UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di Ricerca in Scienze delle Attività Motorie, Coordinatore Prof. Giovanni Zummo

Dipartimento di Biomedicina Sperimentale e Neuroscienze cliniche (BioNec)
Sezione di Fisiologia Umana (SSD BIO/09)

Skeletal muscle regeneration in the adult mouse and rat: study on connexin expression and role in normal and regenerating skeletal muscle and on low-intensity endurance exercise effect in damaged skeletal muscles

Dott.ssa Monica Frinchi

Coordinatore: Prof. G.Zummo Tutor: Prof. N.Belluardo

XXII Ciclo triennale

Cover figure: Schematic representation of skeletal muscle regeneration following a crush injury, showing the potential role of cell expressing Cx39, Cx40, Cx43, and Cx45 during satellite cell activation, proliferation, their differentiation in myoblasts and fusion to form new myotube.

TABLE OF CONTENTS

PAPER INCLUDED IN THIS THESIS

1. Regulation of connexin gene expression during skeletal muscle regeneration in the adult rat.

   Trovato-Salinaro A, Belluardo N, **Frinchi M**, von Maltzahn J, Willecke K, Condorelli DF, Mudò G.

   Am J Physiol Cell Physiol. 2009 Mar;296(3):C593-606. Epub 2009 Jan 7

2. Recovery of damaged skeletal muscle in *mdx* mice by low-intensity endurance exercise

   Frinchi M, Macaluso F, Licciardi A , Mudò G, Morici G, Belluardo N.

   Muscle & Nerve. Manuscript submitted

SUMMARY

The first aim of present work was to perform a comparative spatial and temporal analysis of connexin (Cx) Cx37, Cx39, Cx40, Cx43, and Cx45 expression in developing skeletal muscle and in the adult regenerating skeletal muscle in response to crush injury. Among the Cxs examined, only the Cx39, Cx43 and Cx45 were found expressed during embryonic life and progressively reduced during early postnatal life to become dramatically expressed at very low levels like Cx43 and Cx45, or to be undetectable like Cx39 in the adult muscle. Cx37 and Cx40 were found expressed at low levels and were localized in the endothelial cells. In the adult skeletal muscle, various kinds of trauma promote proliferation of satellite cells that differentiate into myoblasts forming new myofibers, or to repair the damaged one. Within 24h from injury, Cx37 expression was upregulated in the endothelial cells of blood vessels, and, 5 days after injury, Cx37-expressing cells were found inside the area of lesion and formed clusters generating new blood vessels with endothelial cells expressing Cx37. Three days after injury, Cx39 mRNA was selectively expressed in myogenin-positive cells, forming rows of closely apposed cell nuclei fusing in myotubes. Cx40 mRNA-labeled cells were observed within 24h from injury in the endothelium of blood vessels, and, 5 days after lesion, Cx40-labeled cells were found inside the area of lesion-forming rows of myogenin-positive, closely apposed cells coexpressing Cx39. Within 24h from lesion, both Cx43 and Cx45 mRNAs were upregulated in individual cells, and some of them were positive for M-cadherin. Three days after injury, a large number of both Cx43 and Cx45 mRNA-labeled and myogenin-positive cells were found inside the area of lesion.

Taken together, these results show that at least four Cxs, out of five expressed in regenerating skeletal muscle, can be differentially involved in communication of myogenic cells during the process of cell proliferation, aggregation, and fusion to form new myotubes or to repair damaged myofibers.

The second aim of this study was to examine how low-intensity endurance exercise affects the regeneration process in dystrophin-deficient skeletal muscle. The lack of dystrophin in *mdx* mice, an animal model for Duchenne muscular dystrophy, leads to cycles of muscle degeneration and regeneration processes. Male adult *mdx* and wild type mice were subjected to low-intensity endurance exercise by running on a motorized Rota-Rod for 5

days/week, for 4 weeks at progressively increasing loads. Exercised *mdx* mice showed a trend to lower body weight gain and positive effect on the degree of fatigue. Histomorphological analysis showed a significant reduction of both muscle necrosis foci and regeneration processes in the gastrocnemius and quadriceps muscles of exercised *mdx* mice. The reduction of regeneration process was also evaluated by examining the protein expression of Cx39, as a specific gene expressed during regeneration process of injured muscles. While Cx39 was not expressed both in wild type exercised nor in sedentary mice, it was markedly increased in sedentary *mdx* mice, because of active degeneration/regenerating process, and dropped to very low levels in exercised *mdx* mice, suggesting a reduction of muscle regeneration process.

This study has shown that specific low intensity endurance exercise induces a strong beneficial effect on the regeneration of dystrophic muscle and may have therapeutic value at least to decrease progression of muscular dystrophy and in less extend for strengthening dystrophic muscle.

BACKGROUND

Skeletal muscle Development

All the skeletal muscle of the body and the limbs derive from paraxial mesoderm which segments into somites on either side of the neural tube and notochord. The ventral part of the somite, the sclerotome, will contribute the cartilage and bone of the vertebral column and ribs, whereas the dorsal part of the somite, the dermomyotome, give rise to the overlying derm of the back and to the skeletal muscles. Early myogenesis is governed by a complex set of morphological and migratory events which begin with the colonization of the medial myotome by cells originating from the dorsomedial lip of the dermomyotome (Ordahl and Le Douarin 1992; Denetclaw, Jr. *et al.* 1997).

A number of homeobox genes are implicated in the survival, delamination and migration of muscle progenitor cells from the hypaxial dermomyotome to sites of muscle formation elsewhere in the body and limbs. *Lbx1* is one of genes expressed in migrating cells and in the hypaxial myotome of somites. In mice lacking *Lbx1* gene, cells fail to migrate correctly, as evidenced by accumulation beside the somite at the hindlimb level, mislocation of ventral muscle cells to dorsal muscles at the forelimb level, and retarded migration/differentiation in the hypoglossal chord. The *c-Met* receptor tyrosine kinase and its ligand hepatocyte growth factor (HGF) are essential for the delamination of cells from the dermomyotome at all axial levels and are potentially important also for guiding migration. In their absence, infact, migrating muscle progenitors and the muscles form are absent (Birchmeier and Brohmann 2000). *Pax3*, a member of *Pax* gene family, is an other important regulator of myogenesis. *Pax3* have a crucial role in the development of the hypaxial musculature and the long range migration of muscle precursor (Tremblay *et al.* 1998). It also plays a key role in the establishment/survival of cells in the hypaxial dermomyotome, and in the delamination of myogenic progenitors (Tajbakhsh and Buckingham 2000). Moreover *Pax3* is necessary for *MyoD* activation, a member of *MyoD* family of helix-loop-helix transcription factors. This family includes four different members: *MyoD*, *Myf5*, myogenin and the myogenic regulatory factor-4 (*MRF4*) which are expressed

in a hierarchical manner during the myogenesis process. *Myf5,* like *MyoD,* is expressed in the early stage, and it is implicated in the establishment and maintenance of muscle progenitor lineages. Newborn mice mutant for both *MyoD* and *Myf5*, infact, lack skeletal muscle and are devoid of myoblasts (Rudnicki *et al.* 1993). Studies on cultured cells and mutant mouse embryos have demonstrated that *Myf5* and *MyoD* are involved in determining skeletal muscle cell fate with a potential role in remodeling chromatin and regulating the crucial balance between cell proliferation and differentiation. For example, it has been shown an interaction between *MyoD* and the histone transacetylases pCAF and CBP/p300 which function as transcriptional coactivators. Furthermore, acetylation of *MyoD* by pCAF (Sartorelli *et al.* 1999) or p300 (Polesskaya *et al.* 2000) is necessary for myogenic conversion and increases its affinity for muscle specific promoters, hence promoting myogenic differentiation. In contrast, interaction between histone deacetylase *HDACI* and *MyoD* prevents premature activation of the myogenic programme in dividing myoblasts (Mal *et al.* 2001).

As well as *Pax3,* also *Wnt* family members, that are expressed in the neural tube and dorsal ectoderm, synergize with Sonic hedgehog (Shh), produced by the notochord and floorplate, and induces somatic expression of *MyoD* and *Myf5* (Munsterberg *et al.* 1995; Munsterberg and Lassar 1995; Stern and Hauschka 1995; Pownall *et al.* 1996). This suggest that initially precursor cells responding to environmental signal will activate, independently, one or the other gene and that only in the second time the majority of myogenic cells can express either *MyoD* and *Myf5*.

As above mentioned, other two important members of *MyoD* family are Myogenin and *MRF4*, which are required for normal biochemical and morphological differentiation of skeletal muscle, but are not required for commitment of cells to the myogenic lineage (Hasty *et al.* 1993; Nabeshima *et al.* 1993; Olson *et al.* 1996). In the late stage of myogenesis, infact, proliferating myoblasts withdraw from the cell cycle to become terminally differentiated myocytes and express *MRF4*. Myogenin and *MRF4*, and subsequently muscle-specific genes such as myosin heavy chain (MHC) and muscle creatine kinase (MCK). Myogenin-deficient embryos die perinatally, due to a deficit in myoblast differentiation as evidenced by an almost total absence of myofibers in these mutants (Hasty *et al.* 1993; Nabeshima *et al.* 1993). Similarly, *MRF4*-deficient mice display a range of phenotypes consistent with a late role for *MRF4* in the myogenic pathway (Patapoutian *et al.* 1995; Rawls *et al.* 1995; Zhang *et al.* 1995; Yoon *et al.* 1997). Moreover some data show that

Myogenin and *MRF4* are differentially expressed in differentiated muscles and regulate contractile protein target genes (Charbonnier *et al.* 2002; Mak *et al.* 1992; Yutzey *et al.* 1990), that include genes involved in fast and slow fiber differentiation and regeneration (Hinterberger *et al.* 1991; Jacobs-El *et al.* 1995; Nicolas *et al.* 2000).

Not only the member of *MyoD* family are responsible of the skeletal muscle development. Infact, the *MRF*s, are assisted by the myocyte enhancer factor 2 (*Mef2*) family of transcription factors in order to mediate expression of muscle-specific genes (Black and Olson 1998). *Mef2* genes are expressed widely during development. *Mef2* proteins bind to an A/T-rich DNA sequence element (C/TTA(A/T)4TAG/A) which is found in the promoters of many muscle-specific genes (Gossett *et al.* 1989). Mef2 and *MyoD* interact directly in vitro and synergistically activate transfected reporters driven by E boxes and *Mef2* binding sites (Molkentin *et al.* 1995).

Another notable family of transcription factors critical for myogenesis is the *Six* family of homeodomain proteins. There are six members of the *Six* family in mammals, each playing different roles in various developmental contexts (Kawakami *et al.* 2000). One of these members is *Six1*. Some studies had demonstrate that mice lacking *Six1* have impaired primary myogenesis and delayed expression of myogenin and *MyoD* in limb buds, indicating a positive role for *Six1* during muscle development in vivo (Laclef *et al.* 2003).

Myogenesis is also regulated by negative factors. One of these negative regulatory factor is *BMP4*, that inhibits the expression of *MyoD* in the somite. More recently, *BMP4*-mediated inhibition has been shown to be antagonized in the medial somite by Noggin, which is induced by Wnt and Shh (Hirsinger *et al.* 1997; McMahon *et al.* 1998).

All together these signals play a coordinated action that lead to the formation of skeletal muscle (Fig. 1). After the first phase of proliferation and migration, and a second phase of differentiation, finally, mononucleated myocytes specifically fuse to each other to form multinucleated syncytium, which mature into contracting muscle fibers. During the course of muscle development, a distinct subpopulation of myoblasts fails to differentiate, but remains associated with the surface of the developing myofiber as quiescent muscle satellite cells.

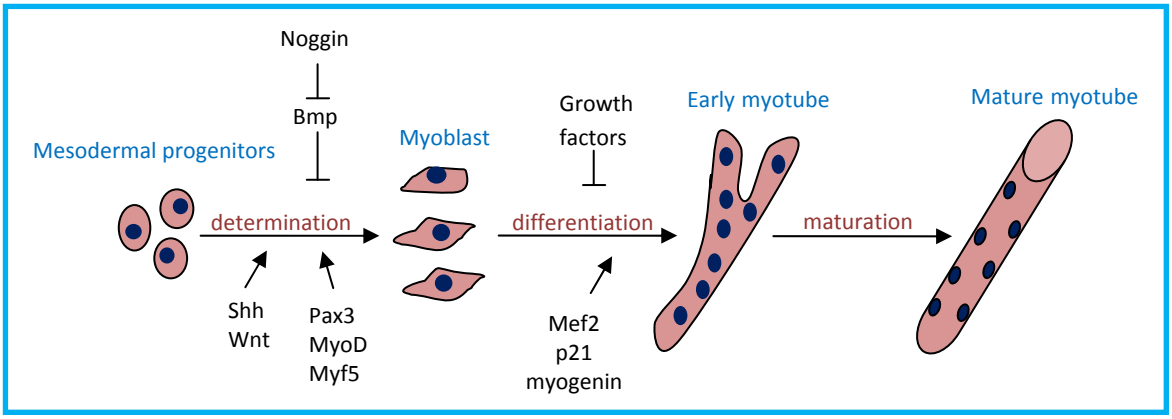

Fig.1
Schematic representation of regulatory network controlling the development of skeletal muscle.

Muscular stem cells

Almost 50 years ago, Alex Mauro observed, by electron microscopy, the presence of mononucleated cells, which he termed "satellite cells", that were intimately associated with skeletal muscle fibers of the frog (MAURO 1961), between the muscle fiber membrane and the basal lamina, which is a component of the extracellular matrix. Satellite cells are rare in uninjured muscles, typically accounting for <2% of the nuclear content of muscle, but their remarkable proliferative potential confer them the property to repair the skeletal muscular damage (Zammit *et al.* 2002). Several studies strongly suggest a correlation among satellite cells, microvasculature and endothelial cells during myogenesis. Infact, the microvasculature seems to play a major role. In humans and mice, respectively 68% and 82% of the satellite cells are located within 5mm from neighboring capillaries or vascular endothelial cells. (Christov *et al.* 2007). In addition, there is also correlation between the number of capillaries per muscle fiber and the number of satellite cells (Christov *et al.* 2007).

Satellite cells are normally quiescent, but become activated in response to injury, proliferate, differentiate, and fuse to repair or replace damaged myofibers. In these processes the stem cell niche, growth factors, cytokines, and neurotrophic factors play a prominent role. The niche is commonly defined the place where stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing (Ohlstein *et al.* 2004). It has

been shown, that mechanical, electrical and chemical signals are involved in satellite cell regulation (Charge and Rudnicki 2004; Kuang *et al.* 2008).

A growing body of evidence suggests that multiple, distinct populations of satellite cells reside in adult muscle (Bischoff 1986; Goldring *et al.* 2002; Hawke and Garry 2001; Schmalbruch and Lewis 2000; Seale and Rudnicki 2000; Zammit and Beauchamp 2001) and this existence of multiple populations of myogenic precursor cells allow muscle tissue to respond differentially to a particular stimulus, type of injury, or physiological demand, and thereby enable a highly controlled response.

Different muscles exhibit distinct characteristics, including anatomical structure, contractile and metabolic properties, fiber composition, blood supply, pattern of innervation and embryonic origin. So, they have different regenerative capacities (Pavlath *et al.* 1998) and are differentially affected in genetic disorders (Emery 2002). One difference is the number or density of the satellite cells expressed by different muscle groups. Beyond the simple property of number or density, evidence suggests that satellite cells resident in different muscle compartments are not identical but differ in terms of embryonic origin, lineage history, gene expression pattern and functional behavior in vitro and in vivo. Moreover, several differences in satellite cells associated with fast or slow fibers/muscles have been reported, including differences in the proliferation rate and differentiation capacity of their progeny in culture (Barjot *et al.* 1995; Lagord *et al.* 1998). Myoblasts from fast or slow muscles differ in the levels of expression of many genes, such as *AChE* (Barjot *et al.* 1993), *FGF*-receptors (Martelly *et al.* 2000), and *Pax7* (Brzoska *et al.* 2009).

The hypothesis that satellite cells are heterogeneous in terms of self-renewal is also supported by evidence from transplantation experiments. Only a small subpopulation of cultured myoblasts was shown to efficiently survive transplantation and contribute to regeneration, exhibiting stem cell-like properties (Beauchamp *et al.* 1999). The finding suggests that an altered environment may select specific subpopulations of satellite cells with different self-renewing ability (Heslop *et al.* 2000).

A recent research also suggests that other precursor cells might play a role in skeletal muscle regeneration (Charge and Rudnicki 2004; Peault *et al.* 2007). These cells can be divided into mesoangioblasts (vessel-associated stem cells), side population cells (SP), muscle-derived stem cells (MDSCs), pericytes, and $CD133^{+}$ stem cells (Dellavalle *et al.* 2007; Asakura *et al.* 2002; Torrente *et al.* 2004; Galvez *et al.* 2006; Peault *et al.* 2007;

Negroni *et al.* 2009), and into muscle- and non muscle-derived stem cells (Cao and Huard 2004).

Mesoangioblasts are multipotent progenitors of mesodermal tissues, physically associated with the embryonic dorsal aorta in avian and mammalian species. Early studies concerning the capacity of mesoangioblasts to differentiate in various mesodermal phenotypes qualified these progenitors as a novel class of stem cells (Cossu and Bianco 2003). Other studies show that it is possible to transplant mesoangioblasts into dystrophic dogs and obtain an extensive reconstitution of fibers expressing dystrophin, an improvement in the contraction force and, in many cases, a preservation of walking ability (Sampaolesi *et al.* 2006).

Pericytes seem to be progenitor cells for mesenchymal cells, such as osteoblasts and adipocytes, and wrap around the vascular tube and interdigitate with the endothelial cells in the basement membrane of the vessels, playing a fundamental role in the maintenance of microcirculation functionality. Some studies demonstrated that pericytes had an high capacity of myogenic differentiation because they gave rise to a high number of muscular fibres when injected into scid/*mdx* mice (Dellavalle *et al.* 2007). Moreover it has been proposed that the pericyte could be released from its position on a vascular tube in the case of a focal injury, functioning as immunomodulatory and trophic mesenchymal stem cell (Caplan 2008; Morgan and Muntoni 2007).

$CD133^+$ cells are considered to be haematopoietic and endothelial stem cells of bone marrow origin, that could give rise to both endothelial cells and myoblasts (Peault *et al.* 2007). It has been demonstrated the stemness of circulating human $CD133^+$ cells and their ability to restore dystrophin expression and eventually regenerate the satellite cells pool in dystrophic mouse after intra-muscular and intra-arterial delivery (Gavina *et al.* 2006).

SP cells in the bone marrow of adult mice are primarily $CD34^-$, and are highly enriched in their hematopoietic content (Goodell *et al.* 1997). It is possible that these cells originate from multipotent mesenchymal stem cells in the bone marrow stroma to give rise to bone, cartilage, and connective tissue (Pereira *et al.* 1995). A particular subpopulation of SP cells was found in the muscle and after transplantation, these cells were able to participate in the formation of skeletal myotubes during regeneration (Gussoni *et al.* 1999). This population of a multipotent muscle-derived stem cell (MDSC) resides within skeletal muscle and has the ability to self renew and to differentiate into other mesodermal cell types (Sarig *et al.* 2006; Tamaki *et al.* 2007). Other studies demonstrated that this population

is distinct from satellite cells (Asakura and Rudnicki 2002; Qu-Petersen *et al.* 2002) and that they can preserve their myogenic potential in vitro even after differentiation into other lineages, such as haematopoietic differentiation, if the appropriate stimulus is given (Negroni *et al.* 2006).

In summary, many different populations of stem cells might be involved in muscle regeneration, and potentially they can be used in the treatment of diseased skeletal muscle.

Connexins (CXs) and GAP junctions in skeletal muscle

Gap junction (Fig.2) are specialized membrane regions composed of aggregates of transmembrane channels that directly connect the cytoplasm of adjacent cells, and that allow intercellular movement of ions, metabolites and second messengers (Bruzzone *et al.* 1996). Each intercellular channel is formed by the conjunction of two hemichannels, or connexons, formed by exameric assembly of subunit protein, called connexins and are characterized by two distinct properties, their conductivity (i.e. permeation of small ions) and their permeability to larger molecules. Gap junction channels are gated by chemicals (including pH) and by membrane potential. Each connexin consists of four α-helical transmembrane domains, two extracellular loops, a cytoplasmic loop, a cytoplasmic aminoterminal and carboxy-terminal domains. Connexins are synthesized in the endoplasmic reticulum and oligomerize in the ER⁄Golgi or trans-Golgi network to form hexameric hemichannels or connexons (Koval 2006). Connexons are subsequently transported to the plasma membrane by vesicular carriers, travelling along microtubules. When two cells are closely apposed, connexons from one cell can dock with their counterparts in the neighboring cell and form a gap junction channel. Depending on the cell type and the connexin expressed, connexons can function as hemichannels, providing a pathway for transmembrane signalling, whereas gap junction channels will enable a direct communication pathway between the cytoplasms of adjacent cells. This type of intercellular communication permits coordinated cellular activity, a critical feature for organ homeostasis during development and adult life of multicellular organisms.

Gap junctions participate in the regulation of diverse functions, including contraction of cardiac and smooth muscle (Huizinga *et al.* 1992; Page and Shibata 1981; Miller *et al.* 1989; Spray and Burt 1990; De Mello 1994), transmission of neuronal signals at electrotonic synapses (FURSHPAN and POTTER 1959; Auerbach and Bennett 1969; Sotelo and Llinas

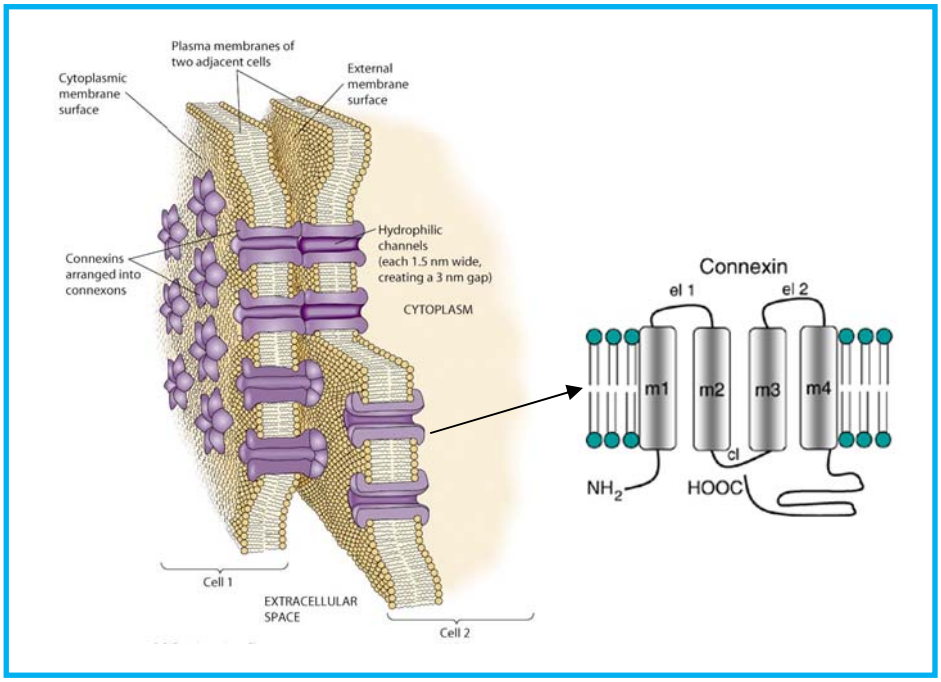

Fig.2
Structure of gap junction channels.

1972; Bennett and Verselis 1992), and metabolic cooperation in development and vascular organs (Larsen and Wert 1988; Goodenough 1992). Permeability of intercellular channels to second messengers may also regulate secretion by both the exocrine and endocrine pancreas (Bruzzone and Meda 1988; Serre-Beinier *et al.* 2000) and plays a critical role in pattern formation during development (Caveney 1985; Guthrie and Gilula 1989; Kidder 1992).

The connexin gene family comprises 20 members in the mouse and 21 members in the human genome, 19 of which can be grouped as sequence-orthologous pairs (Koval 2006). Connexin are commonly named according to their molecular weight (for instance, Cx43 has a mobility of 43 kDa), and their genes have been classified into 4–5 groups (based on sequence homology). Each connexin have distinct expression patterns and regulation properties. Nine of these connexins are expressed in the brain, but only Cx36 (Condorelli *et al.* 2000) and Cx45 are expressed in the neurons. These two connexin are largely expressed during embryogenesis and their expression persist, also during postnatal life, in the specific region of the central nevous system. After the birth Cx36 remain express in many brain area and in particular in the inferior olive and the olfactory bulb (Belluardo *et al.* 2000). The Cx45, instead, is highly expressed during embryogenesis and up to 2 weeks after birth in nearly all brain regions. Afterward its expression is restricted to the thalamus, the CA3

region of hippocampus and the cerebellum (Maxeiner *et al.* 2003). The other connexins present in the nervous system are instead exclusively expressed in the glial cells (Condorelli *et al.* 2003).

Gap junctions are found in virtually all mammalian tissues with the notable exception of adult skeletal muscle, although they have been detected in early developing muscle tissue (Schmalbruch 1982; Balogh *et al.* 1993). This characteristic is due to the intrinsic property of the skeletal muscle. The muscular fibers, infact, must be electrically isolated from each other to prevent the spread of the depolarization current. Myogenic progenitor cells have been shown to adhere and fuse in a highly coordinated way and to form gap junctions, suggesting a role for these channels in early muscle differentiation (Constantin and Cronier 2000). Gap junctions, infact, have been found in embryonic and newborn skeletal muscles of the chick, mouse, and the rat (Dahl *et al.* 1995; Schmalbruch 1982). The importance of intercellular gap junctional communication for myogenesis is also supported by a study by Proulx et al. (1997) showing that after application of pharmacological gap junction blockers, myoblasts do not express myogenin or *MRF4*, both markers of terminal differentiation.

During embryonic development gap junctions have been described among myoblasts, as well as among myoblasts and myotubes, by electron microscopy (von Maltzahn and Willecke 2005). The corresponding connexin proteins are Cx39, Cx40, Cx43 and Cx45(von Maltzahn *et al.* 2004; Belluardo *et al.* 2005; Araya *et al.* 2005; Trovato-Salinaro *et al.* 2009). In myoblasts, Cx43 expression is predominant and a prerequisite for their fusion (Squecco *et al.* 2006). It is expressed first at embryonic stage of 11 days in somite of the dermnomyotome. Then Cx43 is expressed in many cell clusters and their expression varies in dependence on the muscles type. At embryonic stage of 18 days, when the myotube is completely formed, connexin 43 expression is restricted to a scattered cells along the myotube of new formation. Cx45, instead, is largely expressed in many cell of skeletal muscle in the early stage of the myogenesis, then its expression decrease, and after the bird it is limited to a cell along the myotube, probably satellite cells. The distinct time course of connexon expression is important for proper differentiation of skeletal muscle, e.g. overexpression of Cx43 in cultured mouse myotubes, under control of a constitutive active viral promoter, led to significant cell death upon differentiation (Reinecke *et al.* 2004).

The Cx39 gene is localized on chromosome 17 (cytogenetic band q12.1) and has a high level of similarity with other connexins in the extracellular loops, in the aminoterminal domain, and in the transmembrane domains. The Cx39 is expressed both in embryonic

development and in adult life during the process of skeletal muscle regeneration following muscle injury (Belluardo *et al.* 2005; von Maltzahn *et al.* 2004). The first signal of Cx39 mRNA expression was observed at E12, at the myotome level. At E16, Cx39 mRNA was expressed in all the developing skeletal muscles, but at E20, Cx39 expression decreased in several muscles and disappeared within the first week of postnatal life (Belluardo *et al.* 2005), suggesting that this connexin is not involved in the process of postnatal growth of the muscle mass. Our studies also show that this connexin is expressed in regenerating adult skeletal muscle after crush injury, and that its expression is restricted, like during myogenesis, to myogenin positive cells (Belluardo *et al.* 2005). The great intrinsic interest of these results derives from the conclusion that Cx39 is selectively and transiently expressed in cells of the myogenic lineage during skeletal myofiber generation in the embryo and myofiber regeneration in the adult. A very recent study showed that in Cx39 deficient mice skeletal muscles develop regularly and the myogenesis, like regeneration process, is not compromised (von Maltzahn et al, in press). However, Cx39 deficient mice display an earlier onset of markers of differentiation, e.g. *MyoD* and myogenin compared to control littermates. This earlier onset of differentiation is accompanied by an increased expression of Cx43, suggesting that Cx43 may compensate for the loss of Cx39 during myogenesis and regeneration processes (von Maltzahn et al, in press)

Skeletal muscle regeneration following injury

The basic contractile unit of skeletal muscle is the muscle fiber; thousands of muscle fibers form together an individual skeletal muscle. Muscle fibers are limited by a plasma membrane (sarcolemma) surrounded by a basal lamina, and outside of this, a connective tissue composed by scarce extra cellular matrix proteins, capillaries and nerve terminals (Buckingham 2001). The adult skeletal muscle is a highly dynamic tissue that is capable of regeneration following exercise-, immobilization- or chemically-induced damage. In response to exogenous stimuli or to biological factors, the muscle is able to adapt by increasing the size and amount of contractile proteins. This leads to increase in fiber size and their consequent force production. Muscle remodeling occurs throughout the entire life although at different rate considering the developmental stages. This dynamic property is due to satellite cells that are located, as mentioned above, between the basal lamina and the fiber sarcolemma. The satellite cells also confer to skeletal muscle the important property of regeneration in consequent to a muscle damage.

Skeletal muscle regeneration is a complex process, which is not yet completely understood. In human and animal models, increased serum creatine kinase is observed after mechanical stress (e.g., extensive physical exercises) and in the course of muscle degenerative diseases, such as muscular dystrophies (Coulton *et al.* 1988; Nicholson *et al.* 1979; Percy *et al.* 1979; Armstrong *et al.* 1991; LeBlanc *et al.* 1993; Sorichter *et al.* 2001; Zatz *et al.* 1991). It has been hypothesized that increased calcium influx after sarcolemmal or sarcoplasmic reticulum damage results in a loss of calcium homeostasis and increased calcium-dependent proteolysis, that drive tissue degeneration (Alderton and Steinhardt 2000; Armstrong 1990; Belcastro *et al.* 1998). Thus disrupted myofibers undergo focal or total autolysis, depending on the extent of the injury. The healing of skeletal muscle in response to trauma, infact, depends on the type of injury such as contusion, strain, and laceration, and on the severity. However, in general, the healing process consists of three phases: the destruction phase, the repair phase, and the remodeling phase (Huard *et al.* 2002; Charge and Rudnicki 2004; Jarvinen *et al.* 2005; Grefte *et al.* 2007).

The destruction phase is characterized by necrosis, hematoma formation, and the influx of inflammatory cells. The activated immune cells also produce adhesion molecules, such as selectins (Lasky 1995) and cytokines, such as IL-6 and TNF-α. The inflammatory reaction influences the local blood flow and vascular permeability, which accelerates the immune response (Huard *et al.* 2002; Boonen and Post 2008).

As a result of skeletal damage, inflammatory cells, residing within the muscle, release factors which in turn provide the chemotactic signals to circulating inflammatory cells. Neutrophils are the first inflammatory cells to invade the injured muscle, with a significant increase in their number being observed as early as 1-6h after the damage (Fielding *et al.* 1993; Orimo *et al.* 1991). After neutrophil infiltration and 48h postinjury, macrophages become the predominant inflammatory cell type within the site of injury (Tidball 1995).

Recent studies showed that macrophages, which are attracted upon injury, play a crucial role in skeletal muscle regeneration. During the repair phase, the macrophages phagocyte the necrotic debris, and regeneration of myofibers occurs through the action of satellite cells (Wozniak *et al.* 2005; Zammit *et al.* 2006). Different stimuli have been proposed as initiators of satellite cell activation; extracts from the injured fibers, molecules released by the invading macrophages, and soluble factors from connective tissue have all been proposed (Lescaudron *et al.* 1999). After their activation, quiescent satellite cells expressing *Pax7* migrate to the site of injury, up-regulate the MRFs, *MyoD* and *Myf5*, and

become proliferative myoblasts (Smith *et al.* 1994; Yablonka-Reuveni and Rivera 1994; Cornelison and Wold 1997; Cooper *et al.* 1999; Beauchamp *et al.* 2000). Subsequent differentiation of the myoblasts is marked by the down-regulation of *Pax7* (Olguin and Olwin 2004; Zammit *et al.* 2004) and up-regulation of the *MRF4* and Myogenin (Smith *et al.* 1994; Yablonka-Reuveni and Rivera 1994; Cornelison and Wold 1997). Ultimately, these differentiated myoblasts form new multinucleated myofibers (hyperplasia), or fuse to damaged myofibers for muscle regeneration or for muscle hypertrophy process (Hawke and Garry 2001; Charge and Rudnicki 2004). At the end of the regeneration process, the contractile system is perfectly reconstructed, innervated and perfused.

In vivo, macrophage suppression leads to incomplete skeletal muscle regeneration (Segawa *et al.* 2008). Furthermore, the prevention of monocyte recruitment to the site of injury completely inhibits skeletal muscle regeneration (Arnold *et al.* 2007). Malerba (2009) and Segawa (2008) suggest that macrophages directly affect satellite cells by two different mechanisms (Chazaud *et al.* 2009). First, the macrophages can secrete soluble factors affecting satellite cells, and second, macrophages can interact with satellite cells by cell–cell contact, and thereby protect them from apoptosis (Chazaud *et al.* 2003). However, macrophages play a dual role depending on their activity (Arnold *et al.* 2007; Chazaud *et al.* 2009; Villalta *et al.* 2009). Pro-inflammatory macrophages induce myogenic precursor cell proliferation, while anti-inflammatory macrophages induce differentiation and fusion of these cells (Villalta *et al.* 2009). A switch between the pro- and anti-inflammatory macrophages has been observed in vivo after injury, and during the course of muscular dystrophy (Villalta *et al.* 2009; Arnold *et al.* 2007). Depletion of the anti-inflammatory macrophages reduces the diameter of regenerating myofibers (Chazaud *et al.* 2009).

Other important factors involved in the regulation of satellite cells are the cell-cell interaction and the communication with extracellular matrix. The basal side of the satellite cells expresses integrin α7β1, which links the cytoskeleton with laminin in the basal membrane (Song *et al.* 1992; Burkin and Kaufman 1999). Integrin α7β1 plays a major role in the transduction of strain-induced mechanical forces into chemical signals, which are involved in the regulation of myogenesis (Boppart *et al.* 2006). A recent study showed that integrin α7β1 is required for satellite cell migration and that hepatocyte growth factor (HGF) plays a crucial role in the guidance of satellite cells. Furthermore, the results suggests that unrelated and divided satellite cells stay in long contact with each other and co-migrate along the myofiber (Siegel *et al.* 2009). The apical side expresses M-cadherin that attaches

the satellite cell to the adjacent myofiber (Cornelison and Wold 1997; Kuang *et al.* 2008). Both attachment sites are essential for signal transduction between the satellite cell and the two flanking structures (Cornelison and Wold 1997; Burkin and Kaufman 1999). In addition, it has been suggested that M-cadherin plays a significant role in the attachment and fusion of myoblasts to form new and regenerate damaged myotubes (Cifuentes-Diaz *et al.* 1995). This is supported by a significant increase of M-cadherin in activated satellite cells during skeletal muscle regeneration (Irintchev *et al.* 1994).

Growth factors also are crucial in satellite cell regulation, since they can activate intracellular signaling pathways (Charge and Rudnicki 2004; Philippou *et al.* 2007), regulating muscle-specific genes (Hawke and Garry 2001; Charge and Rudnicki 2004). These growth factors secreted by macrophages and T-cell , together with the growth factors released from the extracellular matrix, attract and activate the satellite cells and induce cell differentiation (Huard *et al.* 2002; Grefte *et al.* 2007). Many growth factors, such as HGF, FGF-2 and -6, vascular endothelial growth factor (VEGF), platelet-derived growth factor-AA and -BB (PDGF-AA and -BB), stromal derived factor-1 (SDF-1) and IGF-1 and -2 play a major role in myogenic proliferation and differentiation (Doumit *et al.* 1993; Robertson *et al.* 1993; Allen *et al.* 1995; Haugk *et al.* 1995; Hawke and Garry 2001; Ratajczak *et al.* 2003; Charge and Rudnicki 2004; Boonen and Post 2008). In particular IGF-1 is critical for skeletal muscle growth (Menetrey *et al.* 2000; Sato *et al.* 2003). In vitro, IGF-1, and in a later phase IGF-2, are both able to alter the expression of myogenic regulatory factors and promote the proliferation and the differentiation of SC-derived myoblasts (Allen and Boxhorn 1989; Charge and Rudnicki 2004). Besides IGF-1, also HGF and VEGF are involved (Huard *et al.* 2002). HGF is the primary factor to induce satellite cell proliferation by binding to c-met (Allen *et al.* 1995; Gal-Levi *et al.* 1998; Tatsumi *et al.* 1998). Correlating with this property, HGF expression is increased in proportion with the degree of injury, during the early proliferation phase of muscle regeneration (Tatsumi *et al.* 1998; Suzuki *et al.* 2002; Tatsumi *et al.* 2001). In addition, HGF plays a role in the migration of satellite cells to the site of injury (Bischoff 1997; Suzuki *et al.* 2000). VEGF can improve muscle healing by stimulating angiogenesis to increase the nutrient and oxygen supply, which is essential for the healing process (Springer *et al.* 1998; Gowdak *et al.* 2000). FGF-6 expression, for example, is muscle specific and is up-regulated during muscle regeneration (Delapeyriere *et al.* 1993).

From a morphological point of view, after a skeletal damage, in the injuried area the first three days is caractherized by an accumulation of different cell populations that include, inflammatory cells and satellite cells. Then myoblast cells start to fuse each other to form new myotube. From the fifth day, along edge of damaged area it possible to see cellular aggregate or myotube caractherized by a central nucleus. In the next days the numbers of myotube increase and progressively the tissue appears completely reconstructed and it possible to see only a number of myofibers with central nuclei.

Muscular dystrophy and therapeutic strategies

Duchenne's muscular dystrophy (DMD) is a lethal X-linked myopathy characterized by the near absence of dystrophin protein in skeletal muscles. Dystrophin is a cytoskeletal protein connecting the cytoskeletal actin network to a complex of proteins in the membrane (dystrophin- associated complex, DAC), which further connect to the extracellular matrix by laminin. In DMD, not only dystrophin is generally absent, but the expression of the DAC proteins is greatly reduced (Ervasti and Campbell 1991). It appears that the presence of dystrophin is necessary for the stability of this protein complex. Loss of dystrophin renders multinucleated skeletal muscle cells, known as myofibers, susceptible to exercise induced injury.

Muscle histology in DMD patients is almost normal before the onset of clinical symptoms at 3–5 years of age. The preliminary stage of the disease is characterized by the presence of focal groups of necrotic myofibres, muscle hypertrophy and abnormally high levels of muscle creatine kinase. In the second phase, repeated cycles of degeneration exhaust the regenerative capacity of muscle-specific stem cells and fibrotic mechanisms cause the progressive replacement of contractile muscle tissue with collagenous connective tissue (Deconinck and Dan 2007; Zhou and Lu 2010). This process leads to joint contractures, loss of ambulation by 10–12 years and death following respiratory or cardiac failure (Wells and Wells 2002).

Several different therapeutic strategies have been pursued. The most perfect solution would be to place a normal copy of the dystrophin gene into muscle cells, and hence restore sufficient protein expression to improve structure and function, as successful therapy would require massive and sustained gene transfer, this is a daunting task (Thioudellet *et al.* 2002).

Nevertheless, the availability of high-efficiency viral infection (Roberts *et al.* 2002; Scott *et al.* 2002; Cerletti *et al.* 2003) and nonviral transfection (Thioudellet *et al.* 2002; Gollins *et al.* 2003; Lu *et al.* 2003) methods and the development of functional mini-dystrophin genes still allow it as a possibility (Thioudellet *et al.* 2002; Wells and Wells 2002). Transplantation of normal donor (or genetically corrected host) muscle precursor cells (myoblast transfer) has also been explored as a method for restoring dystrophin protein to dystrophic muscle. Transplanted muscle precursors can differentiate to form dystrophin expressing muscle (Law *et al.* 1988; Partridge *et al.* 1989; Morgan *et al.* 1990). This technique is still constrained by the difficulties associated with treating large volumes of muscle with long-lasting effect. Other strategy includes implementation of exercise programme. In this context various exercise regimes have been used in attempt to improve muscle function and the *mdx* mice have been used to assay the effects of different exercise programme on function and morphology of dystrophic muscle. While models of excessive or otherwise inappropriate activity may induce muscle damage and increase pathology in *mdx* mice (Brussee *et al.* 1997; De *et al.* 2003; Okano *et al.* 2005), low intensity or voluntary exercise may improve muscle pathology. Beneficial effects of regular exercise on dystrophic *mdx* muscle are reported for free wheel running (Dupont-Versteegden *et al.* 1994; Hayes and Williams 1996; Carter *et al.* 1995), swimming, and low-intensity endurance exercise (Hayes and Williams 1998). However, these discrepancies in the literature about the appropriate beneficial exercise in dystrophic muscle may be explained by differences in experimental procedures: duration, intensity and type of exercise, variations in age of *mdx* mice, different environmental conditions.

The most commonly laboratory model used of DMD is the C57Bl/10ScSn *mdx* (*mdx*) mouse (Bulfield *et al.* 1984). Muscle pathology is comparatively moderate and mechanical function is less seriously compromised, resulting in an almost normal lifespan. Muscle pathology is most pronounced in the *mdx* between 2 and 8weeks of age, a period characterized by the presence of necrotic foci, newly regenerated centrally nucleated myofibres and high plasma concentrations of creatine kinase. Detailed analysis of *mdx* muscle pathology has shown that whilst some muscles (such as the masseter) are spared, others (such as the gastrocnemius and in particular, the diaphragm) are severely affected (Muller *et al.* 2001). Currently, the only treatment to prove clinically efficacious is dosage with the steroid drug prednisone/prednisolone, which results in a modest increase in strength

and delays, but does not halt, the progress of the disease (Backman and Henriksson 1995; Dubowitz *et al.* 2002).

AIMS

Aim I

Generally, muscle regeneration may be dependent on myoblasts fusing to existing damaged myofibers (myoblast/myofiber fusion), or on myoblasts that fuse with other myoblasts forming myotubes (myoblast/myoblast fusion). At present, we do not know which signals during muscle regeneration promote fusion of myoblasts to a preexisting myofiber or rather induce formation of a new myofiber. In this context, recent data have shown a relevant role of gap junctions as cell-cell communication and functional synchronization before myoblast fusion (Araya *et al.* 2005; Belluardo *et al.* 2005; von Maltzahn *et al.* 2004).

Myogenic progenitor cells have been shown to adhere and fuse in a highly coordinated way and to form gap junctions, suggesting a role for these channels in early muscle differentiation (Constantin and Cronier 2000). Gap junctions have been found in embryonic and newborn skeletal muscles of the chick, mouse, and the rat (Dahl *et al.* 1995; Schmalbruch 1982). Cx43 and Cx40 have been described in myoblasts of differentiating skeletal muscles, and evidence in favor of a functional role of Cx43 in the differentiation and fusion of myoblasts has been reported by Dahl et al. (Dahl *et al.* 1995). Recently, Cx39 has been found to be expressed during skeletal muscle embryonic development and involved in the myoblast fusion (Araya *et al.* 2005; Belluardo *et al.* 2005; von Maltzahn *et al.* 2004). Indeed, following the chemical block of gap junctions or after deletion of the Cx43 gene, cultured myoblasts do not fuse with each other and do not express specific genes for cellular differentiation, such as myogenin (Constantin and Cronier 2000; Schmalbruch 1982). Moreover, in regenerating skeletal muscle, a transient upregulation of Cx45, Cx43, and Cx39 expression precedes myoblast fusion (Araya *et al.* 2005; Belluardo *et al.* 2005; von Maltzahn *et al.* 2004). Using knockout mice, it has been evidenced that Cx43 is required for normal myogenesis in vitro and adult muscle regeneration in vivo (Araya *et al.* 2005).

A comparative in vivo study of several Cxs in the same model of adult muscle injury provides fundamental information to understand the regulation and role of Cxs in committed myogenic cells during muscle regeneration. To this end, in this study, we analyzed, using in situ hybridization and immunohistochemistry, the spatial and temporal expression pattern of several Cxs (Cx30 Cx37, Cx39, Cx40, Cx43, and Cx45) during adult skeletal muscle

regeneration in response to crush injury. Such mechanical lesion by disruption of myofiber integrity promotes the activation of satellite cells to proliferate and perform both myogenesis and repair of injured skeletal fibers (Schultz and McCormick 1994; Bassaglia and Gautron 1995; Charge and Rudnicki 2004; Creuzet *et al.* 1998; Pavlath *et al.* 1998; Saito and Nonaka 1994).

Aim II

In patients with Duchenne muscular dystrophy, muscle biopsy characteristically demonstrates areas of necrotic or degenerating muscle fibers, often observed in clusters surrounded by macrophages and lymphocytes, and areas with small immature centrally nucleated fibers reflecting muscle regeneration from myoblasts, that results in a balance between necrotic and regenerative processes. (Brunelli *et al.* 2007; Grounds and Torrisi 2004; Hodgetts *et al.* 2006; Radley *et al.* 2007; Spencer and Tidball 2001).

Various strategy have been proposed in order to reduce the muscle wasting associate to muscular dystrophy, such as replacement of functional dystrophin by genetic, cell transplantation or molecular interventions (Odom *et al.* 2007), including implementation of exercise programme. In this context, as above mentioned, voluntary exercise such as free wheel running (Dupont-Versteegden *et al.* 1994; Hayes and Williams 1996; Carter *et al.* 1995) and swimming or low-intensity endurance exercise (Hayes and Williams 1998).

In the present study we designed appropriate exercise parameters that do not worsen the dystrophic condition, namely a low-intensity endurance exercise that consists of exercise bouts performed daily five times per week for a total time period of four weeks at progressively increasing loads (increasing running distance) on a motorized Rotarod.

The aim of this study was to test if a new programmed low-intensity endurance exercise may positively affects the degeneration/regeneration process in dystrophic muscle of *mdx* mice, and may have beneficial effects on dystrophic muscle function, such as fatigue resistance and improvements in strength.

Additionally, in the present study, we evaluated and quantified for the first time the regeneration process in the dystrophic muscle of sedentary and exercised *mdx* mice by monitoring the expression of connexin-39, a specific gene involved in skeletal muscle regeneration process (Belluardo *et al.* 2005).

MATERIALS AND METHODS

Animals

The present study was performed on adult male Wistar rats (350 g body wt) housed under alternating 12h periods of light and darkness, in a temperature-controlled (24 2°C) and humidity controlled room and male Wild-type (C57BL/10ScSn from Harlan Italy), male *mdx* (C57BL/10ScSn-Dmd^{mdx}/J from Jackson Laboratories) and male C57/BL6 (from local stock) adult mice. The mice were kept under environmentally controlled conditions, ambient temperature 24 °C, humidity 40% and 12h light/dark cycle, with food and water *ad libitum*. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. no. 116, G.U., suppl. 40, February 18, 1992) and international laws and policies [European Economic Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*, NIH Publication no. 80–23, 1985, and Guidelines for the Use of Animals in Biomedical Research, *Thromb Haemost* 58: 1078–1084, 1987]. All efforts were made to minimize the number of animals used and all experiments were approved by the local ethical committee.

Animal model of muscle regeneration after injury

Adult males rats and adult C57/BL6 male mice were used for gastrocnemius crush injury of right leg. Gastrocnemius crush injury was achieved under chloral hydrate anesthesia (350 mg/kg intraperitoneally). After skin incision, the right gastrocnemius muscle was exposed, the middle part was crushed with a hemostat clamp closed for 3 s, and the skin was then sutured. The trauma was standardized by using the same muscle level and the same strength to crush the muscle by closing the hemostat clamp. In some rats, the soleus or extensor digitorum longus muscles were crushed. The rats and mice were sacrificed by excess anesthesia, in groups of three at various time points following injury. The injured muscle was dissected, frozen in precooled isopentane, and stored at 70°C. Cryosections were prepared from the mouse muscles in which, under stereomicroscopy, the lesioned area was

dissected and tissue was used for the detection of Cx39 protein levels by western blotting. The rat injured muscles were used for in situ hybridization and immunohistochemical analysis.

Riboprobes labeling

The in situ hybridization procedure was used to examine the spatial and temporal expression pattern of Cx mRNAs in adult regenerating skeletal muscle. The following riboprobes were used: for Cx30 riboprobe, a 360-bp fragment encompassing nucleotides 772-1131 in the Cx30 sequence (GenBank access no. Z70023), subcloned in pCR-Script SK(+) (Stratagene, La Jolla, CA), linearized with *Sac*I, and transcribed with T7 RNA polymerase for the antisense probe, or with *Eco*RI and transcribed with T3 RNA polymerase for the sense probe; for Cx39 riboprobe, a 298-bp fragment encompassing nucleotides 560-859 70-367 in the Cx39 sequence (GenBank access no. X51615), subcloned in pCR-Script SK(+) (Stratagene), linearized with *Sac*I, and transcribed with T7 RNA polymerase for the antisense probe, or with *Eco*RI and transcribed with T3 RNA polymerase for the sense probe; for Cx40 riboprobe, a 455-bp fragment encompassing nucleotides 262-716 in the Cx40 sequence (GenBank access no. AFO21806), subcloned in PCR II TOPO TA (Invitrogen, Carlsbad, CA), linearized with *Eco*RV, and transcribed with SP6 RNA polymerase for the antisense probe, or with *Sac*I and transcribed with T7 RNA polymerase for the sense probe; for Cx43 riboprobe, a 464-bp fragment encompassing nucleotides 373-836 in the Cx43 sequence (GenBank access no. M19317), subcloned in pCR-Script SK(+) (Stratagene), linearized with *Eco*RI, and transcribed with T3 RNA polymerase for the antisense probe, or with *Sac*I and transcribed with T7 RNA polymerase for the sense probe; for Cx45 riboprobe, a 533-bp fragment encompassing nucleotides 625-1157 in the Cx45 sequence (GenBank access no. X63100), subcloned in pCR-Script SK(+) (Stratagene), linearized with *Sac*I, and transcribed with T7 RNA polymerase for the antisense probe, or with *Eco*RI and transcribed with T3 RNA polymerase for the sense probe; for Cx37 riboprobe, a 464-bp fragment encompassing nucleotides 277-740 in the Cx37 sequence (GenBank access no. M76532), subcloned in plasmid vector PCR II TOPO TA cloning (Invitrogen), linearized with *Eco*RV, and transcribed with SP6 RNA polymerase for the antisense probe, or with *Sac*I and transcribed with T7 RNA polymerase for the sense probe.

The radiolabeling of the riboprobes was performed as previously described in Condorelli et al (Condorelli *et al.* 2003)

In situ hybridization

Longitudinal muscle sections (10µm) and whole rat embryonic body, at different developmental stages, were cut at -20°C and thawed onto 3-aminopropyl-ethoxysilane-coated slides. Frozen sections were directly fixed in 4% paraformaldehyde for 1h at 4°C, washed in PBS for 15 min, and incubated for 10 min in methanol at 20°C. Subsequently, the sections were dried at room temperature for 20 min, rehydrated in PBS for 10 min, and processed for the remaining steps of in situ hybridization, as previously described by Condorelli et al. (Condorelli *et al.* 2003). Slides were deproteinated in 0.2M HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1M ethanolamine for 20 min, and dehydrated with increasing concentrations of ethanol. Slides were incubated for 16h in a humidified chamber at 52°C with $8x10^5$ counts/min probe in 80µl hybridization cocktail (50% formamide, 20mM Tris HCl, pH 7.6, 1mM EDTA, pH 8.0, 0.3M NaCl, 0.1M dithiothreitol, 0.5 g/ml yeast tRNA, 0.1 g/ml poly-A-RNA, 1 Denhardt's solution, and 10% dextransulfate), washed twice in 1 SSC (1 SSC 150mM NaCl, 15mM sodium citrate, pH 7.0) at 62°C for 15 min, and then in formamide/SSC (1:1) at 62°C for 30 min. After an additional wash in 1 SSC at 62°C, single-stranded RNA was digested by RNAse treatment (10 g/ml) for 30 min at 37°C in 0.5 M NaCl, 20 mM Tris HCl, pH 7.5, 2mM EDTA. Slides were washed twice with 1 SSC at 62°C for 30 min before dehydration in ethanol and air drying. Hybridized sections were subsequently coated with Emulsion Type NTB (Kodak, catalog no. 8895666) diluted 1:1 in water (Eastman- Kodak, Rochester, NY), and stored in desiccated light-tight boxes at 4°C for 4 wk. Slides were developed with D19 (Eastman-Kodak), fixed with Al-4 (Agfa Gevaert, Kista, Sweden), and counterstained with hematoxylin-eosin, rinsed in PBS, dehydrated through graded alcohols, cleared in xylene, and coverslipped in DPX mountant. A control of the hybridization specificity of the cRNA riboprobes was performed by using sense ^{35}S-labeled riboprobes.

In situ hybridization combined with immunohistochemistry

For the colocalization of Cx mRNA expression in myogenic cells, a double-labeling was performed by combining in situ hybridization and immunohistochemistry. Frozen muscle sections were first processed for the in situ hybridization, as described above, and subsequently used for immunohistochemical identification of cells using specific antibodies. Immediately after the last wash of the in situ hybridization protocol, the sections were rinsed twice in PBS, pH 7.4, for 10 min and subsequently incubated for 20 min in blocking buffer, consisting of 2.5% normal goat serum and 0.3% Triton X-100 in PBS, and incubated overnight at 4°C in the presence of the primary antibodies, rabbit polyclonal antibody anti-myogenin diluted 1:200 (M-225 Santa Cruz Biotechnology), or rabbit polyclonal antibody anti-M-cadherin diluted 1:250 (H-71 Santa Cruz Biotechnology), or mouse anti-rat integrin-(M) (CD11b; M1405 Chemicon International) in PBS supplemented with 1.5% blocking serum and 0.3% Triton X-100. Sections were then washed three times for 5 min in PBS and incubated at room temperature for 1h with a biotinylated anti-rabbit antiserum (Amersham), diluted 1:200. After three short washes with PBS, the sections were incubated for 1h with a horseradish-peroxidase-streptavidin complex (Vector, Burlingame, CA) diluted 1:100 in PBS. After washes in PBS and then in Tris HCl buffer (0.1M, pH 7.4), the peroxidase reaction was developed in the same buffer containing 0.05% 3,3-diaminobenzidine-4 HCl and 0.003% hydrogen peroxide. After being stained and briefly washed in H_2O, the sections were dehydrated, coated in NTB emulsion, and processed as described for in situ hybridization.

Double immunolabeling analysis

Cryostat sections of 10µm thickness of gastrocnemius muscle were thawed onto 3-aminopropyl-ethoxysilane-coated slides and air dried for 15 min. For Cx detection, the sections were then fixed in absolute ethanol for 5 min in RT and rinsed with PBS. After preincubation in blocking solution (5% BSA, Triton 0.1% in PBS) for 30 min, the sections were incubated overnight at 4°C with either rabbit antiCx39 affinity purified antibodies (von Maltzahn *et al.* 2004) diluted 1:100 in blocking solution, goat polyclonal anti-Cx37 antibodies, goat anti-Cx40 antibodies (sc-27715 and sc-20466, Santa Cruz Biotechnology), or rabbit polyclonal anti-Cx45 antibodies (sc-25716, Santa Cruz Biotechnology) diluted

1:400. After three washing steps with PBS for 5 min, the sections were incubated in RT for 1h with specific secondary antibodies Cy2 conjugated, diluted 1:200 (711-225-152; Jackson Immuno Research Laboratories). Following two washings with PBS, the sections were fixed, with exclusion for Cx40 double labeling with Cx39, in 4% paraformaldehyde for 15 min at 4°C, washed in PBS, and then incubated in blocking solution for 2h in RT with the other combination of antibodies for double labeling: the rabbit polyclonal anti-von Willebrand factor (vWF), a marker for endothelial cells, diluted 1:300 (sc-14014; Santa Cruz Biotechnology). After three washing steps with PBS for 5 min, the sections were incubated in RT for 1h with specific secondary antibodies Cy3 conjugated, diluted 1:200 (705–165-003; Jackson Immuno Research Laboratories). Following two washings with PBS, the sections were counterstained by incubation for 10 min with 0.5g/ml of the fluorescent nuclear dye Hoechst 33258 (bisbenzimide, Sigma-Aldrich, Germany). Following a short washing with PBS, sections were coverslipped in a glycerol-based medium with an anti-fading agent, and slides were examined under a fluorescence microscope (DMRBE, Leica Microsystems).

In situ hybridization and bromodeoxyuridine immunolabeling

After crush injury, the bromodeoxyuridine (BrdU; 40mg/kg ip) was injected 2, 3, 4, 5, and 7 days after injury, and rats were killed following 2h of BrdU injection. The dissected gastrocnemius was rapidly frozen and processed first for in situ hybridization, as described above, and subsequently for BrdU immunohistochemistry as follows. Muscle sections after the in situ hybridization were coated with NTB photoemulsion and exposed for 4 wks before being developed for autoradiographic preparation and again fixed in 4% paraformaldehyde for 15 min and rinsed twice in Tris-buffered saline (TBS), pH 7.4, for 10 min. Subsequently, the sections were incubated, for BrdU immunohistochemistry, with 2N HCl (15 min at 37°C), and then in borate buffer for 10 min (0.1M, pH 8.5) to stop the reaction. Subsequently, the sections were washed and incubated for 15 min in blocking serum and 0.3% Triton X-100 in TBS, and, after two washes in TBS, were incubated with a mouse monoclonal anti-BrdU diluted 1:400 (Roche Mannheim, Germany, product no. 11170376001) in TBS with blocking serum and 0.3% Triton X-100. After 24h, sections were washed 1h in TBS and incubated at room temperature for 1h with a biotinylated

universal secondary antibody diluted 1:50 in TBS with blocking serum and 0.3% Triton X-100. After three 5 min washings with TBS, the sections were incubated for 1h with a streptavidin horseradish peroxidase complex (Vector, Burlingame, CA), diluted 1:100 in TBS. After washing in TBS and then in Tris HCl buffer (0.1M, pH 7.4) for 10 min, the peroxidase reaction was developed in the same buffer containing 0.05% 3,3-diaminobenzidine-4 HCl and 0.003% hydrogen peroxide. The reaction was stopped in Tris HCl buffer and, after dehydration through graded alcohols, cleared in xylene and coverslipped with enthelan mountant. The number of BrdU-positive cells was determined by counting, in eight sections of each time point examined, with a micrometric quadriculated reticulum, five random fields (250-μm^2 squares) per section under a light microscope (DMRBE, Leica Microsystems). The percentage of double-labeled cells for each Cx examined was calculated as mean of percentage obtained in each time point studied.

Training protocol

At 10 weeks of age *mdx* mice were randomly assigned to sedentary (n=17; *MDX*-SD) or trained (n=14; *MDX*-EX) groups. Similarly, wild type mice were randomly assigned to sedentary (n=19; WT-SD) or trained (n=16; WT-EX) groups too. Preliminarily all exercised mice underwent a 2-week period of acclimatization trial at very low speed. To avoid the confounding effects of the accelerated necrotic stage of the disease present in young *mdx* mice (3-4 weeks of age) and the hormone effects on skeletal muscle typical of pre-adolescence and puberty in mice (5-7 weeks of age) (Grounds *et al.* 2008) we tested the effects of low intensity endurance training in adult mice (> 8 weeks of age). Training was performed in the same room where the mice were housed using a motorized rotor (Rota-Rod; Ugo Basile, Biological Research Apparatus, Comerio Varese, Italy). Mice ran 5 days/week for 4 weeks at progressively increasing loads. Wild-type and *mdx* mice were weighed at the start time of exercise training protocol and after 2, 3 and 4 weeks of training. Mice were still weighed after the recovery period.

Forelimb strength and fatigue

Wild-type and *mdx* mice were examined for forelimb strength at 1, 3 and 4 weeks of training. Briefly, the mouse was placed on a metal grid attached to a force transducer (Grip strength meter; Ugo Basile, Biological Research Apparatus, Comerio Varese, Italy), and pulled gently from the base of the tail until release. Because the increase in strength paralleled somatic growth in normal animals, we analyzed the strength of the *mdx* and controls as a function of weight. Each mouse underwent five trials and peak grip strength was normalized for body weight (force/BW) and recorded as the average of the three best efforts divided by the mouse's body weight. Grip strength fatigue was calculated by comparing the first two pulls to the last two pulls (Connolly *et al.* 2001). The decrement between pulls 1 + 2 and pulls 4 + 5 gives a measure of fatigue. In the formula (4 + 5)/(1 + 2), animals with no fatigue have a value of 1. For convenience, the value resulted from the formula above was subtracted from the value of "1" so that an animal without fatigue has a calculated fatigue value of 0, and an animal that can only complete three pulls, because completely fatigued, has a value of "1". The fatigue was expressed as percentage so that a value of 0% fatigue would be assigned to mice without fatigue and a value of 100% fatigue to mice with complete fatigue.

Western blotting analysis

Each experimental group of mice was sacrificed by excess of anesthesia. The gastrocnemius and quadriceps muscles were excised under stereomicroscopy, frozen in precooled isopentane and stored at –70°C. From these muscles were prepared cryosections 60 micron used for Cx39 protein levels detection by western blotting. The sampled muscle were homogenized in cold buffer containing 50mM Tris–HCl pH 7.4, 150mM NaCl, 1% triton, 0.1% SDS, H_2O and protease inhibitor cocktail (P8340, Sigma-Aldrich S.r.l., Milan, Italy). The homogenate was left on ice for 30 min and then centrifuged at 13,000 rpm for 30 min at 4°C. The supernatants were stored at −20°C and aliquots were taken for protein determination by the method of Lowry et al.(LOWRY *et al.* 1951). The samples with 30µg of protein and mol.wt. markers (161-0375, Bio-Rad Laboratories S.r.l., Segrate (MI), Italy), were run on 8% polyacrylamide gel at 100 V and electrophoretically transferred onto nitrocellulose membrane (Hybond-C-extra, GE Healthcare, formerly Amersham, Europe

GmbH – Filiale Italiana, Milan, Italy). Following 1h of incubation with 5% nonfat milk, the membrane was incubated overnight at +4°C with anti-CX39 polyclonal antibody (1:1000) raised in rabbit (N-20 Sc-546; Santa Cruz Biotechnology, CA, USA). After washing the membrane was incubated for 1h at room temperature with anti-rabbit IgG horseradish peroxidase-conjugated diluted 1:5000 (Sc 200 4, Santa Cruz Biotechnology) and CX39 band was visualized with chemiluminescence reagent (ECL, GE Healthcare, formerly Amersham, Europe GmbH – Filiale Italiana, Milan, Italy) according to the manufacturer's instructions. The ECL-films were developed in Kodak D19 developer and fixer (Eastman-Kodak, Rochester, NY, USA), and the densitometric evaluation of bands was performed by measuring the optical density (O.D.) using NIH ImageJ software and results expressed as arbitrary units.

Histomorphological analysis

Gastrocnemius or quadriceps longitudinal sections (10μm) were cut at −20°C and thawed onto 3-aminopropyl-ethoxysilane-coatedslides. Frozen muscle sections were directly fixed in 4% paraformaldehyde and stained with hematoxylin–eosin and then dehydrated with ethanol and xylene, mounted with Entellan (Merck, Darmstadt, Germany) and examined by lightmicroscopy.

Statistical analysis

Results are reported as mean ± SE. Analysis of variance (ANOVA) and Fisher's PLDS correction for post hoc comparison were used to test for differences between sedentary wild type (WT-SD), trained wild type (WT-EX), sedentary dystrophic mice (*MDX*-SD) and trained dystrophic mice (*MDX*-EX) at different time points. Time trends in each group were assessed by paired t-test (Statview 5.0.1; SAS Institute, Inc., Cary, NC). Significance was at $p<0.05$.

RESULTS

AIM I

Cxs expression in rat developing skeletal muscle

Autoradiographic film from the in situ hybridization of sagittal sections of whole embryonic body revealed intense labeling of Cx39, Cx43 and Cx45 in developing skeletal muscle tissue (Fig.3).

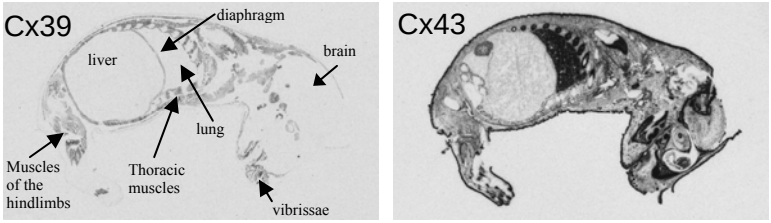

Fig.3
In situ hybridization analysis of Cx39 Cx43 and Cx45 mRNA expression during embryonic development . E18 autoradiogram showing Cx39, Cx43 and Cx45 mRNA expression in a representative section of a whole embryo at E18. Sense: autoradiogram obtained using specific sense probe for Cx43.

Expression of Cx39

Cx39 mRNA was detected in limb and trunk muscles, in respiratory muscles (including diaphragm), in tongue, in masticatory and facial muscles (including intrinsic and extrinsic muscles that drive the vibrissae), and in the muscle group responsible for eye movements. The first signal of Cx39 mRNA expression was observed at E12 at the myotome level (Fig.4A). At E16, Cx39 mRNA was expressed in all the developing skeletal muscles (Fig.4B-F), and the analysis of cresyl-violet-stained sections revealed the presence

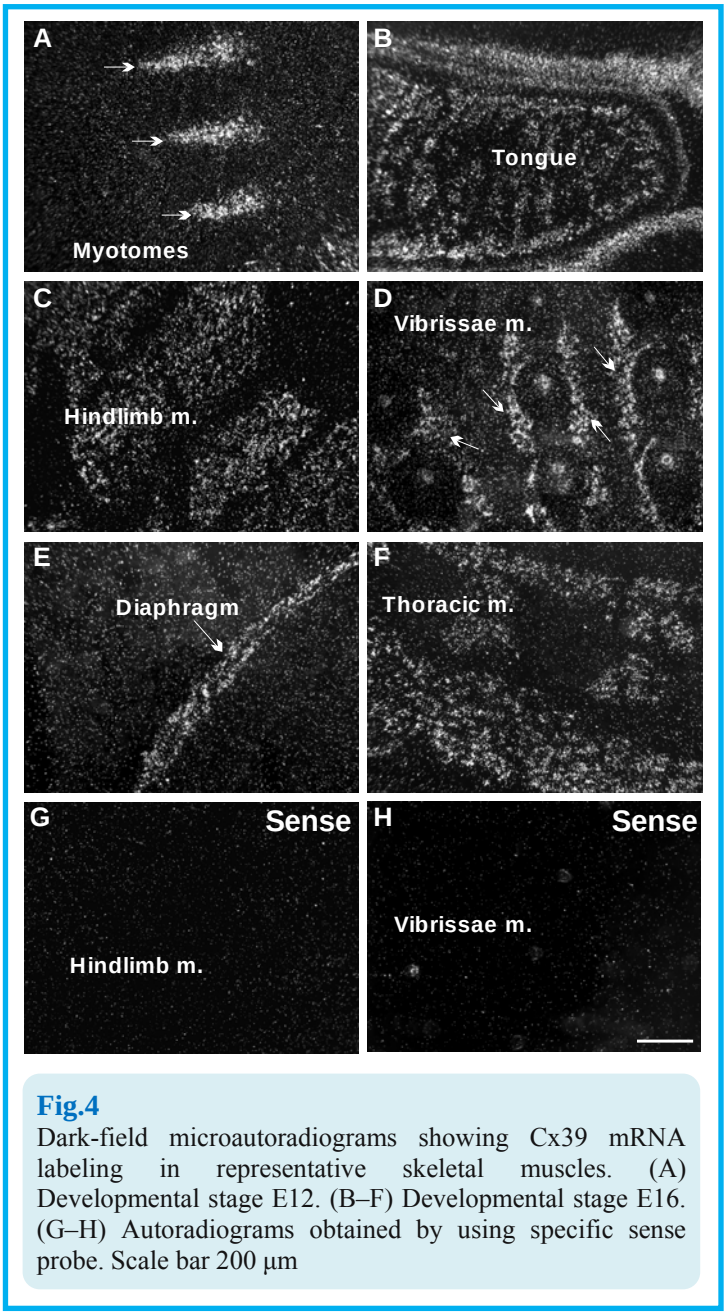

Fig.4
Dark-field microautoradiograms showing Cx39 mRNA labeling in representative skeletal muscles. (A) Developmental stage E12. (B–F) Developmental stage E16. (G–H) Autoradiograms obtained by using specific sense probe. Scale bar 200 µm

of clusters of Cx39-mRNA-positive cells surrounded by Cx39-mRNA-negative cells (Fig.5A). Between E17 and E18, the myotubes were better defined, with a large cytoplasm and central nucleus, and the level of Cx39 mRNA was high with hybridization grains being localized over the nucleus and cytoplasm (Fig.5B). Remarkably at E20, Cx39 expression decreased in several muscles, and progressively disappeared in more differentiated myotubes, identified by their extensive cytoplasm or peripheral nuclei. Between E21 and the first day of postnatal life, the majority of muscles showed a reduction of Cx39 labeling. An analysis of Cx39 expression was also performed in the gastrocnemius muscle at various postnatal days. At P1, a feature of Cx39 mRNA distribution was its presence over residual rows of closely apposed aligned cells or

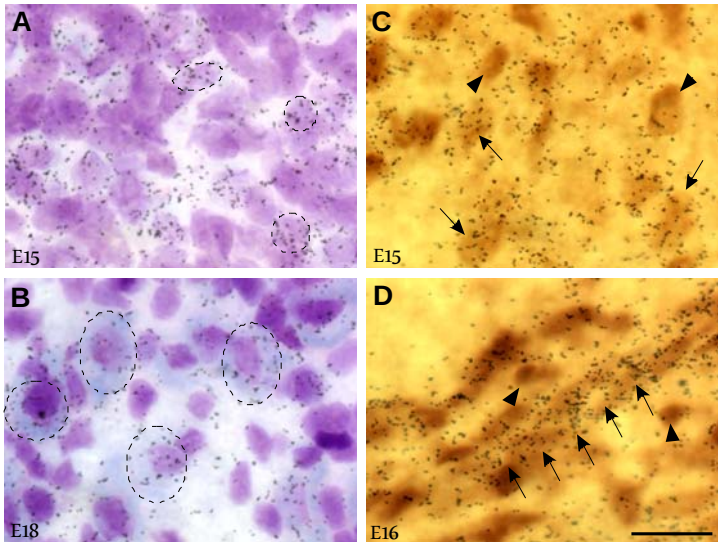

Fig.5
Microautoradiograms showing Cx39 mRNA labeling in cresyl-violet-stained sections of developing hindlimb skeletal muscle at embryonic stages E15 and E18 (dotted rings Cx39 labeled cells with extensive cytoplasm in B and with little cytoplasm in A). C-D, identification of cells expressing Cx39 mRNA in developing skeletal muscles by combining in situ hybridization for Cx39 mRNA and immunohistochemistry for myogenin. The doublelabeling analysis at E15 and E16 revealed that all rCx39-mRNAlabeled cells (black grains) were also positive for myogenin (brown). Arrows indicate colocalization of Cx39 mRNA in myogenin positive nuclei. Arrowheads indicate myogeninpositive cells devoid of Cx39 mRNA labeling. Bar 20 μm

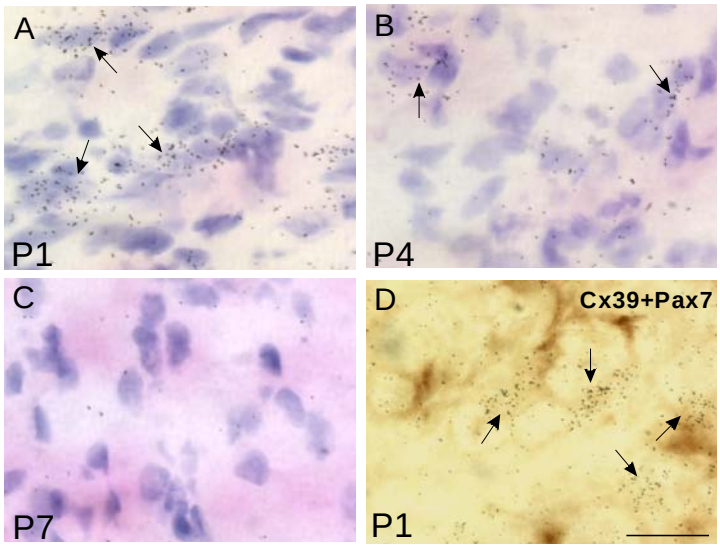

Fig.6
A–C In situ hybridization analysis of Cx39 expression in hematoxylin–eosin-stained sections of postnatal gastrocnemius muscle at various postnatal days (P1, P4, and P7). D Doublelabeling analysis (Cx39 + Pax7) at P1 showing that Cx39 mRNA does not co-localize with Pax7- positive nuclei, a marker for activated satellite cells. Arrows indicate Cx39 mRNA-labeled cells. Scale bar 20 μm (A–C), 25 μm (D)

individual cells (Fig.6A) and its absence in muscle regions containing more differentiated myotubes. At P4, Cx39 expression further decreased (Fig.6B) and disappeared within the first week of postnatal life (Fig.6C).

Expression of Cx43

The first signal of Cx43 mRNA expression was observed at E11 at the myotome level. At E15 the analysis of cresyl-violet-stained sections revealed the presence of clusters of Cx43-mRNA-positive cells surrounded by Cx43-mRNA-negative cells (Fig.7A). At E18

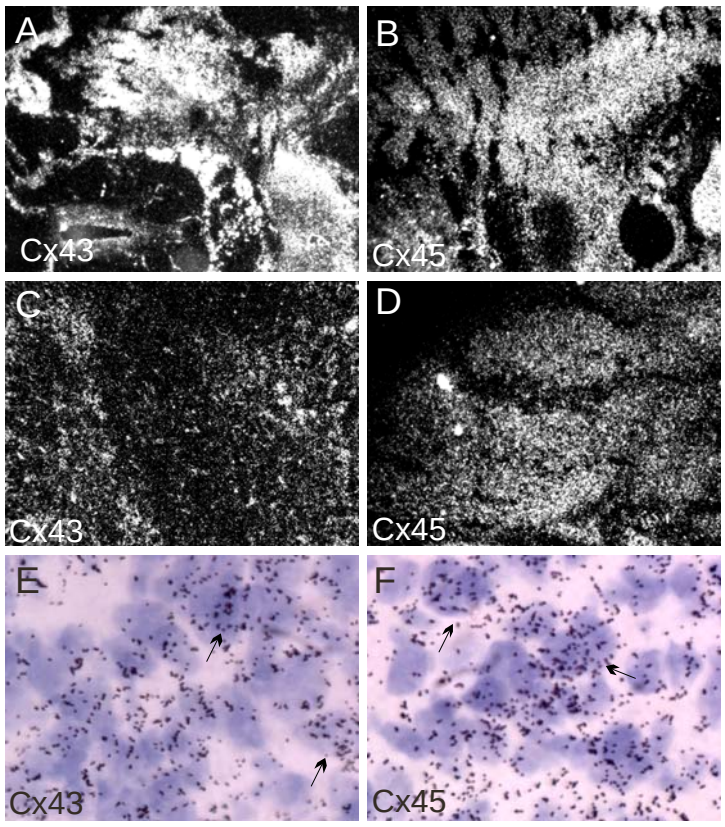

Fig.7
In situ hybridization analysis of Cx43 and Cx45 mRNA expression, during embryonic development. A and B development stage E15. C and D development stage E18. E and F Microautoradiograms showing Cx43 and Cx45 mRNA labeling in cresyl-violet-stained sections of developing hindlimb skeletal muscle at embryonic stages E18.

Cx43 positive cells were located as individual cells only around cluster of myotubes (Fig.7 *C, E*), suggesting a Cx43 expression in satellite cells. At P1, a feature of Cx43 mRNA

positive cell distribution was their presence over individual cells located around 4-5 myofibers in transverse section and in aligned cells in longitudinal section The levels of Cx43 positive cells found at P1 progressively decrease and reach the adult levels at P23.

Expression of Cx45

The first signal of Cx45 mRNA expression was observed at E11 at the myotome level virtually involving all the cells. Between E11 and E17 Cx45 was expressed in a large number of cells and at E18 Cx45 positive cells were located only around the myotubes (Fig...7 *B,D and F*). Between P1 and P7 several Cx45 positive cells forming clusters were observed, with a progressive reduction already at P10. In the adult muscle Cx45 was found expressed in individual cells located along the myofibers, suggesting an expression in satellite cells.

Cxs expression in normal and regenerating muscle of adult rat

Cx (Cx30, Cx37, Cx39, Cx40, Cx43, and Cx45) mRNA expression was analyzed by the in situ hybridization method at different time points (6, 12, and 24h; 2, 3, 5, 7, 9, and 12 days; 2, 3, and 4 wk) from gastrocnemius crush injury.

Cx30 mRNA expression.

Cx30 was not expressed either in the control uninjured or in the regenerating injured muscle (data not shown).

Cx37 mRNA expression.

In control, uninjured muscle, low levels of Cx37 mRNA were detected only in the endothelial cells of blood vessels (Fig.8*A*). Twenty-four hours following the crush injury, Cx37 expression was upregulated surrounding the area of lesion in the endothelial cells (Fig.8*B*). Between 24 and 72h from the crush injury, Cx37 mRNA-labeled cells increased and formed rows of cells migrating from the blood vessels to the area of lesion (Fig.8*C*). Three days after injury, Cx37 mRNA-expressing cells were found inside the area of lesion (Fig.8*D*), and, by 7–9 days after injury, they were distributed in the internal layer of circular cell clusters, some of which differentiated in blood vessels with endothelial cells expressing Cx37 (Fig.8, *E* and *F*). Twelve days after injury, Cx37 mRNA expression in the area of lesion was restricted to endothelial cells of newly generated blood vessels. In all of the time points examined, Cx37 was also expressed in the vessels of undamaged tissue.

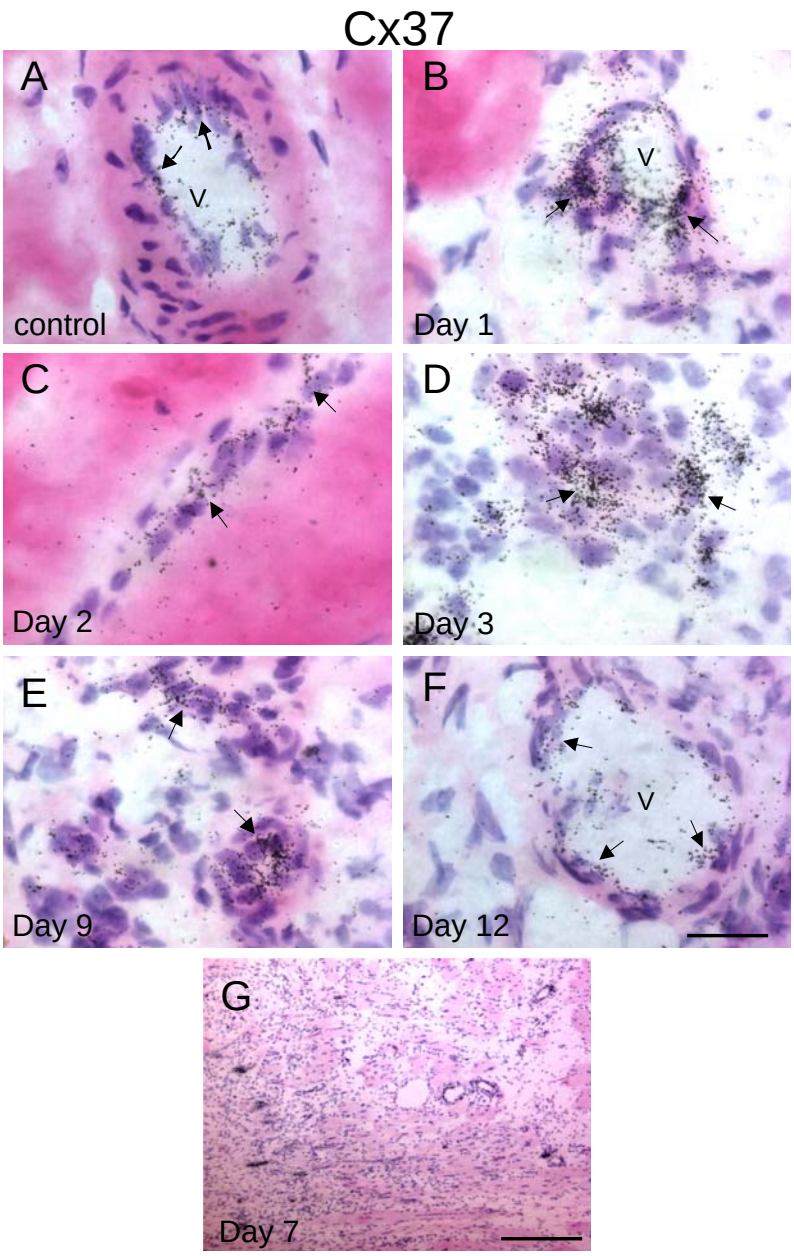

Fig.8
Micro-autoradiograms showing connexin (Cx) 37 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [control (*A*); *day 1* (*B*); *day 2* (*C*); *day 3* (*D*); *day 9* (*E*); *day 12* (*F*); *day 7* (*G*)] from crush injury. Detailed information is given in RESULTS. In *G* is shown an overview of lesioned area. Arrows indicate Cx37 mRNA-labeled cells nuclei (black grains). V, vessel. Scale bar: 25 μm (*A*–*F*); 250 μm (*G*).

Cx39 mRNA expression.

In control, uninjured muscle, Cx39 mRNA was not detectable. Three days after crush injury, we could observe the appearance of Cx39 mRNA-labeled cells along the border between the area of lesion and the uninjured muscle fibers. Cx39 mRNA-labeled cells

increased inside the area of lesion between 4 (Fig.9*A*) and 5 days after injury and were distributed as scattered cells, although small clusters of two and three cells were also observed. From 5 to 9 days after injury, Cx39 mRNA-labeled cells further increased and were mainly forming rows of aligned and closely apposed cell nuclei (Fig.9*B*). By 9 days from injury, in the regenerating area, Cx39 mRNA positive cells formed clusters with

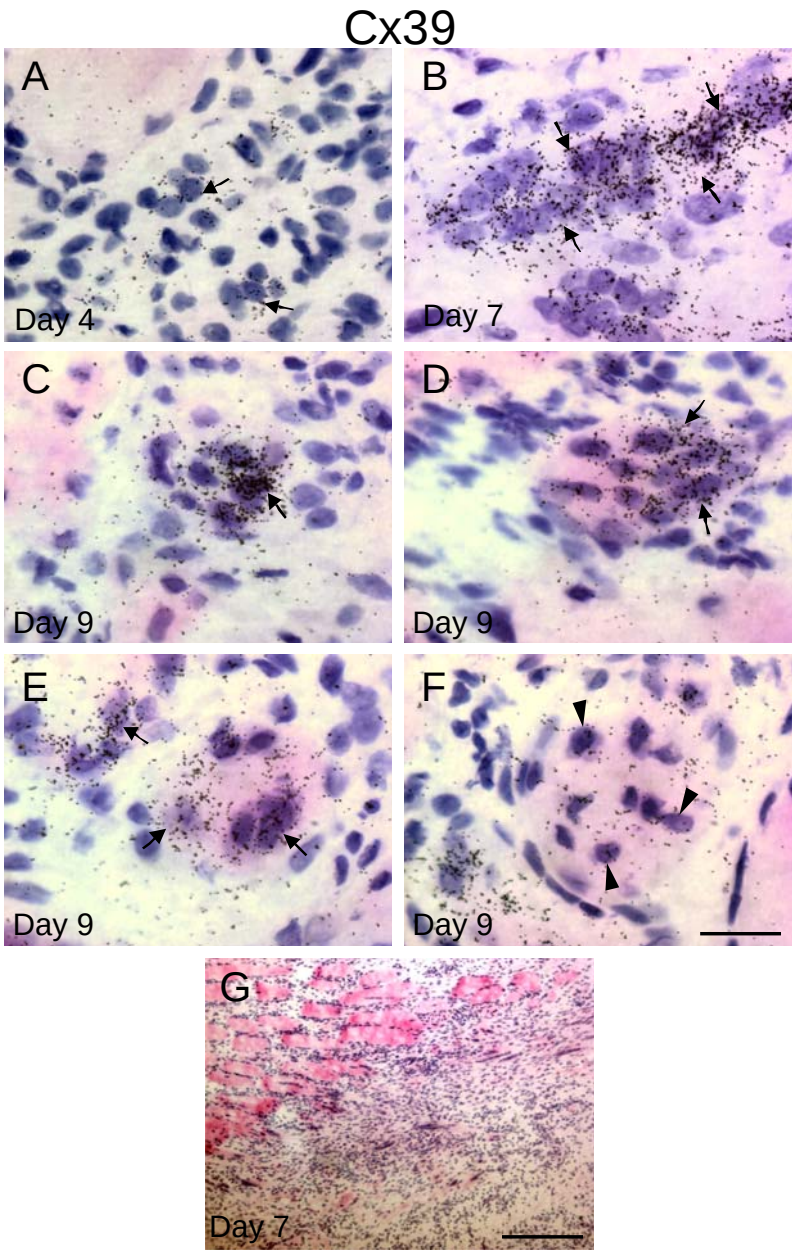

Fig. 9.
Micro-autoradiograms showing Cx39 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [*day 4* (*A*), *day 7* (*B* and *G*), *day 9* (*C–F*)] from crush injury. In *G* is shown an overview of lesioned area. Arrows indicate Cx39 mRNA-labeled cells nuclei (black grains). Arrowheads in *F* indicate cells nuclei poorly labeled with Cx39 mRNA compared with cells nuclei in *C–E*. Scale bar: 25µm (*A–F*); 250 µm (*G*).

heterogeneous morphology: *1*) clusters in which cell nuclei with intense labeling were closely apposed to each other, probably corresponding to an early stage of cell aggregation before cell fusion (Fig.9*C*); *2*) clusters in which cell nuclei were separated by an increasing eosinophilic sarcoplasm, representing a more advanced stage of myotube formation (Fig.9, *D* and *E*); *3*) poorly labeled clusters in which a further increase in size of the eosinophilic sarcoplasm delineated the typical appearance of myotubes with centrally located nuclei (Fig.9*F*). Between 9 and 15 days, the Cx39 expression was still present but progressively decreased until its complete disappearance at 21 days from injury. Cx39 mRNA-labeled cells were always strictly localized inside the area of lesion and were never observed in the surrounding uninjured fibers or in the blood vessels.

Cx40 mRNA expression

In control, uninjured muscle, Cx40 was expressed at very low levels in the endothelial cells of blood vessels (Fig.10*A*). Twenty-four to forty-eight hours after injury, a strong expression of Cx40 mRNA appeared surrounding the area of lesion in the endothelial cells (Fig.10*B*). Three days after the injury, rows of Cx40 mRNA-labeled cells were migrating from the vessels to the area of lesion (Fig.10*C*). Between 4 and 5 days from injury, Cx40 mRNA-labeled cells were inside the area of lesion, mainly distributed as scattered cells, although small clusters of two and three cells were also observed. Between 5 and 9 days from injury, the number of Cx40 mRNA-labeled cells in the area of lesion was further increased, mainly forming rows of closely apposed cell nuclei (Fig.10*D*) that could correspond to an early stage of cell aggregation before cell fusion. Nine days after injury, in the regenerating area, Cx40 mRNA-labeled cells formed clusters with heterogeneous morphology (Fig.10*E*) similar to those above described for Cx39. In the regenerating area, Cx40 was also expressed in clusters of cells probably committed to generate new vessels and in the endothelial cells of new blood vessels (Fig.10*F*). The spatial and temporal patterns of Cx40-expressing cells involved in new blood vessel formation were similar to that described for Cx37. The number of Cx40 mRNA-labeled cells progressively decreased between 15 and 21 days after injury. Four weeks after injury, Cx40 mRNA expression was restricted to endothelial cells.

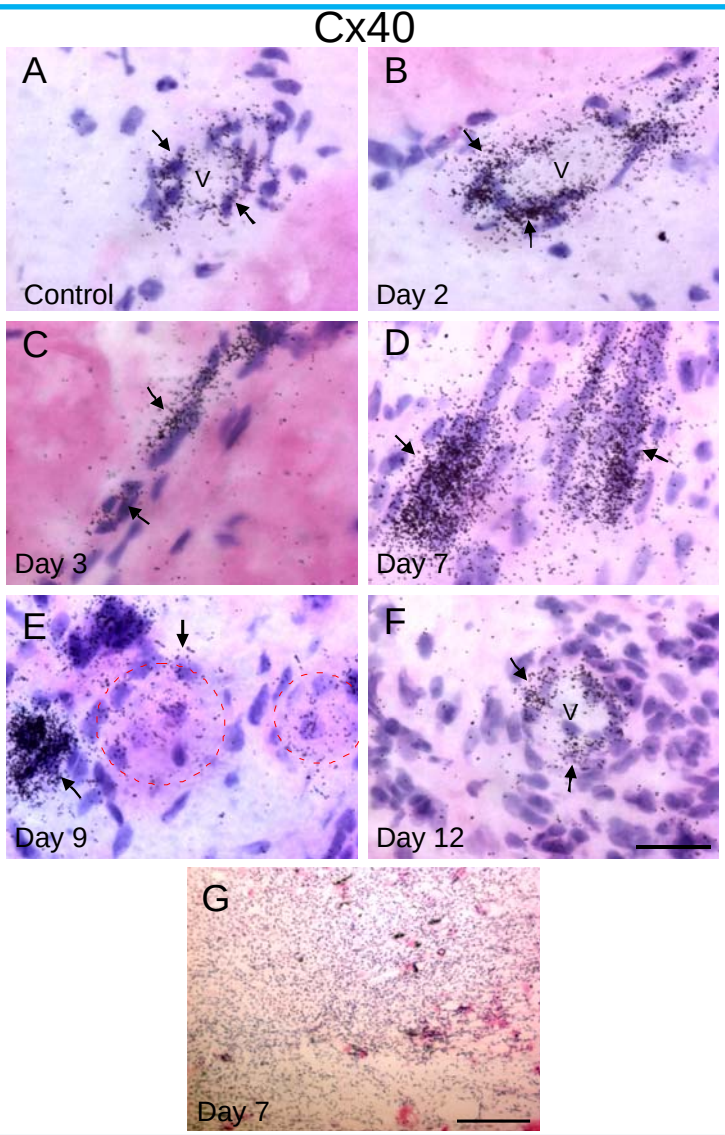

Fig. 10
Micro-autoradiograms showing Cx40 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [control (*A*), *day 2* (*B*), *day 3* (*C*), *day 7* (*D* and *G*), *day 9* (*E*), *day 12* (*F*)] from crush injury. In *G* is shown an overview of lesioned area. Arrows indicate Cx40 mRNA-labeled cells nuclei (black grains). Cluster of cells with different morphology are shown in *E*, where circled area indicates cell nuclei poorly labeled with Cx40 mRNA with increasing eosinophilic sarcoplasm, representing a more advanced stage of myotube formation. Scale bar: 25 µm (*A–F*);250 µm (*G*).

Cx43 mRNA expression

In control, uninjured muscle, Cx43 was weakly expressed in scattered cells along the basal lamina of muscle fibers (Fig.11*A*). An increase of Cx43 mRNA-labeled cells was already observed 3h following the crush injury in the uninjured zones of the muscle. The

number of Cx43 mRNA-labeled cells progressively increased between 6 and 12h from injury, involving region distant from the area of lesion (Fig.11*B*). Between 24 and 48h from injury, Cx43 mRNA-labeled cells accumulated in the region surrounding the lesioned area

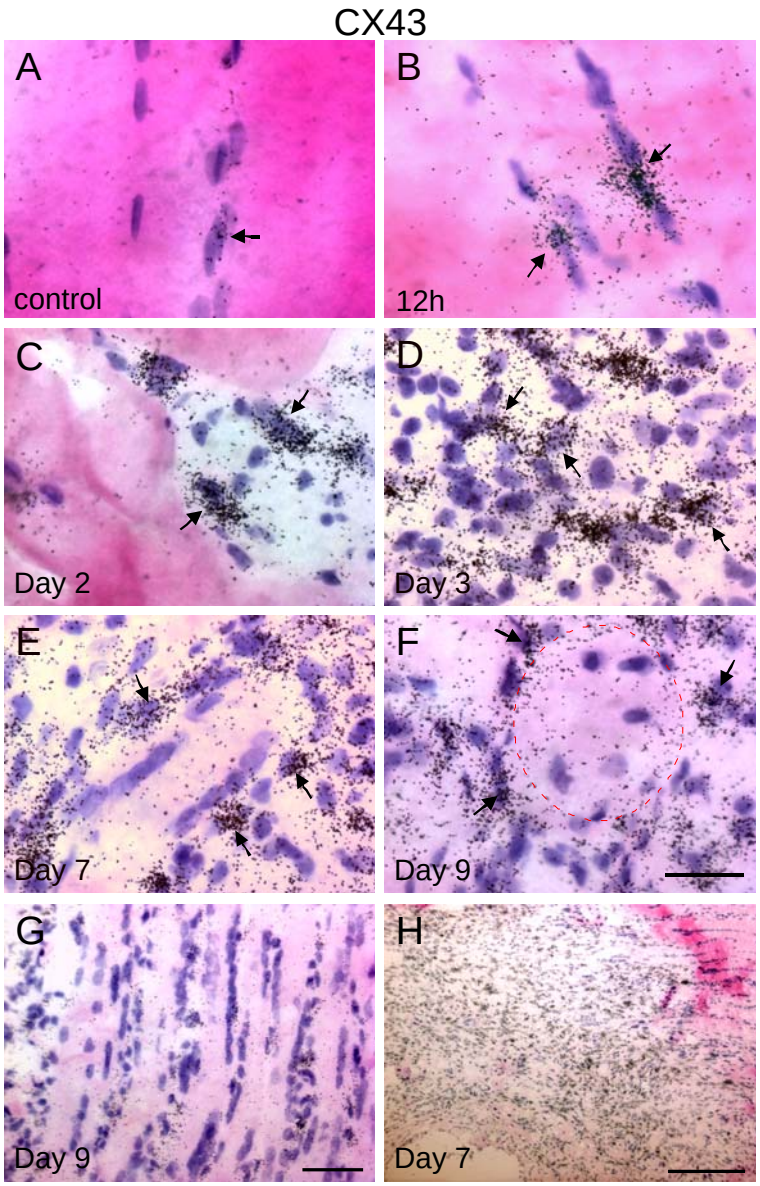

Fig.11
Micro-autoradiograms showing Cx43 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [control (*A*), 12 h (*B*), *day 2* (*C*), *day 3* (*D*), *day 7* (*E* and *H*), *day 9* (*F* and *G*)] from crush injury. In *H* is shown an overview of lesioned area. Arrows indicate Cx43 mRNA-labeled cells nuclei (black grains). In *F*, circled area indicates cell nuclei Cx43 mRNA negative with increasing eosinophilic sarcoplasm, representing a more advanced stage of myotube formation. In *G*, Cx43 mRNA-labeled cells (arrows), probably corresponding to new satellite cells, were located along the basal lamina of new myofibers. Scale bar: 25 µm (*A–F*); 50 µm (*G*); 250 µm (*H*).

(Fig.11*C*). Between 3 and 4 days from injury, a large number of Cx43 mRNA-labeled cells were located inside the area of lesion (Fig.11*D*). Although in large number, the Cx43 mRNA-labeled cells were never forming rows of closely apposed cell nuclei, as we could observe for Cx39 or Cx40 mRNA-labeled cells. However, among the apparent irregular distribution of Cx43-labeled cells, there was a specific localization of Cx43-labeled cells along the growing border of new myotubes (Fig.11*E*). This type of Cx43 mRNA-labeled cell distribution increased between 6 and 9 days and was still present during the subsequent days until there was a complete regeneration of the injured area that took place 4 wk after injury. Interestingly, in the regenerated area, single Cx43 mRNA-labeled cells, probably corresponding to new satellite cells, were located along the basal lamina of new myofibers (Fig.11, *F* and *G*). We never observed Cx43 labeling in the endothelial cells, including the newly formed one in the regenerated area.

Cx45 mRNA expression

In control, uninjured muscle, the Cx45 was weakly expressed in scattered cells along the basal lamina of muscle fibers (data not shown). An upregulation of Cx45 mRNA was already observed at 6h from injury (Fig.12*A*) in individual cells along the uninjured fibers surrounding the lesioned area (Araya *et al.* 2005). Between 12 and 48h from injury, Cx45 mRNA-labeled cells increased and formed chains of cells migrating inside the area of lesion (Fig.12*B*). Between 3 and 4 days from injury, Cx45 mRNA-labeled cells accumulated inside the area of lesion (Fig.12*C*). Between 4 and 9 days from injury, the number of Cx45 mRNA-labeled cells further increased in the area of lesion. Similar to Cx43, Cx45 mRNA-labeled cells were never forming clusters of aligned and closely apposed cell nuclei. However, among the apparent irregular distribution of Cx45-labeled cells, there was specific localization along the growing border of new myotubes (Fig.12*D*). This type of Cx45 mRNA-labeled cells was still present during the subsequent days until there was a complete regeneration of injured area. In contrast to Cx43, we did not observe single Cx45 mRNA-labeled cells along the basal lamina of new myofibers. Although in control, uninjured muscle and also surrounding the area of lesion, Cx45 was never observed in the blood vessels (Fig.12*F*), in the regenerating area, we could observe Cx45-labeled cells in the external layer of cell clusters probably committed to generate new vessels and both in the vascular smooth cells and in endothelial cells of newly formed blood vessels (Fig.12*E*), as also reported by Araya et al. (Araya *et al.* 2005).

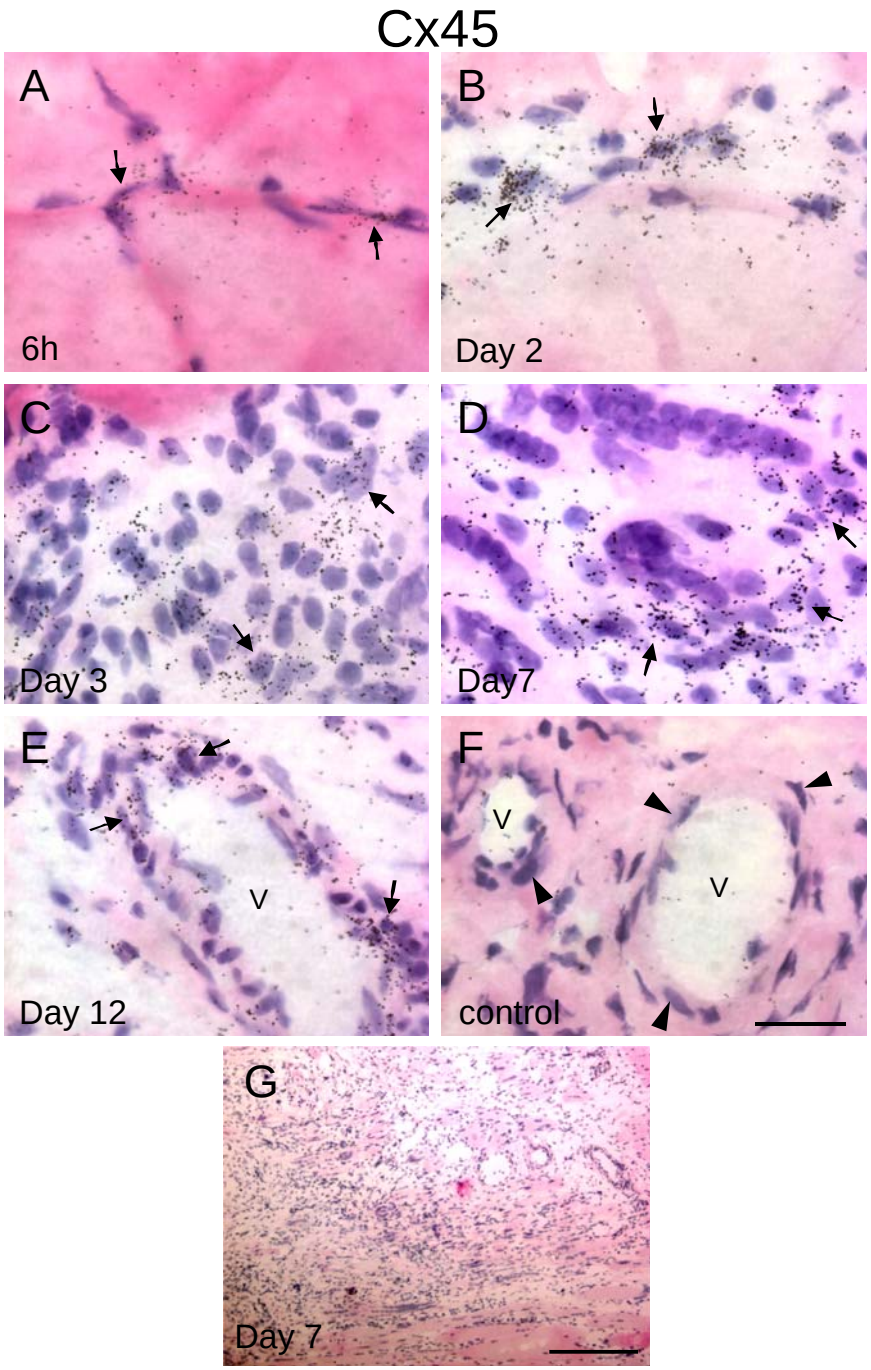

Fig.12
Micro-autoradiograms showing Cx45 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [6 h (*A*), *day 2* (*B*), *day 3* (*C*), *day 7* (*D* and *G*), *day 12* (*E*), control (*F*)] from crush injury. In *G* is shown an overview of lesioned area. Arrows indicate Cx45 mRNA-labeled cells nuclei (black grains). Arrowheads indicate endothelial cells Cx45 mRNA negative. Scale bar: 25 µm (*A–F*); 250 µm (*G*).

Morphological distribution of cells expressing Cx39, Cx40, Cx43, and Cx45 in the area of lesion.

In the regenerating area along the growing border of new myotubes, Cx43 or Cx45 mRNA-labeled cells seemed to form, as shown in figure 13-*C*, -C_1, -*D*, and -D_1, a front of cells that progressively generate chains of aligned and closely apposed cell nuclei. These aligned and fusing cell nuclei were labeled for Cx39 or Cx40 mRNAs (Fig.13*A*, A_1, *B*, and B_1).

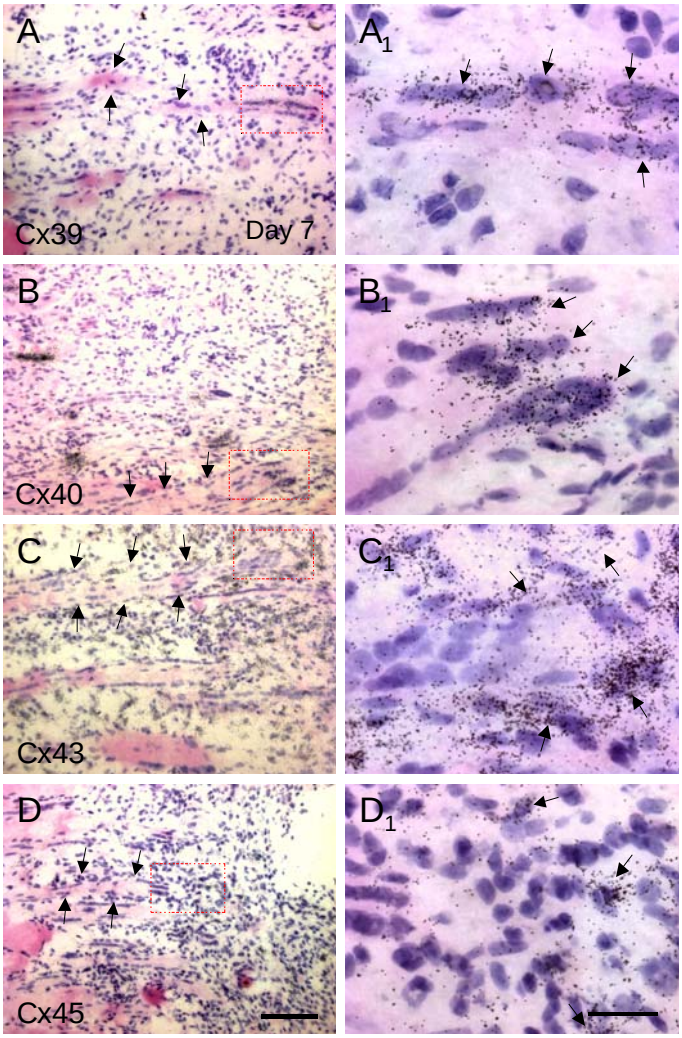

Fig. 13
Micro-autoradiograms showing Cx39 (*A* and *A1*), Cx40 (*B* and *B1*), Cx43 (*C* and *C1*), and Cx45 (*D* and *D1*) mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at 7 days from crush injury. *A–D*: low magnification showing differentiating new myotubes (arrows) in the regenerating area. *A1–D1*: high magnification of the area indicated in the square of *A–D*, corresponding to growing region of selected myotube. Arrows in *A–D* indicate the growing new myotube. Arrows in *A1–D1* indicate Cx mRNA-labeled cell nuclei (black grains). Scale bar: 100 µm (*A–D*); 25 µm (*A1–D1*).

Identification of Cx-expressing cells.

To identify the myogenic cell types involved in Cx mRNA expression, a double-labeling was performed by combining in situ hybridization and immunohistochemistry. A combination of specific markers was used to identify early activated satellite cells or

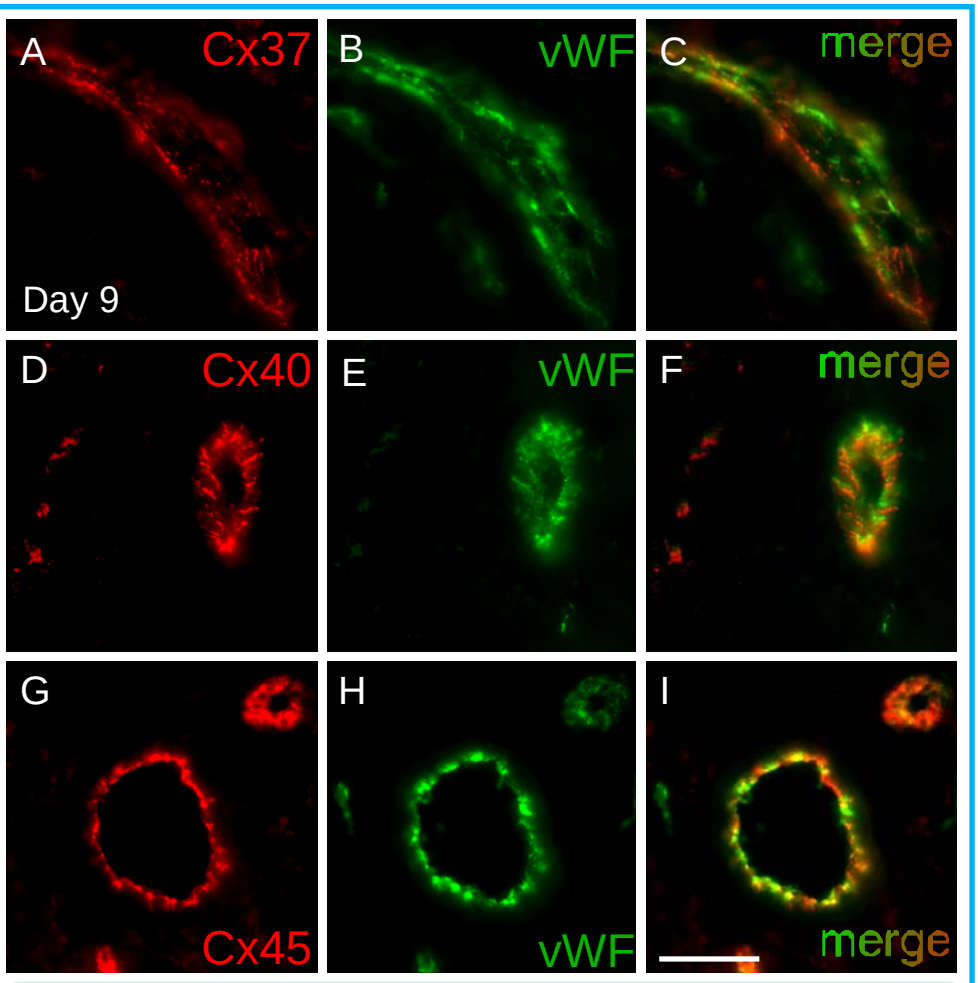

Fig.14
Cx37 (*A*), Cx40 (*D*), and Cx45 (*G*) colocalization with von Willebrand factor (vWF; *B*, *E*, and *H*) in gastrocnemius muscle at 9 days from crush injury. *C*, *F*, and *I*: overlay showing Cx37, Cx40, and Cx45 (red), respectively, colocalizing with vWF (green). Scale bar: 50 µm.

differentiating myogenic cells until their fusion in myotubes: M-cadherin , as marker for quiescent, active, and replicative satellite cells (Irintchev *et al.* 1994), and myogenin, as marker for myogenic cells differentiating in myoblasts and myocytes forming myotubes (Creuzet *et al.* 1998). The double-labeling analysis was performed in injured gastrocnemius muscles at three critical time point (1, 3, and 9 days from injury). Cx37 mRNA was never

colocalized in M-cadherin or myogenin-positive cells (data not shown), in accordance with its expression restricted to endothelial cells of both preexistent and newly formed blood vessels, as identified using anti-vWF antibodies specific for endothelial cells (Fig.14, *A–C*). Cx39 mRNA was always colocalized in myogenin-positive cells mainly forming clusters of aligned and closely apposed cell nuclei or in myogenin-positive cells forming cluster with heterogeneous morphology corresponding to different advanced stages of myotube formation (Fig.15*A*). Virtually all Cx39-labeled cells were myogenin-positive cells. Cx40

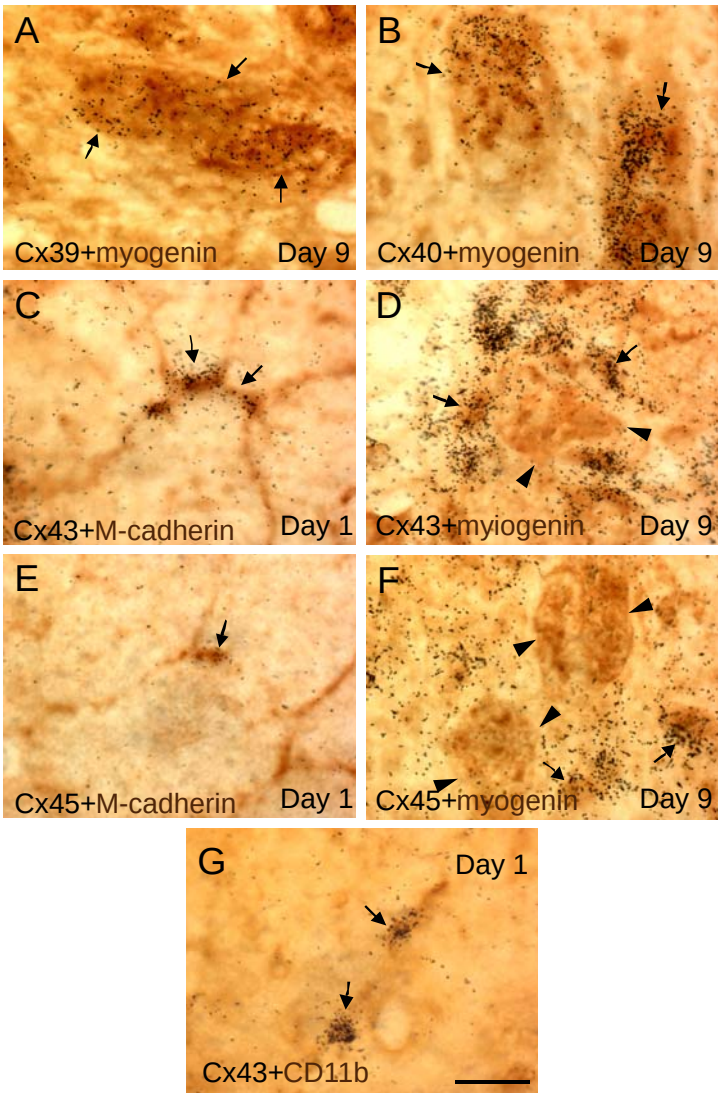

Fig 15
Micro-autoradiograms showing colocalization of Cx43 (*C*) and Cx45 (*E*) mRNAs labeling in cadherin positive cells and Cx39 (*A*), Cx40 (*B*), Cx43 (*D*), and Cx45 (*F*) mRNAs in myogenin-positive cells of gastrocnemius muscle, respectively, at 1 and 9 days from crush injury. *G*: colocalization of Cx43 mRNA labeling (blackgrains) in CD11b-positive cells (brown). Arrows indicate colocalization of Cx mRNA labeling (black grains) in cadherin (brown; *C* and *E*) or myogenin (brown; *A*, *B*, *D*, and *F*) or CD11b-positive cells (brown; *G*). Arrowheads (*D* and *F*) indicate cluster of cells with large sarcoplasm and with myogenin-positive nuclei devoid of specific Cx RNA labeling. Scale bar: 25 µm.

mRNA inside the area of lesion was also colocalized in myogenin-positive cells forming clusters with profiles similar to those described for Cx39 (Fig.15*B*). However, in contrast to Cx39, the Cx40 was also found expressed in endothelial cells of both preexistent and newly formed blood vessels (Fig.14, *D–F*). In control tissue and 1 day after injury, Cx43 mRNA was colocalized in M-cadherin-positive cells (Fig.15*C*), but not all Cx43 mRNA-labeled cells were positive for M-cadherin. Three and nine days after injury, Cx43 mRNA was colocalized in individual myogenin-positive cells, but again not all Cx43-labeled cells were positive for myogenin (Fig.15*D*). Using anti-CD11b antibodies to identify macrophages/monocytes, we could find that Cx43 mRNA is expressed in CD11b-positive cells (Fig.15*G*). However, Cx43 mRNA was not expressed in aligned and closely apposed myogenin-positive cell nuclei or in cluster of cells with large cytoplasm, representing more advanced stages of myotube formation (Fig.15*D*). One day after injury, Cx45 mRNA was expressed in M-cadherin-positive cells (Fig.15*E*), but not all Cx45 mRNA-labeled cells were positive for M-cadherin. Inside the area of lesion, both 3 and 9 days after injury, Cx45 mRNA was colocalized in individual myogenin-positive cells, but not all Cx45-labeled cells were myogenin positive (Fig.15*F*) (Araya *et al.* 2005). Using anti-vWF antibodies, Cx45 was also found coexpressed in new blood vessels (Fig.14, *G–I*). However, Cx45 mRNA was not expressed in aligned and closely apposed miogenin-positive cell nuclei or in cells with large cytoplasm, representing more advanced stages of myotube formation (Fig.15*F*).

Colocalization of Cx39 and Cx40.

Because from 5 to 9 days after muscle injury both Cx39 and Cx40 mRNA-labeled cells were mainly forming clusters of aligned and closely apposed cell nuclei (Figs 9*B* and 10*D*) that could correspond to an early stage of cell aggregation before cell fusion, we argue the possibility that Cx39 and Cx40 were coexpressed in the pre or fusing cells. To this end, we performed, at 7 days from lesion, a double immunolabeling using specific antibodies for Cx39 and Cx40. Both Cxs were detected as puncta localized in clusters of cells in the area of lesion. All cluster of cells expressing Cx40 were also Cx39 positive, whereas several clusters of cells were only Cx39 positive (Fig.16*D*). The developing skeletal muscle at embryonic day 15 was used as internal positive control (Fig.16, *G-I*).

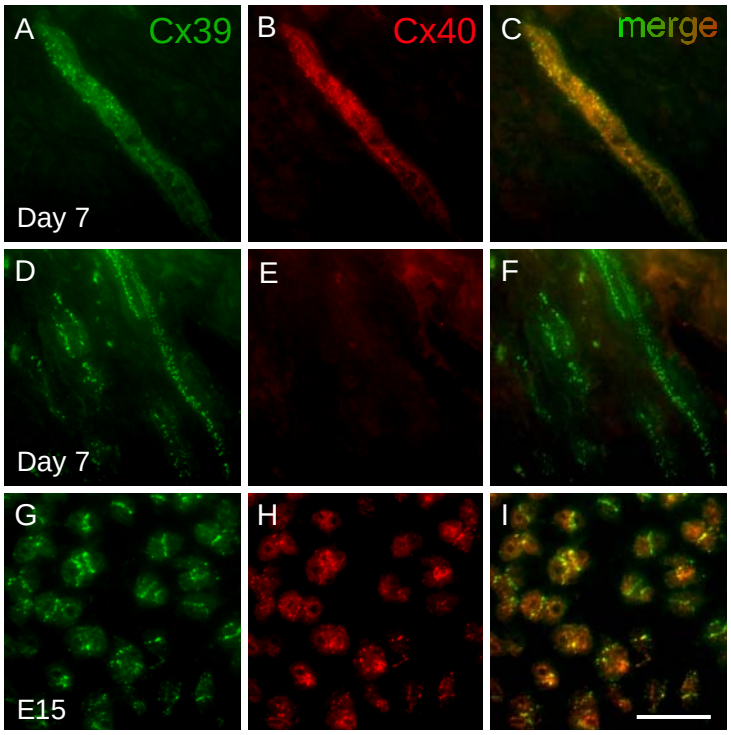

Fig.16
Cx39 (*A*, *D*, and *G*) and Cx40 (*B*, *E*, and *H*) immunolabeling in gastrocnemius muscle at 7 days from crush injury. Note the immunostaining as typical puncta (*A* and *B*, *D* and *E*, *G* and *H*) mainly localized in clusters of aligned and closely apposed cells. *C*: overlay showing Cx40 (red) colocalizing with Cx39 (green). *F*: overlay showing absence of Cx40 (red) in Cx39 (green) positive cells. *G–I*: show, as internal control, immunostaining for Cx40 and Cx39 in developing intervertebral skeletal muscle at embryonic *day 15* (E15). Scale bar: 50 µm.

Cx expression in BrdU-positive cells.

This study was performed to verify the possibility that Cx40 and Cx39 mRNA could be expressed in proliferating myogenic precursor cells. To this end, we labeled proliferating cells by injection of BrdU at three time points (3, 5, and 7 days) from muscle injury. Cx39 mRNA was not expressed in BrdU-positive cells at all time points studied, suggesting that it can be expressed only in differentiating myogenic cells (Fig.17*B*). Three days after lesion, Cx40 mRNA was expressed in 16 ± 3.4% of BrdU-positive cells mainly located around the blood vessels (Fig.17*C*). By contrast, in the same experimental condition, Cx37 and Cx43 mRNAs were expressed, respectively, in about 18 ± 4.3% and 80 ± 8.9% of BrdU-positive cells (17*A*,*C*). The other two time points examined showed a comparable trend.

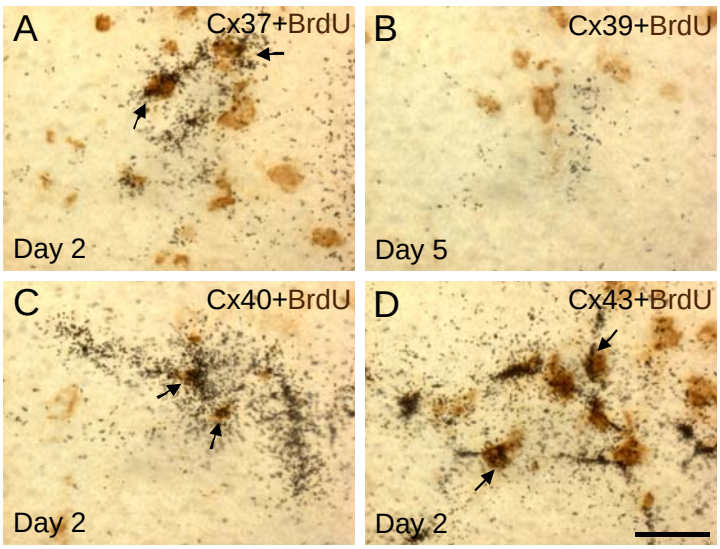

Fig.17
Micro-autoradiograms showing colocalization of Cx37 (*A*), Cx39 (*B*), Cx40 (*C*), and Cx43 (*D*) mRNA labeling in bromodeoxyuridine (BrdU)-positive cells of gastrocnemius muscle at representative time points from crush injury. Note in *B* the absence of colocalization of Cx39 mRNA labeling (black grains) in Brdu positive cells (gray), and in *A*, *C*, and *D* the colocalization, respectively, of Cx37, Cx40, and Cx43 mRNA labeling in BrdU-positive cells. Arrows indicate cells with Cx mRNA labeling (black grains) and BrdUpositive nuclei (gray). Scale bar: 25 µm.

AIM II

Low-intensity endurance exercise effects on damaged skeletal muscles in mouse and rat

Low-intensity endurance exercise

Exercise training protocol (Tab I) on the rotarod apparatus.

Week	Session time (min)	Rotations/min (RPM)	Length (m)	Speed (m/min)
1	30	20	120	4
2	45	20	180	4
3	60	20	240	4
4	60	24	288	4.8

Table I. Training protocol on the rotarod apparatus.

Body weight and exercise

As shown in Figure 18, at the beginning of exercise training protocol (Tab I) on the rotarod apparatus, body weight in *MDX*-EX (26.2 ± 2.2g) and *MDX*-SD (24.8 ± 1.9g) mice was significantly lower compared to WT-EX (27.9 ± 1.8g; $p<0.05$) and WT-SD (28.0 ± 2.0g; $p<0.001$) mice. After 2 weeks of training, body weight was still significantly lower in *MDX*-SD (26.8 ± 2.0g) mice as compared to WT-SD (28.8 ± 2.3g, $p<0.05$) mice. After 3 and 4 weeks of training no significant differences were found between groups; however, in *MDX*-EX mice body weight tended to be lower than in *MDX*-SD mice.

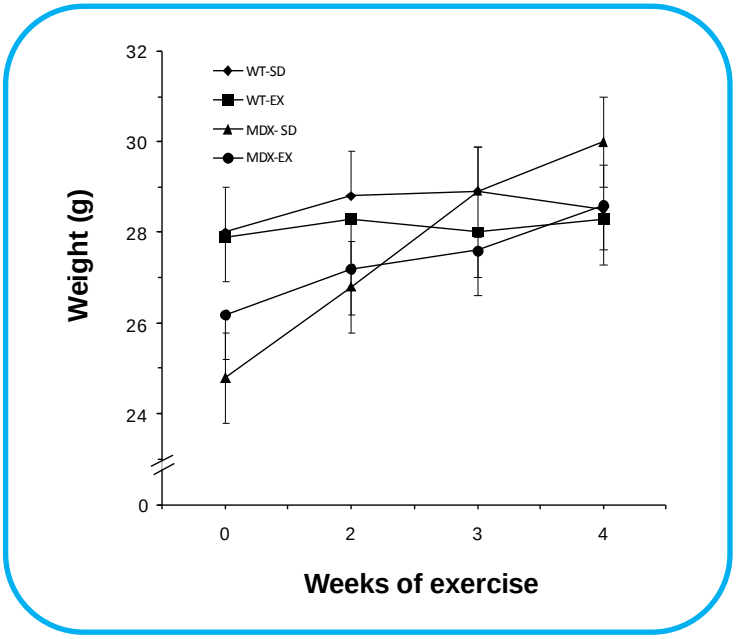

Fig.18
Body weight changes in sedentary and exercised *mdx* and wild type mice during training period. Data are expressed as means. MDX-SD: sedentary *mdx* mice; MDX-EX: exercised *mdx* mice; WT-SD: sedentary wild type mice; WT-EX: exercised wild type mice.

Fatigue

The time course of fatigue resistance was analyzed for each group of mice and in all groups a reduced not significant grip strength fatigue was observed (Fig.19). However, there

was a non significant trend of fatigue resistance improvement in *MDX*-EX mice as compared to *MDX*-SD mice.

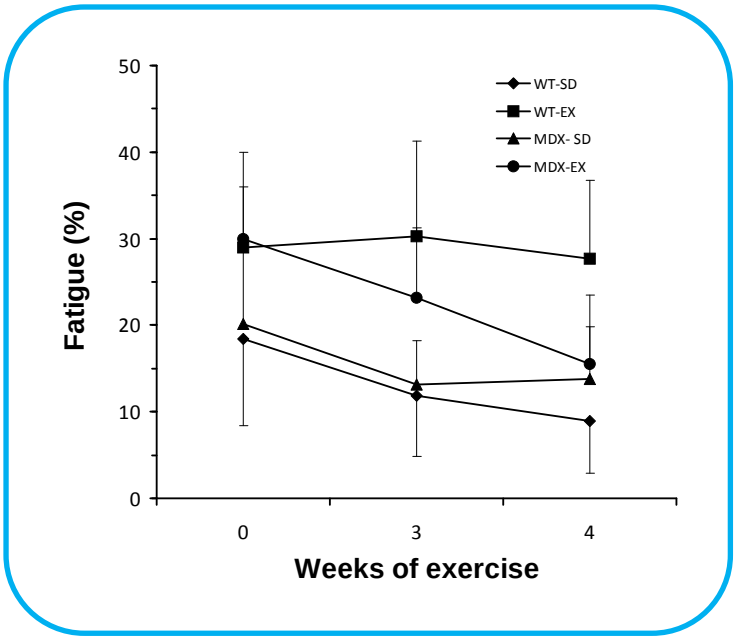

Fig.19
Grip strength fatigue in sedentary and exercised *mdx* and wild type mice during training period. Data are expressed as means. MDX-SD: sedentary *mdx* mice; MDX-EX: exercised *mdx* mice; WT-SD: sedentary wild type mice; WT-EX: exercised wild type mice.

Forelimb strength normalized for weight

Forelimb strength normalized for body weight (FSW, gram strength per gram of body weight) did not show significant difference between all groups at any time point of the training protocol. The time course of FSW was also analysed inside each group of mice and were found significant variations between week 1 and 3, and reported as follows: in *MDX*-EX, FSW was 2.40 ± 0.61g/g at week 1 and 1.88 ± 0.34g/g at week 3 ($p<0.05$); in WT-EX, FSW was 2.29 ± 0.5 g/g at week 1 and 1.76 ± 0.57g/g at week 3 ($p<0.0001$); in WT-SD, FSW was 2.15 ± 0.46g/g at week 1 and 1.64 ± 0.28g/g at week 3 ($p<0.05$). In *MDX*-SD, FSW did not change at any time point.

Histomorphological analysis

Histological analyses on muscle tissue sections were used to assess the effect of low-intensity endurance exercise on muscle pathology progression of *mdx* mice. The Figure 2 shows representative sections for each group, along with the main differences found in the muscle histology as a consequence of exercise. No evident difference was found in muscle of sedentary and exercised wild type mice and was apparently absent both necrosis and regenerating processes in exercised mice (Fig.20, *A*, *B*), suggesting that low-intensity endurance exercise did not cause damage to myofibres. Skeletal muscle of non-exercised (sedentary) *mdx* mice showed in all time points examined, in both gastrocnemius and quadriceps muscles, a typical histopathological signs with necrosis foci, fibrous connective tissue and areas with active processes of degeneration/regeneration of damaged myofibres (Fig.20*C*). By contrast, histomorphological analysis of exercised *mdx* mice showed a progressive almost complete disappearance of areas with inflammatory necrotic myofibres and a parallel increase of regenerating myofibres in the gastrocnemius as well as in the quadriceps muscle (Fig.20*D, F*). Histological evidence of increased areas showing regenerated myofibres, distinguished for the central location of aligned nuclei, was recorded already in muscle of mice exercised for 15 days (Fig.20*D*). The muscle morphology in exercised *mdx* mice for 30 days showed several regenerated myofibres and all necrotic foci under regenerating process (Fig.20*E*). In *mdx* mice exercised for 45 days the analysis showed very few regenerating myofibers and a large number of regenerated myofibres (Fig.20*F*), suggesting a potential complete recovery of damaged muscle. This progressive accumulation of newly regenerated myofibres with nuclei in central location may find explanation with the current observation that central myonuclei may last for about 50–100 days and thereafter move to a peripheral subsarcolemmal position (McGeachie *et al.* 1993).

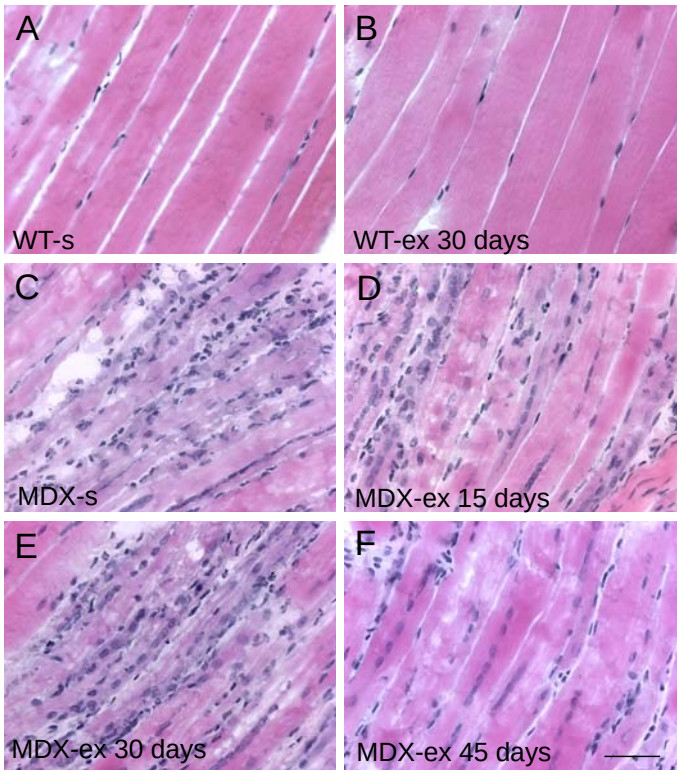

Fig.20
Representative skeletal muscle sections stained with haematoxylin-eosin from wild type and mdx mice. No apparent morphological difference there exist in sedentary and exercised wild type mice (A-B). A consistent myofibre necrosis is present in sedentary mdx mice (C), whereas areas of progressive subsequent regeneration are present in exercised mdx mice with increased new myofibres (D-F) with central nuclei that are a reliable indicator of previously necrotic/regenerated tissue. For detailed explanation see results section. WT-SD, sedentary wild type; WT-EX, exercised wild type; MDX-SD, sedentary mdx; MDX-EX, exercised mdx. Arrows indicate active processes of degeneration with many disorganized nuclei, probably inflammatory cells and cells involved in regenerating process. Arrowed indicate regenerating and regenerated myofibres with aligned central nuclei. Scale bar 50µm

Cx39 protein levels as marker of regenerative process in injured muscle of C57BL6 mice

In the adult skeletal muscle, various kinds of trauma promote proliferation of satellite cells that differentiate into myoblasts to form new myofibers, or to repair the damaged one. In the rat gastrocnemius following injury the Cx39 is upregulated only during the regeneration process and is involved in myogenic cells aggregation and fusion to form new myotubes (Belluardo *et al.* 2005; Trovato-Salinaro *et al.* 2009). Therefore the detection of Cx39 protein can be used as marker associated with active regeneration processes of damaged muscle. Here, we performed a time course of muscle regeneration in C57BL/6

mice following mechanical injury in order to corroborate the critical expression of Cx39 in regenerating muscle. In Figure 21*B* representative gastrocnemius muscle sections from control and following 8 days from injury are shown. Concerning Cx39 protein levels (Fig.21), in control uninjured muscle Cx39 was undetectable, as previously reported for Cx39 mRNA. (Trovato-Salinaro *et al.* 2009). In the injured muscle fibres, Cx39 protein level was undetectable until three days following crush injury, whereas it was found increased after 8 days and showed a strong reduction 11 days after injury (Fig.21). Cx39 disappeared 21 days following injury (Fig.21), when the injured area was completely repaired. Overall, this model confirm that, during muscle regeneration following crush injury, there can be a transient activation of Cx39 in the area of lesion testifying active regeneration process

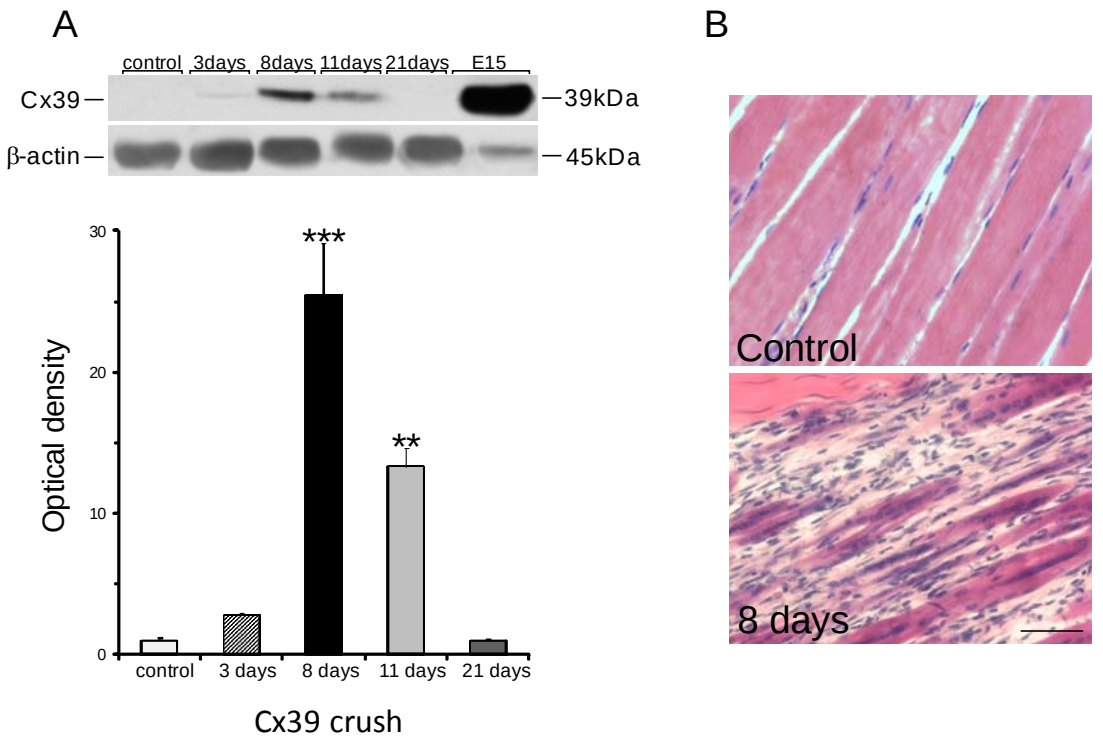

Fig.21
Western blotting analysis of Cx39 protein levels. (A) Time-course (3,8,11 and 21 days from injury) of Cx39 protein levels in the injured gastrocnemious of C57Bl/6 mice as compared to control muscle (uninjured). E15: internal positive control of Cx39 in developing skeletal muscle of E15 embryonic mouse.(B) Representative gastrocnemious muscle sections from control and following 8 days from injury. Scale bar: 50µm

(Belluardo *et al.* 2005).

Evaluation of muscle regenerative process in *mdx* mice by measuring Cx39 proteins levels

In the present work, in order to follow the recovery of damaged muscle in *mdx* mice, we used the detection of Cx39 protein as marker associated with active regeneration processes of damaged muscle. Therefore, in addition to morphological point of view, the levels of regeneration process in the gastrocnemius or quadriceps of exercised *mdx* mice were also evaluated by examining the Cx39 protein levels. Cx39 protein, as expected, was not detected in muscles of wild type C57BL/10 mice, both in sedentary and exercised mice (Fig.22). In sedentary *mdx* mice Cx39 protein levels, both in gastrocnemius and quadriceps muscles, were found markedly increased because of active degeneration/regenerating processes (Fig.22). In exercised *mdx* mice (15, 30 and 45 days), Cx39 protein levels dropped progressively, as compared to sedentary *mdx* mice, to very low levels (Fig.22). These data, according to those of morphological analysis, indicated a strong reduction of regeneration processes in muscle of *mdx* mice exercised for 45 days and suggested almost a full recovery of damaged muscle.

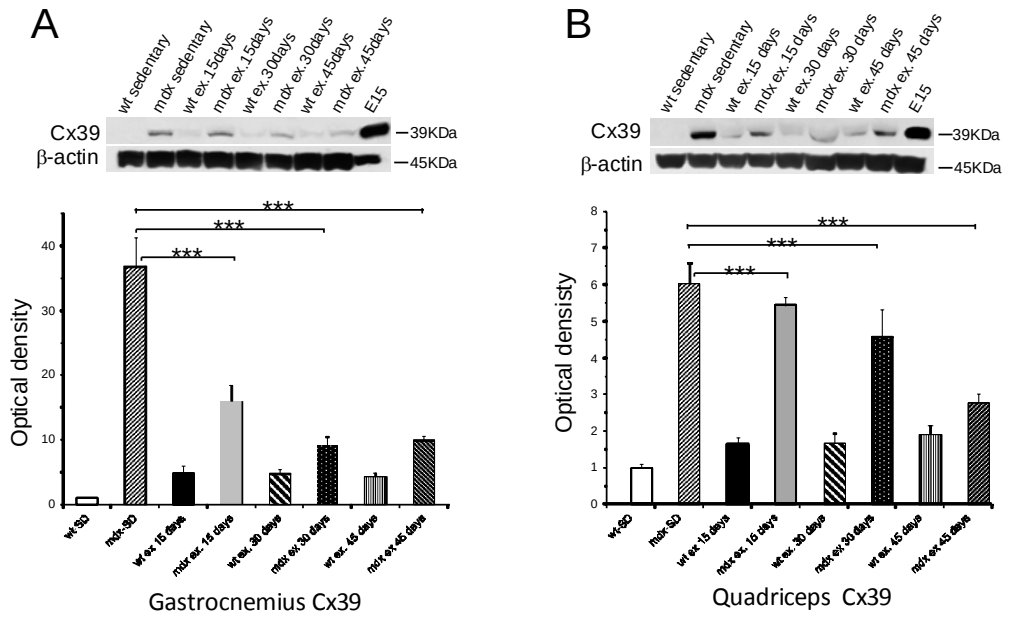

Fig.22
Western blotting analysis of Cx39 protein levels. (A-B) Cx39 protein levels in gastrocnemius and quadriceps skeletal muscles of wild type mice, sedentary (wt-sd) or exercised (wt-ex), and mdx mice, sedentary (mdx-sd) or exercised (mdx-ex) for 15, 30 and 45 days. E15: internal positive control of Cx39 in developing skeletal muscle of E15 embryonic mouse.

DISCUSSION

Role of Cxs expressed in regenerating muscle.

Among the Cxs examined, only the Cx39, Cx43 and Cx45 were found expressed during embryonic life and progressively reduced during early postnatal life until to become dramatically expressed at very low levels like Cx43 and Cx45 or to be undetectable like Cx39 in the adult muscle. The other Cxs, although expressed at low levels, were found localized in the endothelial cells (Cx37 and Cx40) according to previous data (Araya *et al.* 2005; Gorbe *et al.* 2005). Although gap-junctional communication is absent in adult skeletal muscle, the presence of gap junctions during skeletal muscle development has been previously documented by functional and ultrastructural studies (Schmalbruch 1982; Constantin and Cronier 2000; Rash and Staehelin 1974; Kalderon *et al.* 1977; Duxson *et al.* 1989). Multinucleated muscle cells are formed by the fusion of mononucleated myoblasts that are aligned, closely apposed, and interconnected by gap junctions during the pre-fusion phase (Schmalbruch 1982; Constantin and Cronier 2000; Rash and Staehelin 1974; Kalderon *et al.* 1977; Constantin *et al.* 1997; Balogh *et al.* 1993; Araya *et al.* 2003; Mege *et al.* 1994; Proulx *et al.* 1997; Duxson and Usson 1989). Moreover, the presence of gap junctions between myotubes in differentiating skeletal muscles has been reported at the ultrastructural level (Ling *et al.* 1992). Data with respect to the molecular identity of the connexins forming inter-myoblast gap junctions are available. Cx43 and Cx40 have been previously described in myoblasts of differentiating skeletal muscles (Dahl *et al.* 1995), and evidence in favor of a functional role of Cx43 in the differentiation and fusion of myoblasts (Balogh *et al.* 1993; Mege *et al.* 1994; Proulx *et al.* 1997; Araya *et al.* 2003) has been reported in culture systems. Indeed, following the chemical block of gap junctions or after deletion of the Cx43 gene, cultured myoblasts do not fuse with each other and do not express specific genes for cellular differentiation, such as myogenin (Schmalbruch 1982; Constantin and Cronier 2000; Rash and Staehelin 1974; Kalderon *et al.* 1977; Duxson and Usson 1989; Constantin *et al.* 1997; Balogh *et al.* 1993; Araya *et al.* 2003; Mege *et al.* 1994; Proulx *et al.* 1997). The Cx39 is expressed in aligned and closely apposed myogenin-positive myoblasts and in early myotubes and might participate in inter-myoblast and probably inter-myotube

communication in this critical phase of differentiation. The absence of Cx39 in activated satellite cells of early postnatal muscles and its disappearance within the first week of postnatal life suggests that this connexin is not involved in the process of postnatal growth of the muscle mass. Indeed, the postnatal growth of myofibers is dependent on the apposition and fusion of single activated satellite cells to existing myofibers, a process that takes place for several weeks after birth (Moss and Leblond 1971). Recently, using Cx39 knockout mice, it has been showed (von Maltzahn et al, in press) that Cx39 is not indispensable for myoblast fusion and therefore for myofibers formation probably because it is compensate by other Cxs, such as Cx43.

The present study provided a comparative analysis of spatial and temporal mRNA expression of five Cxs in the regenerating skeletal muscle after crush injury. After muscle crush injury, the mRNAs of Cxs examined were upregulated in regenerating muscle with distinct spatial and temporal patterns involving, with exclusion of Cx37, myogenic cells.

The endothelial localization and spatio-temporal distribution of Cx37 mRNA expression clearly revealed an involvement of Cx37 in the angiogenesis of regenerating muscle, although disruption of Cx37 did not result in obvious defects in vasculogenesis or vascular development (Simon and McWhorter 2003), probably because it could be compensated by other Cxs, such as Cx40 (Simon and McWhorter 2002). BrdU incorporation in Cx37 mRNA-labeled cells located in the endothelium of blood vessels, as shown both by histological observation and indirectly by Cx37/vWF colocalization, furthermore, could indicate the source of Cx37-expressing precursor cells involved in the angiogenesis of regenerating muscle.

The expression of Cx39 mRNA in single myogenin-positive cells, in aligned and closely apposed cells, and in newly formed myotubes suggests that Cx39 may be involved in intercellular communication during the process of cell aggregation, coordination, and fusion to form new myotubes, according to previous data (Belluardo *et al.* 2005; von Maltzahn *et al.* 2004). The spatial pattern of Cx39 mRNA expression shows a localization of Cx39 mRNA restricted to the injured area where the formation of myotubes and myofibers takes place (Charge and Rudnicki 2004). The temporal pattern of Cx39 mRNA expression in injured muscle is correlated with the appearance of myogenin-positive cells in the area of lesion (3 days after injury) and with the peak of formation of new myotubes (7–9 days after injury).

In the present work, following muscle injury, was evidenced that Cx40 mRNA expression is upregulated in endothelial cells, as identified by vWF marker, and subsequently, within 3 days from injury, these Cx40 mRNA-expressing cells were migrated to the area of lesion where they followed alignment in closely apposed cells coexpressing Cx39 mRNA fuse in myotube. However, the observation that the majority of cell clusters expressing Cx39 mRNA were Cx40 mRNA negative opens the question about the possible existence in the skeletal muscle of two distinct myogenic precursor cells both committed to form new myotubes. In fact, currently it is believed that Cx39 mRNA is expressed in differentiating and fusing myoblasts generated by myogenic precursor cells or satellite cells expressing Cx43 mRNA and located between the basal lamina and the sarcolemma of their associated muscle fibers (Araya *et al.* 2005; Belluardo *et al.* 2005). Here we could show that Cx40 mRNA-expressing precursor cells located in the endothelium of blood vessels, as shown both by histological observation and indirectly by Cx37/vWF colocalization, may generate myoblasts fusing each other to form myotubes and, as showed by double-labeling immunohistochemistry, coexpressing Cx39. Additionally, myoblast cell line C2C12 cells do not express Cx40, although they express Cx43 and Cx39 (von Maltzahn *et al.* 2006). This is the first report showing, in regenerating muscle, that blood vessels might be a source of Cx40-expressing myogenic precursor cells committed to become myoblasts fusing in myotube. The existence of myogenic cells related to the endothelial cell lineage in human skeletal muscle or in mouse embryo muscle has been reported by Zheng et al. (2007) and by Le Grand et al. (2004), respectively. Additionally, Cx40 mRNA-positive cells could also be related to pericytes associated with microvasculature walls that efficiently contribute to myogenic regeneration (Dellavalle *et al.* 2007), or to mesoangioblast cells, an adult vessel-derived stem cell that possess high myogenic potential (Sampaolesi *et al.* 2006). Furthermore, myogenic precursor cells expressing Cx40 may also derive from the blood. However, specific markers and studies are needed to define the profile of the precursor endothelial Cx40 positive cells and their relationship with myogenic precursor cells generating Cx39-expressing cells. The parallel expression of Cx40 in endothelial cells of new blood vessels further suggests an expression in precursor cell involvement in blood vessel generation, although disruption of Cx40 did not result in obvious defects in vasculogenesis or vascular development (Simon and McWhorter 2003). However, Cx37 and Cx40 could be co-expressed in endothelial precursor cells, and could overlap functionally in generating vessels, as revealed by severe vascular abnormalities after elimination of both

Cxs (Simon and McWhorter 2002). This possibility implicates a Cx40-expressing precursor cell population distinct from that one involved in the new myotube formation.

A few hours after injury, we observed an upregulation of Cx43 mRNA and an increase of Cx43 mRNA-labeled cells in a large area around the lesion, but only a minor percentage of them were myogenic precursor cells, as shown by colocalization with M-cadherin marker. This result is in agreement with the present data showing that Cx43 mRNA is expressed in CD11b-positive cells, a marker for macrophages/monocytes, and with previous observations that, in regenerating muscle, both monocytes and macrophages also express Cx43 (Araya *et al.* 2005; Gorbe *et al.* 2005). The expression of Cx43 mRNA in M-cadherin-positive cells suggests a role in proliferating and differentiating satellite cells (Gorbe *et al.* 2005). Within 3 days, a large number of Cx43 mRNA-labeled cells accumulated inside the area of lesion, and after 4–5 days the Cx43 mRNA-labeled and myogenin-positive cells appear to be organized in clusters or closely apposed to myoblasts forming new myotubes. Cx43 mRNA was never expressed in aligned, closely apposed myogenin-positive cells and in newly formed myotubes, suggesting that its functional role is played in stages preceding the myotube formation. A role for Cx43 in myoblast proliferation and syncytial fusion in regenerating skeletal muscle, or in muscle primary cultures has been recently reported (Araya *et al.* 2005; Gorbe *et al.* 2005). Thus Cx43-positive cells can proliferate inside the area of lesion, generating daughter cells expressing Cx39 and are committed to differentiate in myoblasts fusing in myotubes. This possibility is also supported by the observation that cells expressing Cx39 mRNA appear 3 days after the injury inside the area of lesion. Alternatively, cells expressing Cx39 may directly derivate from Cx43-positive cells after downregulation of Cx43 gene (Gorbe *et al.* 2005; von Maltzahn *et al.* 2004; von Maltzahn *et al.* 2006). This hypothesis correlates with the lack of BrdU incorporation in Cx39 mRNA-positive cells. In the regenerating area, single Cx43 mRNA-positive cells, probably corresponding to new satellite cells, were also observed along the basal lamina of newly myotubes or myofibers identified by the central position and high density of nuclei.

It is well known that satellite cells in injured muscle proliferate and migrate inside the area of lesion, where they further proliferate and generate myogenic precursor cells (self-renewed satellite cells) or myoblasts committed to generate by fusion the multinucleated myotubes (Charge and Rudnicki 2004; Zhao and Hoffman 2004). Similar to Cx43, a few hours after injury, a population of Cx45 mRNA-labeled and M-cadherin-positive cells was observed in a large area around the lesion. The expression of Cx45 mRNA in M-cadherin

cells suggests a role for this Cx in proliferating and differentiating satellite cells. Within 4–5 days after injury, the Cx45 mRNA-labeled and myogenin-positive cells appear as single cells or organized in clusters or closely apposed to myoblasts forming new myotubes. Cx45 mRNA was never found expressed in aligned, closely apposed, myogenin-positive cells and in newly formed myotubes, suggesting that its functional role starts in stages preceding the myotube formation. Although the pattern of Cx45 and Cx43 mRNA expression during muscle regeneration appears to be similar, there are observations supporting their expression in distinct myogenic precursor cell population. For example, in contrast to Cx43 mRNA-labeled cells, we could not observe single Cx45 mRNA-positive cells along the basal lamina of newly formed myofibers. Additionally, myoblast cell line C2C12 cells do not express Cx45, although they express Cx43 and Cx39 (von Maltzahn *et al.* 2006). However, specific investigations are needed to establish if Cx45- and Cx43-labeled cells are distinct myogenic precursor cell population. On the other hand, inside the injured area, Cx45 mRNA was also expressed in M-cadherin or myogenin-negative cells that probably are involved in angiogenesis, as also suggested by expression of Cx45 in the endothelial cells of newly formed blood vessels. This observation is supported by the finding that Cx45-deficient embryos exhibit defects in remodeling and organization of blood vessels after proper initiation of angiogenesis, and fail to form a smooth muscle layer surrounding the major arteries (Kruger *et al.* 2000). Taken together, these data confirm that also Cx45, similarly to Cx37 and Cx40, is involved in blood vessel generation (Kruger *et al.* 2000) and implicate a precursor cell population expressing Cx45 distinct from that one co-expressing Cx45 and myogenin, potentially involved in muscle regeneration, as also reported by Araya et al. (Araya *et al.* 2005).

On the basis of results of the present and related previous studies, it appears that the distinct pattern of Cx40, Cx43, and Cx45 mRNA expression found during muscle regeneration could correlate with different populations of myogenic precursor cells that have been described to reside in the adult skeletal muscle (Bischoff 1986; Goldring *et al.* 2002; Hawke and Garry 2001; Schmalbruch and Lewis 2000; Seale and Rudnicki 2000; Zammit and Beauchamp 2001). By contrast, the Cx39 expression seems to be essential during myoblasts fusion, independently of precursor cell-type-generating myoblasts. As integrate information of the present data, we propose a model of Cx expression during the skeletal muscle regeneration process leading to new myofiber formation or repair of damaged ones (Fig.23). Cx40 mRNA-expressing cells seem to be generated by activated, proliferating

precursor cells localized in the blood vessels, and, subsequently, these Cx40 mRNA-expressing cells migrate to the area of lesion, where, following differentiation in myoblasts and alignment in closely apposed cells, coexpressing Cx39 may generate new myotubes. Cx43 mRNA-expressing cells probably generate by activated proliferating satellite cells and, after migration in the area of lesion, may differentiate in myoblasts that following alignment in closely apposed cells expressing Cx39 mRNA fuse to form new myotubes (Araya *et al.* 2005; Belluardo *et al.* 2005; von Maltzahn *et al.* 2004; von Maltzahn *et al.* 2006). The

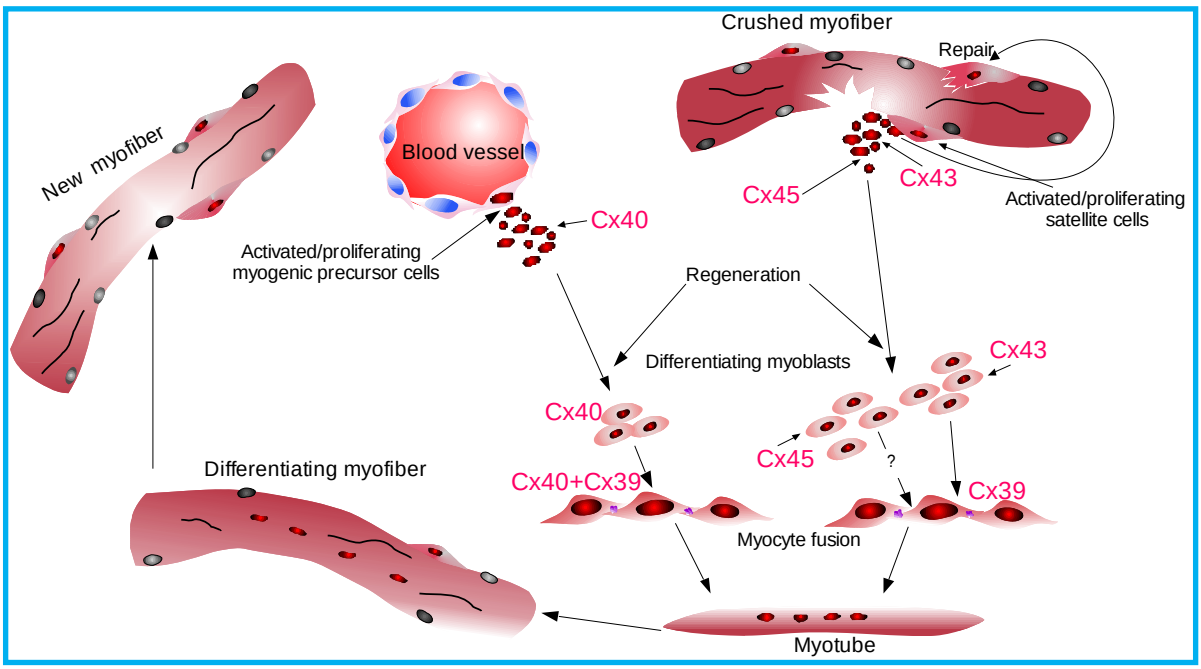

Fig.23
Schematic representation of skeletal muscle regeneration following a crush injury, showing the potential role of cell expressing Cx39, Cx40, Cx43, and Cx45 during satellite cell activation, proliferation, their differentiation in myoblasts and fusion to form new myotube.

potential role of Cx45-expressing myogenic cells in the formation of new myotubes seems to be less clear. Potentially, myogenic cells expressing Cx45 mRNA could represent a distinct precursor cells, a subset of satellite cells, that generate myoblasts committed to repair the injured myofibers and/or to add myonuclei to growing new myofibers.

Overall, in this model we suggest that, during muscle regeneration following crush injury, there can be activated two distinct myogenic precursor cell populations, expressing Cx40 and Cx43, committed to generate new myotubes and, therefore, new myofibers. For both of these myogenic precursor cell populations, the step of myoblast fusion to form new myotubes is characterized by the expression of Cx39. Recently, a functional study during the

regeneration process of skeletal muscle in Cx39 deficient mice evidenced an accelerated onset of *MyoD* and myogenin expression, suggesting an accelerated regeneration of skeletal muscle (von Maltzahn et al, in press). On the basis of increased levels of Cx43 expression in Cx39 deficient mice it has been hypothesized that Cx43 may compensate for the loss of Cx39 during regeneration.

Taken together, these results show that, in regenerating skeletal muscle, at least four Cxs, out of five expressed in the regenerating muscle, can be differentially involved in communication of myogenic cells during the process of cell proliferation, aggregation, and fusion to form new myotubes or to repair damaged myofibers.

Functional and morphological recovery of damaged skeletal muscle following low-intensity endurance exercise

Mdx mice

The results of this study provide evidence for beneficial effects of low-intensity endurance exercise in muscle of *mdx* mice, leading to consistent recovery of damaged muscle and in less extent to muscle function improvement. In fact, despite the severe skeletal muscle degeneration occurring in sedentary *mdx* mice, in skeletal muscle of exercised *mdx* mice there was a marked reduction of dystrophic signs with strong reduction of necrotic foci and increase of regenerating and regenerated myofibres. On the other hand, the low-intensity endurance exercise did not show substantial muscle functional improvement, measured as fatigue resistance and forelimb strength, in exercised *mdx* mice as compared to sedentary *mdx* mice. This weak improvement in muscle function was in contrast to strong muscle morphological changes, with less necrotic fibers and greater increase of fully regenerated muscle fibers. Hence, this study has provided further evidence that appropriate exercise parameters (frequency, intensity, duration, and mode) can indeed be critical for benefit on the recovery of dystrophic muscle and therefore to decrease the progression of muscular dystrophy (Sveen *et al.* 2007; Grange and Call 2007; Dupont-Versteegden *et al.* 1994; Wineinger *et al.* 1998).

Concerning the mechanisms involved in this recovery of dystrophic muscle, we may examine several possibility. Endurance training produces many physiological, metabolic and vascular adaptations in skeletal muscle (Febbraio and Pedersen 2005; Kaczor *et al.* 2007;

Faist *et al.* 2001; Vignos, Jr. and Watkins 1966; McCartney *et al.* 1988), but it is not clear how these changes may improve muscle pathology. Low intensity endurance exercise may have antinflammatory action (Zanchi *et al.* 2010; Nader and Lundberg 2009; Lira *et al.* 2009). It has been proposed that an inappropriate or excessive inflammatory response can directly damage myofibers in *mdx* (Porter *et al.* 2002; Tidball 2005); whereas non steroidal anti-inflammatory or anti TNFα treatment prevents muscular dystrophy pathology, enhances regenerating ability of muscle and delay significantly and persistently the progression of muscular dystrophy (Brunelli *et al.* 2007; Grounds and Torrisi 2004). More likely, several factors released in response to endurance exercise may regulate muscle stem cells and therefore enhance the adaptive response of skeletal muscle (Hawke 2005; Charifi *et al.* 2003).

In our previous studies, in healthy runners was found an high numbers of circulating endothelial progenitors together with reduced hemopoietic progenitor counts, suggesting peripheral utilization of bone marrow-derived cells during endurance exercise (Bonsignore *et al.* 2010), and release of inflammatory and hematopoietically active growth factors (Bonsignore *et al.* 2002). These progenitors cells are tracked by growth factors released from muscle during injury or exercise and gradually change their phenotype becoming muscle stem cells. Further, it has been shown that a release of bone marrow-derived progenitors occurs after exercise and might be involved in repair processes at the level of skeletal muscle (Palermo *et al.* 2005; Labarge and Blau 2002). All together, although the mechanisms involved in the observed effect of regular and low-intensity exercise on skeletal muscle function in the dystrophic condition remains poorly understood (Sveen *et al.* 2007; Grange and Call 2007)**,** it is however relevant that muscle lacking dystrophin can be recovered in response to specific endurance exercise.

It is of interest that a controlled, relatively slow speed of running on a rotarod may induce beneficial adaptations to *mdx* muscles, which currently are susceptible to mechanical stresses that result in small disruptions of the muscle sarcolemma (Brussee *et al.* 1997; Lynch *et al.* 2000). In addition, the present findings showed that a well designed non voluntary endurance exercise may be not deleterious to dystrophic muscle, in agreement with previous data obtained by using similar exercise protocols, such as voluntary low-resistance wheel running (Dupont-Versteegden *et al.* 1994; Hayes and Williams 1996; Radley *et al.* 2007), swimming exercise and low-intensity treadmill running (Hayes and Williams 1998). Because human dystrophic muscle shows several similarities to *mdx*

muscles, the present findings further support the usefulness of a low-intensity exercise as a potential therapeutic adjunct for human patients (Hayes and Williams 1998; Hayes *et al.* 1993).

To better evaluate the potential benefits of low-intensity endurance type of exercise on recovery of damaged dystrophic muscle, we measured also the expression of Cx39 gene that typically occur in response to regeneration process after muscle injury, leading to new myofibre formation (Trovato-Salinaro *et al.* 2009). This evaluation, not only testify for active regenerating process leading to new myofibres in sedentary *mdx* mice, but it also give to us the opportunity to use the Cx39 expression as marker to monitor the muscle regenerative process, and to know when a strong reduction of regenerating processes in muscle of exercised *mdx* mice take place, suggesting almost a full recovery of damaged muscle. Additionally, the disappearance of Cx39 expression in exercised *mdx* mice may also indicate that the designed endurance exercise did not induce damage of *mdx* mice myofibres, that notoriously are susceptible to mechanical stresses, resulting in small disruptions of the muscle sarcolemma and activation of regenerative processes.

In conclusion, this study has shown that specific low intensity endurance exercise induces a strong beneficial effect on the regeneration of dystrophic muscle and may have therapeutic value at least to decrease progression of muscular dystrophy and in less extend for strengthening dystrophic muscle.

ACKNOWLEDGEMENTS

I would like to thank all those persons that during the last three years, in various manners, have supported my work.

Especially thanks to Prof. Natale Belluardo, who introduced me to the world of the research, and guide me with professionalism during this beautiful and exciting experience. Thanks for his contagious passion for the research that stimulate me to do my work in a best way, and for his scientific support that contributed to my professional growth. Thanks for all his teachings which, I am sure, will be precious for my future.

Thanks also to D.ssa Giuseppa Mudò for her essential technical and moral support. Thanks for her words that encouraged me when the experiments do not work.

Thanks to my lab colleagues, Melania (expecially), Alessandra , Valentina, Francesca, Rossella, Maria, Florinda, Lara, Francesca e Federica for the funny moments spent together.

Finally I want also say thanks to my parents who encouraged me and supported all my decisions.

REFERENCES

Alderton J. M. and Steinhardt R. A. (2000) How calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. *Trends Cardiovasc. Med.* **10,** 268-272.

Allen R. E. and Boxhorn L. K. (1989) Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. *J. Cell Physiol* **138,** 311-315.

Allen R. E., Sheehan S. M., Taylor R. G., Kendall T. L. and Rice G. M. (1995) Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. *J. Cell Physiol* **165,** 307-312.

Araya R., Eckardt D., Maxeiner S., Kruger O., Theis M., Willecke K. and Saez J. C. (2005) Expression of connexins during differentiation and regeneration of skeletal muscle: functional relevance of connexin43. *J. Cell Sci.* **118,** 27-37.

Araya R., Eckardt D., Riquelme M. A., Willecke K. and Saez J. C. (2003) Presence and importance of connexin43 during myogenesis. *Cell Commun. Adhes.* **10,** 451-456.

Armstrong R. B. (1990) Initial events in exercise-induced muscular injury. *Med. Sci. Sports Exerc.* **22,** 429-435.

Armstrong R. B., Warren G. L. and Warren J. A. (1991) Mechanisms of exercise-induced muscle fibre injury. *Sports Med.* **12,** 184-207.

Arnold L., Henry A., Poron F., Baba-Amer Y., van R. N., Plonquet A., Gherardi R. K. and Chazaud B. (2007) Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* **204,** 1057-1069.

Asakura A. and Rudnicki M. A. (2002) Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. *Exp. Hematol.* **30,** 1339-1345.

Asakura A., Seale P., Girgis-Gabardo A. and Rudnicki M. A. (2002) Myogenic specification of side population cells in skeletal muscle. *J. Cell Biol.* **159,** 123-134.

Auerbach A. A. and Bennett M. V. (1969) A rectifying electrotonic synapse in the central nervous system of a vertebrate. *J. Gen. Physiol* **53,** 211-237.

Backman E. and Henriksson K. G. (1995) Low-dose prednisolone treatment in Duchenne and Becker muscular dystrophy. *Neuromuscul. Disord.* **5,** 233-241.

Balogh S., Naus C. C. and Merrifield P. A. (1993) Expression of gap junctions in cultured rat L6 cells during myogenesis. *Dev. Biol.* **155,** 351-360.

Barjot C., Cotten M. L., Goblet C., Whalen R. G. and Bacou F. (1995) Expression of myosin heavy chain and of myogenic regulatory factor genes in fast or slow rabbit muscle satellite cell cultures. *J. Muscle Res. Cell Motil.* **16,** 619-628.

Barjot C., Jbilo O., Chatonnet A. and Bacou F. (1993) Expression of acetylcholinesterase gene during in vitro differentiation of rabbit muscle satellite cells. *Neuromuscul. Disord.* **3,** 443-446.

Bassaglia Y. and Gautron J. (1995) Fast and slow rat muscles degenerate and regenerate differently after whole crush injury. *J. Muscle Res. Cell Motil.* **16,** 420-429.

Beauchamp J. R., Heslop L., Yu D. S., Tajbakhsh S., Kelly R. G., Wernig A., Buckingham M. E., Partridge T. A. and Zammit P. S. (2000) Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J. Cell Biol.* **151,** 1221-1234.

Beauchamp J. R., Morgan J. E., Pagel C. N. and Partridge T. A. (1999) Dynamics of myoblast transplantation reveal a discrete minority of precursors with stem cell-like properties as the myogenic source. *J. Cell Biol.* **144,** 1113-1122.

Belcastro A. N., Shewchuk L. D. and Raj D. A. (1998) Exercise-induced muscle injury: a calpain hypothesis. *Mol. Cell Biochem.* **179,** 135-145.

Belluardo N., Mudo G., Trovato-Salinaro A., Le G. S., Charollais A., Serre-Beinier V., Amato G., Haefliger J. A., Meda P. and Condorelli D. F. (2000) Expression of connexin36 in the adult and developing rat brain. *Brain Res.* **865,** 121-138.

Belluardo N., Trovato-Salinaro A., Mudo G. and Condorelli D. F. (2005) Expression of the rat connexin 39 (rCx39) gene in myoblasts and myotubes in developing and regenerating skeletal muscles: an in situ hybridization study. *Cell Tissue Res.* **320,** 299-310.

Bennett M. V. and Verselis V. K. (1992) Biophysics of gap junctions. *Semin. Cell Biol.* **3,** 29-47.

Birchmeier C. and Brohmann H. (2000) Genes that control the development of migrating muscle precursor cells. *Curr. Opin. Cell Biol.* **12,** 725-730.

Bischoff R. (1986) Proliferation of muscle satellite cells on intact myofibers in culture. *Dev. Biol.* **115,** 129-139.

Bischoff R. (1997) Chemotaxis of skeletal muscle satellite cells. *Dev. Dyn.* **208,** 505-515.

Black B. L. and Olson E. N. (1998) Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu. Rev. Cell Dev. Biol.* **14,** 167-196.

Bonsignore M. R., Morici G., Riccioni R., Huertas A., Petrucci E., Veca M., Mariani G., Bonanno A., Chimenti L., Gioia M., Palange P. and Testa U. (2010) Hemopoietic and angiogenetic progenitors in healthy athletes: different responses to endurance and maximal exercise. *J. Appl. Physiol* **109,** 60-67.

Bonsignore M. R., Morici G., Santoro A., Pagano M., Cascio L., Bonanno A., Abate P., Mirabella F., Profita M., Insalaco G., Gioia M., Vignola A. M., Majolino I., Testa U. and

Hogg J. C. (2002) Circulating hematopoietic progenitor cells in runners. *J. Appl. Physiol* **93,** 1691-1697.

Boonen K. J. and Post M. J. (2008) The muscle stem cell niche: regulation of satellite cells during regeneration. *Tissue Eng Part B Rev.* **14,** 419-431.

Boppart M. D., Burkin D. J. and Kaufman S. J. (2006) Alpha7beta1-integrin regulates mechanotransduction and prevents skeletal muscle injury. *Am. J. Physiol Cell Physiol* **290,** C1660-C1665.

Brunelli S., Sciorati C., D'Antona G., Innocenzi A., Covarello D., Galvez B. G., Perrotta C., Monopoli A., Sanvito F., Bottinelli R., Ongini E., Cossu G. and Clementi E. (2007) Nitric oxide release combined with nonsteroidal antiinflammatory activity prevents muscular dystrophy pathology and enhances stem cell therapy. *Proc. Natl. Acad. Sci. U. S. A* **104,** 264-269.

Brussee V., Tardif F. and Tremblay J. P. (1997) Muscle fibers of mdx mice are more vulnerable to exercise than those of normal mice. *Neuromuscul. Disord.* **7,** 487-492.

Bruzzone R. and Meda P. (1988) The gap junction: a channel for multiple functions? *Eur. J. Clin. Invest* **18,** 444-453.

Bruzzone R., White T. W. and Paul D. L. (1996) Connections with connexins: the molecular basis of direct intercellular signaling. *Eur. J. Biochem.* **238,** 1-27.

Brzoska E., Przewozniak M., Grabowska I., Janczyk-Ilach K. and Moraczewski J. (2009) Pax3 and Pax7 expression during myoblast differentiation in vitro and fast and slow muscle regeneration in vivo. *Cell Biol. Int.* **33,** 483-492.

Buckingham M. (2001) Skeletal muscle formation in vertebrates. *Curr. Opin. Genet. Dev.* **11,** 440-448.

Bulfield G., Siller W. G., Wight P. A. and Moore K. J. (1984) X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc. Natl. Acad. Sci. U. S. A* **81,** 1189-1192.

Burkin D. J. and Kaufman S. J. (1999) The alpha7beta1 integrin in muscle development and disease. *Cell Tissue Res.* **296,** 183-190.

Cao B. and Huard J. (2004) Muscle-derived stem cells. *Cell Cycle* **3,** 104-107.

Caplan A. I. (2008) All MSCs are pericytes? *Cell Stem Cell* **3,** 229-230.

Carter G. T., Wineinger M. A., Walsh S. A., Horasek S. J., Abresch R. T. and Fowler W. M., Jr. (1995) Effect of voluntary wheel-running exercise on muscles of the mdx mouse. *Neuromuscul. Disord.* **5,** 323-332.

Caveney S. (1985) The role of gap junctions in development. *Annu. Rev. Physiol* **47,** 319-335.

Cerletti M., Negri T., Cozzi F., Colpo R., Andreetta F., Croci D., Davies K. E., Cornelio F., Pozza O., Karpati G., Gilbert R. and Mora M. (2003) Dystrophic phenotype of canine X-

linked muscular dystrophy is mitigated by adenovirus-mediated utrophin gene transfer. *Gene Ther.* **10,** 750-757.

Charbonnier F., Gaspera B. D., Armand A. S., Van der Laarse W. J., Launay T., Becker C., Gallien C. L. and Chanoine C. (2002) Two myogenin-related genes are differentially expressed in Xenopus laevis myogenesis and differ in their ability to transactivate muscle structural genes. *J. Biol. Chem.* **277,** 1139-1147.

Charge S. B. and Rudnicki M. A. (2004) Cellular and molecular regulation of muscle regeneration. *Physiol Rev.* **84,** 209-238.

Charifi N., Kadi F., Feasson L. and Denis C. (2003) Effects of endurance training on satellite cell frequency in skeletal muscle of old men. *Muscle Nerve* **28,** 87-92.

Chazaud B., Brigitte M., Yacoub-Youssef H., Arnold L., Gherardi R., Sonnet C., Lafuste P. and Chretien F. (2009) Dual and beneficial roles of macrophages during skeletal muscle regeneration. *Exerc. Sport Sci. Rev.* **37,** 18-22.

Chazaud B., Sonnet C., Lafuste P., Bassez G., Rimaniol A. C., Poron F., Authier F. J., Dreyfus P. A. and Gherardi R. K. (2003) Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth. *J. Cell Biol.* **163,** 1133-1143.

Christov C., Chretien F., Abou-Khalil R., Bassez G., Vallet G., Authier F. J., Bassaglia Y., Shinin V., Tajbakhsh S., Chazaud B. and Gherardi R. K. (2007) Muscle satellite cells and endothelial cells: close neighbors and privileged partners. *Mol. Biol. Cell* **18,** 1397-1409.

Cifuentes-Diaz C., Nicolet M., Alameddine H., Goudou D., Dehaupas M., Rieger F. and Mege R. M. (1995) M-cadherin localization in developing adult and regenerating mouse skeletal muscle: possible involvement in secondary myogenesis. *Mech. Dev.* **50,** 85-97.

Condorelli D. F., Belluardo N., Trovato-Salinaro A. and Mudo G. (2000) Expression of Cx36 in mammalian neurons. *Brain Res. Brain Res. Rev.* **32,** 72-85.

Condorelli D. F., Trovato-Salinaro A., Mudo G., Mirone M. B. and Belluardo N. (2003) Cellular expression of connexins in the rat brain: neuronal localization, effects of kainate-induced seizures and expression in apoptotic neuronal cells. *Eur. J. Neurosci.* **18,** 1807-1827.

Connolly A. M., Keeling R. M., Mehta S., Pestronk A. and Sanes J. R. (2001) Three mouse models of muscular dystrophy: the natural history of strength and fatigue in dystrophin-, dystrophin/utrophin-, and laminin alpha2-deficient mice. *Neuromuscul. Disord.* **11,** 703-712.

Constantin B. and Cronier L. (2000) Involvement of gap junctional communication in myogenesis. *Int. Rev. Cytol.* **196,** 1-65.

Constantin B., Cronier L. and Raymond G. (1997) Transient involvement of gap junctional communication before fusion of newborn rat myoblasts. *C. R. Acad. Sci. III* **320,** 35-40.

Cooper R. N., Tajbakhsh S., Mouly V., Cossu G., Buckingham M. and Butler-Browne G. S. (1999) In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. *J. Cell Sci.* **112 (Pt 17),** 2895-2901.

Cornelison D. D. and Wold B. J. (1997) Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev. Biol.* **191,** 270-283.

Cossu G. and Bianco P. (2003) Mesoangioblasts--vascular progenitors for extravascular mesodermal tissues. *Curr. Opin. Genet. Dev.* **13,** 537-542.

Coulton G. R., Morgan J. E., Partridge T. A. and Sloper J. C. (1988) The mdx mouse skeletal muscle myopathy: I. A histological, morphometric and biochemical investigation. *Neuropathol. Appl. Neurobiol.* **14,** 53-70.

Creuzet S., Lescaudron L., Li Z. and Fontaine-Perus J. (1998) MyoD, myogenin, and desmin-nls-lacZ transgene emphasize the distinct patterns of satellite cell activation in growth and regeneration. *Exp. Cell Res.* **243,** 241-253.

Dahl E., Winterhager E., Traub O. and Willecke K. (1995) Expression of gap junction genes, connexin40 and connexin43, during fetal mouse development. *Anat. Embryol. (Berl)* **191,** 267-278.

De Mello W. C. (1994) Gap junctional communication in excitable tissues; the heart as a paradigma. *Prog. Biophys. Mol. Biol.* **61,** 1-35.

De L. A., Pierno S., Liantonio A., Cetrone M., Camerino C., Fraysse B., Mirabella M., Servidei S., Ruegg U. T. and Conte C. D. (2003) Enhanced dystrophic progression in mdx mice by exercise and beneficial effects of taurine and insulin-like growth factor-1. *J. Pharmacol. Exp. Ther.* **304,** 453-463.

Deconinck N. and Dan B. (2007) Pathophysiology of duchenne muscular dystrophy: current hypotheses. *Pediatr. Neurol.* **36,** 1-7.

Delapeyriere O., Ollendorff V., Planche J., Ott M. O., Pizette S., Coulier F. and Birnbaum D. (1993) Expression of the Fgf6 gene is restricted to developing skeletal muscle in the mouse embryo. *Development* **118,** 601-611.

Dellavalle A., Sampaolesi M., Tonlorenzi R., Tagliafico E., Sacchetti B., Perani L., Innocenzi A., Galvez B. G., Messina G., Morosetti R., Li S., Belicchi M., Peretti G., Chamberlain J. S., Wright W. E., Torrente Y., Ferrari S., Bianco P. and Cossu G. (2007) Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat. Cell Biol.* **9,** 255-267.

Denetclaw W. F., Jr., Christ B. and Ordahl C. P. (1997) Location and growth of epaxial myotome precursor cells. *Development* **124,** 1601-1610.

Doumit M. E., Cook D. R. and Merkel R. A. (1993) Fibroblast growth factor, epidermal growth factor, insulin-like growth factors, and platelet-derived growth factor-BB stimulate proliferation of clonally derived porcine myogenic satellite cells. *J. Cell Physiol* **157,** 326-332.

Dubowitz V., Kinali M., Main M., Mercuri E. and Muntoni F. (2002) Remission of clinical signs in early duchenne muscular dystrophy on intermittent low-dosage prednisolone therapy. *Eur. J. Paediatr. Neurol.* **6,** 153-159.

Dupont-Versteegden E. E., McCarter R. J. and Katz M. S. (1994) Voluntary exercise decreases progression of muscular dystrophy in diaphragm of mdx mice. *J. Appl. Physiol* **77,** 1736-1741.

Duxson M. J. and Usson Y. (1989) Cellular insertion of primary and secondary myotubes in embryonic rat muscles. *Development* **107,** 243-251.

Duxson M. J., Usson Y. and Harris A. J. (1989) The origin of secondary myotubes in mammalian skeletal muscles: ultrastructural studies. *Development* **107,** 743-750.

Emery A. E. (2002) The muscular dystrophies. *Lancet* **359,** 687-695.

Ervasti J. M. and Campbell K. P. (1991) Membrane organization of the dystrophin-glycoprotein complex. *Cell* **66,** 1121-1131.

Faist V., Konig J., Hoger H. and Elmadfa I. (2001) Decreased mitochondrial oxygen consumption and antioxidant enzyme activities in skeletal muscle of dystrophic mice after low-intensity exercise. *Ann. Nutr. Metab* **45,** 58-66.

Febbraio M. A. and Pedersen B. K. (2005) Contraction-induced myokine production and release: is skeletal muscle an endocrine organ? *Exerc. Sport Sci. Rev.* **33,** 114-119.

Fielding R. A., Manfredi T. J., Ding W., Fiatarone M. A., Evans W. J. and Cannon J. G. (1993) Acute phase response in exercise. III. Neutrophil and IL-1 beta accumulation in skeletal muscle. *Am. J. Physiol* **265,** R166-R172.

FURSHPAN E. J. and POTTER D. D. (1959) Transmission at the giant motor synapses of the crayfish. *J. Physiol* **145,** 289-325.

Gal-Levi R., Leshem Y., Aoki S., Nakamura T. and Halevy O. (1998) Hepatocyte growth factor plays a dual role in regulating skeletal muscle satellite cell proliferation and differentiation. *Biochim. Biophys. Acta* **1402,** 39-51.

Galvez B. G., Sampaolesi M., Brunelli S., Covarello D., Gavina M., Rossi B., Constantin G., Torrente Y. and Cossu G. (2006) Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. *J. Cell Biol.* **174,** 231-243.

Gavina M., Belicchi M., Rossi B., Ottoboni L., Colombo F., Meregalli M., Battistelli M., Forzenigo L., Biondetti P., Pisati F., Parolini D., Farini A., Issekutz A. C., Bresolin N., Rustichelli F., Constantin G. and Torrente Y. (2006) VCAM-1 expression on dystrophic muscle vessels has a critical role in the recruitment of human blood-derived CD133+ stem cells after intra-arterial transplantation. *Blood* **108,** 2857-2866.

Goldring K., Partridge T. and Watt D. (2002) Muscle stem cells. *J. Pathol.* **197,** 457-467.

Gollins H., McMahon J., Wells K. E. and Wells D. J. (2003) High-efficiency plasmid gene transfer into dystrophic muscle. *Gene Ther.* **10,** 504-512.

Goodell M. A., Rosenzweig M., Kim H., Marks D. F., DeMaria M., Paradis G., Grupp S. A., Sieff C. A., Mulligan R. C. and Johnson R. P. (1997) Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat. Med.* **3,** 1337-1345.

Goodenough D. A. (1992) The crystalline lens. A system networked by gap junctional intercellular communication. *Semin. Cell Biol.* **3,** 49-58.

Gorbe A., Becker D. L., Dux L., Stelkovics E., Krenacs L., Bagdi E. and Krenacs T. (2005) Transient upregulation of connexin43 gap junctions and synchronized cell cycle control precede myoblast fusion in regenerating skeletal muscle in vivo. *Histochem. Cell Biol.* **123,** 573-583.

Gossett L. A., Kelvin D. J., Sternberg E. A. and Olson E. N. (1989) A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Mol. Cell Biol.* **9,** 5022-5033.

Gowdak L. H., Poliakova L., Wang X., Kovesdi I., Fishbein K. W., Zacheo A., Palumbo R., Straino S., Emanueli C., Marrocco-Trischitta M., Lakatta E. G., Anversa P., Spencer R. G., Talan M. and Capogrossi M. C. (2000) Adenovirus-mediated VEGF(121) gene transfer stimulates angiogenesis in normoperfused skeletal muscle and preserves tissue perfusion after induction of ischemia. *Circulation* **102,** 565-571.

Grange R. W. and Call J. A. (2007) Recommendations to define exercise prescription for Duchenne muscular dystrophy. *Exerc. Sport Sci. Rev.* **35,** 12-17.

Grefte S., Kuijpers-Jagtman A. M., Torensma R. and Von den Hoff J. W. (2007) Skeletal muscle development and regeneration. *Stem Cells Dev.* **16,** 857-868.

Grounds M. D., Radley H. G., Lynch G. S., Nagaraju K. and De L. A. (2008) Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy. *Neurobiol. Dis.* **31,** 1-19.

Grounds M. D. and Torrisi J. (2004) Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosis. *FASEB J.* **18,** 676-682.

Gussoni E., Soneoka Y., Strickland C. D., Buzney E. A., Khan M. K., Flint A. F., Kunkel L. M. and Mulligan R. C. (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* **401,** 390-394.

Guthrie S. C. and Gilula N. B. (1989) Gap junctional communication and development. *Trends Neurosci.* **12,** 12-16.

Hasty P., Bradley A., Morris J. H., Edmondson D. G., Venuti J. M., Olson E. N. and Klein W. H. (1993) Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364,** 501-506.

Haugk K. L., Roeder R. A., Garber M. J. and Schelling G. T. (1995) Regulation of muscle cell proliferation by extracts from crushed muscle. *J. Anim Sci.* **73,** 1972-1981.

Hawke T. J. (2005) Muscle stem cells and exercise training. *Exerc. Sport Sci. Rev.* **33,** 63-68.

Hawke T. J. and Garry D. J. (2001) Myogenic satellite cells: physiology to molecular biology. *J. Appl. Physiol* **91,** 534-551.

Hayes A., Lynch G. S. and Williams D. A. (1993) The effects of endurance exercise on dystrophic mdx mice. I. Contractile and histochemical properties of intact muscles. *Proc. Biol. Sci.* **253,** 19-25.

Hayes A. and Williams D. A. (1996) Beneficial effects of voluntary wheel running on the properties of dystrophic mouse muscle. *J. Appl. Physiol* **80,** 670-679.

Hayes A. and Williams D. A. (1998) Contractile function and low-intensity exercise effects of old dystrophic (mdx) mice. *Am. J. Physiol* **274,** C1138-C1144.

Heslop L., Morgan J. E. and Partridge T. A. (2000) Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. *J. Cell Sci.* **113 (Pt 12),** 2299-2308.

Hinterberger T. J., Sassoon D. A., Rhodes S. J. and Konieczny S. F. (1991) Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev. Biol.* **147,** 144-156.

Hirsinger E., Duprez D., Jouve C., Malapert P., Cooke J. and Pourquie O. (1997) Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning. *Development* **124,** 4605-4614.

Hodgetts S., Radley H., Davies M. and Grounds M. D. (2006) Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice. *Neuromuscul. Disord.* **16,** 591-602.

Huard J., Li Y. and Fu F. H. (2002) Muscle injuries and repair: current trends in research. *J. Bone Joint Surg. Am.* **84-A,** 822-832.

Huizinga J. D., Liu L. W., Blennerhassett M. G., Thuneberg L. and Molleman A. (1992) Intercellular communication in smooth muscle. *Experientia* **48,** 932-941.

Irintchev A., Zeschnigk M., Starzinski-Powitz A. and Wernig A. (1994) Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. *Dev. Dyn.* **199,** 326-337.

Jacobs-El J., Zhou M. Y. and Russell B. (1995) MRF4, Myf-5, and myogenin mRNAs in the adaptive responses of mature rat muscle. *Am. J. Physiol* **268,** C1045-C1052.

Jarvinen T. A., Jarvinen T. L., Kaariainen M., Kalimo H. and Jarvinen M. (2005) Muscle injuries: biology and treatment. *Am. J. Sports Med.* **33,** 745-764.

Kaczor J. J., Hall J. E., Payne E. and Tarnopolsky M. A. (2007) Low intensity training decreases markers of oxidative stress in skeletal muscle of mdx mice. *Free Radic. Biol. Med.* **43,** 145-154.

Kalderon N., Epstein M. L. and Gilula N. B. (1977) Cell-to-cell communication and myogenesis. *J. Cell Biol.* **75,** 788-806.

Kawakami K., Sato S., Ozaki H. and Ikeda K. (2000) Six family genes--structure and function as transcription factors and their roles in development. *Bioessays* **22,** 616-626.

Kidder G. M. (1992) The genetic program for preimplantation development. *Dev. Genet.* **13,** 319-325.

Koval M. (2006) Pathways and control of connexin oligomerization. *Trends Cell Biol.* **16,** 159-166.

Kruger O., Plum A., Kim J. S., Winterhager E., Maxeiner S., Hallas G., Kirchhoff S., Traub O., Lamers W. H. and Willecke K. (2000) Defective vascular development in connexin 45-deficient mice. *Development* **127,** 4179-4193.

Kuang S., Gillespie M. A. and Rudnicki M. A. (2008) Niche regulation of muscle satellite cell self-renewal and differentiation. *Cell Stem Cell* **2,** 22-31.

Labarge M. A. and Blau H. M. (2002) Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* **111,** 589-601.

Laclef C., Hamard G., Demignon J., Souil E., Houbron C. and Maire P. (2003) Altered myogenesis in Six1-deficient mice. *Development* **130,** 2239-2252.

Lagord C., Soulet L., Bonavaud S., Bassaglia Y., Rey C., Barlovatz-Meimon G., Gautron J. and Martelly I. (1998) Differential myogenicity of satellite cells isolated from extensor digitorum longus (EDL) and soleus rat muscles revealed in vitro. *Cell Tissue Res.* **291,** 455-468.

Larsen W. J. and Wert S. E. (1988) Roles of cell junctions in gametogenesis and in early embryonic development. *Tissue Cell* **20,** 809-848.

Lasky L. A. (1995) Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu. Rev. Biochem.* **64,** 113-139.

Law P. K., Goodwin T. G. and Wang M. G. (1988) Normal myoblast injections provide genetic treatment for murine dystrophy. *Muscle Nerve* **11,** 525-533.

Le Grand F., Auda-Boucher G., Levitsky D., Rouaud T., Fontaine-Perus J. and Gardahaut M. F. (2004) Endothelial cells within embryonic skeletal muscles: a potential source of myogenic progenitors. *Exp. Cell Res.* **301,** 232-241.

LeBlanc A. D., Jaweed M. and Evans H. (1993) Evaluation of muscle injury using magnetic resonance imaging. *Clin. J. Sport Med.* **3,** 26-30.

Lescaudron L., Peltekian E., Fontaine-Perus J., Paulin D., Zampieri M., Garcia L. and Parrish E. (1999) Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant. *Neuromuscul. Disord.* **9,** 72-80.

Ling Y., Appelt D., Kelly A. M. and Franzini-Armstrong C. (1992) Differences in the histogenesis of EDL and diaphragm in rat. *Dev. Dyn.* **193,** 359-369.

Lira F. S., Koyama C. H., Yamashita A. S., Rosa J. C., Zanchi N. E., Batista M. L., Jr. and Seelaender M. C. (2009) Chronic exercise decreases cytokine production in healthy rat skeletal muscle. *Cell Biochem. Funct.* **27,** 458-461.

LOWRY O. H., ROSEBROUGH N. J., FARR A. L. and RANDALL R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Lu Q. L., Bou-Gharios G. and Partridge T. A. (2003) Non-viral gene delivery in skeletal muscle: a protein factory. *Gene Ther.* **10,** 131-142.

Lynch G. S., Rafael J. A., Chamberlain J. S. and Faulkner J. A. (2000) Contraction-induced injury to single permeabilized muscle fibers from mdx, transgenic mdx, and control mice. *Am. J. Physiol Cell Physiol* **279,** C1290-C1294.

Mak K. L., To R. Q., Kong Y. and Konieczny S. F. (1992) The MRF4 activation domain is required to induce muscle-specific gene expression. *Mol. Cell Biol.* **12,** 4334-4346.

Mal A., Sturniolo M., Schiltz R. L., Ghosh M. K. and Harter M. L. (2001) A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD: inhibition of the myogenic program. *EMBO J.* **20,** 1739-1753.

Malerba A., Vitiello L., Segat D., Dazzo E., Frigo M., Scambi I., De C. P., Boldrin L., Martelli L., Pasut A., Romualdi C., Bellomo R. G., Vecchiet J. and Baroni M. D. (2009) Selection of multipotent cells and enhanced muscle reconstruction by myogenic macrophage-secreted factors. *Exp. Cell Res.* **315,** 915-927.

Martelly I., Soulet L., Bonnavaud S., Cebrian J., Gautron J. and Barritault D. (2000) Differential expression of FGF receptors and of myogenic regulatory factors in primary cultures of satellite cells originating from fast (EDL) and slow (Soleus) twitch rat muscles. *Cell Mol. Biol. (Noisy. -le-grand)* **46,** 1239-1248.

MAURO A. (1961) Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* **9,** 493-495.

Maxeiner S., Kruger O., Schilling K., Traub O., Urschel S. and Willecke K. (2003) Spatiotemporal transcription of connexin45 during brain development results in neuronal expression in adult mice. *Neuroscience* **119,** 689-700.

McCartney N., Moroz D., Garner S. H. and McComas A. J. (1988) The effects of strength training in patients with selected neuromuscular disorders. *Med. Sci. Sports Exerc.* **20,** 362-368.

McGeachie J. K., Grounds M. D., Partridge T. A. and Morgan J. E. (1993) Age-related changes in replication of myogenic cells in mdx mice: quantitative autoradiographic studies. *J. Neurol. Sci.* **119,** 169-179.

McMahon J. A., Takada S., Zimmerman L. B., Fan C. M., Harland R. M. and McMahon A. P. (1998) Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **12,** 1438-1452.

Mege R. M., Goudou D., Giaume C., Nicolet M. and Rieger F. (1994) Is intercellular communication via gap junctions required for myoblast fusion? *Cell Adhes. Commun.* **2,** 329-343.

Menetrey J., Kasemkijwattana C., Day C. S., Bosch P., Vogt M., Fu F. H., Moreland M. S. and Huard J. (2000) Growth factors improve muscle healing in vivo. *J. Bone Joint Surg. Br.* **82,** 131-137.

Miller S. M., Garfield R. E. and Daniel E. E. (1989) Improved propagation in myometrium associated with gap junctions during parturition. *Am. J. Physiol* **256,** C130-C141.

Molkentin J. D., Black B. L., Martin J. F. and Olson E. N. (1995) Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83,** 1125-1136.

Morgan J. and Muntoni F. (2007) Mural cells paint a new picture of muscle stem cells. *Nat. Cell Biol.* **9,** 249-251.

Morgan J. E., Hoffman E. P. and Partridge T. A. (1990) Normal myogenic cells from newborn mice restore normal histology to degenerating muscles of the mdx mouse. *J. Cell Biol.* **111,** 2437-2449.

Moss F. P. and Leblond C. P. (1971) Satellite cells as the source of nuclei in muscles of growing rats. *Anat. Rec.* **170,** 421-435.

Muller J., Vayssiere N., Royuela M., Leger M. E., Muller A., Bacou F., Pons F., Hugon G. and Mornet D. (2001) Comparative evolution of muscular dystrophy in diaphragm, gastrocnemius and masseter muscles from old male mdx mice. *J. Muscle Res. Cell Motil.* **22,** 133-139.

Munsterberg A. E., Kitajewski J., Bumcrot D. A., McMahon A. P. and Lassar A. B. (1995) Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev.* **9,** 2911-2922.

Munsterberg A. E. and Lassar A. B. (1995) Combinatorial signals from the neural tube, floor plate and notochord induce myogenic bHLH gene expression in the somite. *Development* **121,** 651-660.

Nabeshima Y., Hanaoka K., Hayasaka M., Esumi E., Li S., Nonaka I. and Nabeshima Y. (1993) Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364,** 532-535.

Nader G. A. and Lundberg I. E. (2009) Exercise as an anti-inflammatory intervention to combat inflammatory diseases of muscle. *Curr. Opin. Rheumatol.* **21,** 599-603.

Negroni E., Butler-Browne G. S. and Mouly V. (2006) Myogenic stem cells: regeneration and cell therapy in human skeletal muscle. *Pathol. Biol. (Paris)* **54,** 100-108.

Negroni E., Riederer I., Chaouch S., Belicchi M., Razini P., Di S. J., Torrente Y., Butler-Browne G. S. and Mouly V. (2009) In vivo myogenic potential of human CD133+ muscle-derived stem cells: a quantitative study. *Mol. Ther.* **17,** 1771-1778.

Nicholson G. A., Gardner-Medwin D., Pennington R. J. and Walton J. N. (1979) Carrier detection in Duchenne muscular dystrophy: Assessment of the effect of age on detection-rate with serum-creatine-kinase-activity. *Lancet* **1,** 692-694.

Nicolas N., Mira J. C., Gallien C. L. and Chanoine C. (2000) Neural and hormonal control of expression of myogenic regulatory factor genes during regeneration of Xenopus fast muscles: myogenin and MRF4 mRNA accumulation are neurally regulated oppositely. *Dev. Dyn.* **218,** 112-122.

Odom G. L., Gregorevic P. and Chamberlain J. S. (2007) Viral-mediated gene therapy for the muscular dystrophies: successes, limitations and recent advances. *Biochim. Biophys. Acta* **1772,** 243-262.

Ohlstein B., Kai T., Decotto E. and Spradling A. (2004) The stem cell niche: theme and variations. *Curr. Opin. Cell Biol.* **16,** 693-699.

Okano T., Yoshida K., Nakamura A., Sasazawa F., Oide T., Takeda S. and Ikeda S. (2005) Chronic exercise accelerates the degeneration-regeneration cycle and downregulates insulin-like growth factor-1 in muscle of mdx mice. *Muscle Nerve* **32,** 191-199.

Olguin H. C. and Olwin B. B. (2004) Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. *Dev. Biol.* **275,** 375-388.

Olson E. N., Arnold H. H., Rigby P. W. and Wold B. J. (1996) Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell* **85,** 1-4.

Ordahl C. P. and Le Douarin N. M. (1992) Two myogenic lineages within the developing somite. *Development* **114,** 339-353.

Orimo S., Hiyamuta E., Arahata K. and Sugita H. (1991) Analysis of inflammatory cells and complement C3 in bupivacaine-induced myonecrosis. *Muscle Nerve* **14,** 515-520.

Page E. and Shibata Y. (1981) Permeable junctions between cardiac cells. *Annu. Rev. Physiol* **43,** 431-441.

Palermo A. T., Labarge M. A., Doyonnas R., Pomerantz J. and Blau H. M. (2005) Bone marrow contribution to skeletal muscle: a physiological response to stress. *Dev. Biol.* **279,** 336-344.

Partridge T. A., Morgan J. E., Coulton G. R., Hoffman E. P. and Kunkel L. M. (1989) Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature* **337,** 176-179.

Patapoutian A., Yoon J. K., Miner J. H., Wang S., Stark K. and Wold B. (1995) Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* **121,** 3347-3358.

Pavlath G. K., Thaloor D., Rando T. A., Cheong M., English A. W. and Zheng B. (1998) Heterogeneity among muscle precursor cells in adult skeletal muscles with differing regenerative capacities. *Dev. Dyn.* **212,** 495-508.

Peault B., Rudnicki M., Torrente Y., Cossu G., Tremblay J. P., Partridge T., Gussoni E., Kunkel L. M. and Huard J. (2007) Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. *Mol. Ther.* **15,** 867-877.

Percy M. E., Chang L. S., Murphy E. G., Oss I., Verellen-Dumoulin C. and Thompson M. W. (1979) Serum creatine kinase and pyruvate kinase in Duchenne muscular dystrophy carrier detection. *Muscle Nerve* **2,** 329-339.

Pereira R. F., Halford K. W., O'Hara M. D., Leeper D. B., Sokolov B. P., Pollard M. D., Bagasra O. and Prockop D. J. (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc. Natl. Acad. Sci. U. S. A* **92,** 4857-4861.

Philippou A., Halapas A., Maridaki M. and Koutsilieris M. (2007) Type I insulin-like growth factor receptor signaling in skeletal muscle regeneration and hypertrophy. *J. Musculoskelet. Neuronal. Interact.* **7,** 208-218.

Polesskaya A., Duquet A., Naguibneva I., Weise C., Vervisch A., Bengal E., Hucho F., Robin P. and Harel-Bellan A. (2000) CREB-binding protein/p300 activates MyoD by acetylation. *J. Biol. Chem.* **275,** 34359-34364.

Porter J. D., Khanna S., Kaminski H. J., Rao J. S., Merriam A. P., Richmonds C. R., Leahy P., Li J., Guo W. and Andrade F. H. (2002) A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. *Hum. Mol. Genet.* **11,** 263-272.

Pownall M. E., Strunk K. E. and Emerson C. P., Jr. (1996) Notochord signals control the transcriptional cascade of myogenic bHLH genes in somites of quail embryos. *Development* **122,** 1475-1488.

Proulx A., Merrifield P. A. and Naus C. C. (1997) Blocking gap junctional intercellular communication in myoblasts inhibits myogenin and MRF4 expression. *Dev. Genet.* **20,** 133-144.

Qu-Petersen Z., Deasy B., Jankowski R., Ikezawa M., Cummins J., Pruchnic R., Mytinger J., Cao B., Gates C., Wernig A. and Huard J. (2002) Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. *J. Cell Biol.* **157,** 851-864.

Radley H. G., De L. A., Lynch G. S. and Grounds M. D. (2007) Duchenne muscular dystrophy: focus on pharmaceutical and nutritional interventions. *Int. J. Biochem. Cell Biol.* **39,** 469-477.

Rash J. E. and Staehelin L. A. (1974) Freeze-cleave demonstration of gap junctions between skeletal myogenic cells in vivo. *Dev. Biol.* **36,** 455-461.

Ratajczak M. Z., Majka M., Kucia M., Drukala J., Pietrzkowski Z., Peiper S. and Janowska-Wieczorek A. (2003) Expression of functional CXCR4 by muscle satellite cells and secretion of SDF-1 by muscle-derived fibroblasts is associated with the presence of both muscle progenitors in bone marrow and hematopoietic stem/progenitor cells in muscles. *Stem Cells* **21,** 363-371.

Rawls A., Morris J. H., Rudnicki M., Braun T., Arnold H. H., Klein W. H. and Olson E. N. (1995) Myogenin's functions do not overlap with those of MyoD or Myf-5 during mouse embryogenesis. *Dev. Biol.* **172,** 37-50.

Reinecke H., Minami E., Virag J. I. and Murry C. E. (2004) Gene transfer of connexin43 into skeletal muscle. *Hum. Gene Ther.* **15,** 627-636.

Roberts M. L., Wells D. J., Graham I. R., Fabb S. A., Hill V. J., Duisit G., Yuasa K., Takeda S., Cosset F. L. and Dickson G. (2002) Stable micro-dystrophin gene transfer using an integrating adeno-retroviral hybrid vector ameliorates the dystrophic pathology in mdx mouse muscle. *Hum. Mol. Genet.* **11,** 1719-1730.

Robertson T. A., Maley M. A., Grounds M. D. and Papadimitriou J. M. (1993) The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis. *Exp. Cell Res.* **207,** 321-331.

Rudnicki M. A., Schnegelsberg P. N., Stead R. H., Braun T., Arnold H. H. and Jaenisch R. (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75,** 1351-1359.

Saito Y. and Nonaka I. (1994) Initiation of satellite cell replication in bupivacaine-induced myonecrosis. *Acta Neuropathol.* **88,** 252-257.

Sampaolesi M., Blot S., D'Antona G., Granger N., Tonlorenzi R., Innocenzi A., Mognol P., Thibaud J. L., Galvez B. G., Barthelemy I., Perani L., Mantero S., Guttinger M., Pansarasa O., Rinaldi C., Cusella De Angelis M. G., Torrente Y., Bordignon C., Bottinelli R. and Cossu G. (2006) Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* **444,** 574-579.

Sarig R., Baruchi Z., Fuchs O., Nudel U. and Yaffe D. (2006) Regeneration and transdifferentiation potential of muscle-derived stem cells propagated as myospheres. *Stem Cells* **24,** 1769-1778.

Sartorelli V., Puri P. L., Hamamori Y., Ogryzko V., Chung G., Nakatani Y., Wang J. Y. and Kedes L. (1999) Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. *Mol. Cell* **4,** 725-734.

Sato K., Li Y., Foster W., Fukushima K., Badlani N., Adachi N., Usas A., Fu F. H. and Huard J. (2003) Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis. *Muscle Nerve* **28,** 365-372.

Schmalbruch H. (1982) Skeletal muscle fibers of newborn rats are coupled by gap junctions. *Dev. Biol.* **91,** 485-490.

Schmalbruch H. and Lewis D. M. (2000) Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles. *Muscle Nerve* **23,** 617-626.

Schultz E. and McCormick K. M. (1994) Skeletal muscle satellite cells. *Rev. Physiol Biochem. Pharmacol.* **123,** 213-257.

Scott J. M., Li S., Harper S. Q., Welikson R., Bourque D., DelloRusso C., Hauschka S. D. and Chamberlain J. S. (2002) Viral vectors for gene transfer of micro-, mini-, or full-length dystrophin. *Neuromuscul. Disord.* **12 Suppl 1,** S23-S29.

Seale P. and Rudnicki M. A. (2000) A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev. Biol.* **218,** 115-124.

Segawa M., Fukada S., Yamamoto Y., Yahagi H., Kanematsu M., Sato M., Ito T., Uezumi A., Hayashi S., Miyagoe-Suzuki Y., Takeda S., Tsujikawa K. and Yamamoto H. (2008) Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis. *Exp. Cell Res.* **314,** 3232-3244.

Serre-Beinier V., Le G. S., Belluardo N., Trovato-Salinaro A., Charollais A., Haefliger J. A., Condorelli D. F. and Meda P. (2000) Cx36 preferentially connects beta-cells within pancreatic islets. *Diabetes* **49,** 727-734.

Siegel A. L., Atchison K., Fisher K. E., Davis G. E. and Cornelison D. D. (2009) 3D timelapse analysis of muscle satellite cell motility. *Stem Cells* **27,** 2527-2538.

Simon A. M. and McWhorter A. R. (2002) Vascular abnormalities in mice lacking the endothelial gap junction proteins connexin37 and connexin40. *Dev. Biol.* **251,** 206-220.

Simon A. M. and McWhorter A. R. (2003) Role of connexin37 and connexin40 in vascular development. *Cell Commun. Adhes.* **10,** 379-385.

Smith C. K., Janney M. J. and Allen R. E. (1994) Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. *J. Cell Physiol* **159,** 379-385.

Song W. K., Wang W., Foster R. F., Bielser D. A. and Kaufman S. J. (1992) H36-alpha 7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. *J. Cell Biol.* **117,** 643-657.

Sorichter S., Mair J., Koller A., Muller E., Kremser C., Judmaier W., Haid C., Calzolari C. and Puschendorf B. (2001) Creatine kinase, myosin heavy chains and magnetic resonance imaging after eccentric exercise. *J. Sports Sci.* **19,** 687-691.

Sotelo C. and Llinas R. (1972) Specialized membrane junctions between neurons in the vertebrate cerebellar cortex. *J. Cell Biol.* **53,** 271-289.

Spencer M. J. and Tidball J. G. (2001) Do immune cells promote the pathology of dystrophin-deficient myopathies? *Neuromuscul. Disord.* **11,** 556-564.

Spray D. C. and Burt J. M. (1990) Structure-activity relations of the cardiac gap junction channel. *Am. J. Physiol* **258,** C195-C205.

Springer M. L., Chen A. S., Kraft P. E., Bednarski M. and Blau H. M. (1998) VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol. Cell* **2,** 549-558.

Squecco R., Sassoli C., Nuti F., Martinesi M., Chellini F., Nosi D., Zecchi-Orlandini S., Francini F., Formigli L. and Meacci E. (2006) Sphingosine 1-phosphate induces myoblast

differentiation through Cx43 protein expression: a role for a gap junction-dependent and -independent function. *Mol. Biol. Cell* **17,** 4896-4910.

Stern H. M. and Hauschka S. D. (1995) Neural tube and notochord promote in vitro myogenesis in single somite explants. *Dev. Biol.* **167,** 87-103.

Suzuki J., Yamazaki Y., Li G., Kaziro Y. and Koide H. (2000) Involvement of Ras and Ral in chemotactic migration of skeletal myoblasts. *Mol. Cell Biol.* **20,** 4658-4665.

Suzuki S., Yamanouchi K., Soeta C., Katakai Y., Harada R., Naito K. and Tojo H. (2002) Skeletal muscle injury induces hepatocyte growth factor expression in spleen. *Biochem. Biophys. Res. Commun.* **292,** 709-714.

Sveen M. L., Jeppesen T. D., Hauerslev S., Krag T. O. and Vissing J. (2007) Endurance training: an effective and safe treatment for patients with LGMD2I. *Neurology* **68,** 59-61.

Tajbakhsh S. and Buckingham M. (2000) The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. *Curr. Top. Dev. Biol.* **48,** 225-268.

Tamaki T., Okada Y., Uchiyama Y., Tono K., Masuda M., Wada M., Hoshi A., Ishikawa T. and Akatsuka A. (2007) Clonal multipotency of skeletal muscle-derived stem cells between mesodermal and ectodermal lineage. *Stem Cells* **25,** 2283-2290.

Tatsumi R., Anderson J. E., Nevoret C. J., Halevy O. and Allen R. E. (1998) HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev. Biol.* **194,** 114-128.

Tatsumi R., Sheehan S. M., Iwasaki H., Hattori A. and Allen R. E. (2001) Mechanical stretch induces activation of skeletal muscle satellite cells in vitro. *Exp. Cell Res.* **267,** 107-114.

Thioudellet C., Blot S., Squiban P., Fardeau M. and Braun S. (2002) Current protocol of a research phase I clinical trial of full-length dystrophin plasmid DNA in Duchenne/Becker muscular dystrophies. Part I: rationale. *Neuromuscul. Disord.* **12 Suppl 1,** S49-S51.

Tidball J. G. (1995) Inflammatory cell response to acute muscle injury. *Med. Sci. Sports Exerc.* **27,** 1022-1032.

Tidball J. G. (2005) Inflammatory processes in muscle injury and repair. *Am. J. Physiol Regul. Integr. Comp Physiol* **288,** R345-R353.

Torrente Y., Belicchi M., Sampaolesi M., Pisati F., Meregalli M., D'Antona G., Tonlorenzi R., Porretti L., Gavina M., Mamchaoui K., Pellegrino M. A., Furling D., Mouly V., Butler-Browne G. S., Bottinelli R., Cossu G. and Bresolin N. (2004) Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. *J. Clin. Invest* **114,** 182-195.

Tremblay P., Dietrich S., Mericskay M., Schubert F. R., Li Z. and Paulin D. (1998) A crucial role for Pax3 in the development of the hypaxial musculature and the long-range migration of muscle precursors. *Dev. Biol.* **203,** 49-61.

Trovato-Salinaro A., Belluardo N., Frinchi M., von M. J., Willecke K., Condorelli D. F. and Mudo G. (2009) Regulation of connexin gene expression during skeletal muscle regeneration in the adult rat. *Am. J. Physiol Cell Physiol* **296,** C593-C606.

Vignos P. J., Jr. and Watkins M. P. (1966) The effect of exercise in muscular dystrophy. *JAMA* **197,** 843-848.

Villalta S. A., Nguyen H. X., Deng B., Gotoh T. and Tidball J. G. (2009) Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. *Hum. Mol. Genet.* **18,** 482-496.

von Maltzahn J., Euwens C., Willecke K. and Sohl G. (2004) The novel mouse connexin39 gene is expressed in developing striated muscle fibers. *J. Cell Sci.* **117,** 5381-5392.

von Maltzahn J. and Willecke K. (2005) Essential role of gap junctions during development and
regeneration of skeletal muscle, in *Gap Junctions in Development and Disease,* (Elke Winterhager, ed), pp. 13-27. Springer Netherlands.

von Maltzahn J., Wulf V., Matern G and Willecke K (2011) Connexin39 deficient mice display accelerated myogenesis and regeneration of skeletal muscle.

von Maltzahn J., Wulf V. and Willecke K. (2006) Spatiotemporal expression of connexin 39 and -43 during myoblast differentiation in cultured cells and in the mouse embryo. *Cell Commun. Adhes.* **13,** 55-60.

Wells D. J. and Wells K. E. (2002) Gene transfer studies in animals: what do they really tell us about the prospects for gene therapy in DMD? *Neuromuscul. Disord.* **12 Suppl 1,** S11-S22.

Wineinger M. A., Abresch R. T., Walsh S. A. and Carter G. T. (1998) Effects of aging and voluntary exercise on the function of dystrophic muscle from mdx mice. *Am. J. Phys. Med. Rehabil.* **77,** 20-27.

Wozniak A. C., Kong J., Bock E., Pilipowicz O. and Anderson J. E. (2005) Signaling satellite-cell activation in skeletal muscle: markers, models, stretch, and potential alternate pathways. *Muscle Nerve* **31,** 283-300.

Yablonka-Reuveni Z. and Rivera A. J. (1994) Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev. Biol.* **164,** 588-603.

Yoon J. K., Olson E. N., Arnold H. H. and Wold B. J. (1997) Different MRF4 knockout alleles differentially disrupt Myf-5 expression: cis-regulatory interactions at the MRF4/Myf-5 locus. *Dev. Biol.* **188,** 349-362.

Yutzey K. E., Rhodes S. J. and Konieczny S. F. (1990) Differential trans activation associated with the muscle regulatory factors MyoD1, myogenin, and MRF4. *Mol. Cell Biol.* **10,** 3934-3944.

Zammit P. and Beauchamp J. (2001) The skeletal muscle satellite cell: stem cell or son of stem cell? *Differentiation* **68,** 193-204.

Zammit P. S., Golding J. P., Nagata Y., Hudon V., Partridge T. A. and Beauchamp J. R. (2004) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *J. Cell Biol.* **166,** 347-357.

Zammit P. S., Heslop L., Hudon V., Rosenblatt J. D., Tajbakhsh S., Buckingham M. E., Beauchamp J. R. and Partridge T. A. (2002) Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. *Exp. Cell Res.* **281,** 39-49.

Zammit P. S., Partridge T. A. and Yablonka-Reuveni Z. (2006) The skeletal muscle satellite cell: the stem cell that came in from the cold. *J. Histochem. Cytochem.* **54,** 1177-1191.

Zanchi N. E., Lira F. S., de Siqueira Filho M. A., Rosa J. C., de Oliveira Carvalho C. R., Seelaender M., Santos R. V. and Lancha A. H., Jr. (2010) Chronic low frequency/low volume resistance training reduces pro-inflammatory cytokine protein levels and TLR4 mRNA in rat skeletal muscle. *Eur. J. Appl. Physiol* **109,** 1095-1102.

Zatz M., Rapaport D., Vainzof M., Passos-Bueno M. R., Bortolini E. R., Pavanello R. C. and Peres C. A. (1991) Serum creatine-kinase (CK) and pyruvate-kinase (PK) activities in Duchenne (DMD) as compared with Becker (BMD) muscular dystrophy. *J. Neurol. Sci.* **102,** 190-196.

Zhang W., Behringer R. R. and Olson E. N. (1995) Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. *Genes Dev.* **9,** 1388-1399.

Zhao P. and Hoffman E. P. (2004) Embryonic myogenesis pathways in muscle regeneration. *Dev. Dyn.* **229,** 380-392.

Zheng B., Cao B., Crisan M., Sun B., Li G., Logar A., Yap S., Pollett J. B., Drowley L., Cassino T., Gharaibeh B., Deasy B. M., Huard J. and Peault B. (2007) Prospective identification of myogenic endothelial cells in human skeletal muscle. *Nat. Biotechnol.* **25,** 1025-1034.

Zhou L. and Lu H. (2010) Targeting fibrosis in Duchenne muscular dystrophy. *J. Neuropathol. Exp. Neurol.* **69,** 771-776.

PAPERS PUBLISHED DURING THE DOCTORATE

- FGF-2/FGFR1 neurotrophic system expression level and its basal activation do not account for the age-dependent decline of precursor cell proliferation in the subventricular zone of rat brain.
  Frinchi M, Di Liberto V, Olivieri M, Fuxe K, Belluardo N, Mudò G.
  Brain Res. 2010 Oct 28;1358:39-45. Epub 2010 Sep 18.

- Group II metabotropic glutamate receptor activation by agonist LY379268 treatment increases the expression of brain derived neurotrophic factor in the mouse brain.
  Di Liberto V, Bonomo A, **Frinchi M**, Belluardo N, Mudò G.
  Neuroscience. 2010 Feb 3;165(3):863-73. Epub 2009 Nov 10.

- The FGF-2/FGFRs neurotrophic system promotes neurogenesis in the adult brain.
  Mudò G, Bonomo A, Di Liberto V, **Frinchi M**, Fuxe K, Belluardo N.
  J Neural Transm. 2009 Aug;116(8):995-1005. Epub 2009 Mar 17. Review

- Regulation of connexin gene expression during skeletal muscle regeneration in the adult rat.
  Trovato-Salinaro A, Belluardo N, **Frinchi M**, von Maltzahn J, Willecke K, Condorelli DF, Mudò G.
  Am J Physiol Cell Physiol. 2009 Mar;296(3):C593-606. Epub 2009 Jan 7.

- Fibroblast growth factor-2 and its receptor expression in proliferating precursor cells of the subventricular zone in the adult rat brain.
  Frinchi M, Bonomo A, Trovato-Salinaro A, Condorelli DF, Fuxe K, Spampinato MG, Mudò G.
  Neurosci Lett. 2008 Dec 5;447(1):20-5. Epub 2008 Sep 27

- Nicotine-induced fibroblast growth factor-2 restores the age-related decline of precursor cell proliferation in the subventricular zone of rat brain.
  Belluardo N, Mudo' G, Bonomo A, Di Liberto V**, Frinchi M**, Fuxe K.
  Brain Res. 2008 Feb 8;1193:12-24. Epub 2007 Dec 14.

- Analysy of connexin36 and connexin45 expression in the mouse and rat crest-derived neuroendocrine organs: carotid body, adrenal medulla and enteric neurons
  Frinchi M, Di Liberto V, D'Antoni F, Mudò G.
  Brain Research. Manuscript in preparation

- Recovery of damaged skeletal muscle in *mdx* mice by low-intensity endurance exercise
  Frinchi M, Macaluso F, Licciardi A , Mudò G, Morici G, Belluardo N.
  Muscle & Nerve. Manuscript in preparation