nevertheless, the obtained data provide a basis for further investigation into human muscle regeneration and cross specie interaction in vivo.

Repair of tissue damage that requires *in situ* differentiation of MSCs into specialized cell types or their fusion with resident cells has so far only been achieved only with autologous/syngeneic MSCs or in immunocompromised recipients although these cells are held by many to be non-immunogenic[245-247]. Similarly, successful use of MSCs as vehicles for the delivery of therapeutics depends on immunocompatible donor-recipient combinations[248,249]. The involvement of surface MHC class I molecules in graft rejection and the effect of inhibiting MHC class I protein dependant recognition of the transplant on its immunogenicity have been well documented. In **chapter 7**, we described the downregulation of MHC class I protein expression on the surface of human BM-MSCs by retroviral vectors encoding a herpesviral immunoevasin (i.e. the US11 protein) and show the effect of this intervention on their engraftment in immunocompetent recipient mice.

Finally, **chapter 8** contains a general discussion of the results of this thesis and considers the future perspectives of using MSCs for skeletal muscle regeneration.

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