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

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Trovato-Salinario A, Belluardo N, Frinchi M, von Maltzahn J, Willecke K, Condorelli DF, Mudò G. Regulation of connexin gene expression during skeletal muscle regeneration in the adult rat. *Am J Physiol Cell Physiol* 296: C593–C606, 2009. First published January 7, 2009; doi:10.1152/ajpcell.00458.2008.—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. The aim of present work was to perform a comparative spatial and temporal analysis of connexin (Cx) 37, Cx39, Cx40, Cx43, and Cx45 expression in the adult regenerating skeletal muscle in response to crush injury. Within 24 h 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 24 h 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 24 h 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.

connexin 37; connexin 39; connexin 40; connexin 43; connexin 45; myogenic cells; muscle regeneration

DURING POSTNATAL development, satellite cells divide to provide new myonuclei to the growing muscle fibers (20) before becoming quiescent in mature muscle (26). A growing body of evidence suggests that multiple, distinct populations of satellite cells reside in adult muscle and can be activated by damage stimuli for myogenic commitment, proliferation, and differentiation to ultimately form new muscle fibers through syncytial fusion of myoblasts or to repair the damaged myofibers (5, 13, 16, 25, 28, 34). The existence of multiple populations of myogenic precursor cells could allow muscle tissue to respond differentially to a particular stimulus, type of injury, or physiological demand and thereby enable a highly controlled re-

sponse. 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 (2, 4, 32).

Gap junction channels are formed in adjacent cell membranes by the docking of two hemichannels (connexons), each consisting of six connexin (Cx) subunits. The Cxs are a multigene family with at least 21 members in the human genome (31), commonly named according to their molecular weight. Direct cell-cell communication through gap junction channels is thought to play important roles in coordinating cell functions by mediating rapid exchange of electrical potentials, nutrients, ions, metabolites, waste products, and second messengers (12, 14). 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 (8). Gap junctions have been found in embryonic and newborn skeletal muscles of the chick, mouse, and the rat (10, 24). 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. (10). Recently, Cx39 has been found to be expressed during skeletal muscle embryonic development and involved in the myoblast fusion (2, 4, 32). 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 (8, 24). Moreover, in regenerating skeletal muscle, a transient upregulation of Cx45, Cx43, and Cx39 expression precedes myoblast fusion (2, 4, 32). Using knockout mice, it has been evidenced that Cx43 is required for normal myogenesis in vitro and adult muscle regeneration in vivo (2).

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 immuno-

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histochemistry, 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 (1, 3, 6, 9, 21, 22, 27).

METHODS

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 their suffering, and all experiments were approved by the local ethics committee (University of Palermo).

Animals. The present study was performed on adult male Wistar rats (350 g body wt) housed under alternating 12-h periods of light and darkness in a temperature-controlled ($24 \pm 2^\circ\text{C}$) and humidity-controlled room. All of the experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local ethics committee.

Animal model of muscle regeneration after injury. Forty adult males rats were used for in situ hybridization and immunohistochemistry analysis. Gastrocnemius crush injury of right leg 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 were killed by excess anesthesia in groups of three at various time points (6, 12, and 24; 2, 3, 5, 7, 9, and 12 days; and 2, 3, and 4 wk) after injury. The injured muscle was dissected, frozen in precooled isopentane, and stored at -70°C .

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 *SacI*, and transcribed with T7 RNA polymerase for the antisense probe, or with *EcoRI* 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 *SacI*, and transcribed with T7 RNA polymerase for the antisense probe, or with *EcoRI* 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. AF021806), subcloned in PCR II TOPO TA (Invitrogen, Carlsbad, CA), linearized with *EcoRV*, and transcribed with SP6 RNA polymerase for the antisense probe, or with *SacI* 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 *EcoRI*, and transcribed with T3 RNA polymerase for the antisense probe, or with *SacI* 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 *SacI*, and transcribed with T7 RNA polymerase for the antisense probe, or with *EcoRI* 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 *EcoRV*, and transcribed with SP6 RNA polymerase for the antisense probe, or with *SacI* and transcribed with T7 RNA polymerase for the sense probe. The radiolabeling of the riboprobes was performed as previously described in Condorelli et al. (7).

In situ hybridization. Longitudinal muscle sections (10 μm) were cut at -20°C and thawed onto 3-aminopropyl-ethoxysilane-coated slides. Frozen sections were directly fixed in 4% paraformaldehyde for 1 h 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. (7). Slides were deproteinated in 0.2 M HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1 M ethanolamine for 20 min, and dehydrated with increasing concentrations of ethanol. Slides were incubated for 16 h in a humidified chamber at 52°C with 8×105 counts/min probe in 80- μl hybridization cocktail (50% formamide, 20 mM Tris·HCl, pH 7.6, 1 mM EDTA, pH 8.0, 0.3 M NaCl, 0.1 M dithiothreitol, 0.5 $\mu\text{g/ml}$ yeast tRNA, 0.1 $\mu\text{g/ml}$ poly-A-RNA, $1 \times$ Denhardt's solution, and 10% dextran sulfate), washed twice in $1 \times$ SSC ($1 \times$ SSC = 150 mM NaCl, 15 mM 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 $1 \times$ SSC at 62°C , single-stranded RNA was digested by RNase treatment (10 $\mu\text{g/ml}$) for 30 min at 37°C in 0.5 M NaCl, 20 mM Tris·HCl, pH 7.5, 2 mM EDTA. Slides were washed twice with $1 \times$ 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 and 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- α (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 1 h with a biotinylated anti-rabbit antiserum (Amersham), diluted 1:200. After three short washes with PBS, the sections were incubated for 1 h with a horseradish-peroxidase-streptavidin complex (Vector, Burlingame, CA) diluted 1:100 in PBS. After washes in PBS and then in Tris·HCl buffer (0.1 M, 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-2 emulsion, and processed as described for in situ hybridization.

Double immunolabeling analysis. Cryostat sections of 10- μ m thickness of gastrocnemius muscle were thawed onto gelatin-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 (32) 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 1 h 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 2 h 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 1 h 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 0.5 μ g/ml of the fluorescent nuclear dye Hoechst 33258 (bisbenzimidazole, 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; 40 mg/kg ip) was injected 2, 3, 4, 5, and 7 days after injury, and rats were killed following 2 h 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-2 photoemulsion and exposed for ~4 wk 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 2 N HCl (15 min at 37°C), and then in borate buffer for 10 min (0.1 M, 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 24 h, sections were washed 1 h in TBS and incubated at room temperature for 1 h 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 1 h with a streptavidin horseradish peroxidase complex (Vector, Burlingame, CA), diluted 1:100 in TBS. After washing in TBS and then in Tris·HCl buffer (0.1 M, 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 entellan mountant. The number of BrdU-positive cells was determined by counting, in eight sections of each time point examined, with a micrometric quadrilaterated reticulum, five random fields (250- μ m² 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.

RESULTS

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 24 h; 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. 1A). Twenty-four hours following the crush injury, Cx37 expression was upregulated surrounding the area of lesion in the endothelial cells (Fig. 1B). Between 24 and 72 h 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. 1C). Three days after injury, Cx37 mRNA-expressing cells were found inside the area of lesion (Fig. 1D), 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. 1, 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.

Cx39 mRNA expression. In control, uninjured muscle, Cx39 mRNA was not detectable, as previously reported in Belluardo et al. (4). 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. 2A) 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. 2B). By 9 days from injury, in the regenerating area, Cx39 mRNA positive cells formed clusters with 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. 2C); 2) clusters in which cell nuclei were separated by an increasing eosinophilic sarcoplasm, representing a more advanced stage of myotube formation (Fig. 2, 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. 2F). 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. 3A). 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. 3B). Three days after the injury, rows of Cx40 mRNA-labeled cells were migrating from the vessels to the area of lesion (Fig. 3C). Between 4 and 5 days from injury, Cx40 mRNA-labeled cells

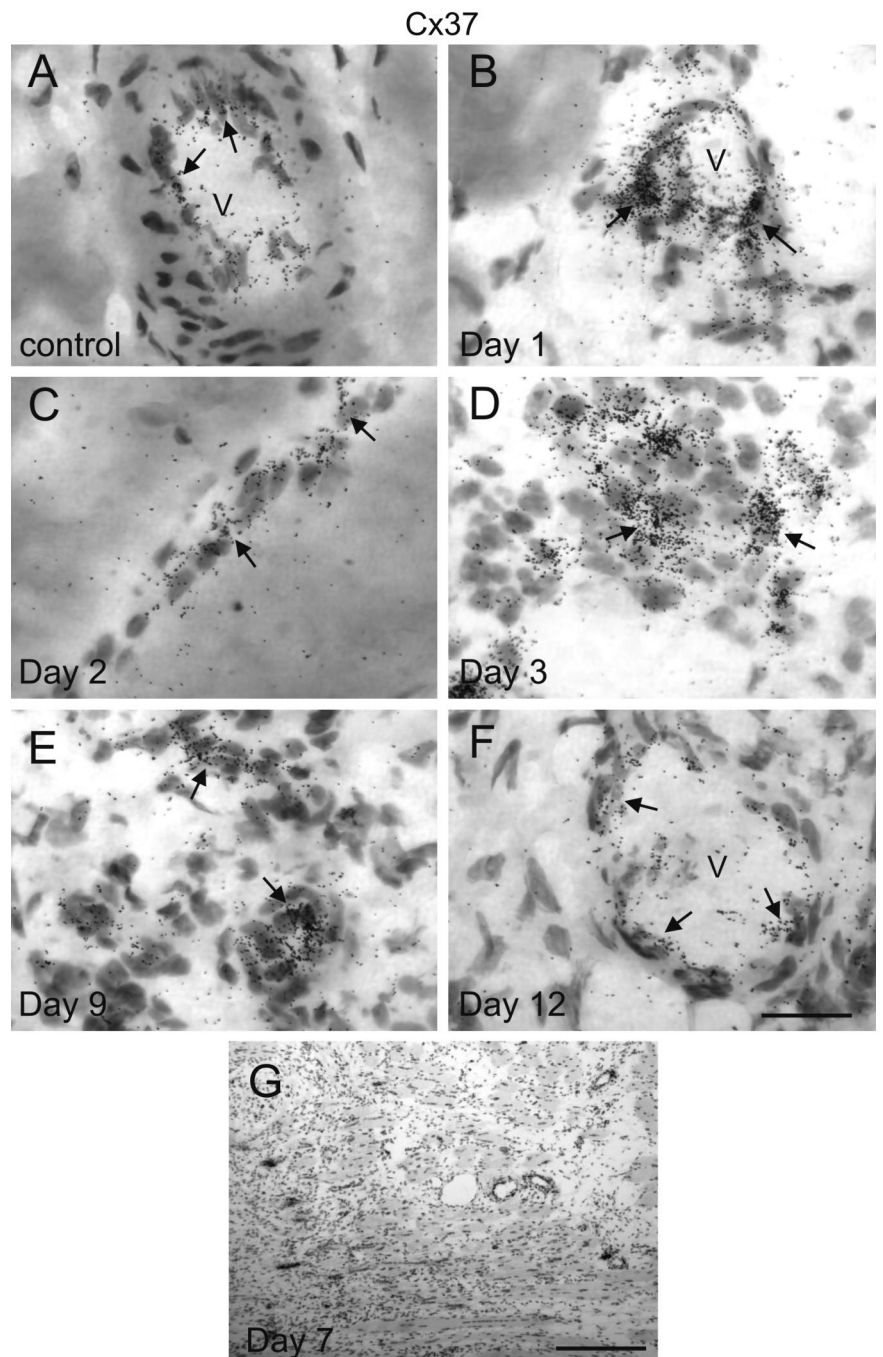


Fig. 1. Micro-autoradiograms showing connexin (Cx) 37 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [control (A); day 1 (B); day 2 (C); day 3 (D); day 9 (E); day 12 (F); day 7 (G)] from crush injury. Detailed information is given in RESULTS. In G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

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. 3D) 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. 3E) 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. 3F). 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.

Cx43 mRNA expression. In control, uninjured muscle, Cx43 was weakly expressed in scattered cells along the basal lamina of muscle fibers (Fig. 4A). An increase of Cx43 mRNA-labeled cells was already observed 3 h following the crush injury in the uninjured zones of the muscle. The number of Cx43 mRNA-labeled cells progressively increased between 6 and 12 h from injury, involving region distant from the area of lesion (Fig. 4B). Between 24 and 48 h from injury, Cx43 mRNA-labeled cells

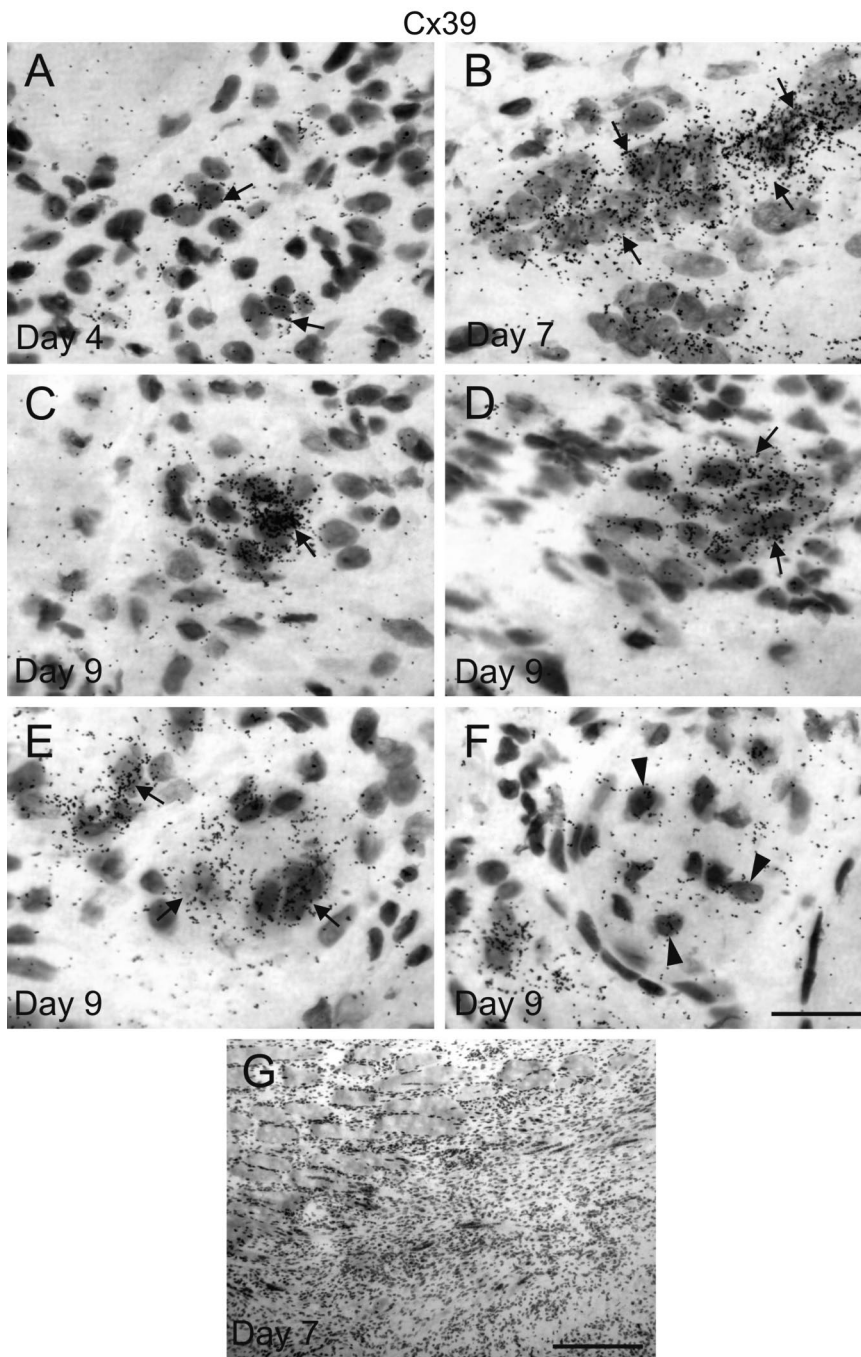


Fig. 2. Micro-autoradiograms showing Cx39 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [day 4 (A), day 7 (B and G), day 9 (C–F)] from crush injury. Detailed information is given in RESULTS. In G is shown an overview of lesioned area. Arrows indicate 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: 25 μm (A–F); 250 μm (G).

accumulated in the region surrounding the lesioned area (Fig. 4C). Between 3 and 4 days from injury, a large number of Cx43 mRNA-labeled cells were located inside the area of lesion (Fig. 4D). 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. 4E). 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. 4, 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 6 h from injury (Fig. 5A) in individual cells along the uninjured fibers surrounding the lesioned area (2). Between 12 and 48 h from injury, Cx45 mRNA-labeled cells increased and formed chains of cells migrating inside the area of lesion (Fig. 5B). Between 3 and 4 days from injury, Cx45 mRNA-labeled cells accumulated in-

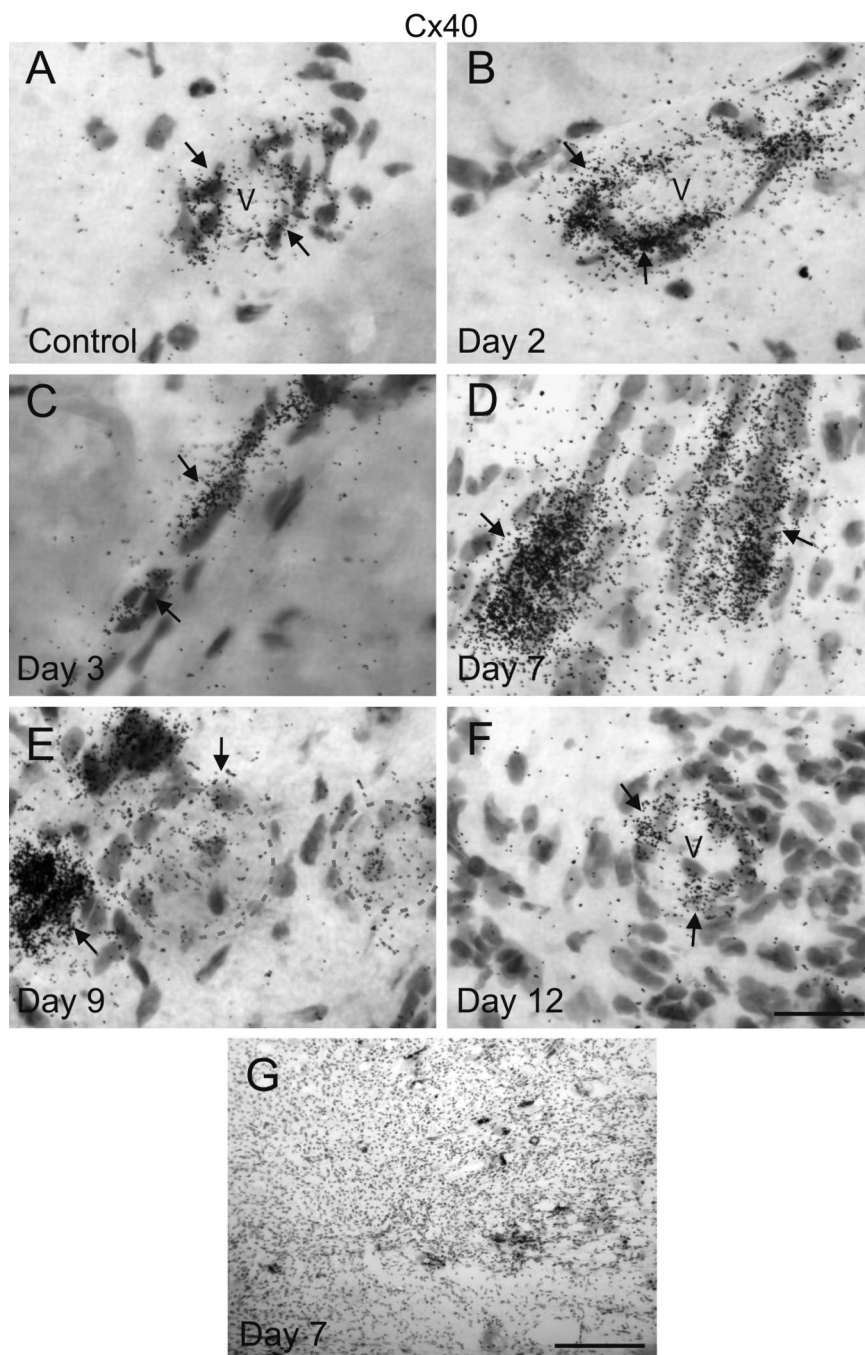


Fig. 3. Micro-autoradiograms showing Cx40 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [control (A), day 2 (B), day 3 (C), day 7 (D and G), day 9 (E), day 12 (F)] from crush injury. Detailed information is given in RESULTS. In G is shown an overview 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).

side the area of lesion (Fig. 5C). 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. 5D). 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. 5F), 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. 5E), as also reported by Araya et al. (2).

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 Fig. 6, C, C₁, D, and D₁, 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. 6, A, A₁, B, and B₁).

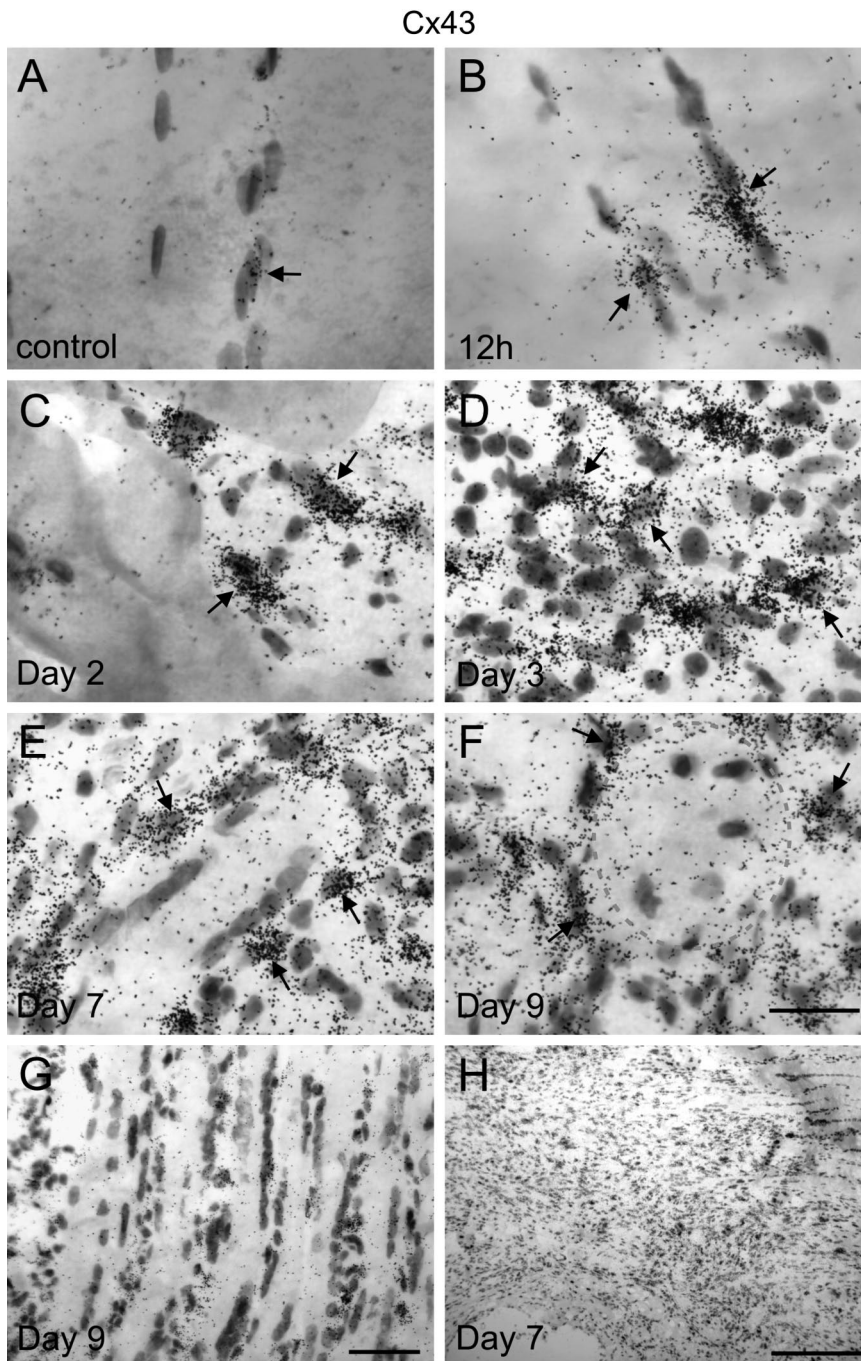


Fig. 4. Micro-autoradiograms showing Cx43 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [control (A), 12 h (B), day 2 (C), day 3 (D), day 7 (E and H), day 9 (F and G)] from crush injury. Detailed information is given in RESULTS. In H is shown an 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).

Cx expression in regenerating fast- or slow-twitch skeletal muscle. To explore the potential differences in regenerating muscles rich in fast or slow fibers, we examined the Cx expression, respectively, in the fast (extensor digitorum longus) and slow (soleus) twitch muscles after crush injury. All of the Cxs examined did not exhibit differences in their spatial and temporal expression pattern in fast- and slow-twitch muscles during the regeneration process (data not shown).

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 differentiating

myogenic cells until their fusion in myotubes: M-cadherin, as marker for quiescent, active, and replicative satellite cells (17), and myogenin, as marker for myogenic cells differentiating in myoblasts and myocytes forming myotubes (9). 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. 7, A–C). Cx39 mRNA was always colocalized in myogenin-positive cells mainly forming clusters of aligned and closely apposed cell

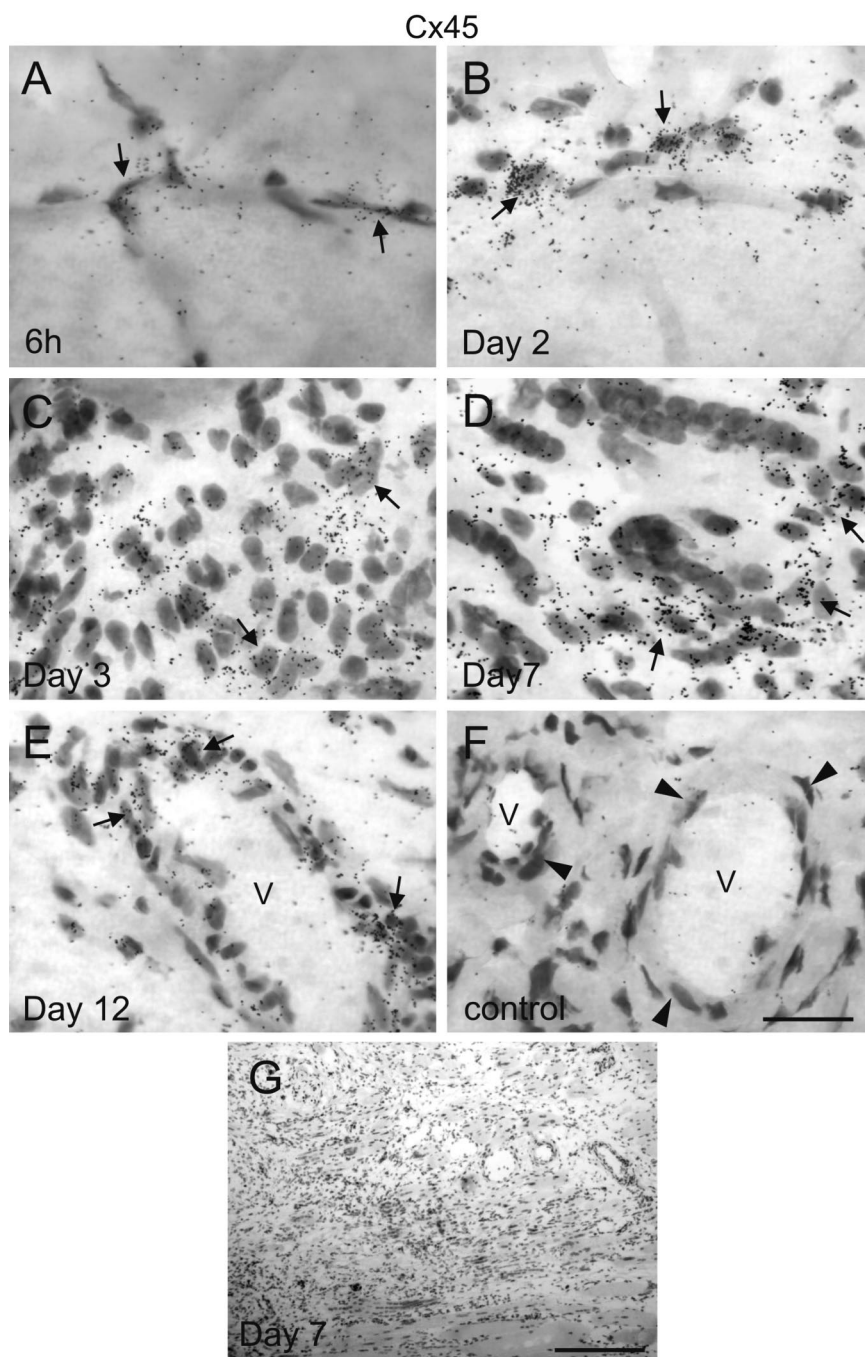


Fig. 5. Micro-autoradiograms showing Cx45 mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at different time points [6 h (A), day 2 (B), day 3 (C), day 7 (D and G), day 12 (E), control (F)] from crush injury. Detailed information is given in RESULTS. In G is shown an overview 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).

nuclei or in myogenin-positive cells forming cluster with heterogeneous morphology corresponding to different advanced stages of myotube formation (Fig. 8A). Virtually all Cx39-labeled cells were myogenin-positive cells. Cx40 mRNA inside the area of lesion was also colocalized in myogenin-positive cells forming clusters with profiles similar to those described for Cx39 (Fig. 8B). However, in contrast to Cx39, the Cx40 was also found expressed in endothelial cells of both preexistent and newly formed blood vessels (Fig. 7, D–F). In control tissue and 1 day after injury, Cx43 mRNA was colocalized in M-cadherin-positive cells (Fig. 8C), but not all Cx43 mRNA-labeled cells were positive for M-cadherin. Three and nine days after injury, Cx43 mRNA was colocalized in indi-

vidual myogenin-positive cells, but again not all Cx43-labeled cells were positive for myogenin (Fig. 8D). Using anti-CD11b antibodies to identify macrophages/monocytes, we could find that Cx43 mRNA is expressed in CD11b-positive cells (Fig. 8G). 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. 8D). One day after injury, Cx45 mRNA was expressed in M-cadherin-positive cells (Fig. 8E), 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

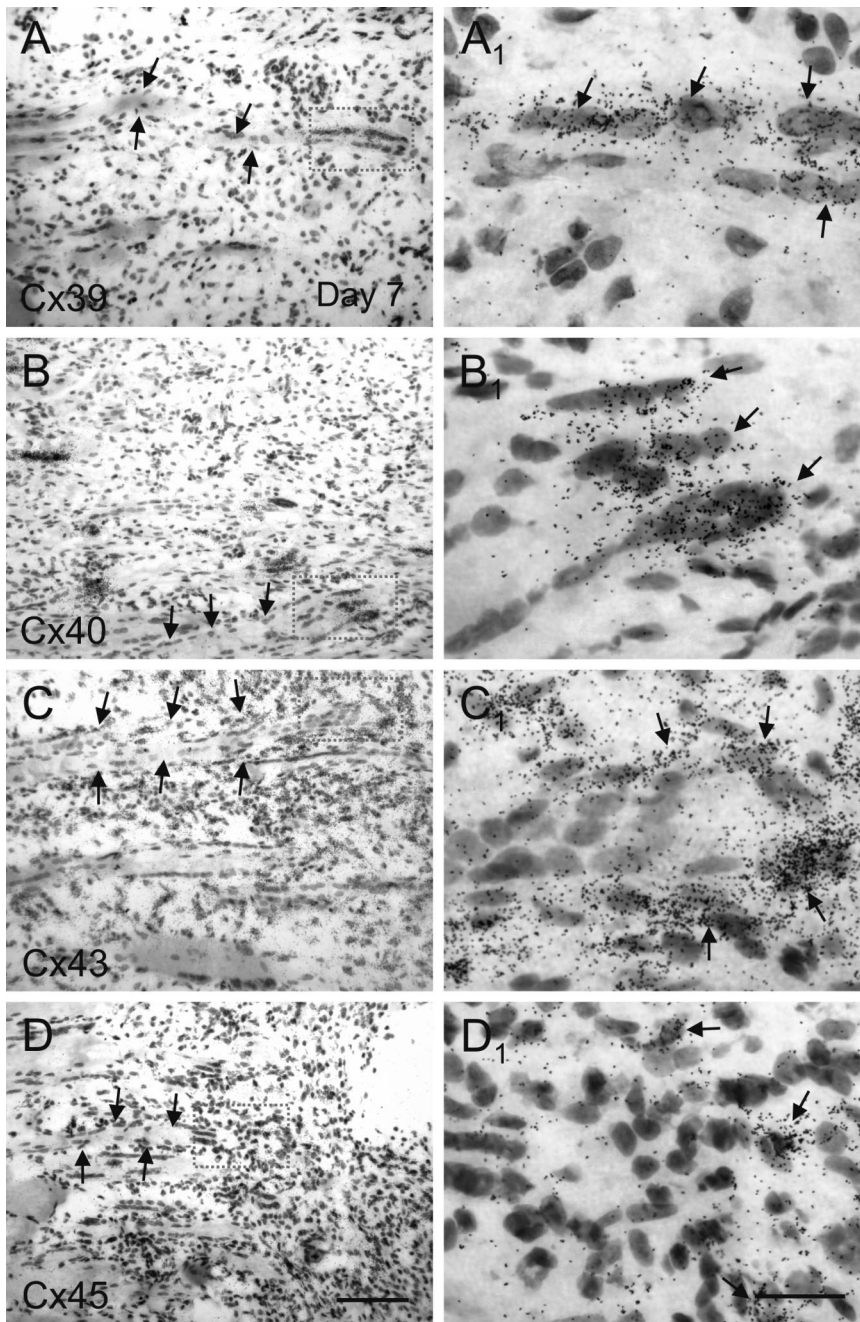


Fig. 6. Micro-autoradiograms showing Cx39 (A and A₁), Cx40 (B and B₁), Cx43 (C and C₁), and Cx45 (D and D₁) mRNA labeling in hematoxylin-eosin-stained sections of gastrocnemius muscle at 7 days from crush injury. A–D: low magnification showing differentiating new myotubes (arrows) in the regenerating 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 μ m (A–D); 25 μ m (A₁–D₁).

positive (Fig. 8F) (2). Using anti-vWF antibodies, Cx45 was also found coexpressed in new blood vessels (Fig. 7, 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. 8F).

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. 2B and 3D) 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. 9). The developing skeletal muscle at embryonic day 15 was used as internal positive control (Fig. 9).

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. 10B). Three days after

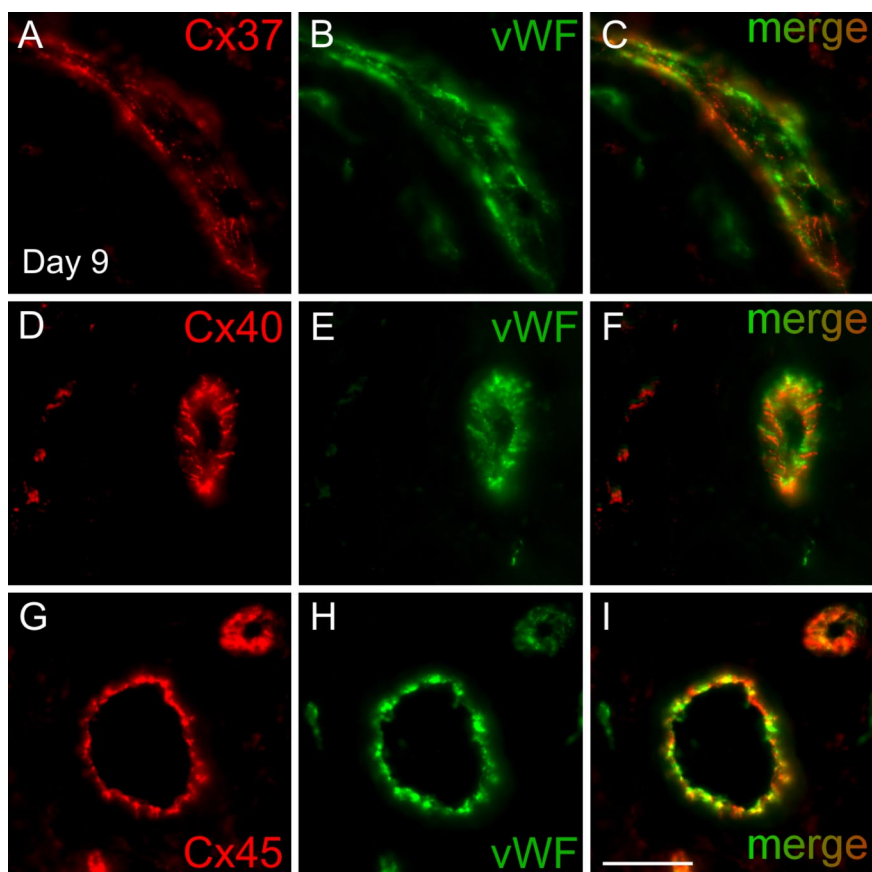


Fig. 7. Cx37 (A), Cx40 (D), and Cx45 (G) colocalization with von Willebrand factor (vWF; B, E, and H) in gastrocnemius muscle at 9 days from crush injury. C, F, and I: overlay showing Cx37, Cx40, and Cx45 (red), respectively, colocalizing with vWF (green). Detailed information is given in RESULTS. Scale bar: 50 μ m.

lesion, Cx40 mRNA was expressed in $16 \pm 3.4\%$ of BrdU-positive cells mainly located around the blood vessels (Fig. 10C). By contrast, in the same experimental condition, Cx37 and Cx43 mRNAs were expressed, respectively, in about $18 \pm 4.3\%$ and $80 \pm 8.9\%$ of BrdU-positive cells (Fig. 10, A and C). The other two time points examined showed a comparable trend.

DISCUSSION

The present study provided a comparative analysis of spatial and temporal mRNA expression of five Cxs in the regenerating skeletal muscle after crush injury. Among the Cxs examined, the Cx39 mRNA was not expressed in control, uninjured muscle, whereas the other Cxs, although expressed at low levels, were localized in the endothelial cells (Cx37 and Cx40) and in individual cells of basal lamina (Cx43 and Cx45), according to previous data (2, 15). 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.

Role of Cxs expressed in regenerating muscle. The endothelial localization and spatiotemporal 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 (29), probably because it could be compensated by other Cxs, such as Cx40 (30). 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 (4, 32). 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 (6). 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

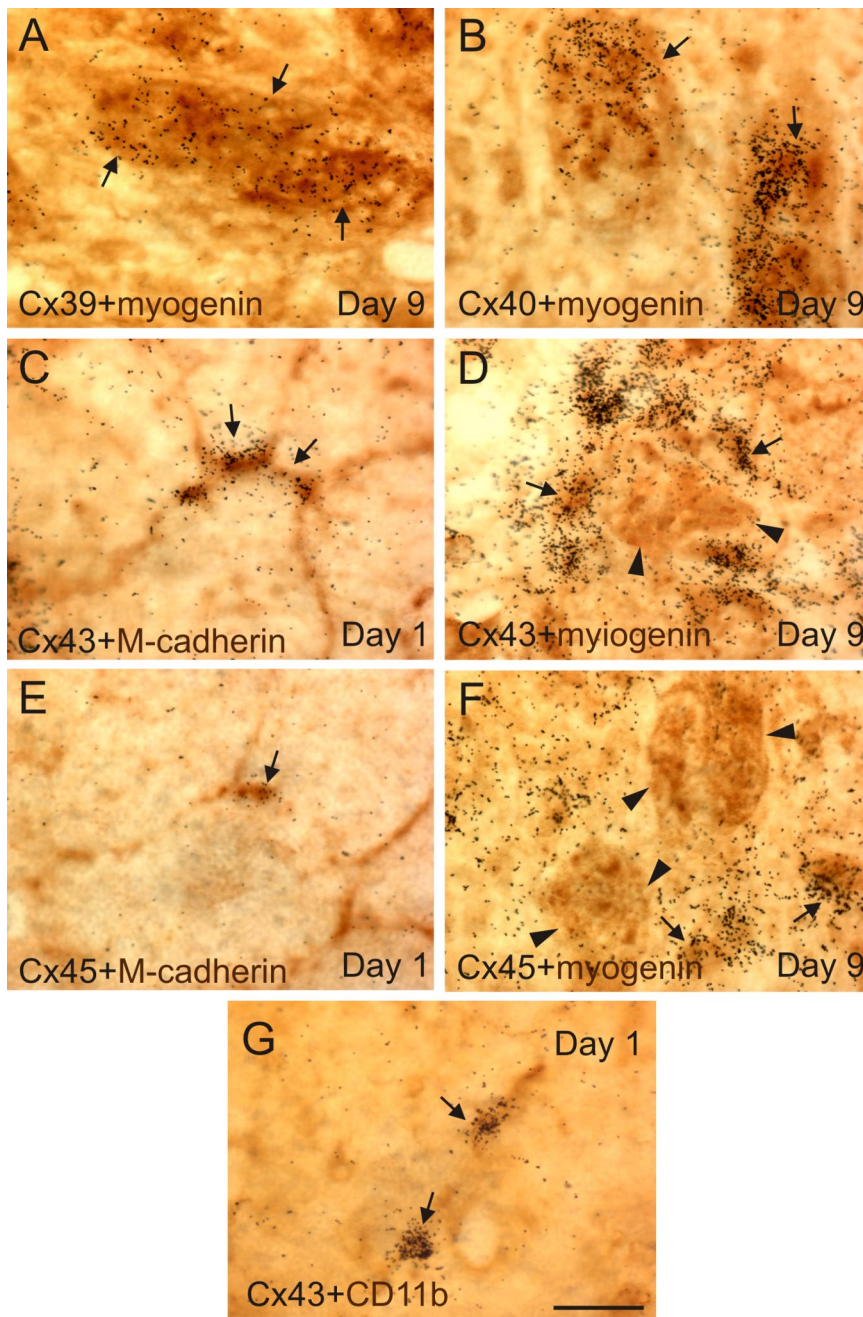


Fig. 8. Micro-autoradiograms showing colocalization of Cx43 (C) and Cx45 (E) mRNAs labeling in cadherin-positive cells and Cx39 (A), Cx40 (B), Cx43 (D), and Cx45 (F) mRNAs in myogenin-positive cells of gastrocnemius muscle, respectively, at 1 and 9 days from crush injury. G: colocalization of Cx43 mRNA labeling (black grains) in CD11b-positive cells (brown). Detailed information is given in RESULTS. 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 mRNA labeling. Scale bar: 25 μ m.

and the sarcolemma of their associated muscle fibers (2, 4). 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 (33). 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. (36) and by Le Grand et al. (19), respectively. Additionally, Cx40 mRNA-positive cells could also be related to pericytes associated with microvasculature walls that efficiently contribute to myogenic regeneration (11) or to mesoangioblast cells, an adult vessel-derived stem cell that possess high myogenic potential (23). 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

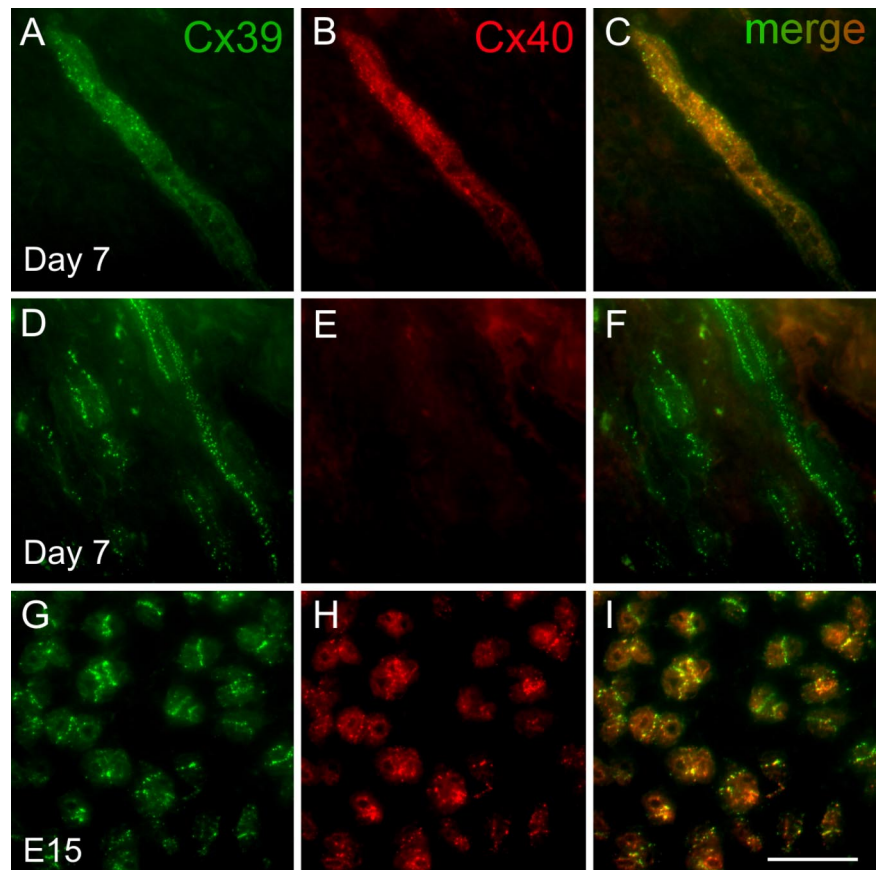


Fig. 9. Cx39 (A, D, and G) and Cx40 (B, E, and H) immunolabeling in gastrocnemius muscle at 7 days from crush injury. Note the immunostaining as typical puncta (A and B, D and E, G and H) mainly localized in clusters of aligned and closely apposed cells. C: overlay showing Cx40 (red) colocalizing with 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 .

defects in vasculogenesis or vascular development (29). However, Cx37 and Cx40 could be coexpressed in endothelial precursor cells and could overlap functionally in generating vessels, as revealed by severe vascular abnormalities after elimination of both Cxs (30). 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

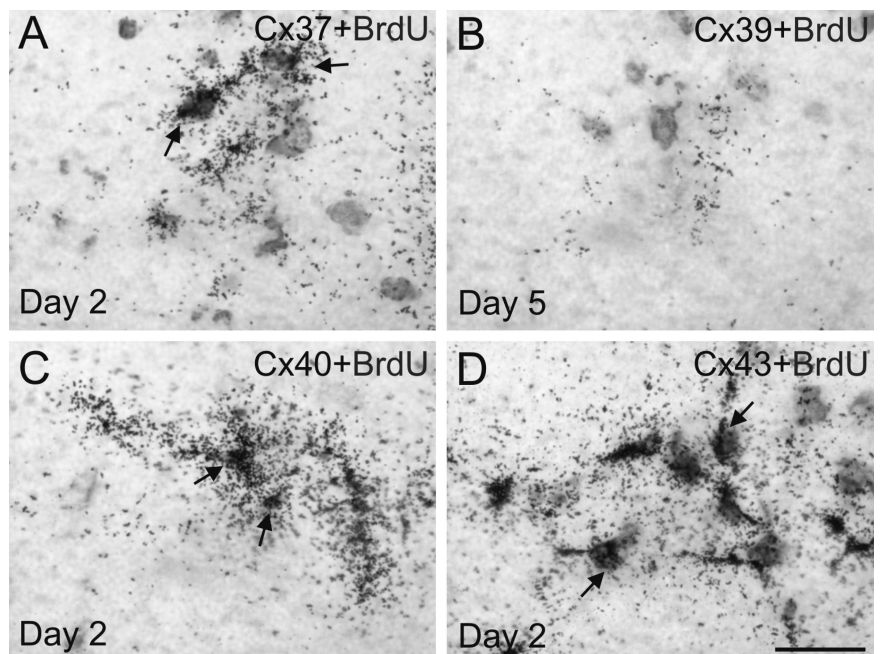


Fig. 10. Micro-autoradiograms showing colocalization of Cx37 (A), Cx39 (B), Cx40 (C), and Cx43 (D) mRNA labeling in bromodeoxyuridine (BrdU)-positive cells of gastrocnemius muscle at representative time points from crush injury. Note in B the absence of colocalization of Cx39 mRNA labeling (black grains) 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 BrdU-positive nuclei (gray). Scale bar: 25 μm .

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 (2, 15). The expression of Cx43 mRNA in M-cadherin-positive cells suggests a role in proliferating and differentiating satellite cells (15). 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 (2, 15). 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 derive from Cx43-positive cells after downregulation of Cx43 gene (15, 32, 33). 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 (6, 35).

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 (33). 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 (18). Taken together, these data confirm that also Cx45, similarly to Cx37 and Cx40, is involved in blood vessel generation (18) and implicate a precursor cell population expressing Cx45 distinct from that one coexpressing Cx45 and myogenin, potentially involved in muscle regeneration, as also reported by Araya et al. (2).

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 (5, 13, 16, 25, 28, 34). 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. 11). Cx40 mRNA-expressing cells seem to be generated by activated, proliferating precursor cells local-

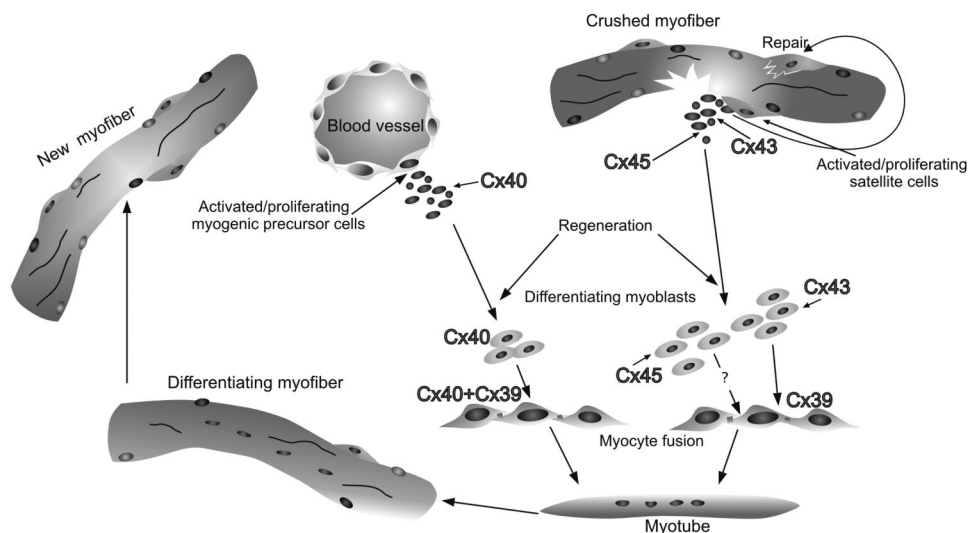


Fig. 11. Schematic representation of skeletal myofiber repair. Shown is the regeneration process following a crush injury with the potential cell expression of Cx39, Cx40, Cx43, and Cx45 during satellite activation-proliferation, myoblast differentiation, and fusion to form myotubes. For detailed information, see DISCUSSION.

ized 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 (2, 4, 32, 33). The 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.

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.

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