Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification

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This work is dedicated to my parents,
Valery Ustanin and Nina Ustanina.

1. SUMMARY

Pax7 is a paired box transcription factor expressed during embryonic and postnatal muscle development and regeneration. In the present work I have investigated of the role of this myogenic transcription factor in the specification and maintenance of muscle satellite cells. Since postnatal growth and regeneration of mammalian skeletal muscles are largely attributed to muscle satellite cells, I wanted to uncover the role of Pax7 in these processes and to gain more insights in the function of Pax7 in complicated signalling network governing specification and differentiation of muscle cells in mammals.

A wide range of modern biological methods were used in the present work: general molecular biological techniques (for example DNA and RNA isolation, molecular cloning, Southern and northern nucleic acids analysis, Real Time PCR), histochemical techniques (paraffin and cryotome tissue sectioning, histochemical and immunohistochemical staining of tissue sections and cell cultures), electron microscopy and mammalian cell culture techniques.

The present analysis of satellite cells in Pax7(-/-) mice has unambiguously established that Pax7 is dispensable for the specification of the satellite cell lineage since we detected a large number of satellite cells in juvenile Pax7(-/-) mutant mice using five different methods (electron microscopy, Pax7-lacZ staining, CD34 staining, *in vitro* cultivation of satellite cells from isolated myofibers and clonal satellite cell analysis). Instead, we detected a continuous decrease of the number of satellite cells in Pax7(-/-) mutants, which strongly suggests a role of Pax7 in the renewal and propagation of satellite cells. In addition, we found an essentially normal degree of muscle formation in adult Pax7 mutant animals that cannot be explained without the contribution of the highly proliferative satellite cell population to the growth of immature muscles. Neither juvenile nor adult Pax7 (-/-) mice displayed a significant reduction of the number and size of myotubes indicating that the remaining number of satellite cells sufficed to allow normal postnatal muscle growth. Moreover, adult Pax7(-/-) mice still own a certain potential for skeletal muscle regeneration although the efficiency of regeneration is severely hampered. The compromised regenerative response of Pax7(-/-) mice came along with an expansion of the remaining Pax7-lacZ satellite cells and resulted in numerous regenerated muscle fibers although faulty regeneration was evident and a complete repair was never achieved.

It is of major importance to understand the mechanisms and pathways that govern muscle repair processes to develop applied therapeutic and clinical tools that will ultimately lead to an improved treatment of muscle disorders. In the current study was the function of

Pax7 as a major regulator of satellite cell renewal and propagation was re-defined. This knowledge will probably help us to manipulate the availability of muscle stem cells for therapeutic purposes in particular in aged and diseased skeletal muscles.

2. INTRODUCTION

2.1. The role of various transcription factors in the formation of skeletal muscle

Development of skeletal muscle in vertebrates begins during early embryonic stages and is a result of a cross-talk between surrounding tissues. Specification and differentiation of myotome is achieved by a complex signalling system. Most of the information about myogenesis is based on the manipulation of chick embryos and chick/quail chimeras (such as ablation, grafting and co-culture) and in general features is applicable also to mammals. Gene manipulations made it possible to investigate the role of various molecular signals leading to the formation of skeletal muscle in mammals and particularly in mice.

2.1.1. Early myogenesis

In vertebrates, the paraxial mesoderm adjacent to the neural tube and notochord gives rise to transient cellular aggregates called somites (Christ and Ordahl, 1995). Somites give rise to vertebrae, ribs, cartilage, back dermis and all skeletal muscles of the body, excluding those of the head, originate from the somites. The musculature of the head is derived from the cephalic mesenchyme and prechordal plate (Noden et al., 1999). An immature somite forms first a spherical epithelial ball and then develops into three distinct compartments: dermomyotome, myotome and sclerotome, which in turn give rise to distinct cell fates (Fig. 1). Somitic cell fates are plastic and are influenced by signals from surrounding tissues. Newly formed somites contain medial and lateral domains that give rise to distinct muscle groups and respond to different inductive signals. The ventromedial part of the somite responds to signals from the notochord and forms the sclerotome which will contribute the axial skeleton and ribs. The dorsal part of the somite responds to signals from the dorsal neural tube as well as the notochord and forms the dermomyotome and the myotome. Additional signals from the ectoderm overlying the somites can also induce the dermomyotome.

Dermomyotomal cells continue to proliferate and are maintained in an undifferentiated state by signals from the lateral plate and surface ectoderm (Pourquie et al., 1996; Amthor et al., 1999). At the dorsomedial and ventrolateral lips of the dermomyotome cells migrate under the dermomyotome to form the myotome, a sheet of differentiating skeletal muscle cells that express high levels of MyoD and Myf5 and eventually give rise to the axial muscles (Kiefer and Hauschka, 2001; Sassoon et al., 1989; Ordahl et al., 2001; Cinnamon et al., 2001; Cinnamon et al., 2001). Cells from both the lateral dermomyotome and the lateral myotome

migrate as a block of tissue to form the ventral body wall muscles (Christ et al., 1983; Cinnamon et al., 1999). Other ventrally situated muscles also arise from the hypaxial lineage. Cells in the dorsal domain of the myotome subsequently form the epaxial (deep back) musculature.

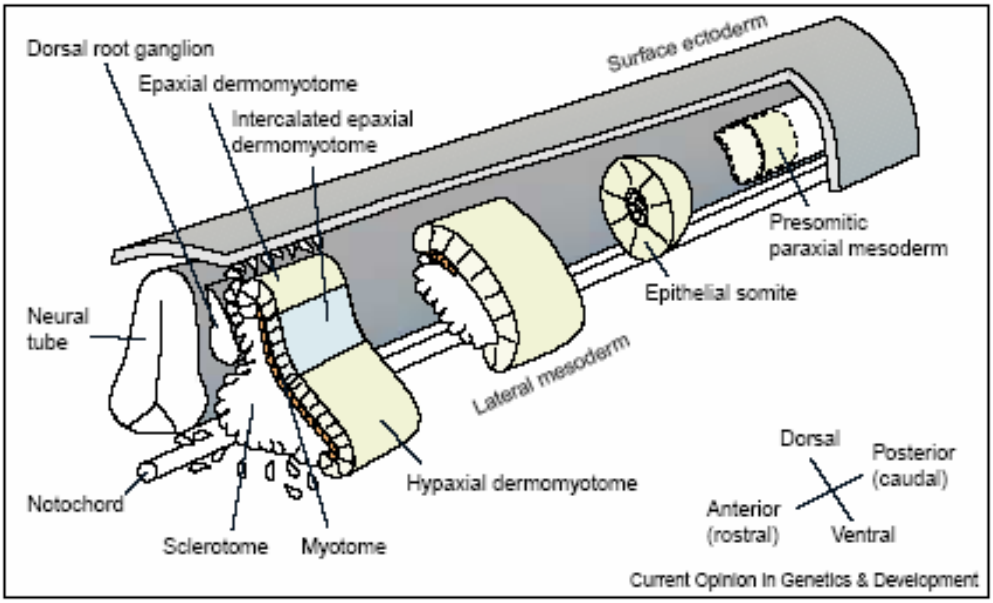

Fig. 1 Schematic representation of vertebrate somitogenesis as it occurs in the mouse embryo. Somites are formed and mature following a rostrocaudal gradient on either side of the axial structures (the picture has been taken from the work of M. Buckingham (Buckingham, 2001)).

2.1.2. Signals regulating myogenesis in the somites

It has been known for many years that signals from the neural tube and notochord induce myogenesis in the somites; several groups have examined the mechanisms involved in this induction by manipulations with chick embryos (Buffinger and Stockdale, 1994; Stern and Hauschka, 1995; Stern et al., 1995; Spence et al., 1996). Their results have shown that the majority of the inducing activity was localized to the dorsal neural tube. The ventral neural tube/notochord possessed only a weak myogenic activity on its own but when combined with the dorsal neural tube there was significantly greater myogenic activity than with either half of the neural tube alone, suggesting that the dorsal and ventral regions contain factors that cooperatively induce myogenesis. In co-cultivation experiments with tissue explants two signals from the axial organs that act in combination were found to induce muscle cell markers in the myotome: one signal originates from the floor plate/notochord, whereas the other originates from more ventral regions of the neural tube (Munsterberg and Lassar, 1995). Le Douarin and colleagues (Rong et al., 1992) demonstrated that excision of the neural

tube/notochord complex from early chick embryos (or physical separation of the neural tube from the somite) results in the striking absence of axial (i.e. vertebral and back) skeletal muscle. Similar analysis performed has shown that excision of either the neural tube alone, or neural tube and notochord together, resulted in the loss of myotomal muscle (Christ et al., 1992). The signals are required to promote myogenesis only in pre-somitic mesoderm and newly formed somites; more mature somites do not need the presence of neighbouring tissues.

Further experiment showed that only precursors of epaxial (back) muscles, located in the dorso-medial domain of the newly formed somites, are dependent upon signals from axial structures. Interestingly, skeletal muscles in the limbs and body wall, hypaxial precursors of which located in the lateral half of the paraxial mesoderm, were unaffected by neural tube/notochord removal, they are rather dependent on signals from dorsal ectoderm (Rong et al., 1992). It was shown that explants of murine paraxial mesoderm, when co-cultured in the presence of axial structures, activate Myf5. In contrast, they activate MyoD when co-cultured with their own dorsal ectoderm (Cossu et al., 1996). This suggests that in mammals axial structures activate myogenesis through a Myf5-dependent pathway, while dorsal ectoderm acts through a MyoD-dependent pathway.

Sonic Hedgehog (Shh), a signalling molecule expressed in the ventral neural tube (i.e. floor plate) and notochord, Wnt-family of growth factors, expressed in the dorsal neural tube, and members of bone morphogenic proteins (BMPs) and Noggin, secreted from the lateral mesoderm and notochord respectively, play key roles in somite patterning (Marcelle et al., 1997) (Fig. 2).

Sonic hedgehog is a ventralizing signal emanating from the notochord and subsequently from the floor plate of the neural tube. Shh has been demonstrated to be a mitogen for somitic cells and to induce the expression of a sclerotomal marker Pax1 in pre-segmental plate mesoderm and specify a sclerotomal fate in conjunction with BMP4, concomitantly inhibiting dermomyotomal markers, Pax3 and Pax7 (Fan and Tessier-Lavigne, 1994). On the other hand, retroviral expression of Shh in the limb bud was shown to sequentially induce an extension of the expression domains of Pax3, MyoD and myosin heavy chain genes concomitant with an increase in the proliferation of myoblasts *in vitro* suggesting that Shh enhances the proliferation of already committed myoblasts (Duprez et al., 1998). It is not clear yet whether Shh can act directly on somite cells or it acts through an intermediate regulator since floor plate/notochord is relatively distal from the location of myogenic cells in the somites.

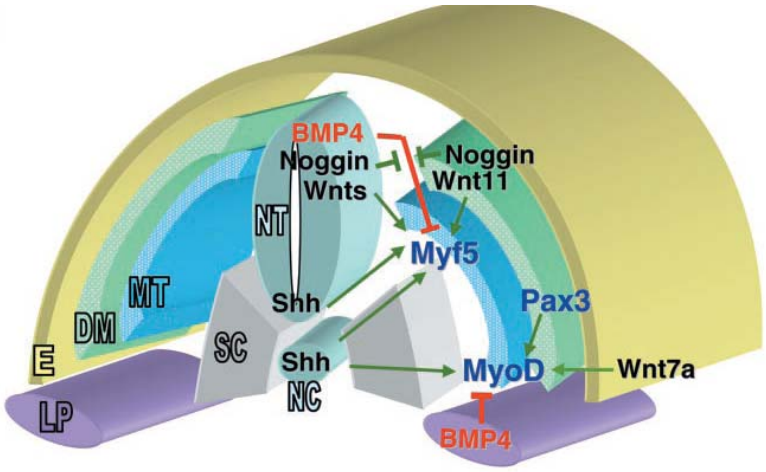

Fig. 2 Factors involved in embryonic skeletal muscle formation. Mesodermal somitic cells located in the dorsal part of the somite [dermomyotome (DM)] receive signals from surrounding tissues, which induce [Wnts, Sonic hedgehog (Shh), Noggin] or inhibit (BMP4) the expression of the primary MRFs (Myf5 and MyoD) and commitment to the myogenic lineage. Committed myoblasts migrate laterally to form the myotome (MT), which eventually forms the skeletal musculature. Pax3 promotes myogenesis in the lateral myotome. E, ectoderm; LP, lateral plate; SC, sclerotome; NC, notochord; NT, neural tube. (the picture has been taken from the work of Charge and Rudnicki (Charge and Rudnicki, 2004))

The removal of the neural tube and notochord leads to the absence of epaxial muscles while hypaxial musculature develops normal. Therefore it has been suggested that Shh is required to control epaxial muscle (back musculature) determination through Myf5 activation, and is less important for the development of hypaxial muscles (Borycki et al., 1999a). However, later it was shown that Shh is required for the maintenance of the expression of myogenic regulatory factors (MRFs) also in hypaxial muscles, and for the formation of differentiated limb muscle myotubes (Duprez et al., 1998; Kruger et al., 2001). Formation of limb muscles in two different Shh-null mouse strains was severely affected, but the limb muscle defect became apparent relatively late. Initial stages of hypaxial muscle development were unaffected or only slightly delayed. On the basis of these data it was suggested that Shh acts similarly in both somitic compartments as a survival and proliferation factor and not as a primary inducer of myogenesis.

Wnts (wingless and integrated) constitute a large family (about 20 Wnt genes are known in vertebrates) of secreted glycoproteins with distinct expression patterns in the embryo and in the adult organism. The Wnt signal transduction pathway is involved in many differentiation events during embryonic development: in the control of embryonic induction, polarity of cell division, cell fate and growth. Wnt family members mediate dorso-ventral patterning of the somite and the decision between sclerotome and dermomyotome development, segmentation, central neural system patterning (Munsterberg and Lassar, 1995; Marcelle et al., 1997) and myogenesis (Ridgeway et al., 2000; Anakwe et al., 2003). Wnts

mainly act on target cells in a paracrine fashion through members of the frizzled receptor family of seven transmembrane spanning proteins (Bhanot et al., 1996). Frizzled were first discovered in Drosophila, but a number of vertebrate homologs with distinct expression patterns have been described. Members of the vertebrate Wnt family have been subdivided into two functional classes according to their biological activities. Some Wnts signal through the canonical Wnt-1/wingless pathway by stabilizing cytoplasmic b-catenin. By contrast other Wnts stimulate intracellular Ca21 release and activate two kinases, CamKII and PKC, in a G-protein-dependent manner (Wodarz and Nusse, 1998).

Wnts are expressed in a tissue-specific manner, and mutant mice with deletions of certain Wnt genes display strong phenotypes. For example, the lack of Wnt-1 results in the deletion of part of the midbrain, Wnt-4 and Wnt-7a affects kidney and limb development, respectively, Wnt-3 knockout mice are deficient in the formation anterior-posterior axis (McMahon and Bradley, 1990; Stark et al., 1994; Parr and McMahon, 1995; Liu et al., 1999).

In cooperation with Shh, other Wnt family members were also shown to induce myogenesis in the dorsal part of isolated somites in vitro (Munsterberg and Lassar, 1995). Myf5-inducing activity of the neural tube can be replaced by cells expressing Wnt1 and Wnt4, while MyoD activation by dorsal ectoderm can be replaced by Wnt7a-expressing cells (Tajbakhsh et al., 1998). It seems to be that Shh in conjunction with Wnt1, and possibly other Wnts, activates myogenesis in the future dermomyotome via a Myf5-dependent pathway. Different Wnts such as Wnt7a may activate myogenesis in the lateral domain, probably through a MyoD-dependent pathway.

BMPs, the members of TGFβ family, counteract Wnt signalling, keeping an undifferentiated state of migrating muscle precursor cells until they reach their targets (Pourquie et al., 1996; Amthor et al., 1999). Noggin, produced by the dorsal neural tube in a Wnt-dependent manner, is an antagonist of BMP signalling. It blocks ligand-receptor interaction, specifically binding the ligand, interfering with signal transduction, inactivates BMP4 (Zimmerman et al., 1996). BMP expression can be induced by Shh, since implantations of Shh-soaked beads in chicken limb buds resulted in an induction of BMP2 and BMP7, and subsequent excessive muscle growth. Fibroblast growth factors (FGFs) also play an important role in myogenesis, regulating proliferation and differentiation of muscle precursor cells (Flanagan-Steet et al., 2000; Itoh et al., 1996).

Activation of these several signalling pathways determines the balance between the determination, proliferation, survival and differentiation of muscle progenitors in the somite. The number of known molecules potentially involved in signalling during mouse

embryogenesis is rapidly growing, so this field needs further investigation to define the model of signal interactions.

2.1.3. Muscle formation in the embryonic limb

At the limb levels muscle progenitor cells in the ventro-lateral dermomyotome delaminate and migrate into the limb buds where they will form an appendicular skeletal muscle (Birchmeier and Brohmann, 2000). This process is dependent on a number of regulatory factors including Pax3, c-met, Tbx1, Mox2, Six1, Six2, Pitx2 and Lbx1h (Fig. 3).

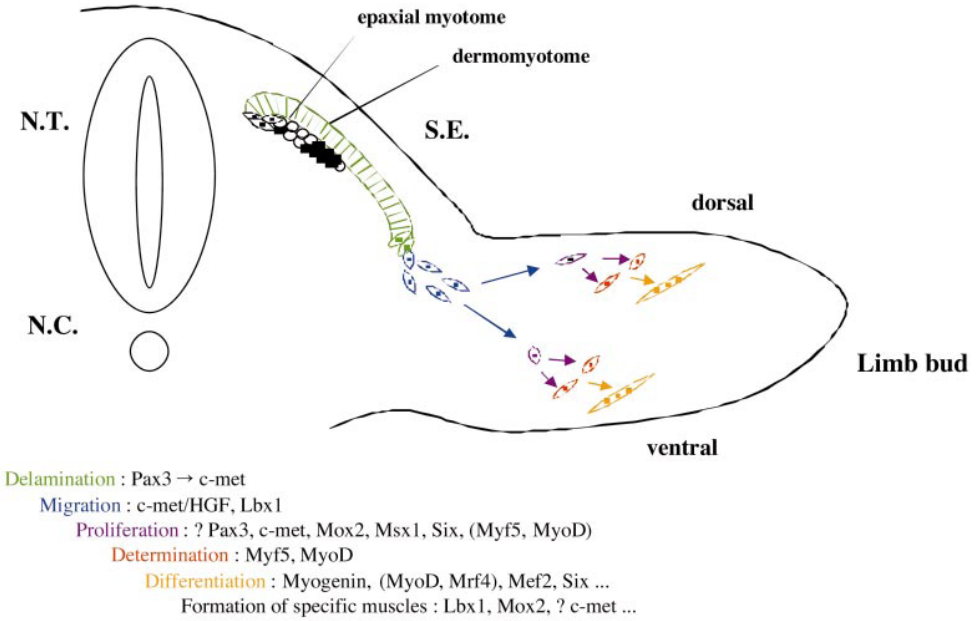

Fig. 3 Schematic representation of skeletal muscle formation in the limb, with the different stages and genes potentially involved at each stage. NC, notochord; NT, neural tube; SE, surface ectoderm (the picture has been taken from the work of Buckingham et al. (Buckingham et al., 2003)).

Initial steps appear to be controlled by the Pax3 gene which is expressed very early in the embryo in the forming paraxial mesoderm, in the dorsal neural tube, and later in the somites where it becomes restricted to the dermomyotome including cells which will migrate to form limb muscle. Among other severe defects, splotch mice (mice with a mutation in Pax3 gene) lack limb muscles as a consequence of impaired migration of muscle progenitors from the lateral dermomyotome, although their differentiation potential is not impaired (Daston et al., 1996; Bober et al., 1994). Migrating Pax3-positive cells do not express members of the myogenic regulatory family genes (MRFs). The expression of Pax3 is highest during embryonic stages and is significantly reduced during foetal life. Mice heterozygous for mutations in Pax3 are characterized by pigmentation defects due to perturbations in neural

crest migration, whereas homozygous embryos have a number of neural defects, including spina bifida and exencephaly (Tremblay and Gruss, 1994). Most homozygous mutants die prior to embryonic day 15 (E15).

Beside the role in hypaxial muscle development Pax3 have a general function in muscle development. The analysis of double splotch/Myf5 mutant has shown that these mice fail to express MyoD in the myotome and lack all body muscles but not the head muscles (Tajbakhsh et al., 1997). Since muscle cells in the absence of Myf5 can only arise through the action of MyoD, these results argue that in the development of body muscle Myf5 and Pax3 act upstream of MyoD and that Pax3 is a critical upstream regulator for MyoD expression in body muscles but not in head muscle, which develop through a Pax3-independent pathway. Moreover, neither Pax3 nor Pax7 is detectable in skeletal muscle progenitors of the head, suggesting that regulation of myogenesis in the head and body are distinct.

These data were confirmed by ectopic expression of Pax3 in cultures of chick embryo tissues using a retroviral expression vector (Maroto et al., 1997). It was shown that Pax3 induces MyoD expression in explants of presomitic mesoderm and maintains Myf5 expression. Moreover, retroviral expression of Pax3 also induces MyoD expression in paraxial and lateral plate mesoderm and neural tube explants, which normally do not express MyoD. However, so far the molecular mechanism of Pax3 action remains unclear.

Both delamination and migration depend on the presence of c-met, a tyrosine kinase receptor which interacts with its ligand HGF, called also scatter factor, produced by non-somitic mesodermal cells, which thus delineate the migratory route (Dietrich et al., 1999; Heymann et al., 1996). In mutant mouse embryos which lack functional c-met (Bladt et al., 1995) or HGF (Schmidt et al., 1995) skeletal muscles are absent in the limbs, diaphragm, and tip of the tongue. In contrast, axial muscles that arise from the myotome were unaffected in these mutants. Transcription of the c-met gene seems to depend on Pax3 (Epstein et al., 1996) since c-met is down regulated in somites and limb buds of *splotch* mice. However, reduced levels of c-met were still detectable by RT-PCR in limb buds of mutant mice (Yang et al., 1996).

Another homeo-domain containing transcription factor, Lbx1, is implicated in the determination of migratory routes of muscle precursor cells in a cell-autonomous manner leading to the formation of distinct limb muscle patterns. Lbx1h is specifically expressed in migrating muscle precursor cells. In Lbx1 mutant embryos muscle progenitor cells delaminate from the dermomyotome but remain in the vicinity of the somite where they may adopt other cell fates (Schafer and Braun, 1999a). The mutation led to a lack of extensor muscles in

forelimbs and absence of muscle in hind limbs; hence not all migrating cells were equally affected by the mutation. Ectopic expression of Lbx1 *in ovo* leads to a strong transient activation of muscle cell markers. Ectopic expression of Lbx1 in explants cultures derived from several tissues induces various muscle cell markers and induces cell proliferation (Mennerich and Braun, 2001). Expression of Lbx1 is strictly dependent on Pax3 expression in lateral parts of somites and in migrating limb muscle precursor cells: in these structures of *splotch* mice Lbx1 expression is completely abolished. However, Pax3 seems to be not sufficient to drive Lbx1 expression since Lbx1 is not expressed in the inter-limb region of chicken embryos where Pax3 is present at the same concentration as in the limb regions (Mennerich et al., 1998).

The homeo-domain factor Mox2 is present in muscle progenitor cells in the limb. In its absence Myf5 transcripts are down-regulated, suggesting that Mox2 may act upstream of this myogenic factor. In this mutant Pax3 is also reduced but MyoD is present (Mankoo et al., 1999). The Six homeo-domain proteins together with co-factors Eya and Dach can influence the transcription of MyoD in the limb (Relaix and Buckingham, 1999).

Msx1 is also expressed in migrating muscle progenitor cells at the forelimb level and has been shown to keep cultured myoblasts dividing (Houzelstein et al., 1999). Indeed ectopic expression of Msx1 can induce dedifferentiation of terminally differentiated murine myotubes in C2C12 cultures. A subset of these myotubes cleave to produce a pool of proliferating, mononucleated cells that are capable to redifferentiate into different cell types that express characteristic markers of chondrocytes, adipocytes, myoblasts and osteoblasts (Odelberg et al., 2000).

Ectopic expression in limbs of chicken embryos has indicated an important role of bone morphogenic proteins (BMP2, BMP4 and BMP7) in the process of limb formation (Pourquie et al., 1996). BMP4 and BMP2 expand the number of Pax3-expressing proliferating muscle precursor cells in a dosage-dependent manner: low concentrations of BMPs maintained the population proliferative, high concentrations prevented expression of Pax3 and MyoD and induced apoptosis (Amthor et al., 1999).

2.1.4. Myogenic regulatory factors (MRFs)

The formation of skeletal muscle during vertebrate embryogenesis requires commitment of mesoderm precursor cells to the skeletal muscle lineage, withdrawal of myoblasts from the cell cycle and transcriptional activation of many muscle structural genes. The three major decisions of a cell, whether to divide, differentiate or die, are influenced by

signals from the environment. Central role in establishment of skeletal muscle lineages in vertebrates belongs to myogenic regulatory factors (MRFs) collectively known as the MyoD family and including the basic helix-loop-helix (bHLH) transcription factors MyoD, Myf5, myogenin and Myf6 (MRF4). MyoD-family proteins bind DNA as heterodimers with ubiquitously expressed bHLH cofactors termed E proteins, such as E12 or E47 (Lassar et al., 1991).

MRFs interact directly with MEF2 family proteins to activate skeletal muscle genes and also to regulate one another. In addition to their roles in activation of muscle-specific genes, the MyoD and MEF2 families serve as end points for diverse intracellular signalling pathways that control myogenesis. These two families of myogenic transcription factors engage the cell cycle machinery to regulate the decision of myoblasts to divide or differentiate (Molkentin et al., 1995; Black and Olson, 1998).

All MyoD family members have been shown to convert a variety of cell lines to myocytes and to activate muscle-specific promoters (Munsterberg et al., 1995). During mouse embryonic development the MRFs are expressed in the myotome in an overlapping pattern. In mice Myf5 mRNA transcripts are the first to be expressed, at embryonic day 8 (E8.0) in cells at the medial edge of the myotome (Ott et al., 1991) and expression spreads ventro-laterally with the expansion of the myotome until E12. Myogenin is transcribed next in the myotome at E8.5 until birth, followed by the transient expression of MRF4 (Sassoon et al., 1989; Bober et al., 1991; Hinterberger et al., 1991). MRF4 is expressed in the somatic myotome between E9 and E11.5 and is later up-regulated in differentiated muscle fibers, where it is the predominant myogenic bHLH factor in adult skeletal muscle. MyoD is expressed first at E10.0. Its expression continues throughout prenatal life. In the somites of the trunk the pattern of MyoD expression is distinct from the other bHLH factors; it is highest in the myoblasts at the lateral edge of the myotome. Transcription of the myogenic bHLH factors in the forelimb buds is delayed until E10.0, when Myf5 is initially detected, and rapidly followed by MyoD and myogenin.

Gene knockout experiments helped to understand the functions these genes in myogenesis. They have revealed that Myf5 and MyoD have redundant functions and required for the commitment of cells to the myogenic lineage. Mice deficient in either Myf5 or MyoD have comparatively normal muscles in the adult stage, while double mutants Myf5/MyoD are completely devoid of cells expressing muscle-specific genes (Braun et al., 1992; Rudnicki et al., 1992; Rudnicki et al., 1993). Double mutant embryos die immediately after birth and contain amorphous connective and adipose tissues in spaces usually occupied by skeletal

muscle. Homozygous Myf-5 mutant embryos lack myocytes during early somite development only until MyoD expression starts, until E10.5. At birth Myf-5 mutants do not have a dramatic muscle deficiency, but, in contrast to mice lacking MyoD, they are not viable, due to a severe truncation of the ribs (Braun et al., 1992).

Mice with inactivated MyoD gene are viable and fertile and have a virtually normal musculature during embryonic development (Rudnicki et al., 1992; Megeney et al., 1996). However, it has been shown that after acute muscle injury during adulthood or when bred into dystrophin deficient mdx background mice lacking MyoD have a marked deficit in satellite cell function and regeneration, resulting in an increased population of precursor myoblasts and a decrease in the number of regenerated myotubes. These results imply that MyoD plays an important but not exclusive role in activation or differentiation of satellite cells. In this mutant strain, however, Myf5 expression remains relatively high and might compensate for the loss of MyoD.

Myf5 is initially expressed in the cells derived from the dorso-medial portion (epaxial musculature) of the dermomyotome, whereas MyoD is initially expressed in cells derived from the ventro-lateral portion (hypaxial musculature) of the dermomyotome. Hypaxial muscle precursors migrate from the ventro-lateral somites to the limb buds, the tongue and the diaphragm (Ordahl and Williams, 1998). It was shown using knockout mice that MyoD-/- embryos display normal but delayed development of the skeletal muscle of the limb buds, brachial arches, tongue and diaphragm, whereas Myf5-/- embryos display normal but delayed development of the back musculature (Kablar et al., 1997; Kablar et al., 1998). The intercostal and abdominal wall musculature development is delayed in both types of embryos. In addition, Myf5+/-/MyoD-/- embryos show 50% reduction in the diaphragm muscle, whereas Myf5-/-/MyoD +/- embryos have normal diaphragm (Rudnicki et al., 1993). These data support the hypothesis that epaxial muscle development is Myf5-dependent, while hypaxial muscle, especially long-range migrating muscle precursor cells for the diaphragm, is MyoD-dependent. MyoD has been recently shown to play a role in determination of contractile properties of the diaphragm, possible by causing a fast-to-slow shift in MyHC phenotype (Staib et al., 2002).

MyoD and Myf5 are expressed in proliferating myoblasts before terminal differentiation, whereas myogenin and MRF4 mark terminally differentiated cells and myotubes. MyoD and Myf5 are shown to be expressed in adjacent but distinct regions of the dermomyotome (Smith et al., 1994). Usage of cell lineage tracing and selective cell ablation techniques helped to suggest that MyoD and Myf5 initially determine two different muscle

cell lineages from independently committed stem cells (Braun and Arnold, 1996). All these findings have led to a model of cellular redundancy, in which Myf5-dependent medial and MyoD-dependent lateral myoblast populations are able to expand and compensate for one another. On the basis of the data available the family of MRFs can be divided into two functional groups. MyoD and Myf5 seem to be required for the determination of skeletal myoblast, myogenin and MRF4 act as differentiating factors (Megeney and Rudnicki, 1995).

Myogenin-null mice die at birth and exhibit a severe skeletal muscle deficiency at birth: they have virtually no muscle fibers but myoblasts appear normal (Hasty et al., 1993; Nabeshima et al., 1993). During myogenin mutant embryogenesis early myogenesis in the myotome is largely normal but, beginning at around embryonic day 13 (E13), further muscle development fails to occur, suggesting that this gene is necessary for terminal differentiation. Interestingly, skeletal muscles in different parts of the embryo were differentially affected in the null mutants: in the latero-ventral body wall, myogenic cells disappeared at day 14.5 (at a stage when myotube formation usually occurs); in the limb bud, most muscle cells arrested as mononucleated myoblasts unable to differentiate; and in the axial muscles (including the intercostal and back muscles) differentiation occurred, although the fibers were disorganized. Thus, the requirement for myogenin to promote skeletal muscle differentiation varies in different muscle groups. Apparently, axial muscle derived from precursor cells from the medial half of the somite can differentiate into mature but disorganized skeletal muscle in the absence of myogenin. The activation of a portion of the myogenic program indicates that myogenin is not necessary for the commitment of skeletal muscle precursors; however, these cells do not fully differentiate into skeletal muscle.

MRF4 is the last member of the MyoD family inactivated in mice. The MRF4 gene is located approximately 8 kb 5'; of Myf5. MRF4 has been inactivated in mice by three groups (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995) through the use of similar but distinct targeting strategies. Remarkably, the phenotypes of the resulting MRF4-null mice range from complete viability to complete lethality. In each case, however, MRF4-null mice have only mild alterations in skeletal muscle development and imbalance of contractile protein isoform expression and a fourfold increased myogenin expression (Olson et al., 1996).

2.2. Muscle satellite cells

Growth, training or injury of musculature require considerable plasticity and ability to adapt to various physiological demands. The mechanical functions of skeletal muscle are

carried out by syncytial myofibers, each containing a highly specialized contractile apparatus maintained by large numbers of post mitotic myonuclei. Adult skeletal muscle fibers are terminally differentiated and cannot divide and repair muscle injuries. Muscle growth and repair in adult animals are largely attributed to a small population of cells, called satellite cells that are physically distinct from myofibers; however, one cannot rule out the possibility that other locally derived cells might also give rise to muscle precursors *in vivo*.

2.2.1. Identification of satellite cells

2.2.1.1. Morphological criteria of satellite cell identification

Satellite cells were first discovered and termed in 1961 (Mauro, 1961) as cells closely associated with the periphery of the uninjured frog myofiber. These mononucleated cells are quiescent, lack myofibrils and reside between the sarcolemma and the basal lamina of adult skeletal muscles (Muir et al., 1965).

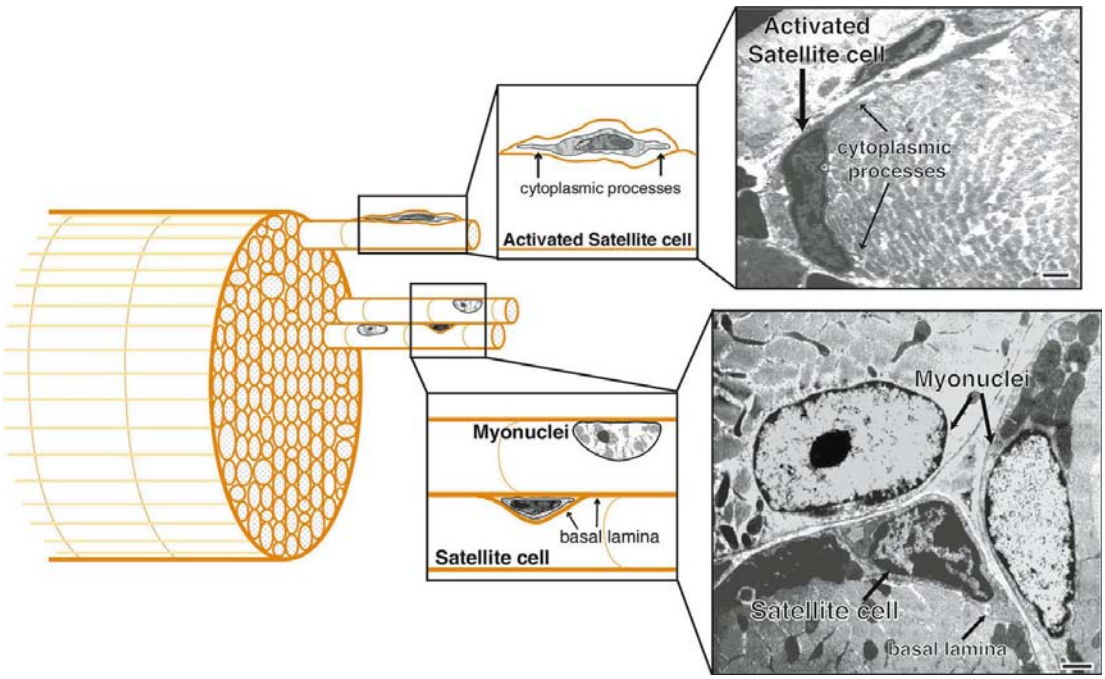

Fig. 4. Satellite cells occupy a sublaminar position in adult skeletal muscle. In the uninjured muscle fiber, the satellite cell is quiescent. The satellite cells can be distinguished from the myonuclei by a surrounding basal lamina and more abundant heterochromatin. When the fiber becomes injured, the satellite cells become activated and increase their cytoplasmic content (the picture has been taken from the work of Hawke and Garry (Hawke and Garry, 2001)).

Nuclei of these cells contain more heterochromatin than nuclei of myotubes (myonuclei). In response to muscle injury satellite cells become activated, proliferate and, finally, fuse to form new myotubes and repair the damaged area (Bischoff, 1994)

Skeletal muscles contain various cell types; including satellite cells, muscle associated fibroblasts, pericytes, smooth muscle and endothelial cells associated with the vasculature. Electron microscopy still remains the most reliable method to identify quiescent satellite cells; however, several immunochemical markers might be used to identify quiescent, activated or proliferating satellite cells. The ultrastructural characteristics of the quiescent satellite cells are the following (Bischoff, 1994): basal lamina surrounds these closely associated with myofibers cells, they have relatively high nuclear-to-cytoplasm ration with few organelles and smaller than in myotubes nuclei, since satellite cells are quiescent and transcriptionally less active, their nuclei have more heterochromatin than myonuclei have (Fig. 4).

2.2.1.2. Molecular markers of satellite cells

Identification of satellite cells by light microscopy is more ambiguous. Pericytes, macrophages or other infiltrating beneath the external lamina cells can be morphologically indistinguishable from satellite cells at the light microscopy level. The use of immunohistochemical markers facilitates the identification. Although the profile of gene expression of quiescent satellite cell as well as their activated and proliferating progeny is largely unknown, a number of molecular markers specifically expressed in satellite cells have been identified (summarized in Table 1., reviewed in (Hawke and Garry, 2001; Charge and Rudnicki, 2004).

Several antibodies which recognize embryonic muscle precursor cells or other cell types have been reported to recognize satellite cells in adult muscle. Isoforms of neural cell adhesion molecule (NCAM) and vascular cell adhesion molecule-1 (VCAM-1) were extensively used to purify muscle precursor cells from fibroblast in culture before more specific antibodies have been identified (Jesse et al., 1998). NCAM is expressed in both myofibers and satellite cells, whereas VCAM-1 is broadly expressed during embryogenesis but limited to satellite cells in adult muscle. Furthermore, VCAM-1 has been shown to mediate satellite cell interaction with leukocytes following injury. M-cadherin, a calcium-dependent cell adhesion molecule, was identify as a unique marker of the satellite cell pool (Cornelison and Wold, 1997; Irintchev et al., 1994). At E11.5 transcripts of M-cadherin mark all myogenic cells and are restricted to the myotome of the somites and the proximal region of the limb buds (Beauchamp et al., 2000).

Molecular Marker	Expression in Quiescent Cells	Expression in Proliferating Cells	Reference
Cell surface molecules			
M-cadherin	+/-	+	(Irintchev et al., 1994; Irintchev et al., 1994; Cornelison and Wold, 1997)
Syndecan-3	+	+	(Cornelison et al., 2000)
Syndecan-4	+	+	(Cornelison et al., 2000)
c-met	+	+	(Cornelison and Wold, 1997)
VCAM-1	+	+	(Jesse et al., 1998)
NCAM	+	+	(Illa et al., 1992)
Glycoprotein Leu-19	+	+	(Illa et al., 1992; Schubert et al., 1989)
CD34	+/-	+/-	(Beauchamp et al., 2000)
Cytoskeletal molecules			
Desmin	-	+	(Cornelison and Wold, 1997; Bockhold et al., 1998)
Transcription factors			
Pax7	+	+	(Seale et al., 2000)
Myf5	+/-	+	(Cornelison and Wold, 1997; Beauchamp et al., 2000)
MyoD	-	+	(Cornelison and Wold, 1997)
MNF	+	+	(Garry et al., 1997)
Myostatin	+	+/-	(Cornelison et al., 2000; Kirk et al., 2000; Mendler et al., 2000)
IRF-2	+	+	(Jesse et al., 1998)
Msx1	+	-	(Cornelison et al., 2000)

Table 1 Expression patterns of satellite cell markers in adult skeletal muscle. Expression of selected molecular markers used to identify the satellite cell population in adult skeletal muscle is outlined. MNF, myocyte nuclear factor; NCAM and VCAM, neural cell and vascular adhesion molecule; IRF-2, interferon regulatory factor-2 (the table has been taken from (Hawke and Garry, 2001) and modified).

In muscles of adult mice M-cadherin is only expressed in a subpopulation of the quiescent cell pool; however, its expression is increased when the satellite cells become activated in response to a stimulus. Two alternatively spliced isoforms of myocyte nuclear factor (MNF), a member of the winged helix transcription factor family, are also expressed in quiescent satellite cells, during myogenesis and muscle regeneration (Garry et al., 1997). Disruption of the MNF locus resulted in a severe growth deficit, a marked impairment in muscle regeneration and decreased number of satellite cells in skeletal muscles of adult mutant mice (Hawke and Garry, 2001).

C-Met, the receptor for hepatocyte growth factor (HGF), is a marker of quiescent satellite cells (Cornelison and Wold, 1997). HGF is a potent mitogen for satellite cells and has been shown to be important in the migration of the myogenic precursor cells from the somite to the developing limb. Desmin, an intermediate filament protein, is present in proliferating muscle precursor cells in cultures and in regenerating skeletal muscles at 24 hours or more after injury (Bockhold et al., 1998). Several other proteins like myostatin (Kirk et al., 2000), syndecan-3, syndecan-4 (Cornelison et al., 2000), glycoprotein Leu-19 (binds proliferative and quiescent human satellite cells and myotubes) (Schubert et al., 1989), interferon regulatory factor-2 (IRF-2) (Jesse et al., 1998) have also be shown to be expressed in myogenic precursor cells. In addition, immunohistochemical staining of the plasmalemma (using antibodies to dystrophin) and of the external basal lamina (using antibodies to collagen IV or laminin) also helps to identify cells, located between these two membranes, at the light microscope level.

Recently the paired box transcription factor Pax7 was identified to be expressed selectively in quiescent and proliferating satellite cells. Analysis of the Pax7 mutant skeletal muscle by the group of M. Rudnicki revealed a complete absence of satellite cells (Seale et al., 2000). The authors claimed that Pax7 is essential for the specification of the satellite cell population.

CD34 is a transmembrane sialomucin, expressed by haematopoietic stem cells (HSC) and progenitors, small-vessel endoepithelium and quiescent satellite cells (Beauchamp et al., 2000). CD34 expression was also found in myogenic cells, both in undifferentiated myoblasts and after differentiation, although levels of expression varied considerably.

Most quiescent satellite cells do not express transcription factors like MyoD, myogenin, MRF4, MEF2 or other known markers of terminal differentiation (Cornelison and Wold, 1997; Megeney et al., 1996; Yablonka-Reuveni and Rivera, 1994). Only Myf5 expression has been reported in a subset of quiescent satellite cells (Beauchamp et al., 2000). *In situ* hybridization, Northern blot analysis or immunohistochemical staining reveal very low or undetectable levels of MyoD and myogenin in mature uninjured mouse skeletal muscle or satellite cells (Grounds et al., 1992; Cooper et al., 1999). MRF4 is expressed at relatively high level in adult differentiated muscles and absent in satellite cells (Cornelison and Wold, 1997). In developing mouse muscles, Northern blot analysis shows that MyoD and myogenin mRNAs decrease to adult low levels between1 and 3 weeks after birth.

Expression of Myf5 in all CD34-positive quiescent satellite cells was shown using $Myf5^{nlacZ/+}$ mice which have a reporter gene encoding nuclear-localizing ß-galactosidase,

targeted to the Myf5 locus (Tajbakhsh et al., 1996a). Previous attempts to detect Myf5 protein in isolated fiber preparations were unsuccessful due to the high levels of non-specific binding encountered with the available antibodies (Yablonka-Reuveni et al., 1999).

Using single myofiber cultures, mass cultures of satellite cells and *in vivo* experiments it was shown that in response to physiological stimuli, quiescent satellite cells become activated, start to proliferate and up regulate first either MyoD or Myf5 genes (Zammit et al., 2002; Cornelison and Wold, 1997). Upon satellite cell activation, MyoD mRNA and protein appear the first within 12 h of activation and is detectable before any sign of cellular division such as proliferative cell antigen nuclear expression (Cooper et al., 1999; Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994). Significantly, MyoD is only observed in cells that are already expressing Myf5. After muscle injury MyoD and myogenin transcripts can be found in mononuclear cells 6 hour, with peaks at 24 and 48 hours, and declines to pre-injury levels by about 8 days (Grounds et al., 1992). Subsequently most cells transcribe both MyoD and Myf5, but there are also subpopulations expressing exclusively MyoD or exclusively Myf5 (Cornelison and Wold, 1997; Cooper et al., 1999) (Fig. 5). It has been found using RT-PCR that all four MRF family members are expressed by 95% of activated satellite cells in mass cultures (Smith et al., 1994). Myogenin- and MRF4-positive cells can be found after 48-72 hours in cultures of myotubes both at the RNA level and using immunohistochemical staining.

The satellite cell compartment is heterogeneous, the majority (about 80%) is Myf5/M-cadherin/CD34-positive (Beauchamp et al., 2000), presumably reflecting commitment to myogenesis, while a minority is negative for these markers. A minor population is not active as indicated by the lack of Myf5 or MyoD expression. While there is no direct evidence linking behavioural and phenotypic heterogeneity, it is possible that Myf5/M-cadherin/CD34-positive cells undergo rapid differentiation following activation. Satellite cells that do not express any myogenic markers may correspond to the slowly activated, proliferative population and may replenish the cells that undergo rapid differentiation.

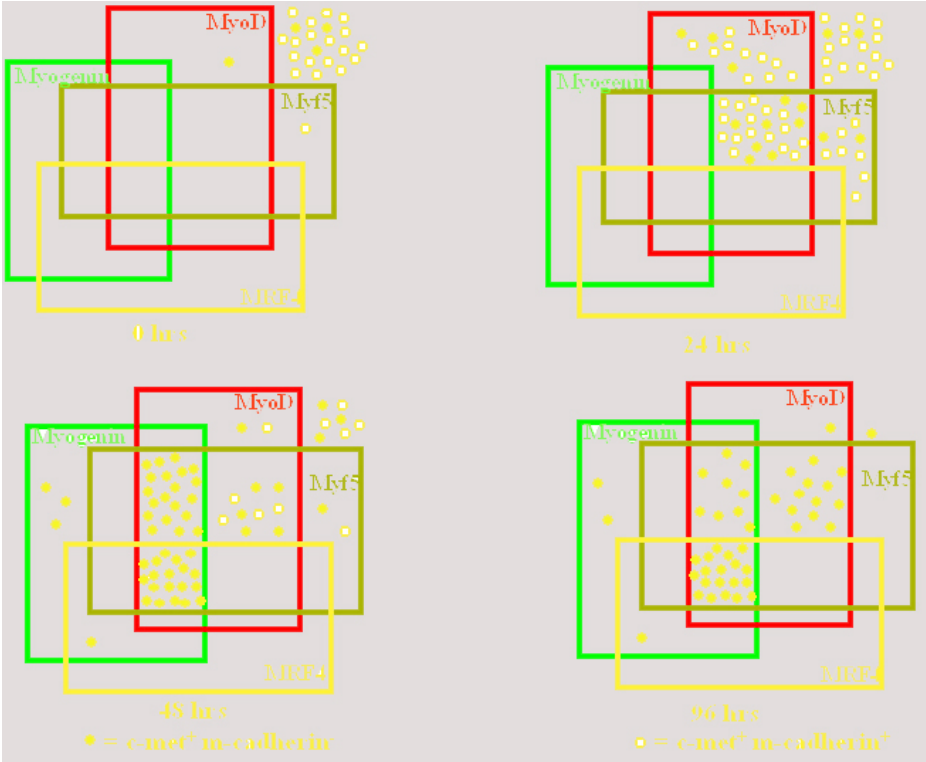

Fig. 5 Summary of the combinatorial expression states of c-met, m-cadherin, MyoD, myf5, myogenin, and MRF4 in wild-type satellite cells during the first 4 days in fiber culture. The results obtained using single cell multiplex PCR do not detect MyoD- and myf5-positive cells among quiescent satellite cells. After 24 hours in culture satellite cells become activated and express MyoD or Myf5 or both these factors. After 48 hours in culture the expression of myogenin and MRF4 (myf6), the differentiation factors, starts. Further cultivation results in expression of all four myogenic factors (the picture has been taken from the work of Cornelison et al. (Cornelison et al., 2000)).

2.2.2. Origin of satellite cells

Previous studies support the hypothesis that muscle precursor cells, including the myogenic satellite cell population, originate from the multipotential mesodermal cells of the somite (Ordahl et al., 2000; Schultz and McCormick, 1994). This hypothesis is supported by avian chimeras or interspecies grafting experiments. When embryonic somites from quail donors were transplanted into host chick embryos (Christ et al., 1974; Le Douarin and Barq, 1969; Armand et al., 1983), quail cells were observed to migrate from the somite and contribute to both the limb muscles and the satellite cell population in postnatal chick skeletal muscle.

Whether the satellite cells migrate from the somite as a distinct lineage or whether they originate from a pre-existing lineage (i.e., embryonic or foetal myoblasts) in the developing limb is unclear. Nevertheless, the current concept postulates that each of the myoblast precursor cells (i.e., embryonic myoblasts, foetal myoblasts, and satellite cells) was a derivative of the somite.

Not all scientists agree with this concept. Recent observations have challenged this view. One group reported that myogenic progenitor cells originating from embryonic dorsal aorta co-express endothelial and myogenic markers (De Angelis et al., 1999). Transplantation of the aorta-derived myogenic cells into newborn mice revealed that this cell population participated in postnatal muscle growth, regeneration, and fusion with resident satellite cells. The authors proposed that satellite cells may be derived from endothelial cells or a precursor common to both the satellite cell and the endothelial cell. Furthermore, the same study shows that myogenic precursor clones can be derived from limbs of c-Met-/- and Pax3-/- mutants, which lack appendicular musculature due to the absence of migratory myoblasts of somitic origin. Thus myogenic precursors derived from these mutant limbs may be of endothelial origin. When directly injected into regenerating host muscles, these cells are incorporated into newly regenerating fibers. When embryonic aortas are transplanted into muscles of newborn immunodeficient mice, they can also give rise to many myogenic cells within the host muscles and also contribute to collateral untreated muscles. Moreover, when foetal limbs are transplanted under the skin of host animals and become vascularized by the host, myogenic cells of host origin are observed within the transplant. Taken together, these results suggest the presence of a multipotential cell population within the embryonic vasculature.

The origin of the satellite cells remains uncertain. It is not clear whether there is a common lineage source for the entire satellite cell population and a common lineage source for cells that have regenerative capacities in both muscle and non-muscle tissues. Thus the presence of myogenic cells within the embryonic dorsal aorta does not rule out the possibility of an indirect somitic origin of the satellite cells. Moreover, the evidence that satellite cells represent a heterogeneous population may be a reflection of their multilineage origin. Both lineages may contribute under physiological or pathological states to the satellite cell population.

2.2.3. Satellite cells as stem cells: self-renewal and proliferative capacity of satellite cells

In contrast to tissues that have constant turnover such as blood, skin, epithelial lining of the gut and nervous system, skeletal muscle is an example of an adult tissue, where new cells are only generated in response to the sporadic demands of growth and repair. Differentiated skeletal muscles are highly specialized and post-mitotic, therefore pre-natal and post-natal development is assured by a population of progenitor cells. Recently interest has focused on whether satellite cells are “true” stem cells.

Two main criteria of stem cells are that (i) they should be clonogenic progenitors of a specific differentiated cell type and (ii) the self-renewal capacity of these cells (Anderson et al., 2001). Satellite cell self-renewal is a necessary process without which recurrent muscle regeneration would rapidly lead to the depletion of the satellite cell pool. In normal muscle, satellite cells are mitotically quiescent, but become activated to divide in response to signals released following damage or in response to increased workload. After division, satellite cell progeny undergo terminal differentiation and form mature muscle fibres. The clonogenic and differentiation potential of muscle satellite cells has been found both *in vivo* and *in vitro*. *In vitro* it has been shown for cultures of mononucleated cells from adult skeletal muscle isolated by enzymatic disaggregation (Bischoff, 1974) and also cultures of isolated single muscle fibres (Rosenblatt et al., 1995). *In vivo* it has been confirmed by studies of revertant fibres in the muscles of mdx mice. The mdx mouse is a genetic and biochemical model of Duchenne muscular dystrophy. Mdx mice do not express dystrophin protein due to a nonsense point mutation in exon 23 of the dystrophin gene (Sicinski et al., 1989). Dystrophin is a major component of the dystrophin-glycoprotein complex, which links the myofiber cytoskeleton to the extracellular matrix. Disruption of this complex leads to increased susceptibility to contraction-induced injury and sarcolemmal damage leading to myofiber necrosis. Although mdx mice are normal at birth, skeletal muscles show extensive degeneration by 3–5 wk of age. Muscles of these mice permanently undergo cycles of degeneration and regeneration. Satellite cells respond to the injury by repopulating the injured skeletal muscle with defective myofibers lacking dystrophin. This process results in continuous exhaustion of the satellite cell pool (Heslop et al., 2000). However, despite the underlying mutation, the muscles of mdx mice contain rare revertant fibers that express dystrophin protein by virtue of exon skipping (Hoffman et al., 1990; Lu et al., 2000). In newborn mdx mice revertant fibres occur as singletons; in older animals, they are found as clusters that increase in frequency, size and constituent fibre number with age. Significantly, each cluster is composed of fibres that share the same pattern of exon skipping, often distinct from that utilized by neighbouring clusters, indicating a clonal origin. Satellite cells are therefore a population of precursors that provide a reserve capacity to replace differentiated, post-mitotic cells required for the functions of adult skeletal muscle.

Not all aspects of the self-renewal capacity of satellite cells are understood. An inability to maintain the satellite cell pool suggests an eventual decrease in the efficiency of self-renewal such that it does not fully compensate for the loss to differentiation over time. Although the above observations demonstrate that the satellite cell pool is maintained, they do

not reveal whether all or a subpopulation of satellite cells are responsible for renewal of the compartment.

In addition to differences of the satellite cel population among different species, age groups and muscles there is also functional heterogeneity within satellite cell populations. Rat satellite cell have different replicative and proliferative capacity: some of the cells produce in cultures colonies with only few myoblasts, whereas some give rise to hundreds cells (Schultz and Lipton, 1982). The whole population can be divided to subpopulations of relatively slowly and rapidly dividing cells. The colony heterogeneity does not depend on the age of animals, although the average size of the colonies decreases with age, reflecting the reduction of the proliferative potential. Only a small population of the cells, about 10-15% of the total population, retains the ability to form large colonies even when derived from aged donors and from extensively damaged regenerating muscles (Schultz and Lipton, 1982; Molnar et al., 1996).

According to one model which explain how satellite cells maintain their population, each satellite cell division is asymmetrical, generating a replacement satellite cell and a daughter cell that later become a myonucleus (Moss and Leblond, 1971). However, it is also possible that satellite cell division is symmetrical and that progeny can withdraw from the differentiation pathway and return to a state of quiescence. Neither hypothesis has been proven wrong.

Data, which support the first model results, are based on *in vitro* investigations of avian muscles. It has been shown that myogenic cells from avian muscle contain two populations: cells committed to terminal differentiation and cells which can undergo either symmetric, self-renewing divisions, or asymmetric divisions that give rise to cell progeny dividing further and after that differentiating (Quinn et al., 1984). Based on EM-BrdU labelling investigations, it has been found that myogenic cells in mammalian muscle are also comprised of at least two subpopulations (Konigsberg and Pfister, 1986). One population may function as a progenitor or stem cells and may give rise to renew daughter progenitor cell by relatively slow or infrequent asymmetric mitotic divisions. Cells of the second population are induced to divide an unknown number of times before entering terminal differentiation or fuse depending on environmental conditions and functional demands. In young growing rats the stem cell like population comprise 15-20% of all muscle precursor cells. In favour of satellite cell asymmetric division is the recent observation by Conboy and Rando (Conboy and Rando, 2002) that Numb, a plasma membrane-associated cytoplasmic protein, is asymmetrically segregated within dividing satellite cells *in vitro*.

Experiments with myoblast transplantation yielded further evidence for self-renewal. After transplantation of cultured myogenic cells into regenerating host muscle, donor cells are incorporated as differentiated myonuclei within newly formed or repaired muscle fibres (Watt et al., 1982). However, some donor-derived cells persist without differentiation within the host muscle. Such cells when isolated, can divide, differentiate in tissue culture and form muscle rvrn after serial transplantation (Yao and Kurachi, 1993; Morgan et al., 1994).

Transplanted primary neonatal myoblasts from the Myf5nlacZ mouse, which has nlacZ targeted to one allele of the Myf5 locus (Tajbakhsh et al., 1996b), have also been shown to give rise to persistent Myf5-positive cells associated with newly regenerated fibres (Heslop et al., 2001). These peripherally located cells continue to express Myf5, which is present in satellite cells but down-regulated after differentiation (Beauchamp et al., 2000), and give rise to clones of myogenic cells *in vitro*, providing further evidence that transplanted myogenic cells can give rise to both differentiated myonuclei and functional satellite cells.

It is a valid assumption that satellite cells are a population of precursors that provide a reserve capacity to replace differentiated, post-mitotic cells required for the functions of adult skeletal muscle. As such, satellite cells fulfil the basic criteria required of an adult stem cell. However, satellite cells have generally been considered as precursors rather than stem cells. This may be due the connotations of multipotentiality derived from brain and blood, where several differentiated cell types are derived from a single stem cell. However, multipotentiality is not an obligate criterion for a stem cell (Tajbakhsh, 2002; Anderson et al., 2001).

2.2.4. Multipotentiality of muscle satellite cells

During vertebrate embryogenesis, mesodermal progenitors give rise to distinct cell lineages, including skeletal myocytes, osteocytes, chondrocytes, and adipocytes, in response to distinct signals derived from surrounding tissues (Brand-Saberi et al., 1996). The existence of multipotential mesodermal progenitors in the embryo has been well studied using the C3H10T1/2 cell line derived from embryonic mesodermal cells. 10T1/2 cells readily differentiate into three distinct mesodermal cell lineages, skeletal myocytes, adipocytes, and chondrocytes following treatment with 5-azacytidine (Taylor and Jones, 1979). Treatment with bone morphogenetic proteins can induce osteogenic, chondrogenic, and adipogenic differentiation of 10T1/2 cells (Katagiri et al., 1990; Asahina et al., 1996). In addition, multipotential mesenchymal stem cells derived from bone marrow can differentiate into skeletal myocytes, adipocytes, osteocytes, and chondrocytes following treatment with various

inducers as well as *in vivo* transplantation (Prockop, 1997; Pittenger et al., 1999; Liechty et al., 2000). Therefore, these results suggest that there are common progenitors which give rise to mesenchymal progenies.

It has been established for several years now that the commitment of skeletal myocytes is reversible under appropriate tissue culture conditions. Primary myoblasts from newborn mice and C2C12 can differentiate into osteogenic or adipogenic cells after *in vitro* treatment with bone morphogenetic proteins (BMP2) or adipogenic inducers (thiazollidinedione or fatty acids), respectively (Katagiri et al., 1994; Teboul et al., 1995).

The adult muscle satellite cell was generally considered a stem cell committed to the myogenic lineage. The multipotent nature of satellite cells has been recently demonstrated when clonal satellite cells, expressing myogenic markers such as MyoD, Myf5, Pax7 and desmin, were driven to adipocytes and chondrocytes (Asakura et al., 2001; Wada et al., 2002) following treatment with BMPs or adipogenic inducers. The osteogenic differentiation of primary myoblasts is characterized by a transient co-expression of myogenic markers (such as MyoD, Myf5 and Pax7) and osteogenic markers (such as alkaline phosphatase), suggesting a direct trans-differentiation from the myogenic lineage to the osteogenic lineage, rather than the passage through a common non-committed progenitor. *In vitro* culture of single myofibers suggests the spontaneous conversion of satellite cells to the osteogenic and adipogenic lineages is a rare phenomenon.

The data support the hypothesis that muscle satellite cells may be involved in the formation of adipogenic and osteogenic tissues under certain *in vivo* circumstances. Aberrant activation of satellite cells during muscle regeneration may lead to such reversal of lineage commitment at the expense of effective muscle regeneration. However, the hypothesis remains to be proven *in vivo*.

Significantly, there is still no evidence of multipotentiality at a clonal level, so that the presence of separate populations cannot be excluded. It remains unknown whether muscle-derived side population stem cells are also capable of differentiation into osteogenic and adipogenic lineages. Further experimentation will elucidate whether satellite cells or muscle-derived stem cells are the origin of adipose cells and osteocytes *in vivo*. However, the possibility that adult skeletal muscle tissue contains a stem cell niche with a resident population of multipotent stem cells raises several fundamental questions regarding the possible identity, origin and location of such cells, their relationship to satellite cells and their relevance to normal muscle regeneration.

2.3. Muscle growth

During early mouse development muscle tissue consists of clusters of myogenic cells separated by loose connective tissue. The first muscle fibers that are known as primary fibers arise at about E11-14 in the mouse limb, around them secondary fibers form at the time when innervation begins to be established (about E14-16) (Ontell and Kozeka, 1984). These processes are still not clearly defined and need further investigation.

Distinct types of myoblasts - termed somitic (obtained from E8.5 somites), embryonic (from E11.5 forelimbs), foetal (from E16.5 limb muscles) and newborn or satellite cell myoblasts (from P1 limb muscles) - appear sequentially at different stages of development, migrate to muscle-forming regions of the embryo, and fuse to form multinucleated muscle fibers (Smith et al., 1993; Miller et al., 1999). The different types of myoblasts can be distinguished based on their different culture requirements, abilities to fuse, and the morphologies and biochemical phenotypes of the myotubes that they form. Studies suggest that distinct lineages generate primary myotubes (embryonic myoblasts) and secondary myotubes (foetal myoblasts). Furthermore, primary and secondary fibers can be distinguished morphologically and show some differences in myosin heavy chain isoforms expression. It has been proposed that some myoblasts remain quiescent in the embryonic limb due to the presence of TGFβ receptors since this pathway blocks differentiation (Cusella-De Angelis et al., 1994). Later on, other signals stimulate a wave of proliferation giving rise to a population of so-called secondary myoblasts which will differentiate to form secondary fibers (Ross et al., 1987). Secondary fibers have the characteristics of fast fibers, whereas primary fibers tend to become slow fibers.

In the foetal period and postnatally the muscle masses undergo very extensive growth. Myofibers increase in length; also increase number of nuclei in postnatal muscle. At about E14-16 numerous mononucleated myogenic cells are closely attached to the myotube, but basal lamina is not present yet, although components of this membrane, such as laminin and agrin, are present. The basal lamina appears only at E18 and encloses clusters of myogenic cells that can be now defined as satellite cells (Godfrey et al., 1988).

Postnatal growth of musculature and maintenance of adult skeletal muscle are thought to be largely accomplished by satellite cells (Moss and Leblond, 1971). Satellite cells proliferate intensively during early development and fuse to growing myofibers. The extent of growth and final number of muscle nuclei vary widely between different species and muscle types. Later, within a few weeks after birth in rodents, the proliferation of these cells and

fusion with myofibers are greatly reduced. Satellite cells withdraw from cell cycle and become quiescent (Bischoff, 1994).

As it was mentioned before, foetal myogenic precursor cells may have not only somitic, but also endothelial origin: they can be readily isolated from explants of the embryonic dorsal aorta and express some endothelial markers (De Angelis et al., 1999). On the basis of there findings the authors hypothesized that when vessels invade the developing muscle field, endothelial derived pluripotent progenitors are induced to become skeletal muscle progenitors by local signals.

Interestingly, in an other study it has been demonstrated that both primary and clonally derived neural stem cells can also generate skeletal muscle cells *in vivo* upon transplantation into the adult. Myogenic conversion of neural stem cells *in vitro* requires a direct exposure of neural stem cells to cultured myoblasts (Galli et al., 2000).

2.4. Muscle regeneration

During adult life of mammals skeletal muscles show a remarkable ability to regenerate. Muscle regeneration includes necrosis of the damaged tissue, inflammation, activation of myogenic stem cells and, as a result of this activation, formation of new myofibers and reconstitution of a functional contractile apparatus (Fig. 6). This highly synchronized process, which requires satellite cell activation, proliferation, migration and terminal differentiation, is activated and controlled by a complex network of signalling pathways and requires collaboration of different cell types (Hansen-Smith and Carlson, 1979; Hansen-Smith et al., 1980). Quiescent satellite cells become active and start to repair muscles under a wide variety of conditions (injury, overwork, denervation, exercise, stretch). Regeneration is regulated by a complex signalling network and requires cell-cell and cell-matrix interactions. Muscle injuries have been shown to cause the release of biologically active molecules into extracellular space. Extracts of crashed muscle contain mitogens for satellite cells (Bischoff, 1986). Peptide signalling molecules that stimulate, inhibit or regulate cellular functions are called growth factors. The action of growth factors can be autocrine (secreted by and acting on the same cell), paracrine (acting on other cells in the local environment), juxtacrine (membrane bound and requiring cell-cell contact) or endocrine (acting on distant target cells).

The first step of the regeneration is necrosis of damaged myofibers that begins with degradation of sarcolemma. Permeability of the sarcolemma, which is increasing as a result of

this degradation, is indicated by the uptake of low-molecular weight dyes like Evans blue or procion orange (Hamer et al., 2002).

Myotrauma initiates an immune response, resulting in attraction of macrophages into the damaged region. Neutrophils are the first inflammatory cells to invade the injured muscle, with a significant increase in their number being observed as early as 1–6 h after injury (Orimo et al., 1991). Within the first two days after injury these blood-borne macrophages infiltrate the damaged area. Macrophages digest necrotic fibers by phagocytosis and secret cytokines that regulate the satellite cell pool (Nathan, 1987). In the absence of a macrophage response, muscle regeneration is also absent; in the presence of an enhanced macrophage response, there is an increase in satellite cell proliferation and differentiation (Lescaudron et al., 1999).

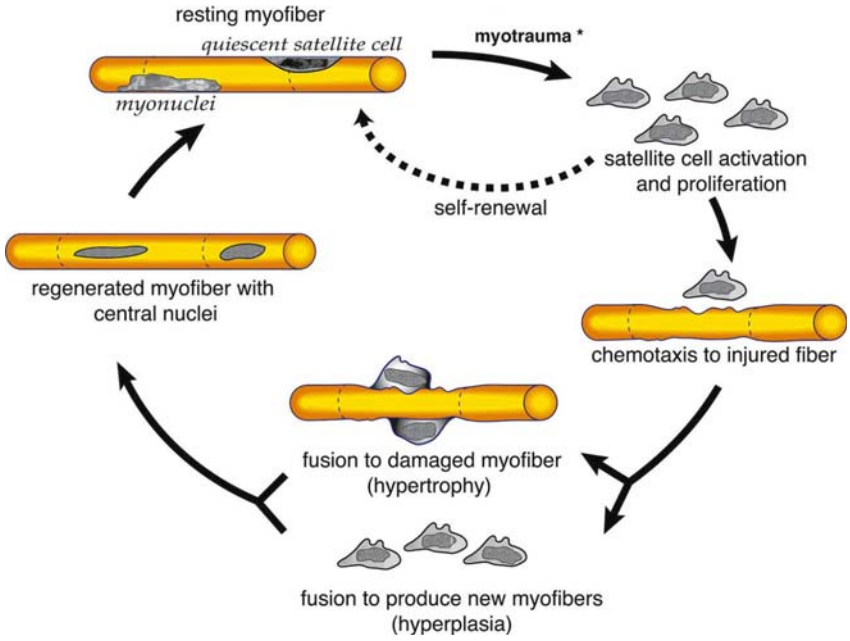

Fig. 6 Satellite cell response to myotrauma. In response to an injury, satellite cells become activated and proliferate. Some of the satellite cells will re-establish a quiescent satellite cell pool through a process of self-renewal. Satellite cells will migrate to the damaged region and, depending on the severity of the injury, fuse to the existing myofiber or align and fuse to produce a new myofiber. In the regenerated myofiber, the newly fused satellite cell nuclei will initially be centralized but will later migrate to assume a more peripheral location (the picture has been taken from the work of Hawke and Garry (Hawke and Garry, 2001)).

Muscle degeneration is followed by activation of the muscle repair process. Damage of the muscles results in liberation of factors leading to activation and proliferation of satellite cells. Activated satellite cells can migrate considerable distances within the muscle towards the crush or even pass into adjacent muscles (Bischoff, 1994). Migration is important for the survival of cells during regeneration of damaged muscles and for participation of large numbers of cells in the repair. After extensive proliferation muscle precursor cells fuse either

with one another to form young multinucleated myotubes or with the ends of damaged myofibers (Robertson et al., 1990). In the regenerated myofiber, the newly fused satellite cell nuclei are initially centrally located but later migrate to aquire a more peripheral location. Newly formed myofibers are often basophilic (reflecting high protein synthesis), express embryonic/developmental forms of MyHC (reflecting *de novo* fiber formation) and are of small calibre. Fiber splitting or branching is also a characteristic feature of muscle regeneration and is probably due to the incomplete fusion of fibers regenerating within the same basal lamina. When fusion of myogenic cells is completed, newly formed myofibers increase in size, and myonuclei move to the periphery of the muscle fiber. Under normal conditions, the regenerated muscle is morphologically and functionally indistinguishable from undamaged muscle.

2.5. Multipotent stem cells in adult skeletal muscle

It has been shown in experiments with intramuscular or intravenous injections of unfractionated mesenchymal stem cells, isolated from the bone marrow stroma of myosin light chain (MyLC)-LacZ transgenic mice, into severe combined immunodeficient (SCID) recipients (mouse strain combining characteristics of SCID animals which lack functional B and T cells and *beige* animals which have intrinsically low natural killer cell activity) that two weeks after injection, small numbers of β-galactosidase stained nuclei had been incorporated into regenerating muscle following chemically induced injury (Ferrari et al., 1998). Another group demonstrated that donor-derived bone marrow cells could be actively incorporated into both the heart and skeletal muscle of mdx mice (Bittner et al., 1999).

Further studies identified subpopulation of pluripotential stem cells within bone marrow cells (Gussoni et al., 1999). These cell are called side population (SP) cells since they are isolated by fluorescence activated cell sorting (FACS), on the basis that these cells actively exclude dyes like Hoechst 33342 due to high expression of *mdr* (multi drug resistant) genes (Fig. 7) (Goodell et al., 1996; Goodell et al., 1997). Bone marrow derived SP cells are positive for Sca-1 (stem cell antigen-1), cKit, CD43 and CD45 and negative for such marker as CD34, B220, Mac-1, Gr-1, CD4, CD5, CD8 (Gussoni et al., 1999).

It has been shown that bone marrow derived SP contains haematopoietic cells as well as cells with myogenic potential which can actively contribute to the regeneration of damaged muscle following intravenous injection into *mdx* mice, with donor-derived nuclei making up to 9% of the total muscle fibers in recipient animals (Gussoni et al., 1999). LaBarge and Blau (2002) showed that bone marrow derived cells not only contribute to regenerating myofibers

but also to the muscle satellite cell pool. Syngeneic mice received whole body irradiation followed by transplantation via tail vein injection of donor GFP-positive bone marrow derived cells. Two to six months after transplantation, GFP-positive cells expressing satellite cell markers were identified at the correct anatomical location for satellite cells. Moreover, clonal progenies of GFP-positive satellite cell isolated from recipient muscles expressed satellite cell markers underwent myogenic differentiation when exposed to low-mitogen media in vitro and contributed to new fiber formation when injected in tibialis anterior muscles of SCID recipient mice.

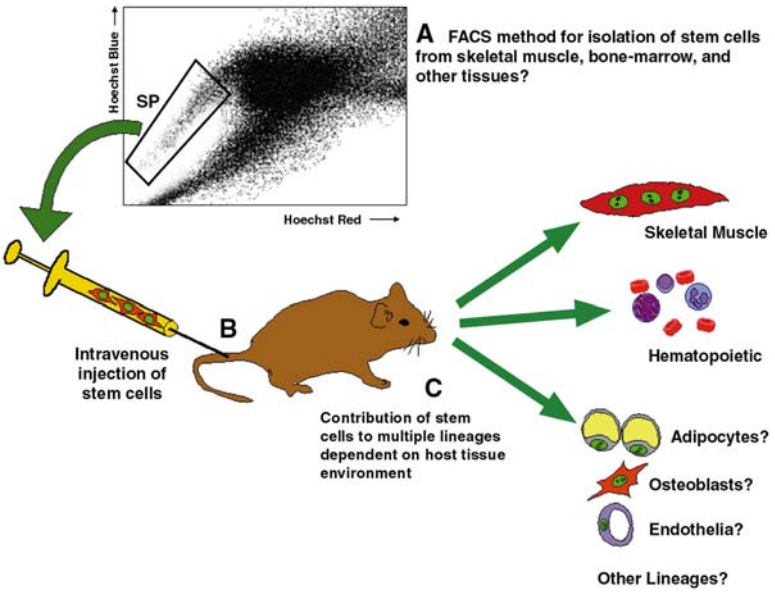

Fig. 7 Pluripotentiality of adult tissue-specific stem cells. (A) Highly purified stem cells are isolated from adult tissue, including bone marrow and skeletal muscle, based on exclusion of Hoechst dye. FACS (fluorescence-activated cell sorting) is used to isolate the side population (SP) of Hoechst-excluding cells. (B and C) Purified stem cells give rise to skeletal muscle cells and haematopoietic cells following intravenous injections in mice. It is possible that such cells could be isolated from many tissues and contribute to multiple lineages following intravenous injection (the picture has been taken from the work of Seale and Rudnicki (Seale and Rudnicki, 2000)).

Recently, the use of more cell-surface markers that are also expressed on haematopoietic cells such as CD45, ScaI, and c-kit have demonstrated that bulk skeletal muscle contains haematopoietic stem cells that can repopulate all major blood lineages both *in vitro* and *in vivo*. Moreover, this activity is probably not attributable to satellite cells (Asakura et al., 2002; McKinney-Freeman et al., 2002; Kawada and Ogawa, 2001). Furthermore, muscle side population cells isolated by FACS, shown to be CD45 positive and to have haematopoietic potential, did not form skeletal muscle autonomously *in vitro*. These cells could give rise to a satellite cell after intramuscular injection or when co-cultured with muscle cells.

Similarly to bone marrow, an enriched population of adult stem cells can be isolated from skeletal muscles by FACS analysis on the basis of Hoechst 33342 staining (Asakura et al., 2002; Gussoni et al., 1999). *In vitro*, muscle SP cells readily form haematopoietic colonies, but do not spontaneously differentiate into muscle cells unless co-cultured with satellite-cell-derived myoblasts. This muscle-derived SP was also found to contribute to differentiated muscle, committing to myogenic conversion *in vivo*, following intravenous injections to mdx mice (Seale and Rudnicki, 2000; Gussoni et al., 1999; Jackson et al., 1999). The ability of muscle-derived populations to contribute to both haematopoiesis and myogenesis might be interpreted as evidence for the presence of multipotent stem cells in adult skeletal muscle capable to adopt alternative lineages in a permissive environment.

2.6. Pax7 and muscle satellite cells

The Pax7 gene is a member of the paired box containing gene family of transcription factors implicated in development of the skeletal muscle of the trunk and limbs, as well as elements of the central nervous system (Mansouri et al., 1996a; Chi and Epstein, 2002; Mansouri et al., 1999). Pax family members function in the transcriptional control of pattern formation during embryogenesis. Each Pax gene has a unique temporal and spatial expression pattern during early development, and some are also expressed with a restricted distribution in the adult. The paired box is a DNA binding domain of 128 amino acids highly conserved during evolution and located close to the amino terminus. Pax7 gene encode a protein containing an N-terminal DNA binding domain consisting of a paired box, octapeptide and complete homeodomain, and a proline-, serine- and threonine-rich C-terminal domain.

Pax7 is detectable at E8.5 in all brain vesicles and later at E11.5 is expressed in mesencephalon with an anterior boundary at the posterior commisure (Jostes et al., 1990). In the neural tube, Pax7is expressed in the dorsal part and Pax7 mRNA is first detected after closure of the neural epithelium. In the somites, Pax7 is first detected in the dermomyotome and later in development is confined to the intercostals muscle (Jostes et al., 1990).

In Pax7(-/-) mice domains where Pax7 is normally strongly expressed (mesencephalon, hindbrain, neural tube and adult brain) appear morphologically normal (Mansouri et al., 1996b). Similarly no embryonic muscle defect has been described in Pax7-mutant mice (Mansouri et al., 1996b), although Pax7 is expressed in myogenic precursor cells (Jostes et al., 1990; Tajbakhsh et al., 1997). However, analysis of skeletal structures showed that newborn Pax7(-/-) have reduced maxilla, some morphological changes of the nose and

other facial skeletal structures; malformations, which might be related to neural crest cell defects.

Pax7 has been recently identified as a gene required for the specification of satellite cell lineage (Seale et al., 2000). Pax7 has been isolated using representational difference analysis of cDNAs as a gene specifically expressed in cultured satellite cells. In addition it is expressed in quiescent and activated satellite cells *in vivo*. Using Northern blot analysis several tissues and cell lines have been tested for Pax7 expression (Seale et al., 2000). Pax7 mRNA has been found in proliferating satellite cell-derived myoblasts, at low level in adult skeletal muscles and in proliferating C2C12 myoblasts, with a rapid down regulation of Pax7 transcripts upon myogenic differentiation. Specific expression of Pax7 within muscle satellite cells *in vivo* was confirmed by *in situ* hybridization and immunocytochemical analyses on fresh frozen muscle sections. Pax7 mRNA and protein were found in a subset of peripherally located nuclei (about 5%) within undamaged wild type skeletal muscle. The number of Pax7-positive cells increased in muscles undergoing regeneration such as in MyoD-/-, mdx and mdx:MyoD-/- skeletal muscles. Centrally located nuclei in regenerating muscles were also Pax7-positive (Seale et al., 2000).

According to previous observations mice carrying a targeted null mutation in Pax7 (Mansouri et al., 1996b) appear normal at birth but fail to grow postnatally (Seale et al., 2000; Mansouri et al., 1996b). Pax7 mutant animals fail to thrive and usually die within 2 weeks after birth. The authors claim that these mice have a decreased skeletal muscle mass resulting from a fiber size decrease rather than a decrease in fiber number. According to their observation satellite cells are completely absent in muscles of Pax7-/- mice. They show that under standard derivation and growth conditions, primary cell cultures from mutant skeletal muscles failed to generate myoblasts; instead, mutant cultures were uniformly composed of fibroblasts and adipocytes. Morphological analysis of mutant skeletal muscles by transmission electron microscopy also indicated a lack of satellite cells in Pax7-deficient musculature. From these data the authors suggested that Pax7 plays a key role in lineage determination, especially in the specification of myogenic progenitors to the satellite cell lineage.

3. RESULTS

3.1. Essentially normal postnatal muscle growth in juvenile and adult Pax7(-/-) mice

3.1.1. Macroscopic features of homozygous Pax7 mutant mice

As reported previously, mice deficient for Pax7 are significantly smaller than their wild-type and heterozygous counterparts and exhibit a general weakness characterized by an abnormal gait and splayed hind limbs (Seale et al., 2000). The body weight of Pax7(-/-) mice at 7 days of age is about 50% reduced in comparison to wild-type littermates. This weight difference increases with age such that at 2 weeks of age mutant animals are about 33% the weight of wild-type littermates. Pax7(-/-) mice usually die within 2-3 weeks after birth. Smaller muscle fiber diameter (about 1,5-fold) and thinner diaphragm muscles were also found in juvenile Pax7(-/-) mice by.

Two available Pax7 mutant strains were used to analyze the physiological role of Pax7 in the control of proliferation and differentiation of muscle satellite and precursor cells. In both strains the Pax7 gene was inactivated by insertion of the neomycin gene into the first exon of the paired box (the paired box is encoded by three exons), which abolishes DNA-binding activity of the paired domain (Chalepakis and Gruss, 1995).

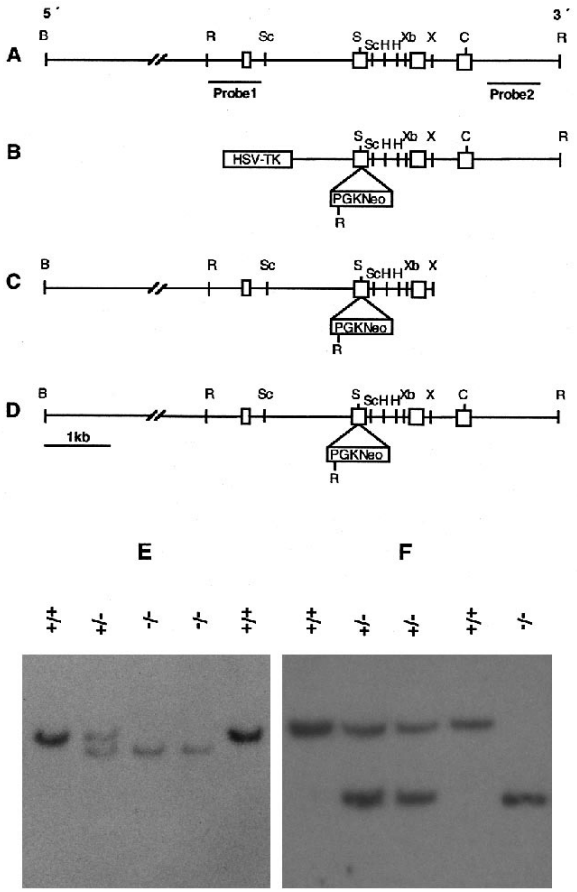

Fig. 8 Strategy for targeted disruption of Pax7 and genotyping of Pax7 mutants. (A) Schematic representation of wild-type Pax7 allele. Boxes: Pax7 exons. (B) First and second targeting construct (constructed using genomic clones from Balb/c or isogenic DNA from the 129Sv strain). (C) Third targeting construct. (D) Mutated Pax7 allele. The PGK-neo expression cassette, which also contains a Poly(A) site and introduces a novel EcoRI restriction site. HSV-TK expression cassette for negative selection. The internal and external probes that lies outside the region of homology are shown as probe1 and probe2. B, BamHI; R, EcoRI; Sc, SacI; S, SalI; H, HindIII; Xb, XbaI; X, XhoI; C, ClaI; kb, kilobase. (E, F) Southern analysis of genomic DNA isolated from mouse tails from heterozygous mating and digested with EcoRI; (E) hybridized with probe 2 – the upper band represents the normal allele (5.4 kb) and the lower band, the mutated allele (4.9 kb); (F) hybridized with probe 1 – the upper band represents the normal allele (5.4 kb) and the lower band the mutated allele (2.3 kb). +/+, wild-type, +/-, heterozygous, -/-, homozygous (the picture has been taken from the work of Mansouri et al. (Mansouri et al., 1996b)).

The Pax7-lacZ strain contains an additional ß-galactosidase reporter gene in front of the neomycin gene to track the fate of Pax7 expressing cells in heterozygous and homozygous mutant animals. The strategy of Pax7 targeted disruption was described by Mansouri et al., 1996 (Fig. 8) (Mansouri et al., 1996b). The Pax7-lacZ strain was maintained on a mixed C57/BL6/129Sv genetic background. As control animals heterozygous Pax7 mice or wild-type C57Bl6 mice were used. Genotyping of Pax7 mice was performed by Southern blot analysis as described by Mansouri et al., 1996 (Fig. 8) (Mansouri et al., 1996b).

Macroscopical examination of both Pax7(-/-) mouse strains used in the present work confirmed the previous observations that mice deficient for Pax7 appear normal at birth but

after a few days after birth start to show a growth delay in comparison to wild type and heterozygous littermates (Fig 9, A, B).

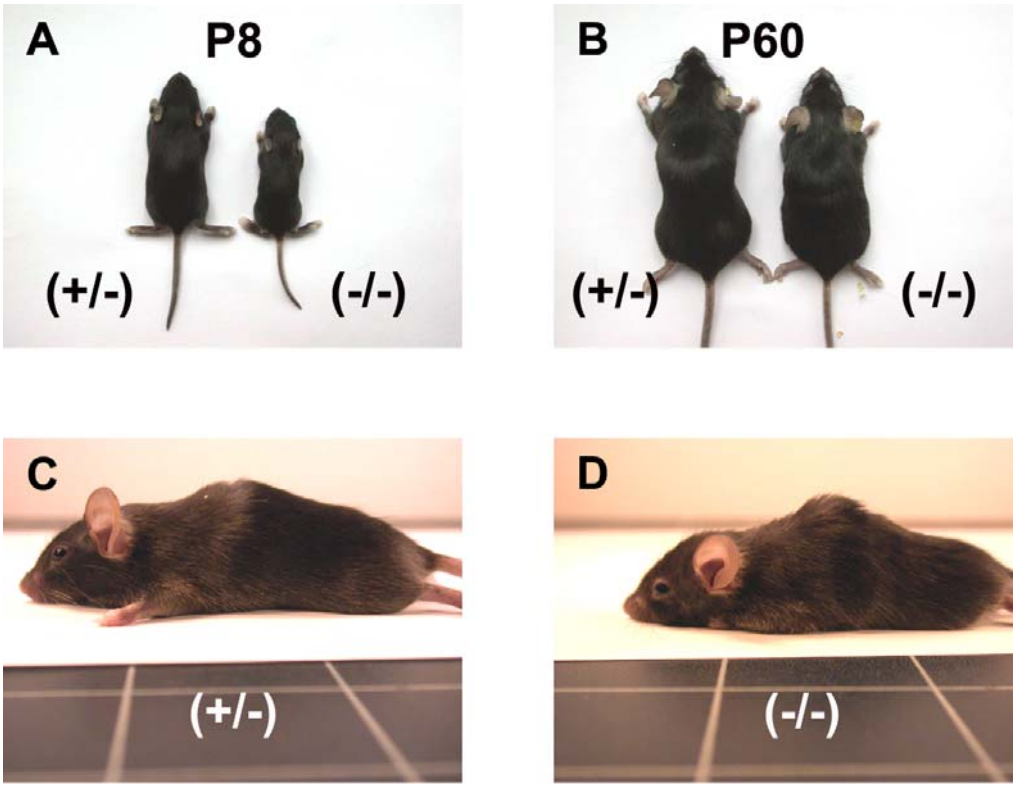

Fig. 9 Growth deficiency and kyphosis of the vertebral column of Pax7(-/-) mice. (A, B) Macroscopic view of Pax7(+/-) and Pax7(-/-) mice at P8 and P60. The growth retardation of homozygous mutant animals is evident. (C, D) Kyphosis of vertebral column of Pax7(-/-) mice at P60 in comparison to normal vertebral column in Pax7(+/-) mice.

Most juvenile (P8) and adult (P30-P60) heterozygous Pax7 animals have normal body size and virtually no differences from wild type counterparts. Only a little percentage of heterozygous juvenile animals looks a bit smaller than their wild type littermates. Normal sized Pax7(+/-) mice were used as control animals in all experiments performed in the present work. As it was published before juvenile Pax7(-/-) mice are weaker then control pups, have abnormal gait and most of them die in the age of 2-3 weeks. However, it has been found that while most of the Pax7-lacZ (-/-) died in first weeks after birth, between 5 to 10% of the Pax7-lacZ(-/-) mutant animals survived until adulthood and were amendable to further analysis. The body weight of adult animals (P30-P60) which survived until adulthood was about 50% reduced in comparison to heterozygous counterparts (Fig 9, A, B), the musculature of homozygous mutant looked generally smaller (Fig. 10) than muscles of control animals. It has been found that homozygous animals have a kyphosis of the vertebral column (Fig 9, C, D), which is often a characteristic of back musculature weakness. However, careful clinical

observation of adult (P30-P60) homozygous Pax7 mutants revealed no particular muscle weakness or abnormal gait.

Previous investigations describing Pax7(-/-) mutant mice demonstrated the important role of Pax7 in skeletal muscle development (Seale et al., 2000). I have found that adult Pax7(-/-) mice have in general smaller muscle mass then Pax7(+/-) mice but no dramatic muscle dystrophy (Fig. 10). Homozygous adult animals have also less fat tissue in the whole body then control animals.

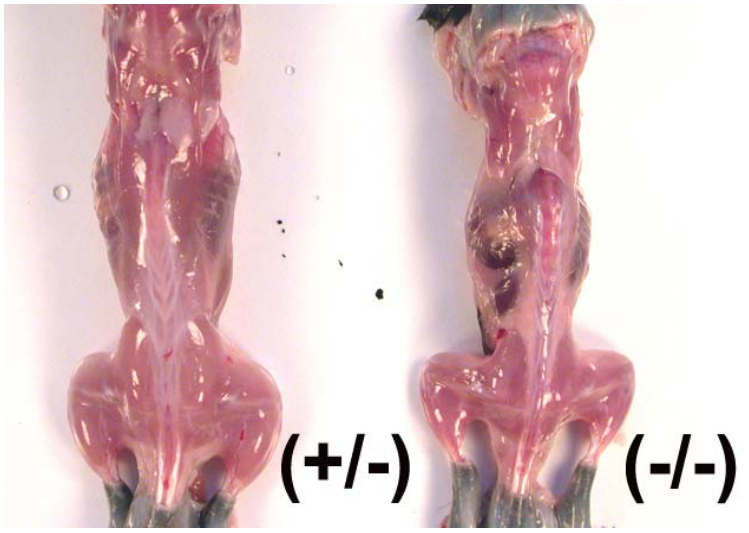

Fig. 10 Macroscopic view of Pax7(+/-) and Pax7(-/-) mice musculature at P60. Muscles of Pax7(-/-) mice look thinner then those of Pax7(+/-) mice but not dramatically.

3.1.2. Histological examination of skeletal muscles of Pax7(-/-) mice

To investigate histological characteristics of skeletal musculature of Pax7(-/-) mice various muscles (M. pectoralis major, M. tibialis anterior, M. gastrocnemius, M. erector spinae, Mm. interossei and Mm. intercostals) from different muscle groups were prepared, fixed and embedded in paraffin.

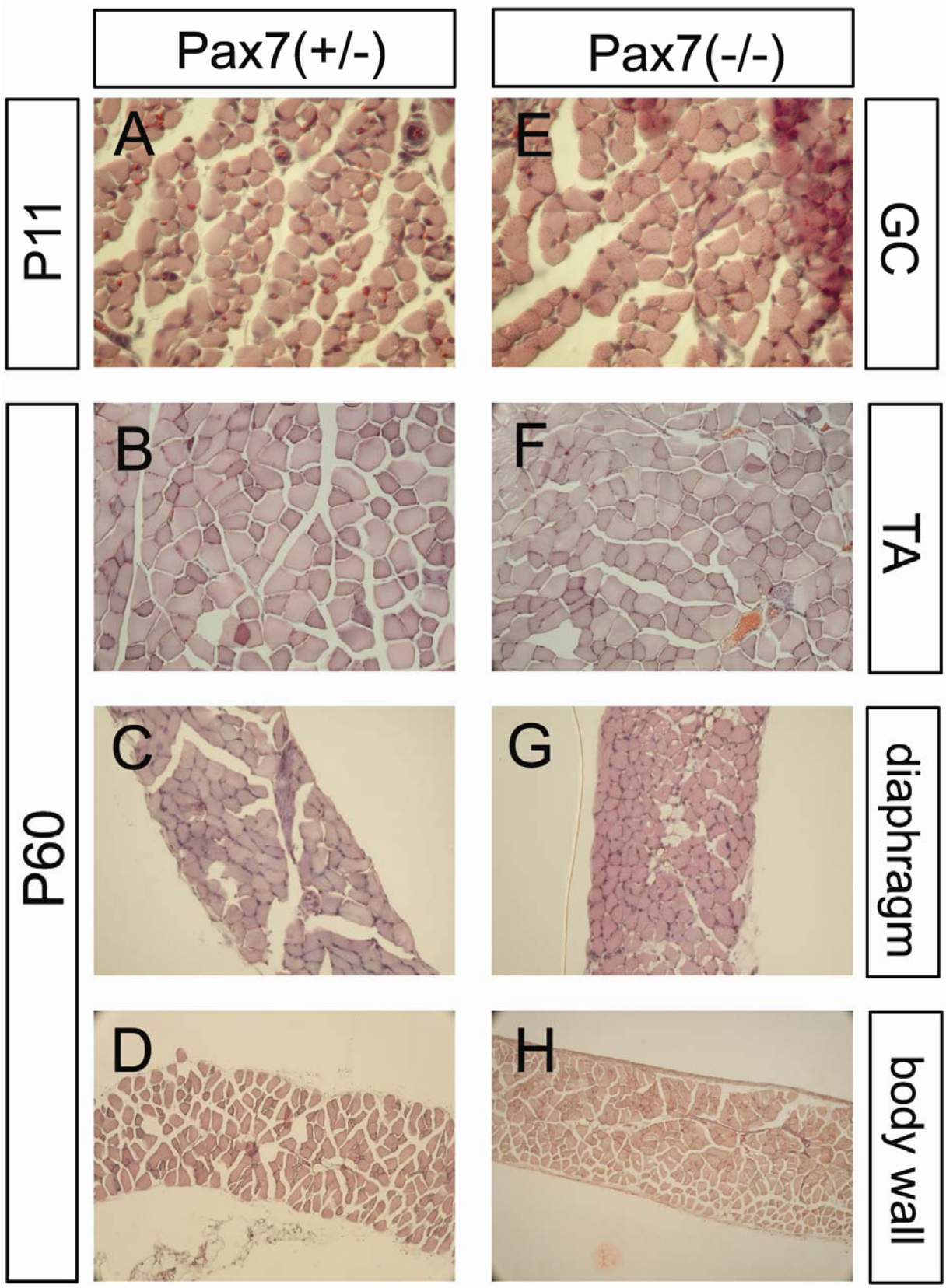

Fig. 11 Microscopic view of Pax7(+/-) and Pax7(-/-) mice musculature at P11 andP60. Haematoxylin and eosin stained paraffin sections of the M. gastrocnemius (GC) at P11 (A, E), the M. tibialis anterior (TA) (B, F), the diaphragm (C, G) and the body wall musculature (D, H) of Pax7(+/-) (A-D) and Pax7(-/-) (E-H) mice at P60. Note that the thickness of individual muscles in Pax7(-/-) mice was only moderately affected although muscles of Pax7(-/-) mice contained more small-sized myofibers.

Serial paraffin sections were stained with haematoxylin (stains nuclei dark violet) and eosin (stains the whole muscle red) dyes, analysed under the light microscope and compared with serial sections from the same muscles of heterozygous Pax7 animals.

The examination revealed quite normal histological characteristics of the musculature in both juvenile (P11-P13) and adult (P30-P60) homozygous Pax7 animals (Fig. 11). No significant reduction of the thickness of the diaphragm or of other muscles that could not be attributed to the reduced bodyweight of Pax7(-/-) mice was observed. I was also unable to observe a significantly reduced diameter of myofibers at P11 and P30 beyond statistical variations. Muscles of Pax7(-/-) mice contained more small-sized myofibers compared to heterozygous controls although it was evident that the distribution of myofiber sizes overlapped extensively. These results contradict the results published by Seale et al., 2000, where the authors claim markedly reduced muscle mass and fiber calibre for Pax7 mutants, and suggest therefore that the postnatal muscle growth normally mediated by satellite cells is deficient in Pax7(-/-) mice.

Based on the rather normal histological appearance of the musculature in juvenile mutant animals it seems unlikely that the cause of death of deceased Pax7 mutants is due to malfunctions of the skeletal musculature. The reduced body weight and early death are most likely caused by malformations of mandicatory organs (probably these mice just cannot eat properly) or neural crest abnormalities, which are known to be present in homozygous Pax7 mutants (Mansouri et al., 1996b). Although adult Pax7(-/-) never reached the size of their wild-type and heterozygous littermates the thickness of individual muscles was only moderately affected. Yet, muscles of adult Pax7(-/-) mice contained more small-sized myofibers than heterozygous or wild-type muscles. It is not clear whether the reduction in muscle mass should be attributed to the effect of the Pax7 mutation on skeletal muscle appearance or simply to the reduced body weight.

The finding of centrally located nuclei in the myotubes of juvenile and adult Pax7(-/-) mice (Fig. 12) was an important for this study finding, since the presence of such nuclei means that some regeneration process is going on in muscles of these mice and they are able to form new myotubes by fusion of mononucleated myogenic cells. At this stage of the investigation it was not clear, what kind of cells take part in the formation of new myotubes, because previous reports claimed the complete absence of satellite cells in Pax7 mutants. The presence of newly formed myotubes, however, clearly indicated that residual muscle stem cells were present in P60 Pax7(-/-) mice.

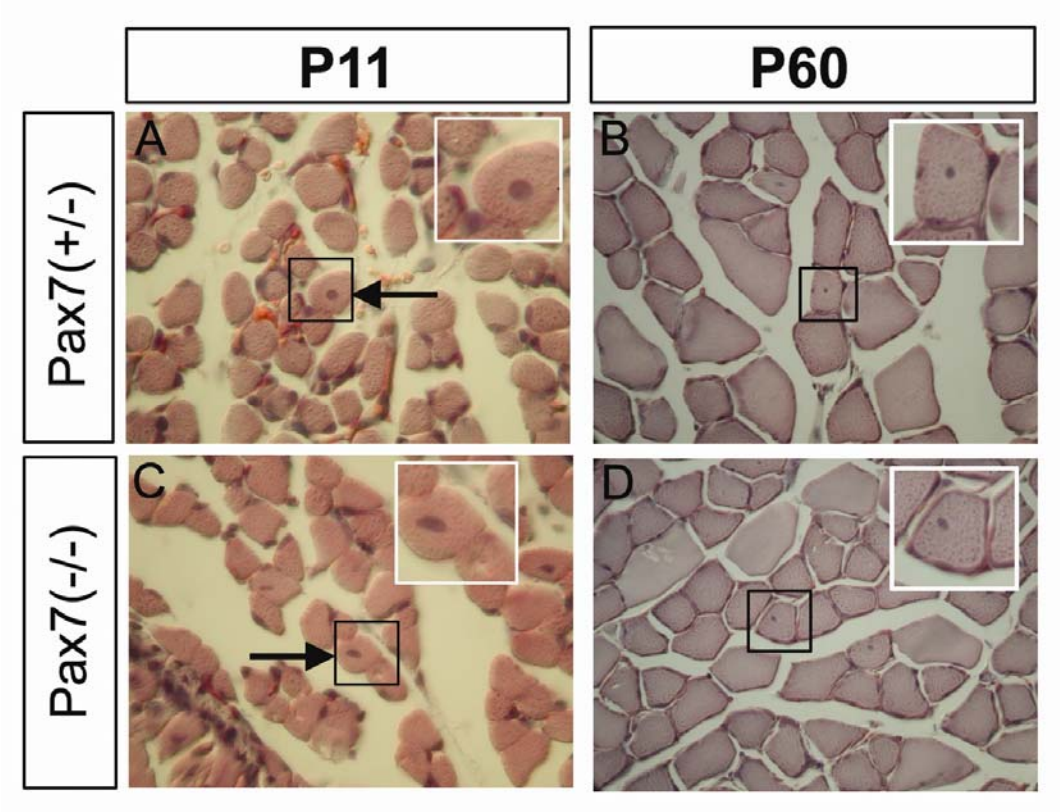

Fig. 12 Presence of centrally located nuclei in skeletal muscle myofibers of Pax7(+/-) and Pax7(-/-) mice at different postnatal stages. Haematoxylin and eosin stained paraffin sections of M. gastrocnemius (A, C) and M. tibialis anterior (B, D) of Pax7(+/-) (A, B) and Pax7(-/-) mutant mice (C, D) at P11 (A, C) and P60 (B, D). Note the presence of centrally located nuclei in myofibers of heterozygous and homozygous mutant animals without prior damage indicating continuous renewal of myofibers. Muscles of Pax7(-/-) mice contained more small-sized myofibers compared to heterozygous controls although it was evident that the distribution of myofiber sizes overlapped extensively.

3.2. Expression of Pax7 is up-regulated in proliferating, satellite cell derived myoblasts

Recently published experiments using Northern blot analysis, *in situ* hybridization and immunocytochemistry have revealed that Pax7 is expressed in proliferating satellite cell-derived myoblasts and at low level in adult skeletal muscles (Seale et al., 2000). Specific expression of Pax7 within muscle tissue was found in peripherally located nuclei within undamaged wild type skeletal muscle, located in the positions characteristic of satellite cells. In addition, it was reported that Pax7 transcripts become rapidly down-regulated upon myogenic differentiation (Seale et al., 2000).

The Pax7-lacZ strain contains an insertion of the ß-galactosidase reporter gene in frame with the Pax7 gene allows tracking of the fate of Pax7 expressing cells in heterozygous and homozygous mice. Further experiments showed, however, that this reporter mouse strain possess only a relatively weak Pax7-lacZ activity. The detection of Pax7-lacZ positive cells in muscles of heterozygous mutants requires extremely long incubation with quite high

concentrations of substrate solutions. The staining intensity becomes stronger in muscles of homozygous mutants which contain two alleles with lacZ insertion, which means two-fold stronger LacZ expression.

The unexpected finding that Pax7-lacZ positive cells located in the positions characteristic of muscle satellite cells are also present in adult Pax7(-/-) (P60) mice, which previously have been thought to have no satellite cells, initiated a careful assessement of the fates of satellite cells in Pax7 mutants. A dramatic reduction of satellite cell number in muscles of homozygous Pax7 mutants was noticeable already at this stage of experiments.

The careful analysis of damaged muscles from both adult Pax7(+/-) and Pax7(-/-) mice has shown that induction of regeneration process by muscle injury leads to a strong increase of the signal and the number of positive cells in the area of regeneration. This finding reflects the expansion of the satellite cell derived myoblasts in the regenerating muscle and an up-regulation of the Pax7 gene expression in proliferating myoblasts. The experiments with homozygous mutants clearly indicate that myogenic cells, which lack Pax7, can divide and are able to respond to physiological cues driving satellite cell expansion.

To study the expression of Pax7 *in situ* in resting and regenerating skeletal muscles I analyzed the expression of the Pax7-lacZ allele by histochemical staining for ß-galactosidase activity. Various hind limb muscles (M. tibialis anterior, Mm. interossei, M. gastrocnemius) were isolated from adult (P30-P60) Pax7(+/-) mice and embedded in Polyfreeze tissue freezing medium and frozen without fixation in liquid-nitrogen cooled isopentane. 10 µm thin sections were stained to reveal LacZ activity using X-gal (5-Brom-4-chlor-3-indolyl-b-D-galactosid) as a substrate. Positive cells normally stain blue in such assays. The first attempts failed to show any positive cells in non-regenerating skeletal muscles taken from adult Pax7(+/-) mice. Only a considerable increase of staining time and substrate concentration facilitated detection of Pax7-lacZ cells located in the positions typical for quiescent satellite cells (cells located on the periphery of myotubes) (Fig. 13 A). Sometimes staining times were extended up to one week using a four-fold increased ß-galactosidase substrate concentration (in comparison to staining solutions which are normally used in staining solutions for the other lacZ assays) were used.

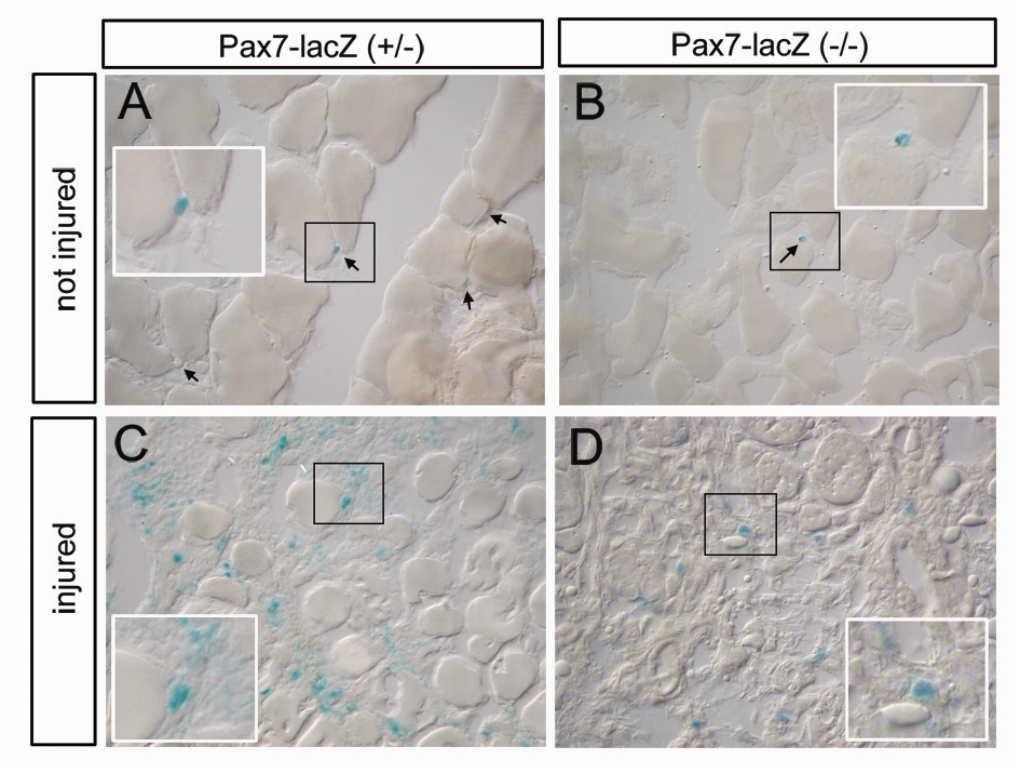

Fig. 13 Increased expression of Pax7-lacZ in activated muscle precursor cells of Pax7(-/-) and Pax7(-/-) mice. LacZ staining of cryostat sections from Mm. tibialis anteriores of Pax7-lacZ(+/-) (A, C) and Pax7-lacZ(-/-) (B, D) mice without prior damage (A, B) and 10 days after cardiotoxin induced muscle injury (C, D). Pax7(+/-) mice do contain more LacZ-positive satellite cells both in uninjured and injured muscles than Pax7(-/-) mutants. Note the increase of LacZ-positive satellite cells and the compromised regeneration in Pax7(-/-) mutant animals.

Pax7 expression in regenerating muscles was investigated using controlled cardiotoxin and freeze-crush induced muscle injuries introduced in hind limb muscles (Mm. tibialis anteriori) of adult (P30-P60) Pax7(+/-) mice. Muscles were isolated 3-10 days after injury, when the proliferative activity of satellite cell derived myoblasts is maximal, embedded in Polyfreeze tissue freezing medium and frozen without fixation in liquid-nitrogen cooled isopentane. 10 µm thin sections were stained to reveal LacZ activity.

After skeletal muscle damage a lot of Pax7-lacZ positive cells were found in the regenerating area. The intensity of staining was much higher than in the experiments with undamaged muscles, and was easily detectable even with an overnight incubation in staining solutions containing low concentrations of the substrate (Fig. 13 C).

The same experiments were repeated with hind limb muscles of adult Pax7(-/-) (P60) mice. Surprisingly, we also found a few Pax7-lacZ positive satellite cells in undamaged muscles of adult homozygous mutants (Fig. 13 B), which previously have been claimed to be completely devoid of satellite cells (Seale et al., 2000). The staining intensity in these rare cells was stronger than in the cells located in uninjured muscles of Pax7(+/-) mice but nevertheless weaker than in regenerating muscles after cardiotoxin injury. Although the

number of Pax7-lacZ positive satellite cells was severely reduced in adult Pax7(-/-) mice a careful examination proved the presence of several Pax7-lacZ positive satellite cells in different muscle groups. In addition, the number and staining intensity of Pax7-lacZ positive satellite cells increased significantly in muscles of adult Pax7(-/-) (P60) mice after cardiotoxin induced muscle damage (Fig. 13 D).

3.3. Detection and quantification of satellite cells in skeletal muscle of Pax7(-/-) mice: severe loss of muscle satellite cells during postnatal development

The normal postnatal growth of skeletal muscles and the presence of Pax7-lacZ positive cells in juvenile and adult Pax7(-/-) mice prompted me to scrutinize vigorously the number of satellite cells in Pax7(+/-) (as a control) and Pax7(-/-) mutant animals at different postnatal stages (P11 and P60) using various established techniques. Mm. interossei are small muscle with very short and unique in length myotubes are very convenient for single myotube preparation. These muscles were used for all quantification methods to avoid variation of satellite cell numbers in different muscles. Mutant and control animals of the same age (littermates) were used for muscle preparations.

3.3.1. Pax7-LacZ-staining

Pax7-lacZ staining was employed to identify satellite cells both on isolated myofibers (Fig. 14 A-F) and on tissue sections (Fig. 13 A-D). Tissue cryosections were prepared from hind limb muscles of Pax7(-/-) and Pax7(+/-) mice of different age. M. tibialis anterior were isolated from adult (P30-P60) mice and M. gastrocnemius from juvenile (P8-P12) mice, embedded in Polyfreeze tissue freezing medium and frozen without fixation in liquid-nitrogen cooled isopentane. 10 μm thin sections were stained to reveal LacZ activity. Numerous Pax7-lacZ positive cells were found on muscle sections from both juvenile Pax7(-/-) and Pax7(+/-) mice (data not shown). The number of positive cells found on the sections of Pax7(-/-) muscles was comparable with that of heterozygous control. A completely different szenario was found in muscles of adult animals. Only a few strongly Pax7-lacZ positive cells were found in samples from adult Pax7(-/-) mice, while a lot of positive cells were found in control samples (Fig. 14 C, A).

The number of Pax7-lacZ-positive cells were counted in cultures of isolated myotubes. Such cultures were prepared from Mm. interossei, attached to glass slides in Matrigel, fixed right after preparation and stained for β-galactosidase activity. All nuclei located on myotubes were counterstained with Hoechst 33258 dye (stained nuclei have a blue fluorescence when

exposed to the UV light). Pax7-lacZ positive cells attached to myotubes and all fluorescently labelled nuclei associated with myotubes (myonuclei) were counted. Several parameters were calculated for the counted numbers: the percentage of LacZ-positive cells to all nuclei that were found on myotubes (myonuclei plus nuclei of LacZ-positive cells), the average number of LacZ positive cells per one myotube, and the average number of myonuclei per one myotube (Fig. 15 A-F). A total number of 1000 to 4000 nuclei per sample were counted.

No significant differences of the average number of LacZ-positive cells per myotube were between Pax7(-/-) and Pax7(+/-) samples from juvenile (P11) mice (Fig. 15 C). Muscles isolated from homozygous juvenile mice contained 0,9 LacZ-positive cells per myotube, while from control heterozygous mice contained 1 LacZ-positive cell per myotube. The average number of myonuclei per myotube was slightly (1,65-fold) reduced in muscles of Pax7(-/-) mice in comparison to muscles of Pax7(+/-) mice (13 in Pax7(-/-) and 21,5 in Pax7(+/-)) (Fig. 15 B). The percentage of LacZ-positive cells of all myonuclei was slightly (1,5-fold) increased in muscles of Pax7(-/-) mice in comparison to muscles of Pax7(+/-) mice. Muscles of juvenile Pax7(-/-) mice contained 6,8% of LacZ-positive cells, whereas Pax7(+/-) control samples contained 4,45% of Pax7-lacZ-positive cells (Fig. 15 A).

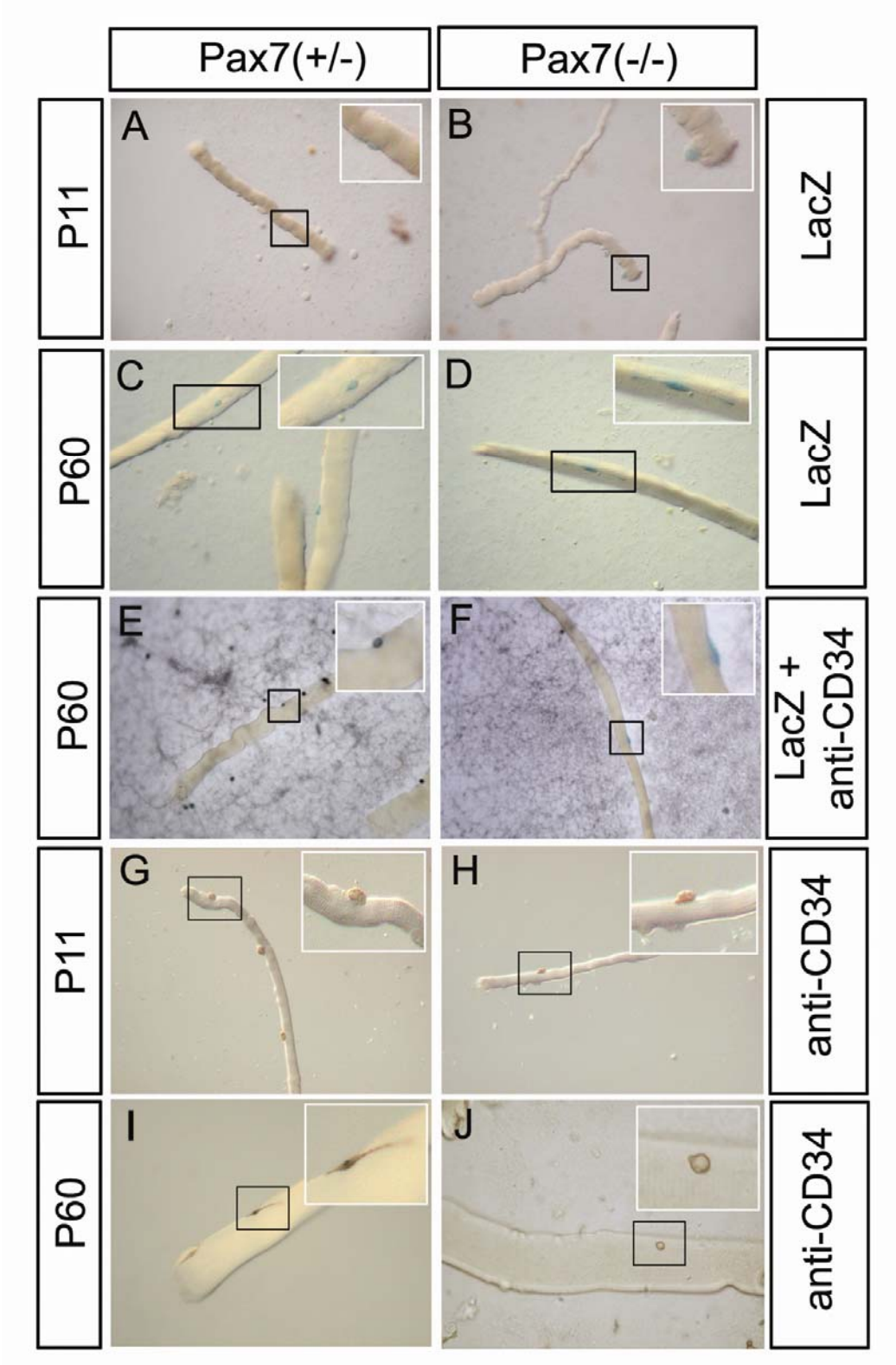

Fig. 14 Expression of Pax7-lacZ and CD34 in satellite cells from Pax7(-/-) and Pax7(-/-) mice at different postnatal stages. Myotubes were isolated from muscles of Pax7(+/-) (A, C, E, G, I) and Pax7(-/-) (B, D, F, H, J) mice at P11 (A, B, G, H) and at P60 (C-F, I, J) and stained for LacZ activity (A-D), reacted with an antibody against CD34 (G-H) or both (E, F). LacZ-positive cells on myotubes from Pax7(-/-) mice show the characteristic morphology of satellite cells (D). Note the abnormal morphology of CD34 positive cells on myotubes from Pax7(-/-) mice at P60 (J). Such cells were not counted as satellite cells. No co-staining was observed for Pax7-lacZ and CD34 in Pax7(-/-) mutants at P60 (F).

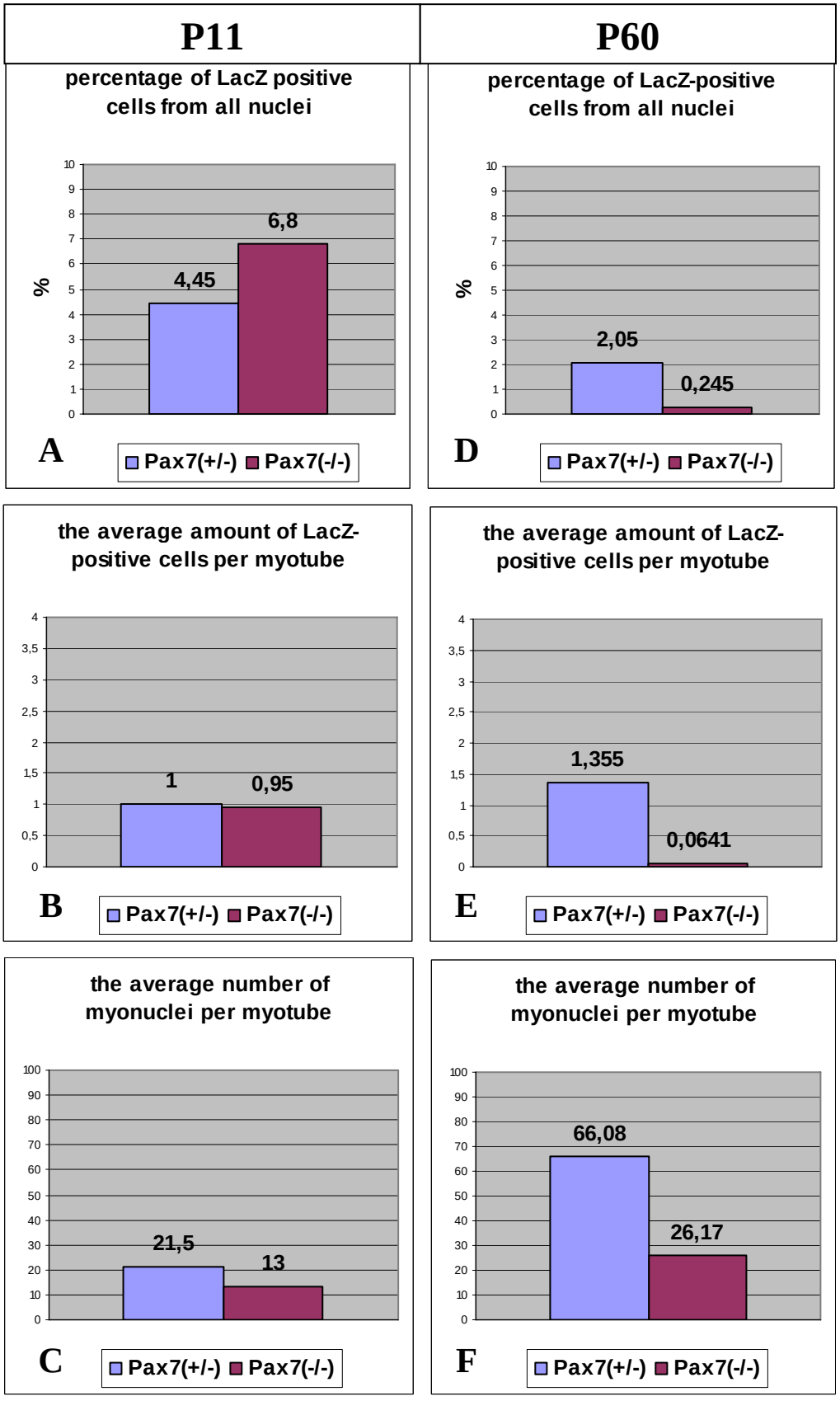

Fig. 15 Severe reduction of Pax7-lacZ positive cells during postnatal development. The number of satellite cells was estimated at P11 (A-C) and P60 (D-F) in Pax7(+/-) and Pax7(-/-) mutant mice using Pax7-lacZ staining and related to different parameters: (A, D) percentage of Pax7-lacZ cells of all myotube nuclei. (B, E) Average number of myonuclei per isolated myotube. (C, F) Average number of Pax7-lacZ cells per isolated myotube. A

drastic reduction of the percentage of Pax7-lacZ cells and of the number of Pax7-lacZ positive cells per myotube was evident at P60 but not at P11.

Completely different ratios of these parameters were found in muscles isolated from adult (P60) mice. A dramatic reduction of all three parameters were found in the samples from adult Pax7(-/-) mice in comparison to control Pax7(+/-) samples. LacZ-positive cells made up only 0,25% of all myonuclei in Pax7(-/-) muscle samples in comparison to 2,05% in the Pax7(+/-) control (8,4-fold reduction) (Fig. 15 D). These results are in agreement with the severe decrease of satellite cell number found using electron microscopy (EM) (see Table 2). The average number of myonuclei per myotube was decreased 2.5-fold in muscles of Pax7(-/-) mice in comparison to control samples. Muscles of Pax7(-/-) mice contained in average 26,17 myonuclei per myotube, muscles of Pax7(+/-) – 66,08 myonuclei per myotube. Finally, the average number of LacZ-positive cells per myotube was 21-fold reduced in muscles of homozygous animals in comparison to heterozygous mice. In Pax7(-/-) samples 0,064 LacZ-positive cell per myotube was found, in Pax7(+/-) samples – 1,35.

3.3.2. Electron microscopic examination

Electron microscopy still remains the most reliable technique to identify muscle satellite cells. Serial ultra thin sections of Mm. interossei were analysed using electron microscopy, with random fields that were chosen for the examination. To avoid double counts of nuclei a 40 μm shift was made between analysed sections. From 500 to 1000 nuclei were counted. The main problem of such counting is to define whether a nucleus belongs to a satellite cell or to myotube. Quiescent satellite cells were recognized according to the following standard parameters: two membranes separate a satellite cell nucleus from the myotube (one of the membranes is the satellite cell plasmalemma, the other is a myotube sarcolemma); the basal lamina surrounds these closely associated with myofibers cells; they have a relatively high nuclear-to-cytoplasm ratio with few organelles and their nuclei are smaller than myotube nuclei; satellite cell nuclei have more heterochromatin than myonuclei have. Myotube nuclei in comparison to satellite cells have only nuclear membranes (Fig. 4, Fig. 17). The percentage of satellite cell nuclei to all nuclei counted (myonuclei, nuclei located inside of myotubes, plus satellite cells nuclei) was calculated.

Satellite cells were found in muscles of both Pax7(-/-) and Pax7(+/-) mice at P11 and P60 (Fig. 17 A-H). Satellite cells from Pax7(-/-) samples were easy to identify and had normal morphology (Fig. 17 C, D, G, and H). The only difference are less heterochromatic nuclei of satellite cells in Pax7(-/-) muscles of adult mice in comparison to Pax7(+/-) control (Fig. 17 G, H)

At P11 I detected a reduced but substantial number of muscle satellite cells in the muscle of Pax7(-/-) mice (3,25% in comparison to 8,25% in Pax7(+/-)). In adult Pax7(-/-) mice at P60 the number of satellite cells was very low indicating a severe loss of satellite cells during postnatal development. Based on EM counting the number of satellite cells dropped to 0.57% from 3.25% at P11 in muscles of Pax7(-/-) mice. The Pax7(+/-) control muscles at P60 contained 6-fold more satellite cells(3,5%) than Pax7(-/-) muscles (0,57%) according to the electron microscopy quantification (Fig. 16).

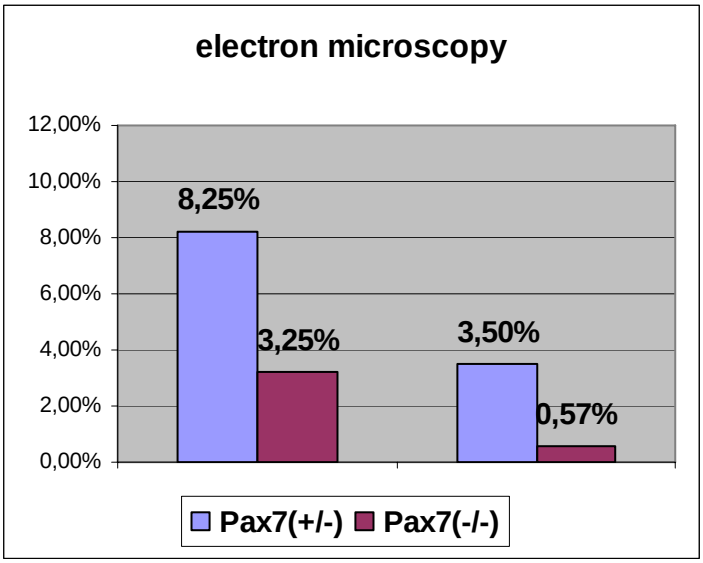

Fig. 16 The numbers of satellite cells identified by electron microscopy in muscles of Pax7(-/-) mice were severely reduced during postnatal development. The number of satellite cells was estimated at P11 (left diagram) and P60 (right diagram) in Pax7(+/-) and Pax7(-/-) mutant mice using electron microscopy and related to all myotube nuclei (the percentage of satellite cells is calculated to all myonuclei).

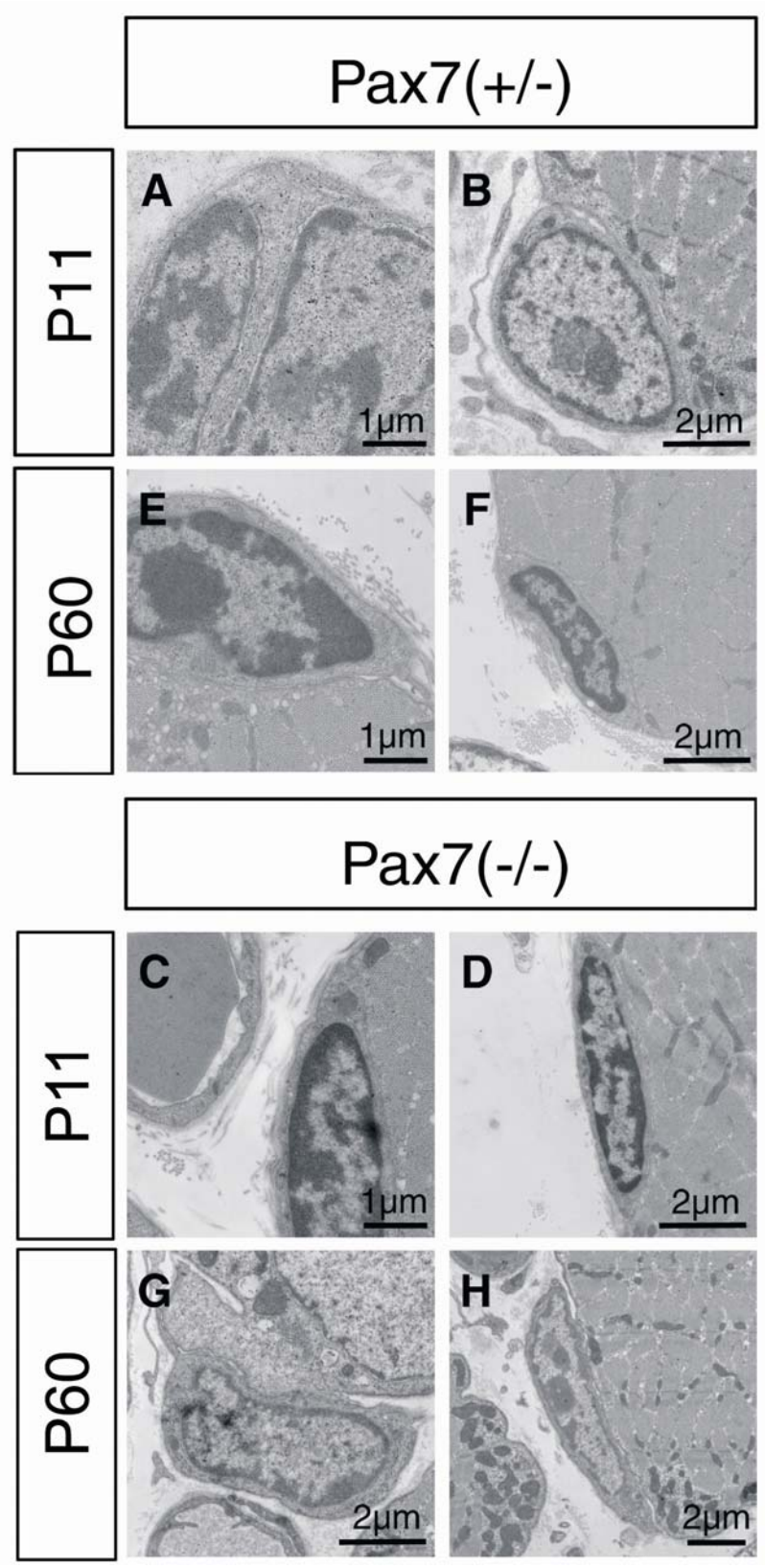

Fig. 17 Electron microscopy microphotographs of satellites cells from interossei muscles of Pax7(+/-) and Pax7(-/-) mice at different postnatal stages. Satellite cells were present in Pax7(+/-) (A, B, E, F) and Pax7(-/-) (C, D, G, H) mice both at P11 (A-D) and P60 (E-H). No morphological abnormalities were noted in satellite cells from Pax7(-/-) mice. The plasma membrane that separates satellite cells from adjacent myofibers, the basal lamina that engulfs satellite cells and myofibers, and the heterochromatic state of the nuclei of satellite cells are clearly visible both in Pax7(+/-) and Pax7(-/-) muscles.

3.3.3. CD34-staining of isolated myotubes

The other method, which was used to evaluate the number of satellite cells in muscles of Pax7 mutants, is the immunohistochemical staining using an antibody to a characteristic marker of satellite cells, CD34. Cultures of isolated myotubes were prepared from Mm. interossei, attached to glass slides in Matrigel, fixed right after preparation and incubated with anti-CD34 antibody with followed by incubation with a secondary biotinilated antibody. All nuclei located on myotubes were counterstained with Hoechst 33258 dye. The percentage of LacZ-positive cells to all nuclei present in myotubes (together with nuclei of LacZ-positive cells) was calculated. Between 1000 and 4000 nuclei per sample were counted.

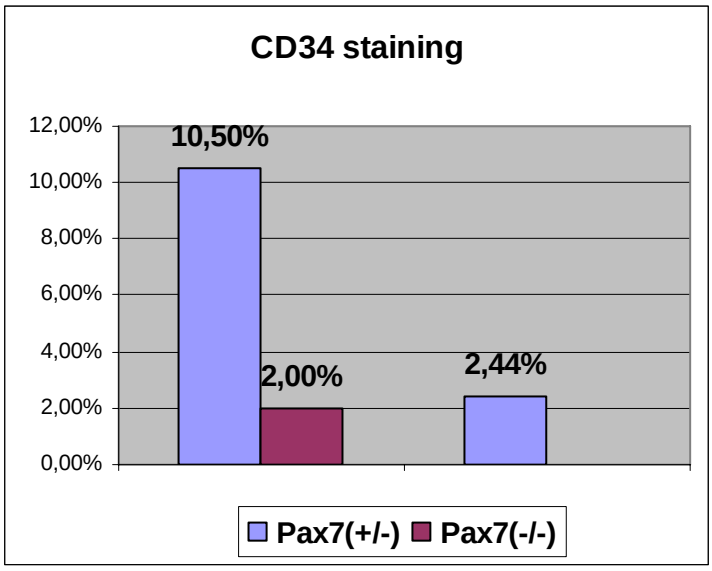

Fig. 18 A reduced but substantial number of satellite cells in the muscle of Pax7(-/-) mice at P11 and absence of CD34-positive cell with normal for satellite cells morphology in the muscle of Pax7(-/-) mice at P60. The number of satellite cells was estimated at P11 (left diagram) and P60 (right diagram) in Pax7(+/-) and Pax7(-/-) mutant mice using antibody staining to CD34 and related to all myotube nuclei (the percentage of satellite cells is calculated to all myonuclei).

Similar to the results obtained by EM counting we observed a high number of CD34-positive satellite cells in Pax7(-/-) mice at P11 (2%) although the amount of satellite cells was clearly lower compared to Pax7(+/-) littermates (10,5%) (Fig. 18 left diagram, Fig. 14 G, H, Fig. 17 A, D). The number of CD34-positive cells in muscles of Pax7(+/-) at P60 (2,44%) also correlated with EM and LacZ counting (Fig. 18 right diagram, Fig. 14 I). Surprisingly, no typical CD34-positive cells with a normal satellite cell morphology were detected in muscles of adult Pax7(-/-) mice at P60. Only a few atypically round weakly CD34-positive cells were detected (Fig. 14 J).

3.3.4. Sca1 and additional LacZ antibody staining on isolated myotubes

Since I found no typical CD34-positive cells in muscles of adult Pax7(-/-) mice at P60, it became interesting to know what kind of cells are stained LacZ-positive in juvenile Pax7(-/-) mice and whether these cells are satellite cells or other types of cell located in muscles (for example mesenchymal stem cells). To answer these questions more careful characterisation of these cells using other immunohistochemical markers was performed.

Myotubes isolated from M. interossei of juvenile mice (P11) were fixed after right preparation and incubated with monoclonal antibodies to CD34. Positive cells were detected using the secondary AlexaFluor488-coupled antibody (green fluorescence). After that the myotubes were incubated with polyclonal anti-LacZ antibody. LacZ-positive cells were detected using the secondary AlexaFluor594-coupled antibody (red fluorescence) (Fig. 19).

A double CD34/LacZ staining was also accomplished by staining for β-galactosidase activity on isolated myotubes using X-gal substrate (blue staining) and incubation of the same myotube samples with an anti-CD34 antibody, followed by incubation with a biotinilated secondary antibody (Fig. 14 E, F).

Double-staining with fluorescently labelled antibodies against Pax7-lacZ and CD34 (Fig. 19 A-F) and CD34-DAB staining of myotubes already stained for LacZ activity (Fig. 14 E, F) revealed that both in Pax7(+/-) and in Pax7(-/-) mice at P11 most Pax7-lacZ-positive cells expressed CD34 and vice versa (Fig. 19). Most of the LacZ-positive cells on myotubes from adult Pax7(+/-) mice were clearly CD34-positive (Fig. 14 E), while all LacZ-positive cells on myotubes from adult Pax7(-/-) mice (P60) were CD34-negative (Fig. 14 F). It should also be pointed out, however, that even in a wild-type situation not all satellite express Pax7 and CD34, simultaneously (Morgan and Partridge, 2003). Double staining for β-galactosidase activity and with the antibody against CD34 has shown that some of the LacZ-positive cells on myotubes of wild type or Pax7(+/-) control samples are only weakly positive or negative for CD34.

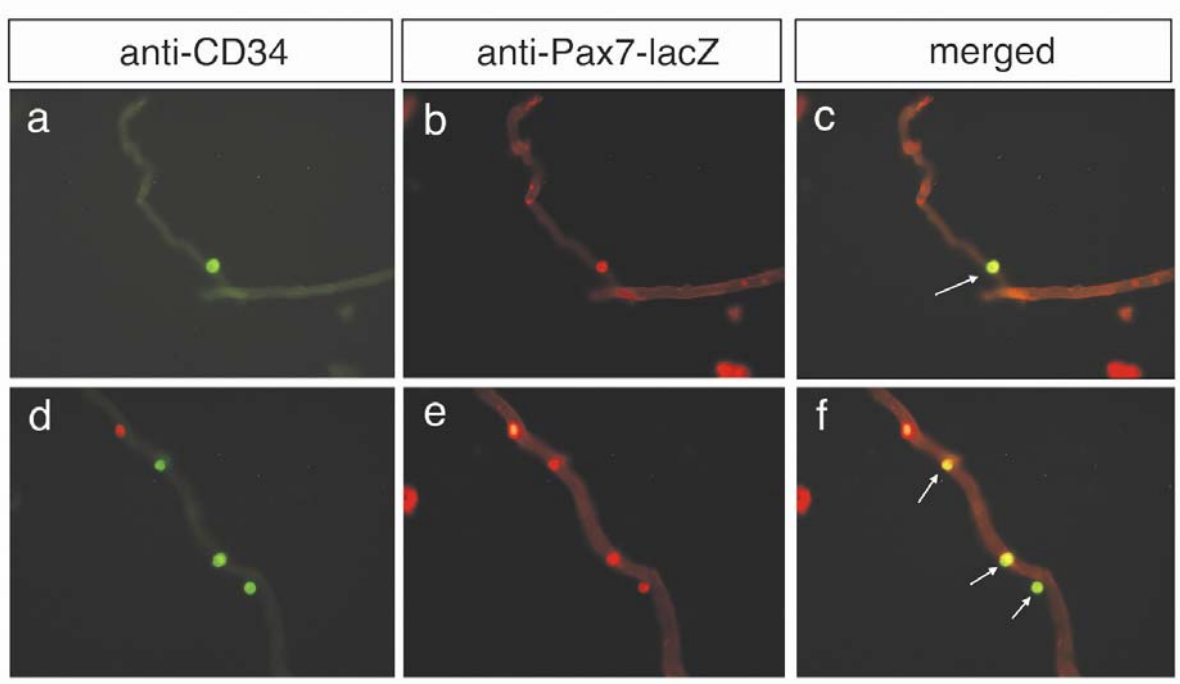

Fig. 19 Co-expression of Pax7-lacZ and CD34 in satellite cells from Pax7(-/-) at P11. Isolated myotubes were reacted with a monoclonal antibody against CD34 (A, D) and a polyclonal antibody against lacZ (B, E) and visualized with secondary antibodies coupled to AlexaFluor488 (green) and AlexaFluor594 (red), respectively. Images from A and B and from D and E were merged to generate C and F, respectively. Satellite cells that co-express both marker molecules are indicated by white arrows in C and F.

Myotubes isolated from muscles of juvenile (P8) and adult (P60) Pax7(-/-) and Pax7(+/-) mice were also incubated with antibodies to Sca1, a marker of multipotent mesenchymal and skeletal muscle-derived stem cells. Sca1-positive cells were detected using the secondary AlexaFluor488 (green fluorescence) labelled antibody. Some Sca1-positive cells were found attached to myotubes of all tested samples, both juvenile and adult, Pax7(-/-) and Pax7(+/-) (Fig. 20 A-D). No significant reduction of Sca1-positive cell numbers was found in samples isolated from Pax7(-/-) either at P8 or at P60 in comparison to Pax7(+/-) samples.

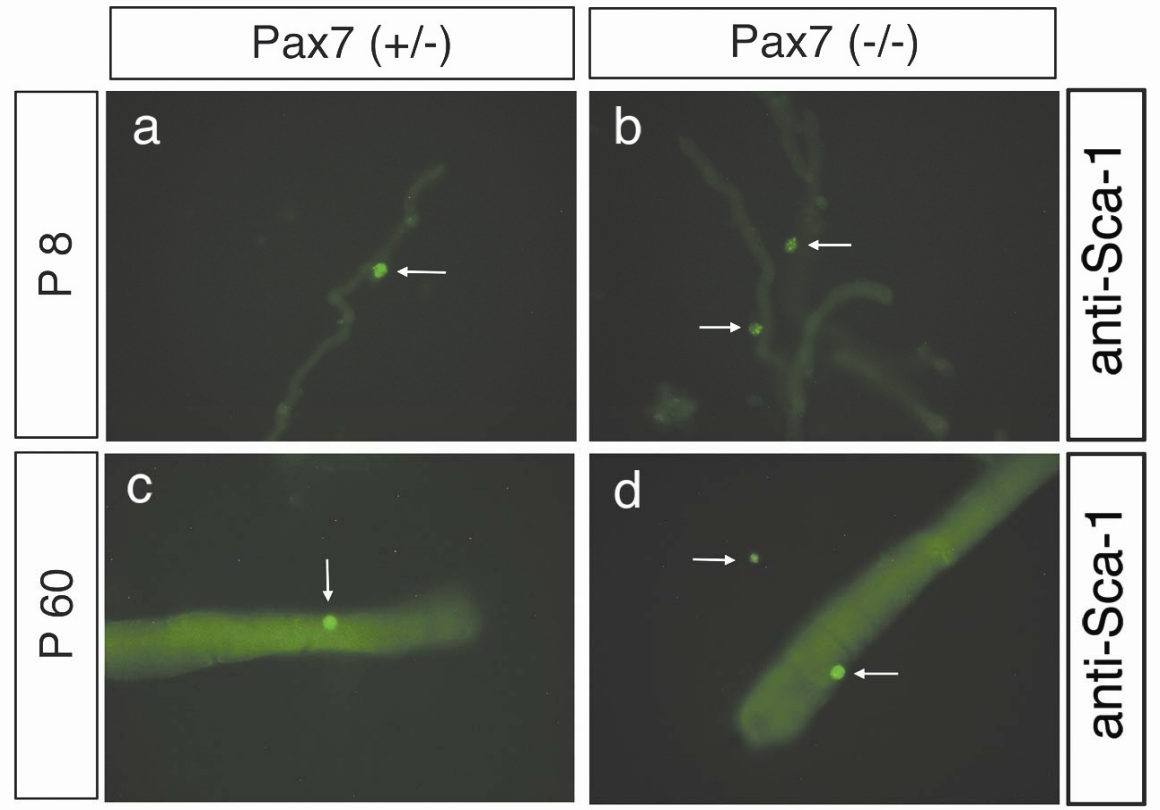

Fig. 20 No reduction of Sca-1 expressing cells, which adhere to isolated myotubes, was evident either in Pax7(+/-) (A, E) or in Pax7(-/-) mice at P8 (A, B) and P60 (C, D).

3.4. Reduced number, impaired maintenance but presumably normal differentiation of satellite cell-derived myoblasts from juvenile and adult Pax7 mutant mice

In vitro systems allow more detailed analysis of specific cellular characteristics of these cells: activation from the quiescent state, migration, proliferation and differentiation. Two primary culture systems are commonly used: monolayer mass cultures of dissociated satellite cells and single muscle fiber cultures with associated satellite cells. Primary monolayer mass cultures of dissociated satellite cells are derived by enzymatic and mechanical liberation of satellite cells from their positions between the muscle fiber plasmalemma and basal lamina. Satellite cells can be released from adult muscle with the aid of enzymes, such as pronase or trypsin, solving the basal lamina. The resulting suspensions of cells are contaminated with fibroblasts and other non-myogenic cells and usually require further purification. Mass cultures allow isolation of large number of satellite cells from any muscle or muscle group. They are convenient systems to perform biochemical and physiological studying of satellite cells.

Single fiber cultures have been used to study the activation of quiescent satellite cells, which requires a culture system that does not result in significant spontaneous activation.

Once activated, the kinetics of entry into the cells cycle and proliferation are similar to that found in monolayer mass culture. The other application of this culture technique is to generate monolayer cultures of relatively pure populations of satellite cells from specific fibers or groups of fibers for the subsequent study of gene expression following activation and differentiation (Rosenblatt et al., 1995).

Clonal analysis allows examination of the replicative capacity of the cells and the maximal number of cell divisions by assessing the number of clones and the final size of each clonal colony. All these techniques, however, do not allow assessing the exact size of the *in vivo* muscle precursor cell population. They are clearly influenced by the ability to extract all cells from the muscle tissue.

3.4.1. Cell cultures derived from muscles of Pax7(-/-) mice contain primary myoblasts

To proof further the presence of satellite cells in muscles of Pax7(-/-) mice at various stages of postnatal development and to evaluate the differentiation capability of Pax7(-/-) satellite cells *in vitro* primary cultures of satellite cells from isolated myofibers of P8 and P60 Pax7(+/-) and Pax7(-/-) mice were prepared.

Isolated myofibers were isolated from Mm. interossei, attached to glass slides in Matrigel, covered with proliferation medium and cultivated for up to 1 week in proliferation medium. The same number of myotubes were plated for Pax7(-/-) samples and Pax7(+/-) control. Cultures were observed daily. Satellite cells normally begin to emigrate from myofiber within 24 hours and increase progressively in number during the next days. Cells were fixed 4 and 7 days after plating. To evaluate the differentiation capability of myoblasts derived from the myofibers, differentiation medium was added after 1 week of incubation and cells were incubated for the additional 2-3 days. Fixed cells were then stained with antibodies against desmin (a characteristic marker for all myogenic cells), MyoD (the myogenic bHLH gene, which is characteristic for activated satellite cells and proliferating myoblasts) and myosin heavy chain (MyHC, a contractile protein expressed by differentiated myotubes). For the detection of positive cells AlexaFluor488- (green fluorescence, for anti-desmin and anti-MyoD staining) and AlexaFluor954- (red fluorescence for anti MyHC staining) labelled secondary antibodies were used Fig. 21, Fig. 22). MyHC-positive cells were also detected using a secondary biotinilated antibody, ABC amplification and DAB (Fig. 22 G, H).

I readily identified satellite cells in cultures of adult (P60) and juvenile (P8) Pax7(+/-) and Pax7(-/-) mice based on anti-desmin staining (Fig. 22 C-F). In addition these cells expressed the myogenic bHLH gene MyoD (Fig. 22 A, B, Fig. 21 E, F, G, H). The absolute

number of satellite cell-derived MyoD positive myoblasts was significantly lower when myotubes from Pax7(-/-) mice were used (Fig. 21 G, H compare to E, G) although at P8 in some areas of the plates similar densities of satellite cell derived MyoD myoblasts were found (Fig. 21 E, F). At P60 the number of satellite cell derived MyoD positive myoblasts further declined paralleling the decrease of satellite cells observed *in vivo*.

The reduced number of satellite cell-derived myoblasts from Pax7(-/-) mice closely matched the reduced formation of MyHC expressing myotubes *in vitro* at P8 and P60, which might indicate that the ability of Pax7(-/-) myoblasts to differentiate was not reduced. Fig. 21 A-C and Fig. 22 G, H show representative examples of differentiated myotubes from Pax7(-/-) and Pax7(+/-) myoblasts. A clear reduction of the number of MyHC-positive myotube numbers was noted in cultures isolated from muscles of juvenile and adult Pax7(-/-). In addition, Pax7(-/-) cultures contained very small two-three nuclei containing differentiated myotubes (Fig. 21 B, D), whereas Pax7(+/-) cultures contained numerous thick multinucleated myotubes (Fig. 21 B, D).

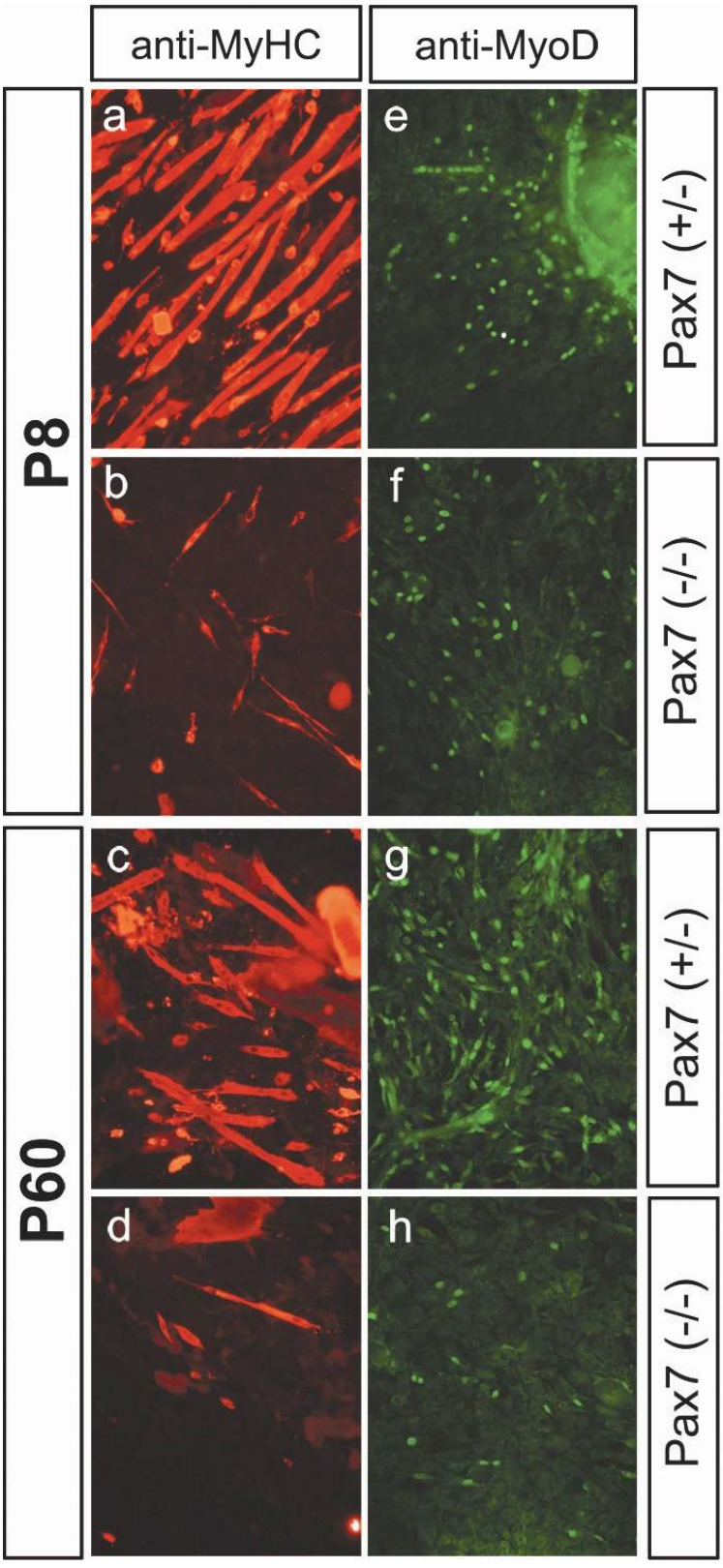

Fig. 21 Expression of MyoD and differentiation of satellite cell derived myoblasts from Pax7(-/-) and Pax7(-/-) mice at different postnatal stages. Primary satellite cell cultures were obtained from isolated myotubes of Pax7(+/-) (A, C, E, G) and Pax7(-/-) (B, D, F, H) mutant mice at P8 (A, B, E, F) and P60 (C, D, G, H) and cultured for 6 days. Cells were reacted with antibodies against MyHC (A-D) and MyoD (E-H). The number of satellite cell derived MyoD positive myoblasts was significantly lower in Pax7(-/-) cultures (F, H) when compared to Pax7(+/-) controls (e, g) giving rise to fewer MyHC positive myotubes (B, D).

3.4.2. Clonal analysis of the proliferation and differentiation capabilities of satellite cell derived myoblasts

To further access the specification and the proliferation potential of satellite cells lacking Pax7 mass cultures of satellite cells were prepared from pooled hind limb muscles of Pax7(-/-) and Pax7(+/-) control animals at P11. Equal numbers of satellite cells were plated at low clonal densities and cultivated in proliferation medium for 1 week. Proliferation medium was changed to differentiation medium to evaluate the differentiation potential of the myogenic cells. Cells were fixed 4, 7 days after plating and after incubation with differentiation medium. Fixed cells were then analyzed for the expression of several markers over a time course of proliferation and differentiation. Antibodies against desmin and MyoD were used to identify proliferating myoblasts and the numbers of positive cells per colony and were counted. Secondary AlexaFluor488-labelled antibodies (green fluorescence) were used for MyoD- and desmin-positive cell detection. Positive cells of about 30-50 colonies per sample were counted. The average numbers of MyoD- and desmin-positive cells per colony were calculated.

Differentiated myotubes were visualized using anti-MyHC antibody with the following detection using the secondary biotinilated secondary antibody, ABC solution and final DAB staining (dark brown staining). The numbers of MyHC-positive colonies per dish (with equal plating density) and the average numbers of myotubes per colony were counted and the average numbers calculated.

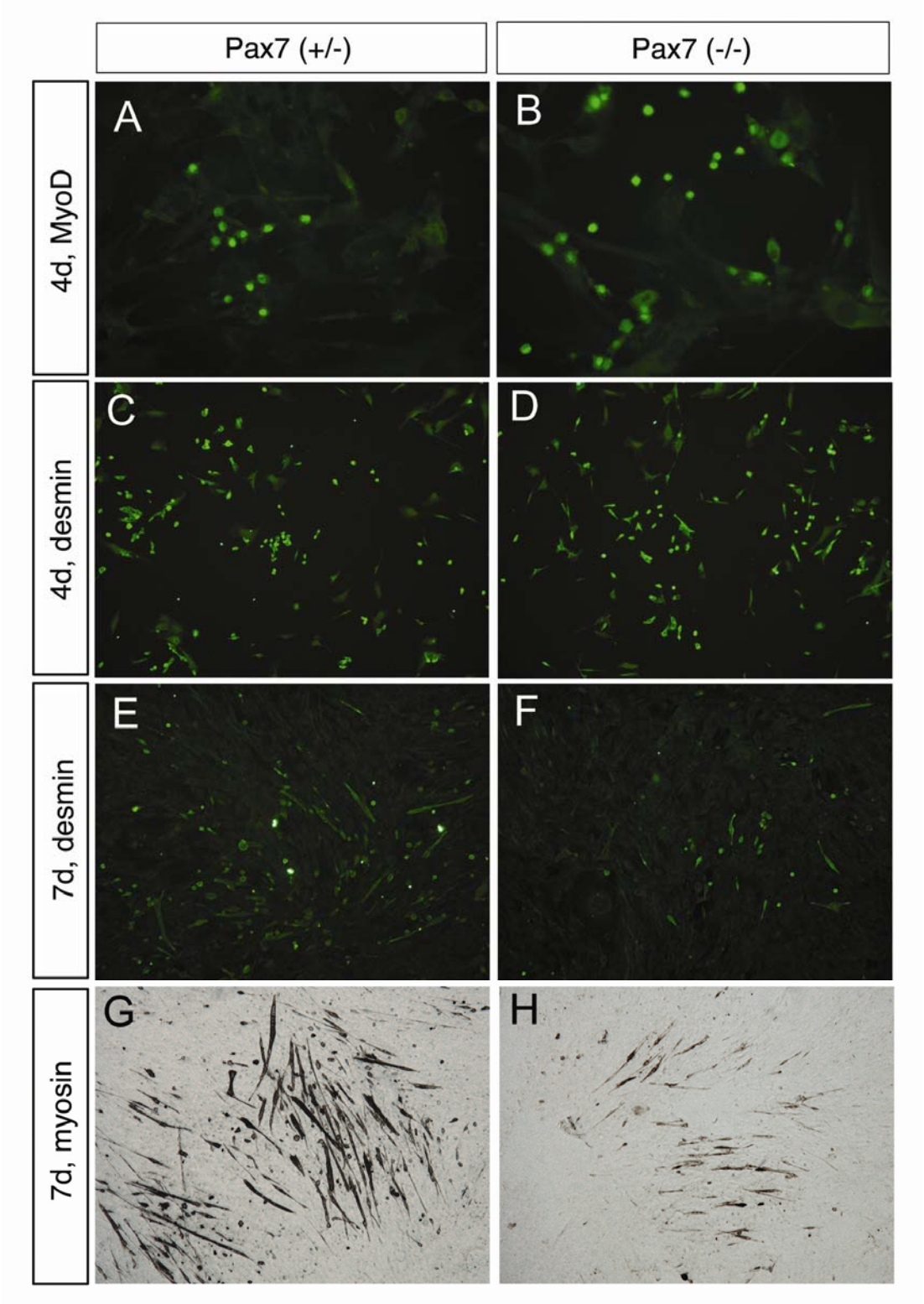

Fig. 22 Clonal analysis of satellite cell derived myoblasts from pooled hind limb muscles of P8 Pax7(+/-) and Pax7(-/-) mice. After 4 days of culture the average number of MyoD (upper panel) and desmin (middle panel) positive cells per colony was not dramatically decreased in cultures derived from Pax7(-/-) mice. After 7 days a considerable decline of the average number of MyoD and desmin positive cells per colony was apparent in Pax7(-/-) cultures. Similarly, the average number of differentiated MF20 positive myocytes (lower panel) at day 7 derived from Pax7(-/-) mice was considerably lower per single colony and per dish compared to Pax7(+/-) cultures.

The average number of MyoD and desmin positive cells derived from Pax7(-/-) mice was not dramatically decreased after 4 days in culture when compared to heterozygous mice. Colonies of myogenic cells from muscles of Pax7(-/-) mice contained 14.1 MyoD- and 11.2 desmin-positive cells, colonies of heterozygous mice contained 19.2 MyoD- and 11.9 desmin-positive cells. However, continued cultivation revealed that the number of MyoD and desmin positive cells per colony was significantly reduced in Pax7(-/-) cultures after 7 days (42.7 and 32.4 in comparison to 101.2 and 66.3 in heterozygous control) (Fig. 23).

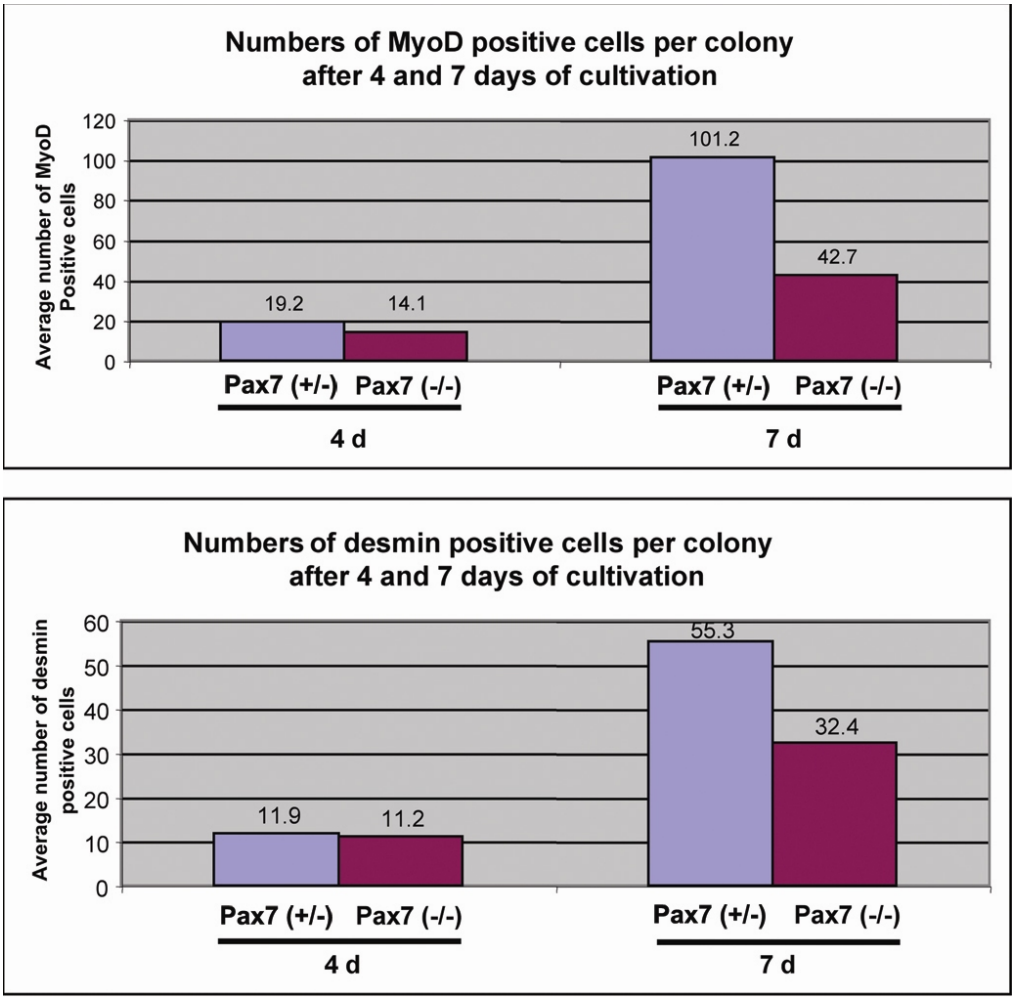

Fig. 23 Impaired maintenance of satellite cell-derived myoblasts from juvenile Pax7 mutant mice. The number of myogenic cells per colony was estimated at P11 using anti-MyoD antibody (upper panel) and anti-desmin antibody (lower panel). Short cultivation (4 days) of monolayer mass cultures revealed no significant difference in colony size of myoblasts from Pax7(-/-) and Pax7(+/-) muscles (left diagrams). Continued cultivation (7 days) revealed that the number of MyoD and desmin positive cells per colony was significantly reduced in Pax7(-/-) cultures.

Likewise, the number of differentiated myocytes at day 7, as indicated by MyHC staining, was considerably lower in Pax7(-/-) colonies. Pax7(-/-) cultures contained 27.6 MyHC-positive cells per colony and 77.1 colonies per plate, Pax7(+/-) cultures contained 36.7

and 120.2 respectively (Fig. 24). Furthermore, differentiated myotubes in cultures prepared from muscles of Pax7(-/-) were smaller (containing one-two nuclei in comparison to multinucleated control myotubes) and the MyHC staining intensity was lower (Fig. 22 G, H).

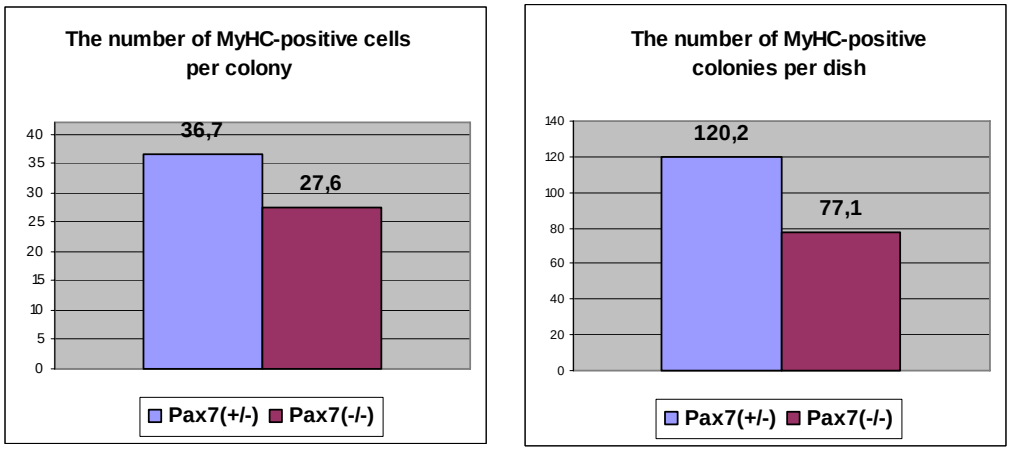

Fig. 24 Reduction of MyHC-positive cells number in monolayer mass cultures from muscles of juvenile Pax7(-/-) mice. The number of differentiated myocytes per dish at day 7 was considerably lower in Pax7(-/-) colonies (right diagram). Pax7(-/-) cultures contain less MyHC-positive cells per colony (left diagram).

Taken together our results strongly suggest that Pax7 is not required for initial proliferation of satellite cells but for their maintenance and the extended proliferation potential of satellite cells.

3.5. Expression profile of muscle-specific genes in muscles of Pax7(-/-) mice

Cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression. Quantification of transcription levels of specific genes is always central to any research into gene function. Among the different methods which are now available for the quantification of transcription northern blot analysis and quantitative real time PCR were found the most suitable for the aims of our study.

3.5.1. Northern blot analysis of muscle specific gene expression in muscles of Pax7(-/-) mice

Northern blot analysis is the only method providing information about mRNA size, amount, alternative splicing and the integrity of RNA samples, although the sensitiviy of this technique is limited. For a further characterization of the musculature of adult Pax7(-/-) mice various muscle specific genes were studied by northern blot analysis. Total RNA was prepared from pooled hind limb muscles of adult Pax7(-/-) and Pax7(+/-) mice at P60. Equal amounts of mutant and control RNA samples were loaded into each line of the gel and final

signals were normalized according to the expression of GAPDH reference mRNA. RNA blots were hybridized with probes containing cDNA of various muscle specific contractile proteins: embryonic myosin heavy chain (MyHC), fast adult MyHC, peri-natal MyHC, MyHCβ, myosin light chain 1 (MLC1), troponin fast and troponin slow. As it is shown at the Fig. 25 A the expression of most muscle specific genes that we studied was slightly and unspecifically down-regulated.

DNA probes containing cDNAs of various myogenic regulatory factors were also used for the northern blot analysis but since the expression levels of these genes in comparatively low in skeletal muscles of adult mice, no reliable signals both for control and mutant samples were obtained.

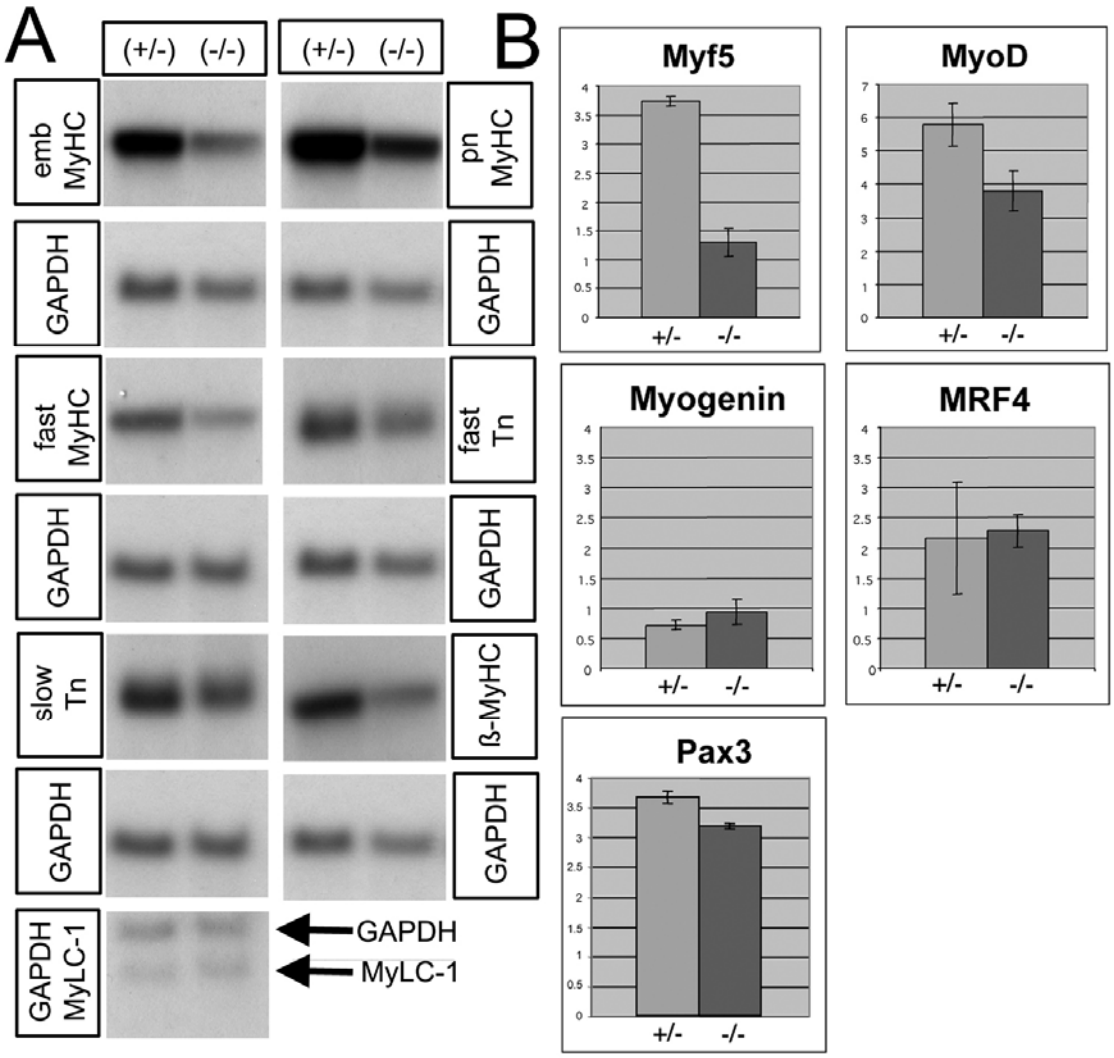

Fig. 25 Reduced expression of Myf5 and MyoD but normal expression of various skeletal muscle markers and of Pax3 in Pax7(-/-) mice. (A) Northern Blot analysis of various muscle specific mRNAs isolated from adult (P60) Pax7(+/-) and Pax7(-/-) mice. (B) Quantitative real time RT-PCR of Myf5, MyoD, Myogenin, MRF4 and Pax3 mRNAs from muscles of adult (P60) Pax7(+/-) and Pax7(-/-) mice . Values on the Y-axis represent the ratio

between the SQ of the mRNA of interest to the SQ of GAPDH. Due to different amplification efficiencies it is not possible to compare between different mRNAs. Note the significant reduction of the expression of Myf5 and MyoD but not Pax3 in mutant animals.

3.5.2. Quantitative real time PCR analysis of the myogenic transcription factors expression in muscles of Pax7(-/-) mice

RT-PCR is the most sensitive and flexible method for the detection of low-abundant mRNA. Currently, the limit of detection when fluorescent dyes such as SYBR Green are used is about 10-100 copies of template DNA in the starting specimen.

To estimate differences in expression levels of various myogenic transcription factors in muscle tissues of mutant mice in comparison to wild type mice real time RT-PCR was used. The same total RNA samples that were used for the Northern blot analysis (pooled hind limb muscles of adult Pax7(-/-) and Pax7(+/-) mice at P60) but the samples were treated with RNase-free DNase before cDNA synthesis. For the PCR reactions primers complementary to cDNA sequences of Myf5, Myf6 (MRF4), Myogenin, MyoD and Pax3 were used. Standard curves for quantification were prepared using plasmid constructs containing cDNA sequences of Myf5, Myf6(MRF4), Myogenin, MyoD and pax3.

For each experiment the amounts of targets and endogenous constitutively expressed housekeeping reference gene glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) was determined from the standard curve. GAPDH is a ubiquitously expressed, moderately abundant message. It is frequently used as an endogenous control for quantitative RT-PCR. The target values (starting quantities) were normalized to the endogenous reference: SQ_{target}/SQ_{GAPDH}.

A clear decrease of the expression of myogenic determination genes Myf5 and MyoD by quantitative RT-PCR was found while the expression of the differentiation genes Myogenin and MRF4 (Myf6) increased to some extent (Fig. 25 B).

Since it has been shown that the function of Pax7 partially overlaps with the paralogous Pax3 gene during development we also analyzed the expression of Pax3 gene using quantitative RT-PCR. Surprisingly we did not find a compensatory increase of Pax3 expression but a minor reduction compared to Pax7(+/-) animals (Fig. 25 B), which argues against a decisive compensatory function of Pax3 in Pax7(-/-) mutant mice. The low quality of available Pax3 antibodies prevented a specific detection of Pax3 in the remaining satellite cells by immunofluorescence (data not shown).

3.6. Muscle regeneration in adult Pax7 mutant mice

Initiation of skeletal muscle injury is a general approach for the study of satellite cell activation, proliferation, regeneration, and self-renewal in vivo. The methods include freeze-crush (Kurek et al., 1997; Creuzet et al., 1998) or chemically induced injury (d'Albis et al., 1988). The most extensive and reproducible muscle injury is the delivery of cardiotoxin (purified from the venom of the Naja nigricollis or N. mossambica snake) into the hind limb skeletal muscle of the mouse. An intramuscular injection of 100 µl of 10 µM cardiotoxin into the M. gastrocnemius results in 80-90% muscle degeneration. After cardiotoxin-induced injury, satellite cells become activated within 6 hours of injury. In response to locally released growth factors from injured myotubes and macrophages satellite cells start to proliferate extensively within 2-3 days of injury Approximately 5 days after injury satellite cells withdraw from the cell cycle and either self-renew or form differentiated myotubes that contain a central nucleus. At this time, newly regenerated myofibers are evident as small, basophilic myofibers with centrally located nuclei. The structure of the injured muscle is largely restored within 10-14 days after injury. The newly regenerated myofiber displays numerous centrally aligned nuclei, demonstrating the fusion of many satellite cells to form a single myofiber. The return to a morphologically and histochemically normal mature muscle is seen at 3–4 weeks after injection (Hawke and Garry, 2001).

To determine the regenerative potential of Pax7-deficient skeletal muscle in response to acute damage, I induced regeneration by freeze-crush injury or by injection of cardiotoxin into the M. tibialis anterior of Pax7(-/-) and Pax7(+/-) mice. Morphological examinations of haematoxylin-eosin stained paraffin sections were performed at 1 day, 4 days, 10 days and 28 days after injection. Necrosis and degeneration of the muscle fibers were observed in both mutant and control mice 4 day after injection (data not present). Myoblasts and cellular infiltration, such as leucocytes and macrophages, were found 4 days after injection (Fig. 26 A, B). Control muscles contained a more or less homogeneous population of newly formed myofibers with centrally located nuclei indicating successful regeneration 10 days after injury. Histological analysis of Pax7(-/-) muscles 10 days after injury in comparison to heterozygous control revealed myofibers of smaller diameter, a high number of mononuclear cells in the muscles, differences in calibre size, and numerous necrotic fibers (Fig. 26 C, D). However, also numerous regenerating myofibers were found in Pax7(-/-) mice indicating that the low number of remaining satellite cells in mice lacking Pax7 was sufficient to mediate an albeit comprised regenerative response. Four weeks after injury the lesion was no longer

detectable in heterozygous mice (Fig. 26 E). In Pax7(-/-) mutants the histological appearance was heterogeneous. Some regions showed a comparatively good regeneration (Fig. 26 F) while in other areas disarranged myotubes and hyaline deposits characteristic of necrotic material were evident (Fig. 26 G).

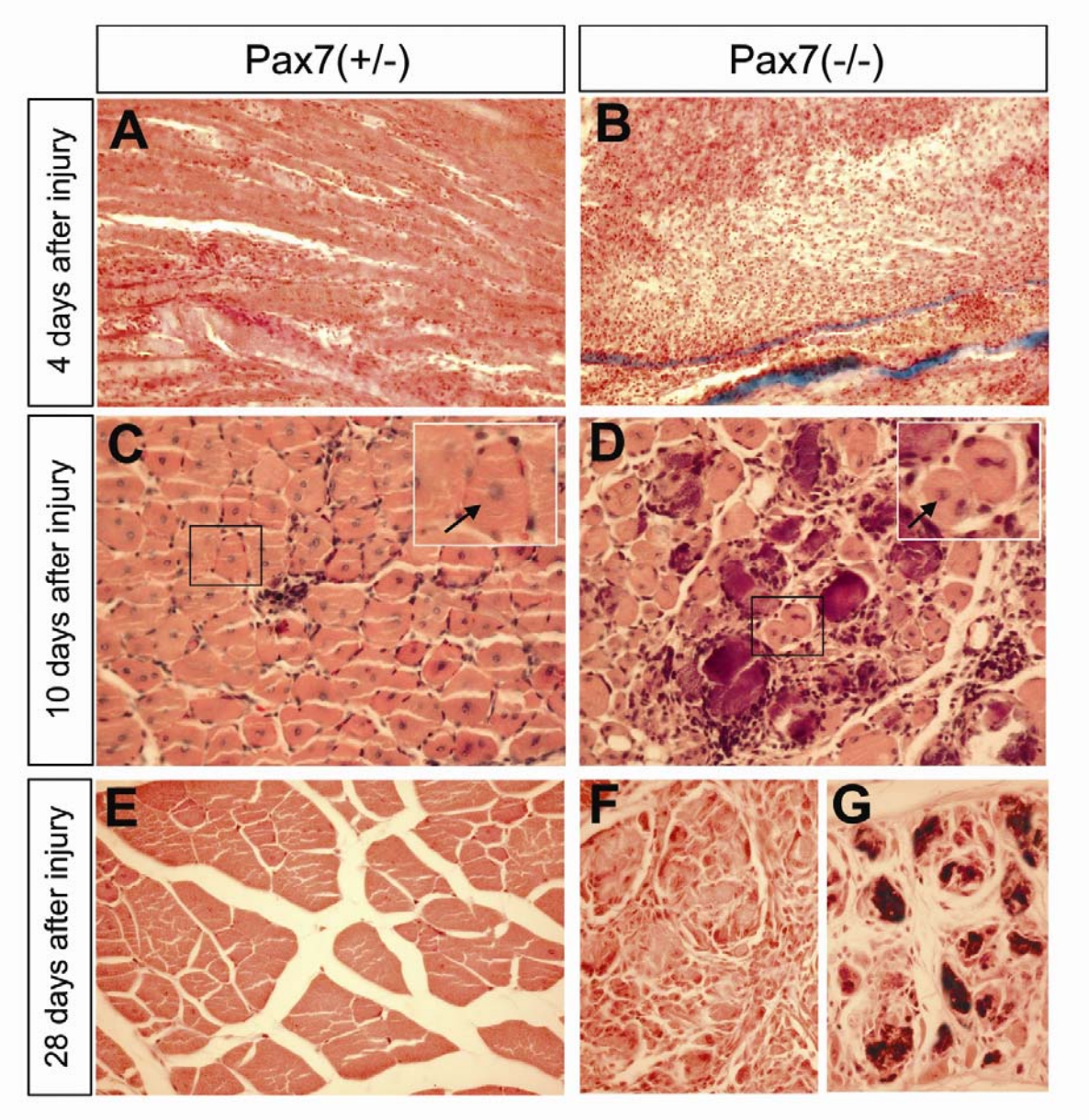

Fig. 26 Impaired skeletal muscle regeneration of adult Pax7(-/-) mice. Paraffin sections from Pax7(-/-) (A, C, E) and Pax7(+/-) (B, D, F, G) were stained with haematoxylin and eosin (HE) 4 days (A, B), 10 days (C, D) and 28 days (E-G) following cardiotoxin injection into the M. tibialis anterior. Note the efficient muscle regeneration in Pax7(+/-) mice as indicated by the presence of centrally located nuclei in virtually all myotubes (C). By contrast Pax7(-/-) lesions contain myotubes of different calibre size and numerous necrotic fibers (D). 1 month after injury the tissue architecture of damaged muscles is virtually restored in Pax7(+/-) mice while Pax7(-/-) mutant muscles still contain numerous necrotic fibers (G) and show a disarranged tissue morphology (F).

4. DISCUSSION

4.1. The problem of satellite cell identification and quantification: heterogeneity of satellite cell population and the presence of other myogenic precursor cells in skeletal muscles.

Satellite cells are present in all vertebrates examined, although the frequency and activity both *in vivo* and *in vitro* varies widely depending upon location, age of an animal, specific muscle, disease or injury, endocrine status, species or other physiological characteristics. A number of studies were performed both *in vitro* and *in vivo* to identify satellite cells and to determine the absolute numbers of muscle precursor cells in muscles and to investigate their physiological activity. *In vivo* studies of the skeletal muscle satellite cells use various criteria of satellite cell identification and are generally dependent on light and electron microscopy. Electron microscopy is time consuming and requires special devices but this is the only method that can detect satellite cells unambiguously according to their classical morphological definition. Light microscopic i*n vivo* analysis of the muscle section allows to localize the cells in muscle tissue based on the analysis of the expression of various immunohistochemical markers and to estimate approximately the number of cells per section area but might be not the best choice for the determination of absolute numbers of satellite cell because the intact muscle contains a lot of other non-myogenic cells. It is also not easy to normalize the counted numbers of satellite cells correctly if they are counted on sections. That is why in addition to classical electron microscopic identification of satellite cells alternative methods were used in this study: isolated myotube cultures in combination with Pax7-lacZ staining and immunohistochemical staining with anti-CD34 antibody to evaluate the number of satellite cells in muscles of mutant animals and to compare it to control samples.

Satellite cells can be placed in culture in association with the myofiber. Such preparations more closely mimic conditions *in vivo*. To obtain single muscle fiber cultures, partial proteolytic digestion and further mechanical disruption of muscle are performed liberating intact muscle fibers with their associated basal lamina and satellite cells but free of blood vessels, nerves, and connective tissue (Rosenblatt et al., 1995). The advantage of this system is the authenticity of the satellite cell environment and the ability to maintain satellite cells in a quiescent state in association with a fiber.

CD34 is a relatively novel but often used and reliable marker of quiescent satellite cells. It is an established marker of haematopoietic stem cells and early blood-cell progenitors and has become the standard criterion for the isolation of HSCs from blood and bone marrow. Immunostainings of freshly isolated fibers revealed co-expression of CD34, Myf5 and M-

cadherin in mononucleated cells attached to the sarcolemma of the muscle fibers (Beauchamp et al., 2000). Seale et al. have shown that Pax7 is one of the molecules which expression selectively marks quiescent satellite cells (Seale et al., 2000).

According to my first results I have found a lot of Pax7-lacZ-positive cells on myotubes of juvenile Pax7(-/-) mice and several Pax7-lacZ-positive cells on myotubes of adult Pax7(-/-) mice. Next was to prove that Pax7-lacZ cells that we have found are really satellite cells according to the presence of other satellite cell markers and morphological criteria.

Using standard electron microscopic criteria satellite cells in muscles from both juvenile and adult Pax7(-/-) mice were clearly identified. These cells seem to have a normal for satellite cells morphology and a normal location. The satellite cells from adult Pax7(-/-) mice had more heterochromatic in comparison to control samples nuclei, which means that these cells are more transcriptionally active then normal quiescent satellite cells. This activation of most satellite cells which are present in the muscles can be caused by the dramatic shortage in satellite cell number in adult Pax7(-/-) mice. Such a general activation can make the muscles of adult Pax7(-/-) mice still to be able to respond to various physiological demands like training or injury.

CD34 marker has been found in a high number of cells located on the myotubes of juvenile Pax7(-/-) mice. Double immunostaining using anti-CD34 and anti-β-galactosidase antibodies has shown that Pax7-lacZ and CD34 are co-expressed in the majority of attached to myotubes Pax7(-/-) and Pax7(+/-) mice at P8-11. That means that most of the cells found in the positions characteristic of satellite cells on myotubes co-express two markers commonly used for satellite cell identification.

A more complicated situation was found in muscles of adult animals. Only weakly positive CD34 cells with abnormal morphology have been found attached to myotubes of adult (P60) Pax7(-/-) animals. In addition, these cells were very rare. We are not sure whether this abnormal morphology is a result of the more active state of these cells as unveiled by electron microscopy. It is also possible that the satellite cell-like location of these cells is a result of imperfect method of myotube isolation. These cells sitting on myotubes might be non-muscle cell (for example blood cells or mesenchymal stem cells) which have not been separated from myotubes during preparation.

All the Pax7-lacZ-positive cells found on myotubes of adult Pax7(-/-) mice were CD34 negative. However, it has also been found that not all Pax7-lacZ-positive cells on myotubes of control adult Pax7(+/-) mice were CD34-positive. The majority was strongly

positive for both Pax7-lacZ and CD34 but some Pax7-lacZ cell were only weakly CD34-positive, some – clearly CD34-negative. These results are in agreement with the previous observations showing that satellite cells population consists of different not very well characterised subpopulations. Pax7-positive/CD34-negative cells found in muscles of Pax7(-/-) mice may represent one of such uncharacterised subpopulations.

Currently, we do not know whether satellite cells represent a separate myogenic lineage either of somitic or non-somitic origin that is established early during embryogenesis and laid aside for future usage or whether satellite cells originate from embryonic or foetal myoblasts, which encounter a temporary differentiation block and are therefore prevented to be used up for myotube formation during development. Despite their disputed origin satellite cells seem to rely on the same network of transcription factors for determination and differentiation than myogenic precursor cells during embryonic development. Like their embryonic cousins satellite cells express MyoD and Myf5 and combined disruption of these genes leads to the ablation of satellite cells similar to the annihilation of other myogenic cells (Braun et al., 1992; Rudnicki et al., 1993). Although satellite cells can be characterized by expression of characteristic marker genes such as M-cadherin, CD34, Msx1, cMet, MNF or Foxk1 (Hawke and Garry, 2001) and Pax7 (Beauchamp et al., 2000; Cornelison et al., 2000; Cornelison and Wold, 1997; Seale et al., 2000) and by their typical morphological appearance it has been proposed that they do not represent a unique cell type but a rather heterogeneous population of muscle precursor cells.

However, various investigations have shown satellite cell population is phenotypically and functionally heterogeneous (see “Introduction”). CD34/Myf5/M-cadherin positive cell population represent only a part (about 80%) of satellite cells associated with individual isolated myofibers. The other, minor part is negative for these markers. According to one hypothesis satellite cells that do not express any myogenic markers might correspond to the slowly activated population, which is responsible for satellite cell self-renewal and may replenish the cells that undergo rapid differentiation (Beauchamp et al., 2000).

It remains unclear whether all satellite cells are potential stem cells or whether the defining properties are restricted to a particular subpopulation. It now seems likely that not all satellite cells are equivalent and that the regenerative compartment of skeletal muscle contains phenotypically and functionally different subsets of precursor.

Adult stem cells isolated from various tissues appear to differentiate *in vitro* and *in vivo* into multiple lineages depending on environmental cues. The fact that satellite cells alone facilitate postnatal growth and regeneration of adult muscle has recently been challenged by

the finding that the other kinds of stem cells, isolated from bone marrow stroma (Ferrari et al., 1998; Bittner et al., 1999; Gussoni et al., 1999), adult skeletal muscle (Asakura et al., 2002; Gussoni et al., 1999; Qu-Petersen et al., 2002; Torrente et al., 2001) the neuronal compartment (Clarke et al., 2000; Galli et al., 2000) and various mesenchymal tissues (Young et al., 2001a; Young et al., 2001b), exhibit considerable myogenic potential and participate in muscle regeneration after damage.

Bone marrow derived side population (SP) cells are mostly positive for Sca1 (stem cell antigen-1), c-Kit, CD43 and CD45 and negative for such marker as CD34, B220, Mac1, Gr1, CD4, CD5, CD8 (Gussoni et al., 1999). Muscle-derived SP represent a separate cellular population than satellite cell population. SP cells do not express the satellite cell markers Myf5-nlacZ, Pax7 or desmin (Asakura et al., 2002). Various cell surface markers have been employed to purify adult stem cell populations from skeletal muscle, including c-kit, Sca1, CD34, and CD45. Most of the muscle derived SP cells (92%) express Sca1, only 16% express CD45 consisting of both Sca1+ (9.2%) and Sca1- (6.8%) (Asakura et al., 2002; Gussoni et al., 1999). Almost all muscle-derived haematopoietic progenitor and blood reconstitution activity is derived from CD45+ cells (Asakura et al., 2002). Muscle-derived CD45+ cells purified from uninjured muscle are uniformly non-myogenic *in vitro* and do not form muscle *in vivo*. However, co-culture experiments indicate that CD45+ SP, as well as CD45- SP, cells possess myogenic potential A fraction of both CD45+ (9%) and CD45- (5%) SP cells undergo myogenic conversion when co-cultured with myoblasts *in vitro*.

Independent of an isolation via Hoechst dye efflux or differential adhesion to collagen-coated plastic, the only consensus marker of multipotent skeletal muscle-derived stem cells remains the expression of Sca-1. Sca-1 is widely known as a marker of HSCs in the mouse, but is also expressed on peripheral lymphocytes, parenchymal cells of the thymus, spleen and kidney, and on vascular cells (Spangrude et al., 1988; van de et al., 1989; van de Rijn M. et al., 1989). According to immunostaining using sections of normal adult mouse skeletal muscle Sca-1 is expressed in the vascular network (Zammit and Beauchamp, 2001). In larger vessels, Sca-1 is expressed in the outer layers and no expression has been found in the endothelium. However, immunostaining of capillaries associated with isolated muscle fibres clearly shows that Sca-1 is expressed by the capillary endothelium. Sca-1 expression has never been observed in satellite cells, defined by expression of Pax7 or Myf5 (Zammit and Beauchamp, 2001; Asakura et al., 2002).

To prove that the Pax7-lacZ-positive cells which have been found in muscles of Pax7(-/-) mice are satellite cells but not other kind of muscle derived stem cells we also

checked Pax7-lacZ-positive cells for the expression of Sca1. Isolated myotube cultures have been used for this purpose to minimize participation of non-muscle tissue cells in the analysis. Although Sca1-positive cells were found attached to myotubes of adult and juvenile Pax7(-/-) and Pax7(+/-) mice, such cells are most likely not satellite cells for the following reasons: (i) no significant reduction of Sca1-positive cell numbers was observed in samples isolated from Pax7(-/-) either at P8 or at P60 in comparison to Pax7(+/-) samples; (ii) Sca1 staining has never been observed in Pax7-lacZ-positive cells. We do not know what kind of Sca1-positive cells has been found attached to myotubes. To our knowledge there is no data in the literature about such Sca1-positive cells attached to myotubes and so far there is no clear data about the location of SP cells in muscle tissue. It is possible that these cells represent a contamination of non-muscle cells that co-purify with myotubes.

The vascular localization of Sca-1 in adult skeletal muscle raises the possibility that the vasculature may be the source of multipotent muscle stem cells rather than the satellite cell compartment. It has been suggested that primordial perithelial elements could give rise to multipotent cells in addition to the smooth muscle and connective tissue layers of developing vessels (Bianco and Cossu, 1999). Thus, multipotent stem cells isolated from whole skeletal muscle may also be derived ultimately from the endothelium. It is important to note that myogenic cells, which co-express both muscle and endothelial markers can be derived from embryonic vessels such as the dorsal aorta and furthermore, that satellite cells, but not foetal myoblasts also express a number of endothelium-associated markers prior to differentiation (De Angelis et al., 1999). This suggests that some precursor cells responsible for muscle development do not share a common origin.

The data available so far suggest that muscle-derived progenitors, other than muscle satellite cells, can be capable of myogenic differentiation and of integration into the regenerating musculature *in vivo*. It is not known yet whether muscle derived SP cells represent satellite cell progenitors or myogenic progenitors capable of direct myogenic fusion. Stem cell populations are derived by enzymatic disaggregation of whole muscle tissue. Although predominantly consisting of myofibers and satellite cells, skeletal muscle tissue also contains a wide range of other cell types including connective tissue, fat, nerves and supporting cells, an extensive vascular system, and blood. It is therefore possible that multipotent cells present within the muscle tissue are not a part of myogenic cells. The SP phenotype results from the efficient efflux of Hoechst dye and therefore provides a mixed cellular population with little information on cellular characteristics or origin. The concept of the multipotent muscle stem cell may therefore be premature and possibly misleading. Further

clonal and phenotypical characterization is required to investigate properties of different subpopulations within muscle derived side population.

4.2. Impaired muscle regeneration in adult Pax7 mutant mice.

The ability of skeletal muscles of adult mammals for regeneration is largely attributed to a satellite cell population. Upon injury satellite cells become activated and turn into proliferating myoblasts that eventually fuse to pre-existing myotubes or to each other to form new myotubes (Bischoff, 1994). Myotubes in mammals, in contrast, are post mitotic and cannot re-enter the cell cycle. Although there is a wide agreement that satellite cells represent the main source of muscle stem cells and are required for efficient muscle regeneration it has been questioned whether they represent the sole source of muscle precursor cells. Recently, other cell populations collectively called adult stem cells have been proposed to contribute to muscle regeneration (Ferrari et al., 1998; Gussoni et al., 1999; Jackson et al., 1999; Polesskaya et al., 2003). Some studies have demonstrated the ability of non-muscle resident cells to follow the myogenic lineage For example, bone marrow-derived myogenic cells are able to participate in skeletal muscle regeneration, although at low frequency, when injected intravenously, suggesting that some myogenic cells with similar functional characteristics as satellite cells originate from bone marrow-derived stem cells (Ferrari et al., 1998; Bittner et al., 1999). Another group has shown that embryonic vasculature can give rise to myogenic population able to participate in postnatal muscle growth, regeneration, and fusion with resident satellite cells (De Angelis et al., 1999).

However, the view that non-committed bone marrow or muscle-derived stem cells participate in muscle regeneration after (trans)-differentiating into muscle precursor cells (LaBarge and Blau, 2002; Seale and Rudnicki, 2000) is not commonly accepted (Camargo et al., 2003; Wagers et al., 2002). It has been put forward that the low contribution of bone marrow cells to muscle regenerating after bone marrow transplantation is due to infrequent stochastic events that are caused by an entrapment of inflammatory cells (most likely macrophages) into fusogenic processes during muscle regeneration (Camargo et al., 2003). Other groups have reported little evidence of developmental plasticity of bone marrow derived haematopoietic cells re-enforcing the traditional view of skeletal muscle regeneration (Wagers et al., 2002). Interestingly, none of the non-muscle cell types reported so far has been shown to contribute significantly to muscle growth in undamaged muscle supporting the idea of low efficiency fusion mediated process that mimics regular muscle growth and regeneration (Camargo et al., 2003; Ferrari and Mavilio, 2002).

At present the situation is far from clear and requires a careful analysis of the molecules and mechanisms that determine the identity of muscle stem cells as well as their renewal and contribution to the regeneration process.

The data presented in this work clearly show a severe but not overwhelming regeneration deficit in Pax7(-/-) mice, which parallels the postnatal decline of satellite cells. The remaining Pax7 deficient satellite cells, however, seem to be capable to expand and complete a partial regeneration of damaged skeletal muscles.

The strong reduction of satellite cells in Pax7 mutant mice during postnatal development represents an interesting model to access the contribution of satellite cells and the hypothesized alternative muscle stem cells for skeletal muscle regeneration. Interestingly, a clear correlation of the decreased number of satellite cells with impaired muscle regeneration in Pax7 mutant mice has been found. In our view this argues against a significant contribution of an alternative muscle regeneration pathway based on adult stem cells.

Although it cannot be excluded that Pax7 might also play a role for adult stem cell mediated muscle regeneration this possibility does not seem plausible for several reasons: (i) the formation of muscle satellite cells and differentiation of muscle precursor cells was not dependent on Pax7, hence a putative alternate stem cell population would probably also require Pax7 for propagation and renewal of committed stem cells but not their specification. However, no clear evidence for the presence of non-satellite stem cells responsible for muscle repair was found in Pax7 mutants. (ii) We detected a clear reduction of the expression of Myf5 and MyoD in Pax7 mutant muscles concomitant with the reduction of satellite cells. Since both genes are required for muscle cell determination a putative alternative adult stem cell population would also require an up-regulation of either of these genes, which was not the case. This decrease can be explained by larger proportion of non-muscle tissues (fat or connective tissue, for example) in pooled hind limb muscles of Pax7(-/-) in comparison to twice bigger Pax7(+/-) animals. Since Myf5 and MyoD are expressed in satellite cells and myoblasts derived thereof, respectively, the down-regulation of Myf5 and MyoD suggests a postnatal decline of myoblasts in Pax7(-/-) mice. In addition, it illustrated that a major contribution of non-satellite muscle stem cells for postnatal muscle growth seems unlikely because the determination and differentiation of all cells would also depend on Myf5 and MyoD. It should be mentioned, however, that our real time PCR data represent the average expression in all cells present in skeletal muscle and hence cannot be used as a precise marker for distinct cell populations. (iii) The relative increase of the number of Pax7-lacZ cells in regenerating muscle of homozygous Pax7 mutant mice compared to heterozygous Pax7-lacZ

mice correlated with the number of resting Pax7-lacZ cells, which remained in homozygous Pax7 mutants and not with a proposed non-satellite muscle stem cell population.

4.3. The severe reduction of satellite cells in adult Pax7 mutant mice does not seem to initiate compensatory or alternate muscle repair mechanisms

According to the genomic organisation and sequence similarities in the paired domain, Pax genes can be subdivided into subgroups which share common expression domains. Pax7 form such a paralogous group with Pax3, a key regulator of somitic myogenesis, based on highly similar protein structures and partially overlapping expression patterns during embryonic development (Jostes et al., 1990; Goulding et al., 1991). Pax3 is important in cell migration, formation of the central nervous system, brain regionalization, and cellular differentiation. The mutation of Pax3 in splotch mice leads to abnormalities of the neural tube, neural crest-derived structures and peripheral musculature (Epstein et al., 1991).

In situ hybridization analysis of mouse embryos revealed that Pax3 and Pax7 are both expressed in the developing nervous system and in somite compartments that give rise to skeletal muscle progenitors (Goulding et al., 1991; Jostes et al., 1990). In the developing neural tube and in the somites, Pax7 and Pax3 expression extensively overlaps but the expression patterns are not completely identical.

In the developing neural tube Pax3 expression precedes that of Pax7 which starts after neural tube closure. Therefore, Pax3 might partially complement the function of Pax7 in neural development (Goulding et al., 1991; Mansouri et al., 1996b). Pax7 expression is activated later and persists longer than that of Pax3. In addition, Pax3 becomes localized to the lateral dermomyotome while Pax7 is more prominent in the medial dermomyotome. Pax3 but not Pax7 is expressed by myogenic progenitors that migrate to the limbs (Bober et al., 1994; Goulding et al., 1991). Finally, while Pax3 expression was not detected in the adult mouse, Pax7 expression was detected in muscle satellite cells of the adult mouse (Seale et al., 2000). Therefore, Pax3 and Pax7 are expressed with overlapping but distinct patterns in myogenic precursors. Pax3 is also expressed in satellite cells (Conboy and Rando, 2002; Buckingham et al., 2003); however, due to the early death of Pax3 mutants (at E13.5-14.5), its role in adult muscle remains to be elucidated. Comparison between muscles of splotch Pax3(-/-) and Pax7-/- mice demonstrates that although both proteins are highly similar neither compensates fully for the other.

In a recent work Relaix et al. have replaced Pax3 by Pax7 using gene targeting in the mouse to investigate the mechanism of functional redundancy of these two proteins (Relaix et

al., 2004). A key question in understanding the relative functions of Pax3 and Pax7 is whether these are determined by biochemical differences between the proteins or by differences in the spatiotemporal expression of the two genes. In mutant mouse embryos with one or two alleles of Pax7 replacing Pax3, Pax7 can functionally substitute Pax3 in the dorsal neural tube, in neural crest cells, and in somite development. However, Pax3 function in the long-range migration of muscle progenitor cells is only partially rescued. Different combinations of the Pax7 replacement alleles act as hypomorphic alleles of Pax3 in the formation of limb muscles, and reveal a multi step requirement for Pax3 activity in appendicular muscle development. In limbs in which Pax3 is replaced by Pax7, the severity of the muscle phenotype increases as the number of Pax7 replacement alleles is reduced, with the forelimb more affected than the hind limb. It has been concluded that functions already prefigured by the single ancestral Pax3/7 gene present before vertebrate radiation are fulfilled by Pax7 as well as Pax3, whereas the role of Pax3 in appendicular muscle formation has diverged in evolution, reflecting the more recent origin of this mode of myogenesis.

The dermomyotome of developing Pax7(-/-) mice appear morphologically normal, as demonstrated by normal expression of myogenic markers (Mansouri et al., 1996b). This indicates that the Pax7 mutation does not affect somite formation. The other explanation is a compensation of Pax7 by an alternative regulatory pathway. Pax3 might be a candidate for such a substitutive factor.

In the current study the expression of Pax3 gene in postnatal muscles of adult Pax7(-/-) mice was analyzed using quantitative RT-PCR. Surprisingly we did not find a compensatory increase of Pax3 expression but a minor reduction compared to adult Pax7(+/-) animals, which argues against a decisive compensatory function of Pax3 in Pax7(-/-) mutant. Together with the continuous elimination of satellite cells during postnatal live this finding makes an important postnatal compensation of Pax7 by Pax3 less likely although this conclusion has to be confirmed by combined postnatal knockouts of both genes.

4.4. Possible role of Pax7 in maintaining satellite cell compartment

The activation of satellite cells from quiescence to proliferation and myogenic differentiation are controlled by various transcription factors, chief among which are the myogenic regulatory factors Myf5, MyoD, myogenin, and MRF4. The expression of these MRFs in satellite cells provides a series of molecular landmarks for the transition from quiescence to activation and differentiation (Yablonka-Reuveni and Rivera, 1994; Beauchamp et al., 2000). The role of Pax7 during satellite cell activation and muscle regeneration has not

yet been fully investigated. Little is known also about potential regulatory interactions between Pax7 and the MyoD transcription factor family.

Borycki et al. reported that disruption of Pax3 expression by antisense oligonucleotides significantly impaired MyoD activation by signals from neural tube/notochord and surface ectoderm in cultured presomitic mesoderm, and is accompanied by a marked increase in programmed cell death (Borycki et al., 1999b). Nevertheless, in vivo, in Pax3 mutant (Splotch) embryos, MyoD is activated normally in the hypaxial domain of somites, but MyoD expressing cells are disorganized and apoptosis is prevalent in newly formed somites.

However, apoptosis did not occur in dorsal neural tube or in more mature somites where Pax7 becomes ectopically expressed and upregulated in the absence of Pax3 function. It was shown that Pax3 can repress Pax7 expression in C2C12 cultured myoblast cells. These results established that Pax3 has complementary functions for MyoD activation and inhibition of apoptosis in the somitic mesoderm and in negative regulation of Pax7 during neural tube and somite development, explaning the expanded expression of Pax7 in neural tube and somites of Splotch embryos. The misexpression of Pax7 in Pax3 mutant embryos provided a likely compensatory mechanism for cell survival and for cell fate determination in neural tube and in mature somites. Such cross-gene repression was also observed for two other closely related Pax genes, Pax1 and Pax9, which are expressed in the ventral sclerotome domain of somites, based on the observation that Pax9 expression is upregulated in homozygous Pax1 mutant mice (Peters et al., 1999). These crossregulatory mechanisms amongst closely related factors provide a sophisticated layer of functional redundancy that protects the embryo from individual gene mutation or inactivation. There is evidence for similar regulatory networks amongst other transcription factor gene families, such as GATA genes and bHLH genes, whose functions are critical for the embryo (Kuo et al., 1997; Rudnicki et al., 1993; Weiss et al., 1994). Consistent with this idea, the paired and homeobox domains of Pax3 and Pax7 are 91% and 95% identical, respectively (Goulding et al., 1991; Jostes et al., 1990). Structural homology and the expression data presented here suggest that Pax7 may be able to compensate, at least partially, for loss of Pax3, as has been suggested for GATA factors and bHLH myogenic factors.

The continuous decline of satellite cells in Pax7 mutant mice makes Pax7 an attractive candidate to control renewal of the satellite cell pool. The expression of Pax7 in the majority if not all satellite cells and the enhanced expression of Pax7 in regenerating muscle tissue do not necessarily exclude such a concept. The presence of Pax7 in activated satellite cells might

permit a subset of these cells either by cell autonomous decisions or by local signalling events to “fall back” and refill the original stem cell pool. Similar examples of transcription factors that direct expansion and propagation of cell lineages without specifying them include GATA-2, which is required for proliferation/survival of early haematopoietic cells, but not for erythroid and myeloid terminal differentiation (Tsai and Orkin, 1997) and SOX10, which maintains multipotency and inhibits neuronal differentiation of neural crest stem cells (Kim et al., 2003).

The minor reduction of MyoD and desmin positive cells in satellite cell derived colonies of Pax7(-/-) after 4 days of cultivation seems to exclude a simple proliferation defect of satellite cells. Similarly, the ratio between satellite cells and myoblasts derived thereof was not changed drastically in myotube cultures grown in Matrigel although the total number of satellite cells was severely decreased in adult Pax7 mutant mice. Instead, continued cultivation of equal numbers of satellite cells from P11 Pax7(+/-) and Pax7(-/-) mice at clonal densities revealed a decline of the number satellite derived myoblasts isolated from Pax7(-/-) mutant mice although the initial amplification of these cells occurred normally. Similarly, in regenerating skeletal muscles of adult homozygous Pax7 mutant mice Pax7-lacZ cells expanded noticeably suggesting a rather normal response of Pax7 mutant cells to proliferative stimuli. Hence, Pax7 might be required neither for the specification nor the initial proliferation of satellite cells but for their maintenance and extended proliferation.

These conclusions are in line with the current models of satellite cell activation. Zammit et al. (Zammit et al., 2004) used isolated myotube cultures to follow the fate of satellite cells without any selection or any potential exogenous sources of myogenic cells such as connective tissue and blood supply. They showd that satellite cells can adopt divergent fates (Fig. 27). Quiescent satellite cells become synchronously activated to co-express both Pax7 and MyoD. Most satellite cells then undergo limited proliferation before down-regulating Pax7 and differentiation. Alternatively, satellite cell progeny may maintain Pax7 but lose MyoD. These Pax7+ve/MyoD-ve cells are typically located in clusters together with Pax7-ve cells destined for differentiation. Pax7+ve/MyoD-ve cells persist and eventually divide slowly or not at all. Significantly, although most cells within a cluster express myogenin and differentiate, some retain the ability to be reactivated and re-enter the cell cycle. Thus, dividing satellite cells can either enter terminal differentiation or regain characteristics of quiescence. This finding suggests that the satellite cell pool is maintained via self-renewal, involving withdrawal from the terminal myogenic program, and may not require a contribution from elsewhere.

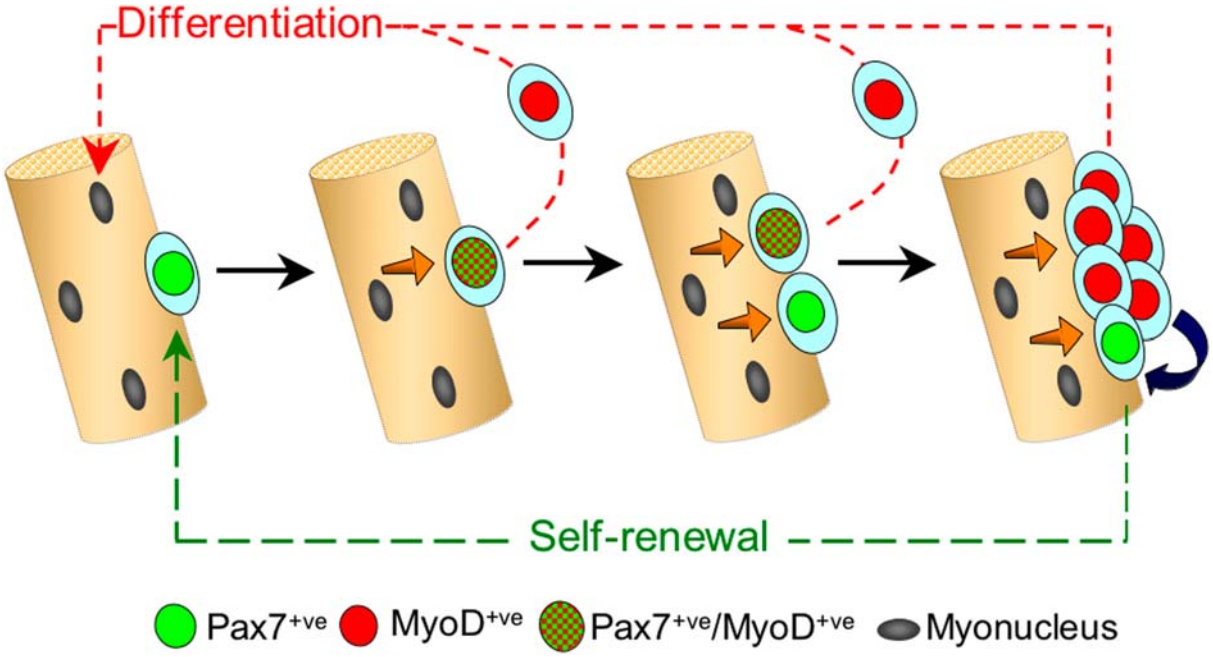

Fig. 27 A model of satellite cell self-renewal. Quiescent satellite cells (green) activate to co-express Pax7 and MyoD (green and red tartan), and then most proliferate, down-regulate Pax7, maintain MyoD (red), and differentiate (red pathway). However, activated Pax7+ve/MyoD+ve (green and red tartan) satellite cells can also divide to give rise to cells that adopt a different fate. These give rise to clusters of cells containing both Pax7-ve/MyoD+ve (red) progeny, whereas others down-regulate MyoD expression and cycle while maintaining only Pax7 (green). These clusters may grow by the further generation of cells with divergent fates. Pax7+ve/MyoD-ve cells (green) become quiescent, thus renewing the satellite cell pool (green pathway), whereas the MyoD+ve cells (red) differentiate to produce myonuclei (red pathway). Signalling from the myofiber (orange arrows) and/or between cells within the clusters (blue arrow) may dictate which fate the satellite cell adopts (the picture has been taken from (Zammit et al., 2004)).

Recent studies of Olguin and Olwin has also supported the view that Pax7 plays a role in satellite cell maintenance (Olguin and Olwin, 2004). When Pax7 expression in quiescent and activated satellite cells was analysed, they revealed that Pax7 protein was undetectable in 30% of satellite cells associated with freshly harvested myofibers. Substantial heterogeneity was also detected within daughter cells of the same individual clones strongly suggesting that Pax7 is differentially expressed in activated satellite cells. The heterogeneity observed for Pax7 did not correlate with detectable differences in MyoD staining early after activation, where MyoD staining within a clone of satellite cells was uniform and Pax7 staining is highly heterogeneous. This result suggested that Pax7 may be not a universal marker for satellite cells and supported the view that Pax7 is not necessarily required for satellite cell specification.

Further analyses performed by the same group (Olguin and Olwin, 2004)showed that Pax7 and myogenin expression were mutually exclusive during differentiation, where Pax7 appeared to be up-regulated in cells that escape differentiation and exit the cell cycle,

suggesting a regulatory relationship between these two transcription factors. Pax7 overexpression caused MyoD down-regulation, prevented myogenin induction and blocked MyoD-induced myogenic conversion of 10T1/2 cells. Ectopic expression of Pax7 seemed to inhibit MyoD function and MyoD dependent transcription not by alteration of subcellular localization of MyoD protein but more likely interfering MyoD protein or directly by competition for MyoD DNA binding sites. Thus, Pax7 did not appear to be capable of overcoming the effect of the MyoD-E47 tethered dimmer in induced myogenic conversion, suggesting that the inhibition of MyoD may not involve competition for MyoD-binding sites on DNA but could involve competition for proteins necessary for MyoD-dependent transcription.

Forced down-regulation of Pax7 neither induced myogenin in proliferating MM14 myoblasts nor interfered with myogenin induction upon differentiation. Thus, the loss of Pax7 protein in MM14 cells does not appear to either induce differentiation or affect the endogenous differentiation program, suggesting that the proposed role for Pax7 during satellite cell renewal may be transient, functioning in a subset of the activated satellite cell population. Overexpression of Pax7 reduced BrdU incorporation into DNA by at least 80%, compared to myoblasts that express endogenous levels of Pax7, promoting cell cycle exit even in proliferation conditions. Together, these results suggested that Pax7 may play a crucial role in allowing activated satellite cells to reacquire a quiescent, undifferentiated state.

Olguin and Olwin also provided a model of the Pax7 role in satellite cell biogenesis, where Pax7 allows satellite cells to exit the cell cycle, down-regulate MyoD, and prevent myogenin induction (phenotypes characteristic of the quiescent satellite cell) (Olguin and Olwin, 2004). Thus, allowing satellite cells to self-renew from cycling myoblasts or a subpopulation of satellite cells (Fig. 28).

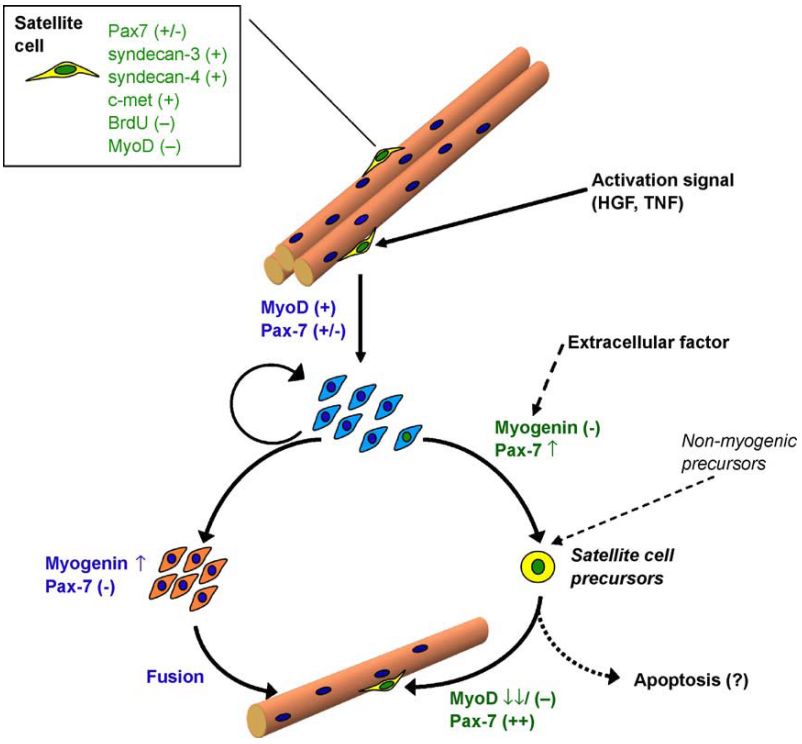

Fig. 28 A model for the role of Pax7 in satellite cell physiology. Mitotically quiescent satellite cells (yellow cell on myofiber) express a subset of characteristic proteins including the markers syndecan-3, syndecan-4, and c-met but are heterogeneous for Pax7 protein. Upon activation, satellite cells proliferate and up-regulate MyoD. Proliferating myoblasts that are positive for both Pax7 and MyoD (blue cells) behave as a heterogeneous population where a small fraction of cells are prone to precocious differentiation inducing myogenin and losing Pax7 expression (orange cells/blue nuclei) while a small number retain precursor characteristics (blue cell/green nucleus). Co-expression of Pax7 and MyoD might be required to retain myoblasts in a proliferative state and prevent premature differentiation. As the myogenic program proceeds, MyoD family transcription factors are up-regulated and Pax7 is down-regulated in cells committed to differentiation (orange cells/blue nuclei). A small number of precursor cells (blue cell/green nucleus) up-regulate Pax7 and down-regulate MyoD, exit the cell cycle, and form a new satellite cell pool (yellow cell on myofiber). Additional events might be involved to evade apoptosis and acquire the final satellite cell position beneath the basal lamina of regenerated muscle fibers (the picture has been taken from (Olguin and Olwin, 2004)).

Another group got similar result analysing the pattern of Pax7 protein expression in relation to the expression of MyoD and myogenin in myogenic cultures derived from chicken (Halevy et al., 2004). This study shows that Pax7 is an early marker also for chicken satellite cells. Foetal myoblasts, isolated from the pectoralis muscle of 10-day-old chicken embryos, like adult myoblasts and satellite cells expressed Pax7. Thus, Pax7 expression cannot be used to distinguish between the different myoblast populations identified during muscle histogenesis in embryonic and posthatch chicken.

Pax7+/MyoD- cells were present at all time points analyzed, and their number increased in advanced cultures. In parallel, the frequency of Pax7+/MyoD+ cells declined and a large number of cells transited into the myogenin+ state and/or fused into myotubes in advanced cultures. The authors propose that the Pax7-/MyoD+ cells found at all time points represent cells that have transited into the Pax7-/myogenin+ state. Pax7 and myogenin

expression appeared to be mutually exclusive throughout the cell culture analysis. However, at all time points, a small number of cells exhibited a Pax7+/myogenin+ phenotype. These cells may represent an intermediate population facing the onset of differentiation. The Pax7+/MyoD-cells identified in this study were proposed to represent reserve myoblasts. The model of satellite cell dynamics proposed by Halevi et al. (Halevy et al., 2004) is presented at the Fig. 29.

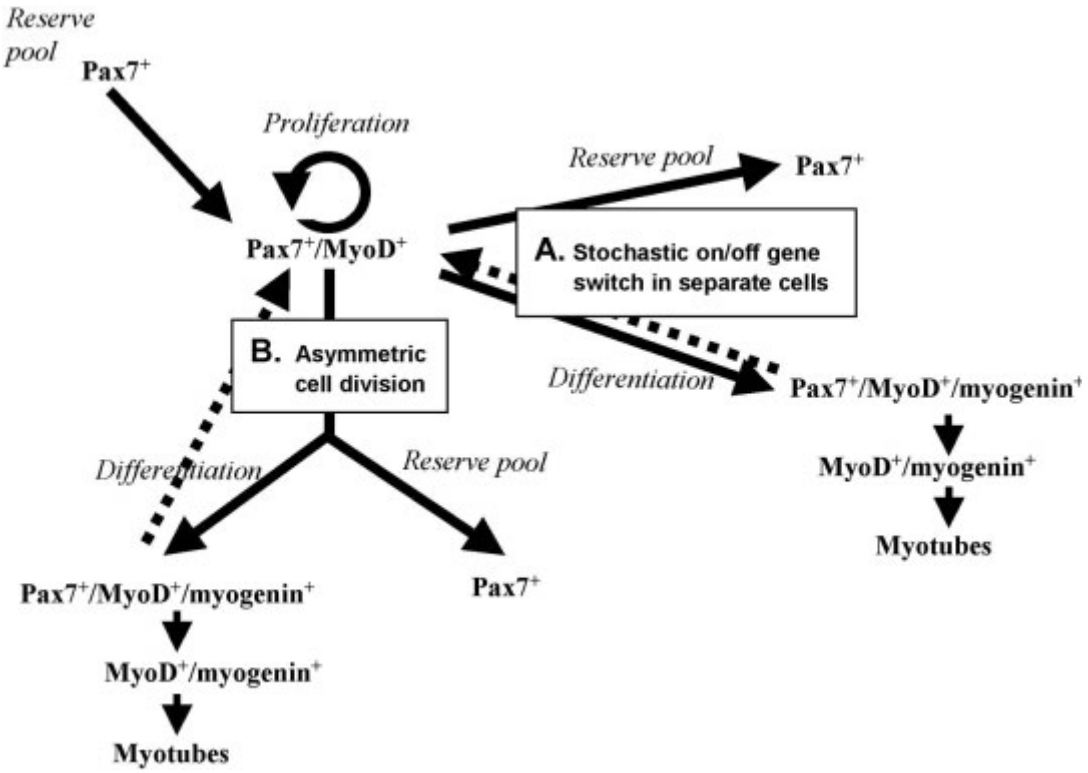

Fig. 29 A model depicting satellite cell dynamics during myogenesis in early posthatch muscle development. Quiescent satellite cells expressing Pax7 only, are driven to the cell cycle during muscle growth. A,B: Fully proliferating cells express both Pax7 and MyoD and undergo either (A) stochastic on/off gene switch in separate cells or (B) asymmetric divisions, leading to subsequent differentiation or return to the reserve pool. The "decision" to undergo differentiation is accompanied by the induction of myogenin expression and those cells that express Pax7 and MyoD as well as myogenin are probably at the turning point to differentiation. Cells will differentiate (black arrows) when myogenin expression increases and Pax7 levels are decreasing and eventually will be shut off all together. However, under specific signals, cells undergoing differentiation may go back into the cell cycle to their Pax7+/MyoD+ position (dashed arrows**)** fibers (the picture has been taken from (Halevy et al., 2004)).

Seale et al. claimed that Pax7 is necessary and sufficient for the myogenic specification of CD45+/Sca1+ stem cells from injured muscle (Seale et al., 2004). CD45+/Sca1+ adult stem cells isolated from uninjured muscle did not display any myogenic potential, whereas those isolated from regenerating muscle gave rise to myoblasts expressing Pax7 and the bHLH factors Myf5 and MyoD. By contrast, CD45+/Sca1+ isolated from injured Pax7(-/-) muscle were incapable of forming myoblasts. Infection of CD45+/Sca1+

cells from uninjured muscle with retrovirus expressing Pax7 efficiently activated the myogenic program. The resulting myoblasts expressed Myf5 and MyoD and differentiated into myotubes that expressed myogenin and myosin heavy chain. Although CD45+ cells from Pax7(-/-) muscle were unable to undergo myogenesis, ectopic Pax7 induced expression of Myf5 and myogenic specification in Pax7-deficient CD45-/Sca1- cells.

Myf5 was expressed at high levels in proliferating CD45+/Sca1+ cells after retrovirally mediated Pax7 expression. Moreover, these cells continued to express Myf5 protein during their differentiation. They also expressed MyoD but at low levels relative to primary myoblasts. MyoD was transiently upregulated as the cells entered their differentiation program.

The primary myogenic regulatory factor expression profile in CD45+/Sca1+ cells after retrovirally mediated Pax7 expression contrasted with the pattern observed in satellite-cell-derived primary myoblasts. Primary myoblasts expressed higher levels of MyoD and lower levels of Myf5 and downregulated Myf5 immediately upon differentiation. Myogenin was upregulated during the differentiation of these cells, albeit at lower levels compared with differentiating satellite-cell derived myoblasts.

The authors proposed that expression of Myf5 in Pax7-infected CD45+/Sca1+ cells is due to a preferential activation of Myf5 by Pax7, while Pax3 has been implicated in myogenesis specifically upstream of MyoD (Tajbakhsh et al., 1997). Based on these observations the authors hypothesized that Pax3 and Pax7 might specify distinct myogenic lineages through the preferential activation of MyoD and Myf5, respectively and that Pax7 might activate expression of Myf5 to promote adult myoblast expansion whereas Pax3 might preferentially induce MyoD and differentiation.

Although it remains possible that Pax7 may regulate myogenesis via the MyoD family of transcription factors, role of Pax7 in the specification or the survival of the satellite cell progenitor pool remains unclear. Understanding the molecular pathways regulated by Pax7 should be useful in understanding the early event of satellite cell development.

4.5. Pax7 and cell survival

Our hypothesis that Pax7 plays a major role for the renewal and propagation of satellite cells but not their specification is in line with previous reports that Pax3 and possibly also Pax7 maintain cells in a deregulated undifferentiated and proliferative state (Mansouri, 1998). As it was mentioned before, Pax7 and Pax3 proteins are closely related, and both genes likely arose by gene duplication from a common ancestor (Noll, 1993). Both genes strongly

stimulate cell proliferation in various tissues (Mansouri, 1998). During normal myogenesis, Pax3 is expressed in migrating myoblasts and is believed to inhibit their differentiation until they reach their destination. It has been suggested that dysregulated expression of Pax3 and Pax7, and/or their normal target genes, is involved in tumorogenesis (Bober et al., 1994; Epstein et al., 1996).

It was shown that neural tube defects in Splotch embryos are associated with neuroepithelial apoptosis (Phelan et al., 1997). This suggested that disruption of Pax3 may cause apoptosis in malformed structures. An alternative explanation is that Pax3 directly or indirectly inhibits apoptosis. Several studies support the latter interpretation. For example, apoptosis is prevalent in somites of Splotch embryos (Borycki et al., 1999b), and inhibition of Pax3 expression with antisense oligonucleotides, or expression of an engineered PAX3 fused to a transcriptional repressor domain, causes apoptosis in cultured presomitic mesoderm, pediatric rhabdomyosarcoma, and melanoma (Borycki et al., 1999b; Barr et al., 1993; Galili et al., 1993; Shapiro et al., 1993; Bernasconi et al., 1996; Scholl et al., 2001).

Translocations in humans resulting in the in-frame fusion between the undisrupted Pax3 and Pax7 genes and FKHR gene lead to the formation of alveolar rhabdomyosarcomas, a paediatric tumour of skeletal muscle (Barr, 2001). These novel chimeric genes encode for a transcription factor containing the paired box and homeodomain DNA-binding domains from the transcription factor Pax3 fused to the potent transactivation domain of the FKHR transcription factor (Sorensen et al., 2002). Such fusions result in an enhanced transcriptional activity and probably also change protein stability of Pax7 or Pax3. It is tempting to speculate that the role of Pax genes in the pathogenesis of primary muscle tumours represent an exaggerated reflection of the normal function of Pax genes to keep muscle cells in a proliferative state that enables maintenance of the muscle stem cell pool.

Pax3/FKHR is believed to result in a molecular gain of function that may initiate a deregulated muscle development program in the affected cells. Bernasconi et al. presented evidence (Bernasconi et al., 1996) that specific down-regulation of the PAX3/FKHR fusion protein after antisence RNA treatment can trigger physiological tumor cell death. Similarly, induction of cell death was observed after downregulation of the wild-type PAX3 or PAX7 proteins, suggesting that PAX gene products might, in these embryonal rhabdomyosarcoma cells, be able to suppress apoptosis and are required for cell survival.

It was also reported that ectopic expression of several Pax genes in murine embryonal fibroblasts is able to induce transformation and tumor formation in nude mice, suggesting that deregulated Pax proteins can induce tumorigenesis (Maulbecker and Gruss, 1993). It was

proposed that this effect is, in part, due to loss of expression of the tumor suppressor protein p53 at the transcriptional level (Stuart et al., 1995). A conserved Pax binding site is located in the first untranslated exon of the human p53 gene (Stuart et al., 1995). Pax2, Pax5, and Pax8 can transcriptionally inhibit p53 expression (Stuart et al., 1995). However, Bernasconi et al. presumed that in their experiments (Bernasconi et al., 1996), inactivation of p53 by down-regulation of Pax3 and Pax7 does probably not play a major role for two reasons. (i) Transcriptional repression as a way to inactivate p53 is specific for Pax2 and Pax5 and is not observed by Pax3 (Stuart et al., 1995). (ii) The two cell lines analyzed in this study already carry inactivating mutations in p53. The investigators proposed that these regulators contribute to suppression of the apoptotic pathway possibility by either activation or repression of specific, so far unknown, target genes.

On the other hand Pani et al. (Pani et al., 2002) found that inactivation of p53, caused by germ-line mutation or by pifithrin-α, an inhibitor of p53-dependent apoptosis, rescues not only apoptosis, but also neural tube defects, in Splotch embryos. Pax3 deficiency had no effect on p53 mRNA, but increased p53 protein levels. These results suggest that Pax3 regulates neural tube closure by inhibiting p53-dependent apoptosis, rather than by inducing neural tube-specific gene expression.

A number of known oncogenes and tumor suppressor genes are involved in the regulation of apoptosis, among them bcl-2 and its family members (Hockenbery, 1994), c-myc (Evan et al., 1995), pRB (Cordon-Cardo, 1995), and WT1 (Englert et al., 1995). Therefore, genes such as growth factors or growth factor receptors might potentially be positively regulated by Pax proteins. On the other hand, an alternative possibility is that specific protein–protein interactions play a major role. Such interactions seem to be important for the control of apoptosis by c-myc (Kohlhuber et al., 1995) and in the bcl-2 protein family (Hockenbery, 1994). In the case of rhabdomyosarcoma, they would most likely involve the common constituents of PAX3, PAX7, and PAX/FKHR encompassing the two DNA binding domains, namely the paired box and the homeobox.

To investigate the role of the translocation-associated gene Pax3/FKHR in alveolar rhabdomyosarcomas, Keller et al. (Keller et al., 2004) generated a Cre-mediated conditional knock-in of Pax3/FKHR into the mouse Pax3 locus, causing dominant-negative effects on Pax3 and paradoxical activation of the Pax3 target gene, c-Met. Ectopic neuroprogenitor cell proliferation also occured. To study the consequences of later embryonic activation of Pax3/FKHR in the overlap of Pax3 and Pax7 expression domains, the researchers bred conditional Pax3/FKHR mice to Pax7–Cre mice. $Pax3^{P3Fa/wt}/Pax7^{ICNm/wt}$ pups were

represented at normal Mendelian ratios and displayed the mild birth defects of a narrowed nose. However, later the animals showed a postnatal growth defect and a moderately decreased Pax7+ muscle satellite cell pool, phenocopying Pax7 deficiency. The pups had reduced muscle mass, maxillary and lacrimal bone hypoplasia, nasal turbinate abnormalities but did not show an enchanced tumor formation. Satellite cells were present in normal weight $Pax3^{P3Fa/wt}/Pax7^{ICNm/wt}$ mutant P0 pups in fewer numbers than control littermates. More precise investigation revealed a significant reduction in the percentage of satellite cells in vastus lateralis muscle of P9 mutant mice compared with a control.

To determine whether the Pax3/FKHR allele was directly suppressing Pax7 transcription, a ROSA26 LacZ reporter gene (Soriano, 1999) was bred into $Pax3^{P3Fa/wt}$ $Pax7^{ICNm/wt}$ mutant mice to indicate Pax7 promoter activity and cell survival in E10.5 and E11.5 embryos. The Pax7 promoter activity of $Pax3^{P3Fa/wt}/Pax7^{ICNm/wt}$ mice was either equivalent or increased as compared with $Pax3^{wt/wt}/Pax7^{ICNm/wt}$ control mice. Such increased expression of Pax7 mice might be the result of Pax3/FKHR-mediated suppression of Pax3 expression, because Pax3 is normally a repressor of Pax7 expression (Borycki et al., 1999b) (in other words, Pax7 expression increases because Pax3 no longer suppresses Pax7 transcription). At the protein level, Pax7 was also found to be increased in the neural tube and dermomyotome of $Pax3P^{3Fa/wt}/Pax7^{ICNm/wt}$ E10.5 embryos. The authors suppose that the Pax7 knockout phenocopy, despite increased Pax7 expression, can be explained by dominant-negative effects of Pax3/FKHR on Pax7 targets, rather than effects on expression of Pax7 itself.

Recent transfection studies have shown that Pax3 level is increased and Pax7 level is decreased in embryonal rhabdomyosarcoma cells forced to express the alveolar rhabdomyosarcoma oncogene Pax3/FKHR, (Tomescu et al., 2004). Keller et al. have no clear explanation for the differences between these studies but presume that embryonal rhabdomyosarcomas may not be the starting point or an equivalent cellular context for alveolar rhabdomyosarcomas.

A general function of Pax gene products in promoting cell survival is supported by several observations but it is not clear so far whether Pax3 or Pax7 directly or indirectly represses genes in the apoptotic pathway during the development of the paraxial mesoderm and whether inhibition of apoptosis is an essential, or even the sole, function of these genes during development or transformation.

4.6. What are the reasons for a different perception of Pax7 mutant mice?

Previously, Pax7(-/-) mice have been described to be completely devoid of satellite cells probably due to the inability of mutant animals to specify the satellite cell lineage (Seale et al., 2000). These findings are difficult to explain in the light of our current investigations in particular since the same mutant strain on the same genetic background has been used for the investigation. However, some differences in the analyses are evident and should be pointed out: (i) we have analyzed different muscle groups from several different animals whereas Seale et al. apparently only used muscles derived from the hind limb. (ii) In the current study several mice between P8 and P11 were studied. Although Seale et al. also analyzed mice within this range a low number of analyzed mice and the analysis of single individuals that were not exactly age-matched might have inappropriately amplified minor differences. (iii) We have counted a large number of myonuclei (several thousand) to avoid statistical deviations and used a number of different techniques to corroborate our results and finally, (iv) in addition to the Pax7-strain without lacZ we have utilized a Pax7-lacZ strain that allowed the easy identification of cells that normally express Pax7 (i. e. satellite cells). Taken together these differences might culminate in substantial differences in the perception of the Pax7 phenotype and in a rather different interpretation. It should also be emphasized that we did not phenotypically selected Pax7(-/-) mice prior to analysis and that all Pax7(-/-) mice showed craniofacial abnormalities and the retarded growth as described originally (Mansouri et al., 1996b). Only adult Pax7(-/-) mice represented a “selected” population of individuals since only approximately 5-10% of all Pax7(-/-) mice survived into adulthood. Nevertheless, the rather normal growth and morphological appearance of the musculature of juvenile Pax7(-/-) mice seem to exclude a selection based on malfunctions of the muscular-skeletal system. It is clear that neither the generation of satellite cells nor their ability to regenerate damaged myotubes rely strictly on Pax7 since satellite cells develop normally in juvenile Pax7 mutant mice and satellite cells derived from adult Pax7 mutant mice express MyoD and differentiate normally in culture.

4.7. Conclusions

The present analysis of satellite cells in Pax7(-/-) mice has unambiguously established that Pax7 is dispensable for the specification of the satellite cell lineage since a large number of satellite cells was detected in juvenile Pax7(-/-) mutant mice using five different methods (electron microscopy, Pax7-lacZ staining, CD34 staining, *in vitro* cultivation of satellite cells

from isolated myofibers and clonal satellite cell analysis). Instead, I detected a continuous decrease of the number of satellite cells in Pax7(-/-) mutants, which strongly suggests a role of Pax7 in the renewal and propagation of satellite cells. In addition, an essentially normal degree of muscle formation in adult Pax7 mutant animals was found that cannot be explained without the contribution of the highly proliferative satellite cell population to the growth of immature muscles. Moreover, adult Pax7(-/-) mice still own a certain potential for skeletal muscle regeneration although the efficiency of regeneration is severely hampered. The compromised regenerative response of Pax7(-/-) mice came along with an expansion of the remaining Pax7-lacZ satellite cells and resulted in numerous regenerated muscle fibers although faulty regeneration was evident and a complete repair was never achieved.

It is of major importance to understand the mechanisms and pathways that govern muscle repair processes to develop applied therapeutic and clinical tools that will ultimately lead to an improved treatment of muscle disorders. In the current study the function of Pax7 as a major regulator of satellite cell renewal and propagation was re-defined. This knowledge will probably help us to manipulate the availability of muscle stem cells for therapeutic purposes in particular in aged and diseased skeletal muscles.

5. EXPERIMENTAL PROCEDURES

5.1. Reagents and materials

Unless otherwise stated, the reagents used were in analytical grade. Most of the chemicals for the present work were ordered from the following firms: Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Sigma (Deisenhofen, Germany), Eppendorf (Wessening-Berzdorf, Germany), New England Biolabs (Frankfurt/Main, Germany), Invitrogen (Karlsruhe, Germany), Promega (Mannheim, Germany), Stratagene (Heidelberg, Germany), Boehringer (Mannheim, Germany) Quiagen (Hilden, Germany), Roth (Hamburg/Karlsruhe, Germany), Molecular Probes-Invitrogen (Karlsruhe, Germany), Pharmacia (Freiburg, Germany), Roche Diagnostics (Mannheim, Germany). Radioactive substances were ordered from Amersham Buchler (Braunschweig, Germany) und NEN Life Science (Köln, Germany), BD Biosciences (Heidelberg, Germany).

Biodyne B nylon transfer membrane	Pall (Dreieich, Germany)
Diaminobenzidin (DAB)	Sigma (Deisenhofen, Germany)
Dimethylsulfoxid, Dimethyformamid	Sigma (Deisenhofen, Germany)
Dithyotreithol (DTT)	Promega (Mannheim, Germany)
DMEM	Invitogen (Karlsruhe, Germany)
Filters Minisart NML (0.2 und 0.45 µm)	Sartorius (Göttingen, Germany)
Filters	Schleicher & Schüll (Hannover, Germany)
Fluorescein	Bio-Rad (Munich, Germany)
Hoechst 33258 dye	Molecular Probes (Karlsruhe, Germany)
Glass slides and cover slides	Plano (Wetzlar, Germany)
Glassware	Schütt (Göttingen, Germany)
Ion exchange resin	Bio-Rad (Munich, Germany)
$K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$	Sigma (Deisenhofen, Germany)
Kodak D-19 developer	Kodak (Frankfurt/Main, Germany)
Matrigel	BD Biosciences (Heidelberg, Germany)
Mowiol	Merck (Darmstadt, Germany)
NAP-5 columns (Sephadex G-25-columns)	Pharmacia Biotech (Freiburg, Germany)
PCR oligonucleotides	Roth (Hamburg/Karlsruhe, Germany)
Penicillin/Streptomycin mix solution	Invitrogen (Karlsruhe, Germany)
Phenol, pH 4-4,5	Roth (Karlsruhe, Germany)

Plastic ware	Nunc (Wiesbaden, Germany)
Polyfreeze tissue freezing medium	Polysciences (Warrington PA, USA)
Rapid-Fix	Kodak (Frankfurt am Main, Germany)
SDS	Bio-Rad (Munich, Germany)
SYBR green	Sigma (Deisenhofen, Germany)
2,2,2-Tribromoethanol	Sigma (Deisenhofen, Germany)
Trizol reagent	Invitrogen (Karlsruhe, Germany)
Tween-20	USB (Bad Homburg, Germany)
Vectabond reagent	Camon (Wiesbaden, Germany)
Whatman 3MM Chr for blotting	Whatman International (Maidstone, England)
X-gal	Sigma (Deisenhofen, Germany)
X-ray film	Kodak (Frankfurt/Main, Germany)

5.1.1. Enzymes

Collagenase P	Roche (Mannheim, Germany)
DNA polymerase I large (Klenow enzyme)	Promega (Mannheim, Germany)
Expand Reverse Transcriptase	Boehringer (Mannheim, Germany)
Protease from Streptomyces griseus	Sigma (Deisenhofen, Germany)
Proteinase K	Boehringer (Mannheim, Germany)
Restriction endonucleases	Boehringer (Mannheim, Germany), New England Biolabs (Schwalbach, Germany)
RNase A	Boehringer (Mannheim, Germany)
RNase H	Promega (Heidelberg, Germany)
RNasin Plus (RNase Inhibitor)	Promega (Heidelberg, Germany)
RQ1 Rnase-free DNase	Promega (Heidelberg, Germany)
Taq-DNA-Polymerase	Eppendorf (Wesseling-Berzdorf, Germany)

5.1.2. Antibodies

anti-MyoD rabbit anti-mouse polyclonal, Santa Cruz Biotech. Inc. (SC-304), 1:100-1:2000

anti-MyHC mouse monoclonal MF20, (Schafer and Braun, 1999b), 1:20.

anti-CD34 rat monoclonal, PharMingen, BD Biosciences (550537), 1:25

anti-β-galactosidase rabbit polyclonal, Cappel, ICN Pharmaceuticals (55976), 1:1000

anti-desmin rabbit polyclonal, Sigma (D8281), 1:15

anti-Sca1 rat monoclonal, PharMingen, BD Biosciences (553333), 1:25

anti-Pax3 rabbit polyclonal , Active Motive Inc. (39335), never worked in our hands
anti-Pax3 mouse monoclonal, from Dr. Ordahl, UCSF, USA, never worked in our hands
Alexa Fluor 488 labelled chicken anti-rabbit IgG, Molecular Probes (A-21441), 1:1000
Alexa Fluor 594 labelled chicken anti-rabbit IgG, Molecular Probes (A-21442), 1:1000
Alexa Fluor 488 labelled goat anti-mouse IgG, Molecular Probes (A-11001), 1:1000
Alexa Fluor 594 labelled chicken anti-mouse IgG, Molecular Probes (A-21201), 1:1000
Alexa Fluor 488 labelled donkey anti-rat IgG, Molecular Probes (A-21208), 1:100
Biotinilated rabbit anti-rat IgG, Vector Laboratories (BA-4001), 1:100
Biotinilated horse anti-mouse, anti-rabit IgG, Vector Laboratories (BA-1400), 1:1000

5.1.3. Kits

DNA Cycle Sequencing Kit (Abi, Weiterstadt)
Qiagen Plasmid Mini- und Maxi-Kit (Qiagen, Hilden)
Vectastain Elite ABC Kit (Vector Laboratories, CA, USA)

5.1.4. Origin of mouse mutant strains

The generation of Pax7 and Pax7-lacZ mutant mice has been published by Mansouri et al. (Mansouri et al., 1996b). Mutant mice were maintained on a mixed 129Sv/C57/BL6 background and genotyped by Southern blot analysis.

5.1.5. PCR primers

CW-MyoD IR:5′-GGT CTG GGT TCC CTG TTC TGT GT
CW-MyoD IF:5′-CCC CGG CGG CAG AAT GGC TAC G
CW-Myf5 IR: 5′-CGC TGG TCG CTG GAG AG
CW-Myf5 IF: 5′-GAG GGA ACA GGT GGA GAA CTA TTA
CW-MRF4 OR: 5′-ATG GAA GAA AGG CGC TGA AGA CTG
CW-MRF4 OF: 5′-CTG CGC GAA AGG AGG AGA CTA AAG
CW-myogenin OR: 5′-AGG AGG CGC TGT GGG AGT T
CW-myogenin OF: 5′-GGG CCC CTG GAA GAA AAG
Pax3 OR: 5′-GAT CCG CCT CCT CCT CTT CTC CTT
Pax3 OF: 5′-GCC AGG GCC GAG TCA ACC AG
GAPDH forward: 5′-GTG GCA AAG TGG AGA TTG TTG CC
GAPDH reverse: 5′-GAT GAT GAC CCG TTT GGC TCC

5.1.6. Plasmids

pGEM-T vector (Promega, Heidelberg)

pBluescript KSII+ (pKSII+) (GenBank-Nr.:X52327 [KS(+)]), Stratagene, Heidelberg)

pMHC2.2 recombinant plasmid containing part of embryonic MyHC cDNA and described by Weydert et al., 1985

pMHC16.2A recombinant plasmid containing part of peri-natal MyHC cDNA and described by Weydert et al., 1985

pMHC32 recombinant plasmid containing part of fast adult MyHC cDNA and described by Weydert et al., 1983

pGAPDH recombinant plasmid containing the GAPDH cDNA fragment obtained as a result of RT PCR using mouse muscle total cDNA as a template and GAPDH forward and reverse primers. The fragment cloned pGEM-T vector (constructed by the author of the present work)

pMyf5 recombinant construction on the basis of pBluescript containing full-length mouse myf5 cDNA sequence, laboratory plasmid collection

pMyf6 recombinant construction on the basis of pBluescript containing full-length mouse myf6 (MRF4) cDNA sequence, laboratory plasmid collection

pMyogenin recombinant construction on the basis of pBluescript containing rat myogenin cDNA sequence, laboratory plasmid collection

pMyoD recombinant construction on the basis of pV2CIIα containing mouse MyoD cDNA sequence, laboratory plasmid collection

pPax3 recombinant plasmid containing the pax3 cDNA fragment obtained as a result of RT PCR using mouse muscle total cDNA as a template and Pax3 forward and reverse primers. The fragment cloned pGEM-T vector (constructed by the author of the present work)

pMLC 1, laboratory plasmid collection

pTroponin fast, laboratory plasmid collection

pTroponin slow, laboratory plasmid collection

pMyHCβ, laboratory plasmid collection

5.1.7. Probes for Northern blot hybridization

GAPDH: DNA fragment obtained as a result of PCR using pGAPDH as a template and GAPDH forward and reverse primers

Embryonic MyHC: PstI-insert fragment of the pMHC2.2

Peri-natal MyHC: PstI-insert fragment of the pMHC16.2A

Fast adult MyHC: PstI-insert fragment of the p32

Myosin light chain: 1 EcoRI-insert fragment of pMLC1

Troponin fast: EcoRI-insert fragment of pTroponin fast

Troponin slow: EcoRI-insert fragment of pTroponin slow

MyHCβ: EcoRI-insert fragment of pMyHCβ

5.1.8. Solutions

10 x BPTE electrophoresis buffer	100 mM PIPES free acid, 300 mM Bis-Tris free base, 10 mM EDTA, pH 8,0. Final pH 6,5. The solution was treated with DEPC at final concentration 0,1% overnight at room temperature and then autoclaved.

Glyoxal mixture	6 ml of DMSO, 2 ml of deionised glyoxal, 1,2 ml of 10 x BPTE buffer, 0,6 ml of 80% glycerol in H_2O, 0,2 ml of 10 mg/ml ethidium bromide, store at -80^{0}C)
RNA gel loading buffer	95% the best quality formamide, 0,025% bromophenol blue, 0,025% xylene cyanol FF, 5 mM EDTA, pH 8,0, 0,025% SDS)
Glyoxal deionization	was mixed several times with mixed-bed ion-exchange resin Bio-Rad AG-510-X8 to pH ≥5,5, filtrated, aliquoted and stored at -80^{0}C)
6 x DNA gel loading buffer	0,25% bromophenol blue, 0,25% xylene cyanol FF, 15% Ficoll400)
Mowiol	6 g of glycerol was mixed with 2,4 g Mowiol and 6 ml H_2O, incubated for 4 hours at room temperature, mixed with 12 ml 0,2 M Tris, pH 8,5, heated 10 minutes at 50^{0}C, aliquoted and stored frozen.
10 x PBS buffer	137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4
20 x SSC buffer	3 M NaCl, 0,3 M sodium acetate, final pH 7,2
TE buffer	10 mM Tris-HCl, pH 7,5, 1 mM EDTA, pH 8,0
1 x TAE electrophoresis buffer	40 mM Tris-acetate, 1 mM EDTA
4% PFA	40 g of PFA was solved in 800 ml of water, warmed up to 60^{0}C with stirring, a few drops of

	10 M NaOH were added to clear the solution. Next 100 ml of 10 x PBS buffer was added, the solution final volume adjusted to 1000 ml and pH to 7,4. The solution was filtered and stored in aliquots at -20^{0}C.
Proliferation medium	DMEM with 20% foetal calf serum, 10% horse serum, 1% chick embryo extract and 1 x penicillin/streptomycin mix
Differentiation medium	2% FCS and 1 x penicillin/streptomycin mix

5.2. Methods

5.2.1. General molecular biological methods

All standard molecular biological procedures (isolation of plasmid DNA, purification of DNA, DNA electrophoresis in agarose gels, elution of DNA fragments from agarose gels, digestion of DNA with restriction nucleases, ligation of DNA fragments, preparation of *E. coli* competent for transformation cells, transformation of competent cells with plasmid DNA, polymerase chain reaction) were performed according to protocols described in "Molecular Cloning" (Sambrook and Russel, 2001). Composition of the solutions and protocols used during the present work are provided below only if they differ from the reference. All solutions used in this work were prepared using bidistilled water or water purified with Milli-Q device and sterilized by autoclaving or filtration.

5.2.2. DNA sequencing

Sequences of DNA fragments cloned in plasmid vectors were performed using polymerase chain reaction with fluorescently labelled dNTPs and Abi Prism TM 310 Genetic Analyzer (Perkin Elmer) to separate the products of sequencing reactions. A sequencing reaction mix contained 0,2-0,5 µg of plasmid DNA, one of the universal T3, T7 or SP6 primers and other components of the DNA Cycle Sequencing Kit (Abi, Weiterstadt). The reaction was performed according to the protocol of the supplier. Perkin Elmer Cetus Cycler "Gene Amp PCR-System 9600" was used for amplification of DNA.

5.2.3. Mouse genotyping by Southern blot analysis

Biological material to define mouse genotypes (DNA) was obtained by clipping 0,3-0,5 cm of tail tips. Pieces of tails were incubated overnight at 56^0C in 0,5 ml proteinase K solution containing 100 mM Tris-HCl, pH 8,5; 5 mM EDTA; 0,2% SDS; 200 mM NaCl; 200 mg/ml proteinase K. Tail DNA was precipitated by mixing with an equal volume of isopropanol at room temperature and then "fished" with a needle, transferred in 200 µl of TE buffer and solved for 30 minutes at 65^0C. 10-20 µl of tail DNA was digested with 30-40 U of EcoRI restriction enzyme overnight at 37^0C.

DNA products of digestion were separated using 1% agarose gels and TAE electrophoresis buffer, then according to a protocol of alkaline capillary Southern blotting was performed (Sambrook and Russel, 2001). To facilitate the transfer of big DNA fragments, the gels were treated for 15 minutes at room temperature with 0,25% HCl solution (depurination of DNA) and washed with water for 10 minutes. The transfer was carried out in alkaline 0,4 M NaOH solution overnight. The membranes were then washed in 2 x SSC and dried.

DNA transferred to nylon membranes was hybridized with the radioactively [α-^{32}P] dCTP labelled using multiprime protocol DNA probes (Feinberg and Vogelstein, 1983). 50 ng of probe DNA and random hexanucleotides as primers were denaturated at 95^0C for 5 minutes, cooled down on ice, mixed with the other components of the reaction mixture (buffer, dNTPs, BSA, Klenow enzyme, 50 µCi [α-^{32}P] dCTP) and incubated for 2 hours at 37^0C.

Before hybridization the probe was purified from not incorporated radioactive nucleotides using NAP-5^{TM} columns. Hybridization was performed at 65^0C in Church and Gilbert hybridisation buffer (0,5M NaH_2PO_4; 1 mM EDTA; 7% SDS) containing 0,1 mg/ml denaturated herring sperm DNA. After hybridization the membranes were washed 3 times at 65^0C in a solution containing 40 mM NaH_2PO_4; 1% SDS. Radioactive bands were visualized using X-ray films. Hybridisation with the probe containing a part of the pax7 cDNA and described by Masouri et al. results in appearance of 5,4 kb wild type band and 4,9 kb mutant band (Mansouri et al., 1996b).

5.2.4. Total RNA isolation from mouse tissues

Total RNA was isolated according to the protocol of Invitrogene for Trizol reagent. RNase free reagents and sterile, disposable plastic ware were used for isolation. Working parts of the homogenizer were treated with 0,5 M NaOH for 15-30 minutes at room

temperature and rinsed with RNase-free water before RNA isolation to avoid RNase contamination. Muscle tissues were homogenized in Trizol reagent (1 ml of Trizol reagent per 100 mg of tissue) at 0^0C (on ice) using an Ultra Turrax homogenizer (IKA Works, Wilmington, USA). The suspensions were centrifuged for 10 minutes at 12000 g at $+4^0$C to separate fat and insoluble material from liquid RNA containing phase. Liquid phase was transferred to a fresh tube and incubated 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Then 0,2 ml of chloroform was added per 1 ml of initially added Trizol reagent, tubes were mixed vigorously by hand, incubated for 3 minutes at room temperature and centrifuged at 12000 g for 15 minutes at $+4^0$C. Following centrifugation, the mixture separates into a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase. Since RNA remains exclusively in the aqueous phase, this phase was transferred to a fresh tube and RNA was precipitated with 0,5 ml of isopropanol per 1 ml of initially added Trizol reagent. Samples were incubated for 10 minutes at room temperature and centrifuged at 12000g for 10 minutes at $+4^0$C. Invisible gel-like RNA pellets were washed with 75% ethanol, briefly dried and solved in RNase-free water.

5.2.5. Quantitative Real Time PCR

Quantitative Real Time PCR (Bustin, 2000) was used estimate differences in expression levels of various myogenic transcription factors in muscle tissues of mutant mice in comparison to wild type mice. Real time PCR uses commercially available fluorescence-detecting thermocyclers to amplify specific nucleic acid sequences and measure their concentration simultaneously. There is no need to withdraw aliquots during the reaction or to process them. The instrument plots the rate of accumulation of amplified DNA over the course of an entire PCR. The greater the initial concentration of target DNA in the reaction mixture, the fewer the number of cycles required to achieve a particular yield of amplified product. The initial concentration of target sequences can therefore be expected as the fractional cycle number (Ct) required to reach a present threshold of amplification. A plot of Ct against the $\log_{10}$ of the initial copy number of a set of standard DNAs yields a straight line. The target sequences in an unknown sample may be easily quantified by interpolation into this standard curve. Unlike other forms of quantitative PCR, internal standards are not required in real time PCR.

The real time PCR instruments measure the enhanced fluorescence by dyes that intercalate into, or bind to the grooves of double-stranded DNA. The yield of amplified DNA may be estimated at any point during exponential phase of a PCR from the amount of

fluorescence emitted by dyes such as ethidium bromide, SYBR Green or oxazole derivatives. DNA-binding dyes allow detection of any double-stranded DNA generated during PCR, independent of the template and primers used in the reaction.

As RNA cannot serve as a template for PCR, PCR quantification technically demands cloning steps (to generate templates for standard curves) and generation of complementary DNA (cDNA). The first step in a real time PCR assay is the reverse transcription of the RNA template into cDNA pool, followed by its exponential amplification in PCR reaction. The application of fluorescence techniques to the RT-PCR, together with automatic instrumentation allow to combine amplification, detection and quantification. In the present work SYBR Green fluorescent dye was used for the detection step. The unbound dye exhibits little fluorescence in solution, but during elongation increasing amounts of dye bind to the nascent double-stranded DNA.

A standard curve using fluorescence is easily generated, thanks to the linear response over a large dynamic range. A relative standard consists of a sample, the calibrator, which is used to create a dilution series with arbitrary units. In the present work plasmids containing insertions of cDNA sequences of interest (a gene which expression is to quantify) were used as calibrator sequences to prepare 1:10 dilution series and to generate standard curves.

RNA used for quantitative RT-PCR was treated with RQ1 RNase-free DNase to remove the rest of DNA from samples. 1 unit of DNase was added per 1 μg of RNA. The reaction was performed in the optimal for the enzyme conditions recommended by Promega (using RQ1 RNase-free DNase reaction buffer and nuclease-free water) for 30 minutes at 37^0C and stopped by adding RQ1 DNase stop solution and incubation for 10 minutes at 65^0C. After DNase treatment RNA samples were deproteinized using water saturated acidic phenol, pH4-4,5. Equal volume of phenol was added to RNA solution, the mixture was mixed vigorously by hand, centrifuged at 12000 g for 5 minutes to separate phases, RNA containing water phase was transferred to a fresh tube. RNA was precipitated by mixing samples with LiCl added at final concentration 0,8 M, 2,5 volumes of 100% nuclease free ethanol and incubation for 1 hour at -80^0C.The samples were centrifuged for 10 minutes at $+4^0$C at 12000 g, the RNA pellets washed twice with 75% ethanol, dried at room temperature and solved in RNase-free water.

DNase treated RNA samples were used as templates for first-strand cDNA synthesis performed according to the protocol of Boeringer (Mannheim) for the Expand Reverse Transcriptase. On the first step of the reaction 1 μg of RNA was mixed with 50 pmoles of random hexanucleotide primer, denaturated for 10 minutes at 70^0C and cooled down on ice.

Then the other components of the reaction mixture were added: Expand reverse transcriptase buffer, DTT at final concentration 10 mM, a mixture of dNTPs (dCTP, dATP, dGTP, dTTP) at 1mM final concentration of each, 20 units of RNasin Plus RNase inhibitor (Promega), 50-100 units of Expand Reverse Transcriptase and RNase-free water up to the final volume 20 µl of the reaction mixture. The mixtures were first incubated for 10 minutes at 25^0C then for 50 minutes at 42^0C to allow random primer annealing and first-strand cDNA synthesis. When the reaction was finished the reverse transcriptase was inactivated by heating the samples at 70^0C for 15 minutes. As an optional step the samples were cooled down on ice and incubated with 1,5 units of RNaseH for 20 minutes at 37^0C to remove RNA from DNA-RNA hybrids. cDNAs resulted from such reactions were frozen for storage or used directly as templates for quantitative PCR.

Quantitative PCR was performed using the iCycler iQ Multi Colour Real Time PCR machine (BioRad Laboratories, Munich) in 25µl of reaction mix containing 1 x TaqPol buffer (Eppendorf), 1 x Enhancer (Eppendorf), 1,5 mM $MgCl_2$, deoxynucleoside triphosphates at 200 µM each, primers at 100-600 nM each, *Taq* polymerase (Eppendorf) at 0.05 U/µl, 1 µl of cDNA, 0.5 µl of SYBR green (at a 1:1000 dilution of original stock), fluorescein at 10 nM. Relative quantification of MyoD, Myf5, MRF4, myogenin and Pax3 expression was done by using starting quantity parameter.

For each experiment the amounts of targets and endogenous constitutively expressed housekeeping reference gene glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) was determined from the standard curve. The target values (starting quantities) were normalized to the endogenous reference: SQ_{target}/SQ_{GAPDH}.

5.2.6. Northern Blot analysis

Northern blot analysis was performed according to the protocol recommended by Sambrook and Russel (Sambrook and Russel, 2001) and used to estimate differences in expression levels of various muscle-specific contractile proteins. The method includes isolation of intact total RNA, separation of RNA according to size through a denaturing agarose gel, transfer of RNA to the solid matrix, hybridization of the immobilized RNA to probes complementary to the sequence of interest, removal of probe molecules that are non-specifically bound to the solid matrix and finally detection, capture, analysis of an image of the specifically bound probe molecules.

Total RNA isolated from mouse hind limb muscles was denaturated with glyoxal and separated according to size through an agarose gel. Equal amounts of all RNA samples were

loaded into each line of the gel and final signals were normalized according to their content of GAPDH reference mRNA. In general the procedure of RNA separation was the following. 50 µg of RNA was mixed 50 µl of glyoxal mixture denaturated for 10 minutes at 65^0C, cooled down on ice, mixed further with 12 µl of RNA loading buffer and loaded immediately into the wells of 1,2% agarose gel, prepared with 1 x BPTE electrophoresis buffer and without ethidium bromide. The electrophoresis was carried out in 1 x BPTE electrophoresis buffer at 5 V/cm. Capillary transfer of RNA at alkaline pH was performed to nylon Biodyne B membranes. 0,01 M NaOH/3 M NaCl solution was used for the overnight transfer. The membranes were washed then for 5 minutes in 6 x SSC, pH 7,2, dried, stained in 0,02% methylene blue/0,3 M sodium acetate (pH 5,5) to visualize RNA on a membrane and distained with 0,2 x SSC/1% SDS.

RNA blots were hybridized with radioactively labelled probes as it is described in "Mouse genotyping by Southern blot analysis". Probe removal for re-hybridization was made by boiling of the blots in 0,1% aqueous SDS for a few minutes.

5.2.7. Electron microscopy of muscles

Electron microscopy analysis of mouse muscles was performed in collaboration with Dr. Gerd Hause, Biocenter, Microscopy Unit, University of Halle-Wittenberg.

Small pieces of muscle tissues (about 1-2 mm) were prepared using fine blades and fixed directly after preparation for 3 hours in 3% glutaraldehyde/0,1 M sodium cacodylate buffer at room temperature. The fixative was removed by 6 times washing in 0,1 M sodium cacodylate buffer. Then the tissues were incubated for 1 hour with 1% osmium tetroxid/0,1 M cacodylate buffer, washed 3 times 10 minutes in water, dehydrated for 30 minutes in serial ethanol dilution 10%, 30%, 50% with following incubation in 1% uranyl acetate/70% ethanol for 1 hour. Uranil acetate was removed by washing in 70% ethanol overnight. Further dehydration of sections was performed in 70%, 90% and twice in 100% ethanol for 30 minutes in each solution. After dehydration the samples were infiltrated with Epoxidharz, ethanol:SPURR=3:1 for 3 hours; Epoxidharz, EtOH:SPURR = 1:1 for 4 hours, overnight with Epoxidharz; EtOH:SPURR = 1:3 and twice 8 hours in pure SPURR, which polymerizes finally. The blocks were sectioned with an ultramicrotome. The sections were placed on copper grids. Satellite cells and myonuclei in randomly chosen fields were counted.

5.2.8. Embedding tissues in freezing medium and cryotome sections

Muscle tissues for immunohistochemical staining were fixed in 4% PFA/PBS overnight at $+4^0$C, washed with PBS buffer, incubated 2-10 hours at $+4^0$C in 30% saccharose/PBS solution and briefly rinsed with PBS. Then tissue samples were put in small plastic boxes containing polyfreeze tissue freezing medium and immersed in liquid nitrogen cooled isopentan. Muscles used for further LacZ-staining were frozen in polyfreeze medium without fixation and saccharose treatment. The blocks were stored at -20^0C in plastic bags and sectioned at -20^0C using a cryotome (Leica, Germany). The cryosections were collected on Vectabond-coated glass slides, dried at room temperature and stored at -20^0C.

5.2.9. Embedding tissues in paraffin and preparation of paraffin sections

Muscle tissues after preparation were washed in PBS buffer and fixed overnight at $+4^0$C in 4% PFA. After fixation the fixative was removed by washing in PBS buffer and the tissues were dehydrated by incubation for 60 minutes in serial ethanol dilutions 25%, 50%, 75%, 96%, twice in 100%. Then the tissues were infiltrated at room temperature for 1 hour twice with xylene, for 1 hour with a mixture of equal volumes of xylene/paraffin at 60^0C, twice with melted paraffin for 1 hour at 60^0C. After infiltration the tissues were put in prewarmed plastic boxes and embedded in melted at 60^0C paraffin. The blocks were kept at room temperature overnight to let them cool down and stored at $+4^0$C or used to prepare 10 μm sections with a microtome (Leica, Germany). The sections were attached to glasses coated with Vectabond solution. The procedure of coating glasses included washing with acetone for 5 minutes, incubation with 7 ml of Vectabond solution in 343 ml of acetone, short washing in distilled water and drying.

5.2.10. LacZ-staining

Single myotube cultures or 10 μM muscle cryosections (muscles frozen without fixation) were fixed for 5 minutes at room temperature in 0.2% glutaraldehyde (in PBS buffer with 5 mM EGTA and 2 mM $MgCl_2$). Then washed 3 times 10 minutes in PBS buffer containing 0.01% Na desoxycholate, 0.02% Nonidet P40, 5 mM EGTA and 2 mM $MgCl_2$. After washing samples were incubated 3-5 days at 37^0 in PBS buffer containing 0.01% Na desoxycholate, 0.02% Nonidet P40, 5 mM EGTA and 2 mM $MgCl_2$, 10 mM $K_3[Fe(CN)_6]$, 10 mM $K_4[Fe(CN)_6]$ and 1,5-2 mg/ml X-gal (3-4 x more than usually is used for staining). Staining reaction was stopped by washing in PBS buffer. Cell nuclei were counterstained with

5 µg/ml solution of Hoechst 33258 dye in PBS buffer (stained nuclei have a blue fluorescence when exposed to UV light).

5.2.11. Immunohistochemical staining with antibodies

Unless otherwise stated all the procedures for immunostainings were performed at room temperature. Single myotube cultures, cells in monolayer or 10 µM muscle cryosections were fixed in 4% PFA for 10 minutes, and then washed 3 times with PBS buffer. Endogenous peroxidase activity was reduced by incubation with 0,5% H_2O_2 in PBS buffer for 10 minutes. After that samples were washed 3 times in PBS buffer containing 0,3% Triton X-100 and incubated with blocking solution (0,3% Triton X-100; 1% BSA; 5% of a serum from an animal, in which the secondary antibody was made) for 1 hour. After blocking step samples were incubated overnight at $+4^0$C with a specific primary antibody diluted in blocking solution. On the next day samples were washed 3 times in PBS containing 0,1% Triton X-100 and 0,3% BSA (day 2 buffer) and incubated for 1 hour with a secondary antibody diluted in day 2 buffer. If a secondary antibody was fluorochrome coupled, direct fluorescent detection was performed after this step. If further amplification of signal was needed a biotinilated secondary antibody was used, with the following amplification and detection. Samples were washed 3 times in day 2 buffer, incubated for 1 hour with ABC-solution containing avidine-coupled horse radish peroxidase (ABC-kit, Vector Laboratories, CA, USA) and washed again. During incubation the biotin-coupled antibody makes avidine-biotin-horseradish peroxidase complexes, allowing further detection of the complexes using colour reaction with diaminobenzidin (DAB) substrate. 10 ml of the final detection solution contained 1 mg/ml DAB; 0,1 M Tris-HCl, pH 7,2; 5 µl of 30% H_2O_2. The reaction is performed in darkness for 5-10 minutes and leads to the formation of brown (or black if 50 µl of 8% NiCl is added) precipitate. To stop the reaction samples were washed in PBS buffer. Nuclei were counterstained by incubation with 5 µg/ml solution of Hoechst dye. Sections were covered with glass slides using Mowiol solution for mounting.

5.2.12. Haematoxylin-eosin staining of paraffin sections

Paraffin sections were deparaffinized before staining 2 times for 5 minutes in xylen, then washed twice from xylen with 96% ethanol, re-hydrated by incubation for 5 minutes in serial ethanol dilutions (two times in every solution) 100%, 75%, 50%, 25%, finally in PBS buffer. Then the sections were washed several times with distilled water and stained for 10 minutes in haematoxylin solution (Merck, Germany). After 10 minutes under running tap

water the access colour was removed for 1 second in 1% HCl in 70% ethanol with following washing for 10 minutes under running tap water. After that sections were stained in 1% eosin/70% ethanol solution for 5-10 minutes, washed again and finally mounted using Mowiol solution.

5.2.13. Regeneration assay

Before introduction of muscle injury mice were anaesthetized intraperitoneally with 2,2,2-tribromoethanol. The stock solution of 1 g/ml was made in isobutane. The working solution for injections contained 120 µl of the stock solution in 10 ml of sterile PBS buffer (12 mg/ml). To anaesthetize mice 25 µl of the working solution per 1 g of body weight was injected intraperitoneally using a 27-gauge needle.

To introduce a muscle injury 50 µl of 0,06% cardiotoxin from *Naja mossambica mossambica* (Sigma, Germany, catalogue number C-9759) in PBS buffer was injected into the M. tibialis anterior of adult (1-2 months old) mice using a 27-gauge needle and a 1 ml syringe. The needle was inserted deep into the muscle longitudinally towards the knee from the ankle. The anterior tibial muscles were isolated 1, 4, 10 and 28 days after the injection, embedded either in Polyfreeze tissue freezing medium without fixation (for the further sectioning and LacZ staining) or fixed overnight in 4% PFA and embedded in paraffin. Serial transversal 10 µm sections were stained with haematoxylin and eosin.

5.2.14. Preparation of satellite cell mass cultures

Primary myoblasts (satellite cells) were prepared according to recommendations of Bischoff (Bischoff, 1974) with modifications using muscles of both hind limbs of a mouse. Muscles was minced using scissors and then incubated 1 hour at 37^0C with 10 ml of 1,25 mg/ml protease from Streptomyces griseus (Sigma, catalogue number P 8811) in PBS. After incubation the muscles were triturated 50 times with a glass pipette to release satellite cells attached to muscle fibers and the digestion was stopped by adding 10-20 ml of serum containing medium (normal medium which was used further for the myoblasts cultivation). The suspension was filtered through a 70 µm nylon cell strainer (BD Labware, catalogue number 352350) to remove muscle debris. After filtration the cell suspension was concentrated in 1 ml PBS (centrifuged for 5 minutes at 200g) and satellite cells were either plated on collagen coated dishes or purified before plating from non-myogenic cells using a Percoll gradient centrifugation (Percoll, Amersham Biosciences, catalogue number 17089102). Discontinuous gradients contained 35%, 50% and 70% Percoll in PBS and

50%/70% interphase which contained almost pure satellite cells was collected. Collected cell suspension was washed with PBS, re-suspended in proliferation medium (DMEM with 20% foetal calf serum, 10% horse serum, 1% chick embryo extract and 1 x penicillin/streptomycin mix), plated on a collagen coated 3,5 cm dishes and incubated at 37^0C in an atmosphere of 10% CO_2. The medium was first changed after 2 days after plating and then changed daily and split normally once a week. To promote differentiation of myogenic cells DMEM medium containing 2% FCS and 1 x penicillin/streptomycin mix was used.

5.2.15. Preparation of single myotubes cultures

Cultures of isolated single myotubes were prepared according to the method published by Rosenblatt et al. (Rosenblatt et al., 1995) with modifications using Mm. interossei which is a small muscle situated between mouse toes. The muscle has been chosen for the preparation because its myotubes are short, uniform in length and easy to isolate. Before muscle preparation mice were soaked in 70% ethanol for 5 minutes and then washed carefully with sterile water to remove ethanol from the skin. Isolated under sterile conditions Mm. interossei were incubated with 0,2% collagenase P (Roche, catalogue number 1213873) in DMEM medium for 2 hours at 37^0C. After incubation muscles were transferred to 1 ml of the serum containing medium (DMEM with 10% FCS and penicillin/streptomycin) to stop the digestion and then triturated 40-60 times with a glass Pasteur capillary to release single myotubes. Single myotubes were attached to glass cover slides using Matrigel basement membrane matrix (BD Biosciences, catalogue number 354234) and fixed for further staining. Aliquots of Matrigel were stored frozen at -20^0C and defrozen on the day of use on ice according to the recommendations of BD Biosciences. Culture plates with glass slides were kept on ice. The ready-to-use Matrigel solution was spread with a sterile cell scraper on a surface of an ice-cold glass slide (30-40 µl per 24 mm in diameter round glass slide). A few drops of isolated myofiber suspension were added on the top of the Matrigel coated slides. Culture plates were incubating for 30 minutes at room temperature to gel the Matrigel with myotubes and then PBS buffer or proliferation medium was added.

6. ABBREVIATIONS

bHLH	basic helix-loop-helix
Bis-Tris	bis[2-hyroxyethyl]iminotris[hydrohymethil]methan
BMP	bone morphogenic protein
Bp	base pair
BSA	bovine serum albumin
DAB	3,3´-diaminobenzidine
DEPC	diethylpyrocarbonate
DMEM	Dulbecco´s modified Eagle medium
DMSO	dimethylsulfoxide
DTT	dithiothreitol
E	ectoderm (abbreviations in the figures)
E	embryonic day *post coitum*
EDTA	ethylenediamine tetraacetic acid
EM	electron microscopy
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
HE staining	haematoxylin and eosin staining
HGF	hepatocytes growth factor
HSC	hematopoetic stem cells
IRF	interferon regulatory factor
LacZ	ß-galactosidase
LP	lateral plate
M.	muscle (lat.)
mdr	multi drug resistence
MLC	myosin light chain
MNF	myocyte nuclear factor
Mm.	muscles (lat.)
MRF	myogenic regulatory factor

mRNA	messenger RNA
MyHC	myosin heavy chain
NC	notochord
NCAM	neural cell adhesion molecule
NT	neural tube
P	days after birth
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PIPES	piperazine-1,4-bis[2-ethanesulfonic acid])
RT PCR	reverse transcriptase polymerase chain reaction
SC	sclerotome
SCID mice	severe combined immunodeficient mice
SDS	sodium dodecyl sulphate
SE	surface ectoderm
Shh	sonic hedgehog
SP	side population
TAE	Tris-acetate buffer
TGF	transforming growth factor
UV	ultra violet
VCAM	vascular cell adhesion molecule
Wnt	wingless and intergated factor
Wt	wild type
X-gal	5-Brom-4-chlor-3-indolyl-b-D-galactosid

7. CURRICULUM VITAE

Personal data

Name	Svetlana Ustanina (Oustanina in French transcription)
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1997-1999	Master of Sciences Degree, Honours Degree in Biology, Pushchino State University, Russia Thesis topic: Investigation of the role of the site-specific endonuclease F-Tfl I encoded by bacteriophage T5 in exchanging genetic information in T5-like phages.
1999-2000	PhD student at Pushchino State University, the study is not finished
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8. PUBLICATIONS AND PRESENTATIONS

Published

Neuhaus,P., Oustanina,S., Loch,T., Kruger,M., Bober,E., Dono,R., Zeller,R., and Braun,T. (2003). Reduced mobility of fibroblast growth factor (FGF)-deficient myoblasts might contribute to dystrophic changes in the musculature of FGF2/FGF6/mdx triple-mutant mice. Mol. Cell Biol. *23*, 6037-6048.

Oustanina,S., Hause,G., and Braun,T. (2004). Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. EMBO J. *23*, 3430-3439.

Posters

Oustanina,S., Hause,G., and Braun,T. Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. ELSO congress in Nice, September 2004

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10. ERKLÄRUNG

Hiermit erkläre ich, daß ich mich mit der vorliegenden wissenschaftlichen Arbeit erstmals um die Erlangung des Doktorgrades bewerbe, die Arbeit selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt und die den benutzen Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Halle/Saale, November 2004 Svetlana Ustanina

11. ZUSAMMENFASSUNG

Das Ziel der Arbeit war die Untersuchung der Rolle des myogenen Transkriptionsfaktors Pax7 bei der Spezifizierung und Aufrechterhaltung von Muskelsatellitenzellen. Da Satellitenzellen größtenteils für das postnatale Wachstum und die Regenerierung von Skelettmuskeln der Säugetiere verantwortlich sind, war diese Arbeit ein Versuch, die Rolle von Pax7 in diesen zwei wichtigen Prozessen der Entwicklung und Homöostase der Skelettmuskulatur zu verstehen. Ein weiteres Anliegen war die Bestimmung der Position von Pax7 in der komplizierten Signalkaskade, welche die Spezifizierung und Differenzierung der Muskulatur in Säugetieren reguliert.

In der Arbeit wurde ein weites Spektrum biologischer Methoden angewendet: allgemeine molekularbiologische Methoden (zum Beispiel DNA- und RNA-Isolierung, Klonierung, Southern- und Northernanalyse, Real Time PCR), histochemische Techniken (Paraffin- und Kryotomschnitte, histochemische und immunhistochemische Färbung der Gewebeschnitte und Zellkulturen) Elektronenmikroskopie und Zellkulturtechniken.

Die vorliegende Analyse von Satellitenzellen in homozygoten Pax7 Mäusen hat ergab, dass Pax7 für die Spezifizierung der Satellitenzelllinie nicht notwendig ist, da unter Verwendung fünf verschiedener Methoden (Elektronenmikroskopie, Pax7-LacZ-Färbung, CD34-Färbung, in vitro Kultivierung von Satellitenzellen aus isolierten Muskelfasern und Analyse von Satellitenzellklonen) eine große Anzahl von Satellitenzellen in juvenilen homozygoten Pax7 Mäusen gefunden wurde. Stattdessen fanden sich eine kontinuierliche Verringerung der Anzahl der Satellitenzellen bei der postnataler Entwicklung von homozygoten Pax7 Mutanten, was stark darauf hindeutet, dass Pax7 eine Rolle bei der Erneuerung und Verbreitung der Satellitenzellen spielt. Außerdem fanden sich eine Wesentlichen normale Muskulatur in erwachsenen Pax7 Tieren, welche nicht ohne Beitrag der hochproliferierten Satellitenzellpopulation zum Wachstum der unreifen Muskeln erklärt werden kann. Zudem zeigte sich keine signifikante Reduzierung der Anzahl und Größe der Myotuben in juvenilen und erwachsenen homozygoten Pax7 Mäusen, was darauf hindeutet, dass die verbliebene Zahl der Satellitenzellen ausreicht, um ein normales Muskelwachstum zu erlauben. Überdies besitzen erwachsene Pax7 Mäuse noch ein gewisses Potential zur Skelettmuskelregenerierung, obwohl die Effizienz der Regeneration stark eingeschränkt ist. Die Muskelregeneration in Pax7 Mäusen geht einher mit der Verbreitung der verbliebenen Pax7-LacZ Satellitenzellen und resultiert in zahlreichen regenerierten Myotuben, obwohl eine unvollständige, defekte Regeneration deutlich ist und eine vollständige Reparatur nie erreicht wurde.

Es ist von großer Bedeutung, die Mechanismen und Abläufe zu verstehen, die die Muskelregeneration regulieren, um Therapien und klinische Methoden zu entwickeln, die zu einer verbesserten Behandlung von Muskelerkrankungen führen. In der vorliegenden Studie wurde die Funktion von Pax7 als bedeutender Regulator der Satellitenzellerneuerung und des Wachstums neu definiert. Dieses Wissen wird helfen, die Verfügbarkeit und den Ersatz von Muskelstammzellen für therapeutische Zwecke, besonders im alten und kranken Muskel zu verbessern.

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