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Expression of connexins during differentiation and regeneration of skeletal muscle: functional relevance of connexin43

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Summary

The molecular mechanisms regulating skeletal muscle regeneration and differentiation are not well understood. We analyzed the expression of connexins (Cxs) 40, 43 and 45 in normal and regenerating tibialis anterior muscle and in primary cultures of differentiating myoblasts in adult and newborn mice, respectively. Cxs 45 and 43, but not 40, were strongly expressed in normal muscle and their expression was upregulated during regeneration. Furthermore, the functional role of Cx43 during differentiation and regeneration was examined after induced deletion of Cx43 in transgenic mice. In vivo, the inducible deletion of Cx43 delayed the formation of myofibers and prolonged the expression of myogenin during regeneration. In primary cultures of satellite cell-derived myoblasts, induced deletion of Cx43 led to decreased expression of myogenin and MyoD, dye coupling, creatine kinase activity and myoblast fusion. Thus, the expression of Cx45 and Cx43 is upregulated during skeletal muscle regeneration and Cx43 is required for normal myogenesis in vitro and adult muscle regeneration in vivo.

Key words: Connexins, Myoblasts, Myogenesis, Regeneration

Introduction

The formation of skeletal muscle during development occurs via a series of cellular and molecular steps that lead to the formation of multinucleated myofibers. Members of the MyoD family of transcription factors, including MyoD (Davis et al., 1987), Myf5 (Braun et al., 1989), myogenin (Wright et al., 1989) and MRF4/hereculin/Myf6 (Rhodes and Konieczny, 1989; Miner and Wold, 1990; Braun et al., 1990) are key regulators in skeletal muscle ontogeny. MyoD and Myf5 are expressed during myogenesis and are markers of commitment to a muscle fiber fate (Braun et al., 1992; Rudnicki et al., 1992; Rudnicki et al., 1993; Weintraub, 1993). The onset of the terminal differentiation process is characterized in part by cell cycle withdrawal and myogenin expression. These muscle regulatory factors activate the expression of muscle-specific genes such as the acetylcholine receptor α-subunit (Piette et al., 1990) and creatine kinase (CK) (Lassar et al., 1989). Adult skeletal muscle fibers have the ability to regenerate after injury, a process that in many aspects recapitulates skeletal muscle development. After injury, quiescent mononucleated satellite cells closely associated with injured fibers are activated, leading first to their proliferation and later to cell fusion, culminating in the formation of new myofibers (Grounds et al., 2002). Activation of satellite cells in different injury models is characterized by the expression of MyoD, Myf5, myogenin and MRF4 at early stages after muscle injury (Grounds et al., 1992; Füchbauer and Westphal, 1992; Koishi et al., 1995; Kami et al., 1995; Cooper et al., 1999; Launay et al., 2001; Casar et al., 2004). However, many of the mechanisms that orchestrate myogenesis remain unknown.

During development and regeneration of skeletal muscle, proliferation, differentiation and growth are coordinated by diverse intercellular signaling mechanisms. One of them is mediated by gap junctions, clusters of intercellular channels that enable direct cell-cell signaling and propagation of electrical activity as well as allowing for the exchange of ions and small molecules such as second messengers (Sáez et al., 2003). A gap junction channel is formed by the docking of two hemichannels, each of which is composed of six protein subunits termed connexins. In the mouse, connexins are encoded by a gene family that consists of at least 19 genes (Willecke et al., 2002).

The possible role of gap junctional communication in myogenesis has been previously studied (Constantin and Cronier, 2000). Ultrastructural analyses in developing rat and chicken skeletal muscle demonstrated the presence of gap junctions between myoblasts and between myoblasts and myotubes (Rash and Staehelin, 1974; Kalderon et al., 1977; Duxson et al., 1989). Functional gap junctions have been detected in early developing myoblasts (Schmalbruch, 1982; Balogh et al., 1993; Proulx et al., 1997). Dahl and coworkers (Dahl et al., 1995) showed that connexin 40 (Cx40) is transiently expressed in axial skeletal muscles of mouse embryos during myoblast fusion. Moreover, Cx43 is expressed in vitro in prefusional myoblasts of the C_2C_{12} cell line (Constantin and Cronier, 2000). Myogenin mRNA expression and myotube formation during myoblast differentiation in the L6 cell line is reversibly blocked by 18-β-glycyrrhetinic acid or octanol, which inhibit gap junction channels (Proulx et al., 1997). However, these and other gap junction blockers are not specific and have many side effects (Lee et al., 1996; Horigome et al., 1999; Horigome et al., 2001; Jeong and Kim, 2002). Analysis of the complete transcriptome has recently revealed that Cx43 mRNA is upregulated in cardiotoxin-injected mouse skeletal muscle (Bakay et al., 2002). However, direct demonstration of the cell types that express Cx43 and the role of this protein in skeletal muscle myogenesis and regeneration is unknown.

In this study, the expression of both Cx43 and Cx45 was analyzed in differentiating myoblasts and in regenerating skeletal muscles. In addition, using inducible Cx43-deficient mice, we found that Cx43 was required for myogenesis in vitro and for the normal timing of skeletal muscle regeneration.

Materials and Methods

Generation of transgenic mice

Mice were kept in standard housing conditions with a 12-hour:12-hour dark:light cycle, and with food and water ad libitum. Heterozygous $Cx43^{del/+}$ and $Cx45^{+/-}$ mice carried in the 'del' and '–' allele a *lacZ* gene, encoding nuclear or cytoplasmic β-galactosidase respectively, in place of the Cx43 or Cx45 coding region. Floxed Cx43 ($Cx43^{fl/fl}$) animals and heterozygous $Cx43^{del/+}$ and $Cx45^{+/-}$ mice were generated as described previously (Theis et al., 2000; Theis et al., 2001; Krüger et al., 2000). Homozygous $Cx43^{del/del}$ mice were generated by intercrossing $Cx43^{del/+}$ mice. For directed ablation of the Cx43 coding region, $Cx43^{fl/fl}$, *Mx-cre* mice (see Inducible Cx43 gene deletion section) were generated by intercrossing the following parental generations: F1, $Cx43^{fl/fl}$ × $Cx43^{+/+}$, *Mx-cre* (Kühn et al., 1995) mice; F2, $Cx43^{fl/fl}$ × $Cx43^{fl/+}$, *Mx-cre* mice.

The generation of $Cx43^{Cre\text{-}ER(T)/fl}$ mice is described in detail by Eckardt et al. (Eckardt et al., 2004). Briefly, a double replacement strategy for the generation of the Cx43 knock-in Cre-ER(T) allele was used. First, the Cx43 coding region was replaced by an HPRT minigene. Second, the minigene was replaced by the Cre-ER(T) coding region (Feil et al., 1996). The expression of Cre-ER(T) was then controlled by the endogenous Cx43 promoter. For the generation of $Cx43^{Cre\text{-}ER(T)/fl}$ mice, $Cx43^{Cre\text{-}ER(T)/+}$ mice were intercrossed with $Cx43^{fl/fl}$ mice. Successful matings were identified by the presence of a vaginal plug on the morning after mating.

Genotyping of mice

Genomic DNA was prepared from tail biopsies to characterize the transgenic mice. Transmission of the Mx-cre transgene was identified by the Mx-cre PCR, using the primers 5′-CAT GTG TCT TGG TGG GCT GAG-3′ and 5′-CGC ATA ACC AGT GAA ACA GCA T-3′, generating an ~600 bp amplicon. PCR conditions were: 30 cycles of 2 minutes at 94°C, 0.5 minutes at 60°C, 1 minute at 72°C. The Cre-ER(T) PCR was performed using the primers 5′-AAA GTA TTA CAT CAC GGG GGA GGC AGA GGG-3′ and 5′-TGG CCA CTG CAA GCA GCA ACA TAC CAT TGC-3′, generating a ~1.9 kb amplicon. PCR conditions were: 35 cycles of 1 minute at 92°C, 1 minute at 70°C, 2.5 minutes at 72°C. The $Cx43^{fl}$ allele was detected by $Cx43^{fl}$ PCR (Theis et al., 2001) and the $Cx43^{del}$ allele by $Cx43^{del}$ PCR (Theis et al., 2001). The status of the *Cx45* allele was detected by PCRs as previously described (Krüger et al., 2000).

Skeletal muscle injury model

Mice (4 months old) of both genders were anesthetized with intraperitoneal injection of a combination of rompun and ketamin (10 and 70 μg/g of body weight, respectively; Bayer, Germany). A single injection of 60 μl 1.2% (w/v) $BaCl_2$ in saline solution was administered along the center of tibialis anterior (TA) of each hindlimb. Mice were sacrificed by quick cervical dislocation and muscles were collected at days 3, 5, 7, 10 and 14 post $BaCl_2$ injection (PI). Five different mice were obtained at each day PI.

Inducible Cx43 gene deletion

For inducible inactivation of Cx43 under in vivo and in vitro conditions we used $Cx43^{fl/fl}$, *Mx-cre* mice and $Cx43^{Cre\text{-}ER(T)/fl}$ mice, respectively. Under in vivo conditions, Cre-mediated Cx43 deletion was induced by intraperitoneal administration of double stranded RNA poly(I)-poly(C) (pI-pC) (Amersham Pharmacia Biotech, Germany) that promoted the interferon production. Interferon activated the Mx-1 promoter (Hug et al., 1988) that controls the expression of Cre (Kühn et al., 1995), which specifically deleted the floxed Cx43 coding region. The deletion of the floxed Cx43 gene elements led to *lacZ* activation in cells that express Cx43 mRNA. Before site-specific deletion of the floxed gene, Cx43 was expressed. The *lacZ* gene became expressed under control of the Cx43 promoter only after the floxed cassette had been deleted. Specific deletion of Cx43 was detected by 5-bromo-4-chloro-3-indoyl-β-galactosidase (X-gal) staining (Roth, Karlsruhe, Germany) (Krüger et al., 2000; Theis et al., 2001). pI-pC (250-300 μg) was injected every 48 hours for a total of five times. After the last injection, $BaCl_2$ was injected in both TAs.

For in vitro inactivation of Cx43, primary cultures of satellite cell-derived myoblasts were prepared (see Cell culture section) from $Cx43^{Cre\text{-}ER(T)/fl}$ mice. Then, cultures (~20% confluence) were treated with 2 μM 4-OH-tamoxifen every 24 hours (five times) prior to the onset of differentiation (~70% confluence). The tamoxifen-induced Cre-ER(T) system has been previously described (Feil et al., 1996; Schwenk et al., 1998). Deletion of the floxed Cx43 allele was detected by X-gal staining at 0, 24 and 48 hours of differentiation and by immunofluorescence and western blot analysis of total cell homogenates at 24 hours of differentiation.

Histological analysis

Freshly dissected TAs were embedded in tissue mounting solution OCT (Electron Microscopy Sciences, Washington, PA, USA) and fast frozen in liquid-nitrogen-cooled isopentane (Merck, Darmstadt, Germany). Serial cryostat sections of 10 μm or 25 μm thickness were obtained for Hematoxylin and Eosin and Eosin/X-gal staining, respectively. Sections were placed on glass slides (SuperFrost Plus, Menzel-Glaeser, Germany) and fixed for 5 minutes with 0.2% glutaraldehyde (Sigma-Aldrich, St Louis, MO, USA) for X-gal staining, or 2% paraformaldehyde (Sigma-Aldrich Inc.) for simultaneous X-gal staining and immunofluorescence or immunohistochemistry.

Indirect immunofluorescence and immunohistochemical analyses

For indirect immunofluorescence of X-gal-stained TA sections, samples were washed three times in phosphate-buffered saline (PBS) solution, pH 7.4, and then incubated in blocking solution (hamster serum diluted 1:1 with carragenin (0.7%)-Triton X-100 (0.5%) in 5 mM Tris, pH 7.8) for 30 minutes at room temperature. Sections were incubated at 4°C overnight with either primary rabbit anti-CD14, rabbit anti-myogenin or goat anti-M-cadherin antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:400, or rabbit anti-desmin or rabbit anti-VWF antibodies (Sigma-Aldrich

Inc.) at a dilution of 1:500. Then, sections were rinsed three times with PBS at room temperature and incubated with FITC-conjugated goat anti-rabbit or rabbit anti-goat IgG secondary antibodies for 1 hour at room temperature. Sections were rinsed and mounted with fluoromount G (Electron Microscopy Sciences, Washington, PA) on glass slides and observed under a xenon arc lamp on a Nikon Labophot-2 microscope equipped with epifluorescence illumination and photographed.

For immunohistochemistry of X-gal-stained sections, samples were washed three times with PBS and incubated for 10 minutes in 3% (v/v) H_2O_2 in 10% methanol/Tris-buffered saline (TBS) (pH 7.4) to inhibit endogenous peroxidase activity. Then they were washed three times with PBS and non-specific protein binding sites were blocked with 2% normal goat serum in TBS. Sections were incubated with rabbit anti-CD14 antibodies at a dilution of 1:400, washed three times with TBS and incubated for 30 minutes with secondary goat anti-rabbit IgG (Sigma-Aldrich Inc). Then they were washed three times in TBS and incubated for 30 minutes with rabbit peroxidase-anti-peroxidase soluble complex (PAP) (Sigma-Aldrich Inc.) at a 1:100 dilution. Sections were washed three times with TBS and diaminobenzidine (1 mg/ml) (Sigma-Aldrich Inc.) plus 1 μl/ml H_2O_2 added until staining was observed.

Serial sections from three to five different wild-type and transgenic mice were used for all these analyses.

Quantitative evaluation of CD14-positive cells during regeneration

The number of CD14-positive cells was analyzed by counting 10 random 250 μm^2 squares fields (10× ocular) per TA section (five sections per experiment) under a light microscope (Nikon Labophot-2 microscope) equipped with a micrometric quadriculated reticulum (Nikon, USA). Statistical analysis was performed using Student's *t*-test with Sigmaplot (Software).

Cell culture

Primary cultures of satellite cell-derived myoblasts were prepared as described previously (Springer et al., 1997). Cells were obtained from newborn (post-natal days 1-5) wild-type, *Cx43*$^{Cre\text{-}ER(T)/fl}$ or *Cx43*$^{fl/fl}$ mice or from embryos (E18-20) of *Cx43*$^{del/del}$ mice. Briefly, animals were decapitated, limbs removed and muscles dissected away and minced to a slurry. This was then incubated for 15 minutes at 37°C in a solution (0.5 g tissue per ml) containing 1.5 U/ml collagenase D/2.4 U/ml dispase II/2.5 mM $CaCl_2$. Collagenase D and dispase II were from Boehringer Mannheim Corp. (Indianapolis IN, USA). Dissociated cells were plated on collagen-coated Petri dishes in growth medium (GM) [F10 nutrient mixture (Gibco-BRL, Rockville MD, USA), 20% fetal calf serum (HyClone Laboratories, Inc., Logan, UT, USA), 50 μl (25 μg/ml) human recombinant mouse basic fibroblast growth factor (bFGF) (ReproTech Inc., Rocky Hill, New Jersey, USA)]. Using immunofluorescence desmin detection it was determined that in primary cultures of muscle cells 90-95% of the cells were myoblasts. Once myoblasts cultures were ~70% confluent, cells were fed with fusion medium (DMEM nutrient mixture and 5% horse serum; both from Gibco-BRL).

Cell fusion was scored by counting the number of nuclei present in myotubes divided by the total number of nuclei present in a 400× microscope field multiplied by 100. An average number of 15 fields in four different experiments were scored per condition tested.

Creatine kinase activity

Cells were washed twice with PBS, pH 7.4 and lysed in 1 ml ice cold PBS containing 0.1% Triton X-100 for 10 minutes. Then, lysed cells were scraped and creatine kinase activity was measured in an aliquot using a creatine kinase assay kit (VALTEK, Santiago, Chile).

Western blot analysis

TAs frozen on dry ice were pulverized, lyophilized and resuspended in lysis buffer [PBS containing proteases inhibitors: 2 mM phenylmethanesulfonyl fluoride (PMSF), 200 μg soybean trypsin protease inhibitor, 1 mg/ml benzamidine, 1 mg/ml ε-aminocaproic acid, and 500 μg/ml leupeptin and phosphatase inhibitors: 20 mM $Na_4P_2O_7$ and 100 mM NaF] and sonicated. Primary cultures of satellite cell-derived myoblasts, C_2C_{12} cells and Cx45 HeLa transfectants were washed twice with ice cold PBS (pH 7.4) and then harvested by scraping with a rubber policeman in 100 μl lysis buffer and then lysed by sonication. Proteins were measured according to the method of Smith et al. (Smith et al., 1985). Western blot analyses were performed as described previously (Martínez and Sáez, 1999). Blots were incubated overnight with either rabbit anti-rat myogenin antibodies (1:400; Santa Cruz Biotechnology Inc), rabbit anti-rat MyoD antibodies (1:400; Santa Cruz Biotechnology Inc.), rabbit anti-mouse Cx43 (1:1,500) (Traub et al., 1994), rabbit anti-mouse Cx45 (1:500), or a monoclonal α-tubulin antibody (1:5000) (Sigma-Aldrich, Inc.) diluted in TBS with 5% non-fat milk. Then, blots were rinsed with TBS and incubated for 1 hour at room temperature with either: (a) alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies diluted 1:2,000 in TBS with 5% non-fat milk (for Cx43 antibody in blot from Fig. 2B); (b) horse radish peroxidase(HRP)-conjugated goat anti-rabbit IgGs antibodies (Dianova, Germany) diluted 1:30,000 for myogenin, MyoD, and Cx43 antibodies or (c) with an anti-mouse-IgG-HRP conjugate (1:1500) (BioRad, Hercules, CA, USA). After repeated rinses, blots were incubated with: (a) alkaline phosphatase substrate (BCIP/NBT tablets; Sigma-Aldrich Inc.), until reactive bands were clearly observed, and with (b) and (c) and ECL detection reagents (Amersham Pharmacia Biotech). Loading equivalence was confirmed by protein staining with Ponceau Red as previously described (Martínez and Sáez, 1999) and/or levels of α-tubulin measured in stripped blots. Gels showing equal amounts of protein were destained as described and used for analysis.

Dye coupling assay

Cells plated on collagen-coated no. 1 glass coverslips (Marienfeld, Germany) were placed into Petri dishes and bathed with recording medium (HCO_3^- free F12 medium buffered with 20 mM Hepes). Cells were microinjected at 37°C with LY (5% w/v in 150 mM LiCl). After dye injection, cells were observed for 2 minutes to determine whether dye transfer occurred, as described previously (Martínez and Sáez, 1999). The incidence of dye coupling was scored by dividing the number of injections that resulted in dye transfer to at least two mononuclear adjacent cells by the total number of injections performed in each experiment multiplied by 100. The coupling index was calculated by dividing the total number of stained cells when dye diffused to two or more cells from the injected one divided by the number of injection that revealed dye coupling. In all experiments dye coupling was tested with a minimum of 20 microinjected cells. The set-up used to monitor dye coupling was as previously described (Elfgang et al., 1995).

Connexin gene nomenclature

The nomenclature used for connexins in the manuscript follows the most accepted one employed by the gap junction community and it was used to avoid confusion to readers. However, this nomenclature did not follow the rules and guidelines established by the International Committee on Standardized Genetic Nomenclature for Mice that was implemented through the Mouse Genomic Nomenclature Committee (MGNC).

Results

Regenerating skeletal muscle expresses Cx45 and Cx43

To determine whether connexin-composed channels are present during skeletal muscle regeneration, we first studied the time course of connexin expression in the tibialis anterior muscle (TA) after $BaCl_2$-induced muscle degeneration. In cross sections of TAs 3 days post $BaCl_2$ injection (PI), fibers with normal appearance were found only immediately below the epimysium (not shown). All other regions of each muscle cross section (~80%) were covered mainly by fibers of large diameter (> approx. 50 μm) and small mononucleated cells (between 10-15 μm), indicative of necrosis (Caldwell et al., 1990; Sakamoto et al., 1996; Casar et al., 2004) (Fig. 1A, day 3 PI, arrows and arrowheads, respectively). At 5 days PI, several different stages of fiber formation were evident (Fig. 1A, day 5 PI). In this system, fiber regeneration took about 7 days PI (Fig. 1A, day 7 PI). At this time, the diameter of the regenerated fibers (~ 90% of the total fibers) was smaller (~30 μm) than that of fibers from control, undamaged TAs (~50 μm). Furthermore, numerous small mononucleated cells were still present between the newly formed fibers (Fig. 1A, day 7 PI). Myotubes continued maturing and by day 15 PI they were ~50 μm in diameter and the nuclei lateralization occurred at even later stages (not shown). Similar events have been reported to occur following $BaCl_2$-induced injury (Casar et al., 2004) as well as in other models of skeletal muscle regeneration (Hawke and Garry, 2001).

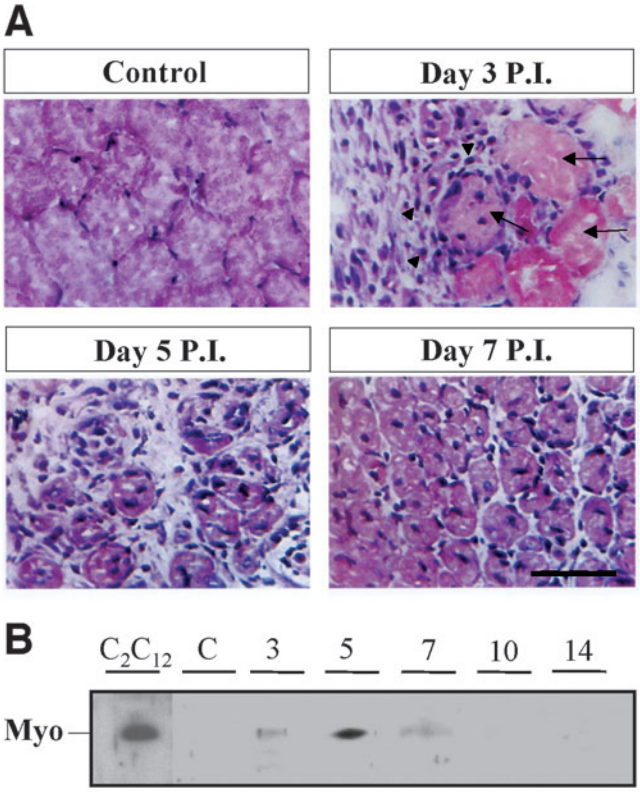

Fig. 1. Time course of $BaCl_2$-induced regeneration of tibialis anterior muscle (TA). TAs from C57BL/6 mice under control conditions or after 3, 5 and 7 days of $BaCl_2$-induced injury were dissected, counterstained and examined histologically (A) or analyzed for myogenin (Myo) levels (B). (A) The Hematoxylin and Eosin stained sections show that in the control most stained nuclei were located in the cell periphery. At 3 and 5 days post $BaCl_2$ injection (PI), fibers probably undergoing necrosis (arrows) and small mononucleated cells (<15 μm; arrowheads) were abundant in the muscle core. At 7 days PI, nearly 90% of the muscle section area was occupied by centrally nucleated myotubes. Scale bar: 60 μm. (B) Myogenin (Myo) was measured by western blot analysis in 100 μg protein aliquots of homogenates from control TA (C) and from TA at different days PI (3, 5, 7, 10 or 14). Differentiated C_2C_{12} myoblasts were used as positive control.

Myogenin is expressed during regeneration and is downregulated when full regeneration is achieved (Launay et al., 2001). During $BaCl_2$-induced TA regeneration, myogenin expression was almost undetectable at days 3 and 7 PI, however at 5 days PI expression levels were high (n=3) (Fig. 1B). Therefore, both the downregulation of myogenin expression and the morphological changes described above suggest that after degeneration following treatment with $BaCl_2$, fibers were regenerated at 7 days PI.

The expression of three connexins (Cx45, Cx43 and Cx40) was evaluated in control and regenerating TAs. When tested by immunoblots, Cx40 was not detected in control or $BaCl_2$-treated TAs (not shown). In contrast, Cx45 and Cx43 were found in both control and regenerating TAs. While the highest levels of Cx45 were detected 3 days PI, maximal Cx43 expression levels were present between 5 and 7 days PI. Thereafter, levels of both connexins decreased towards control values (Fig. 2). Densitometric analyses of Cx43 (the Cx43 value divided by the corresponding α-tubulin value and normalized with respect to control) revealed its up-regulation after 3, 5, 7 and 10 days PI [1.64±0.10; 3.22±1.20; 5.57±1.20 and 2.26±1.00 (mean±s.d.; n=3) increase from control level at day 3, 5, 7 and 10, respectively].

Heterozygous Cx45 (*Cx45*$^{+/-}$) (Krüger et al., 2000) and

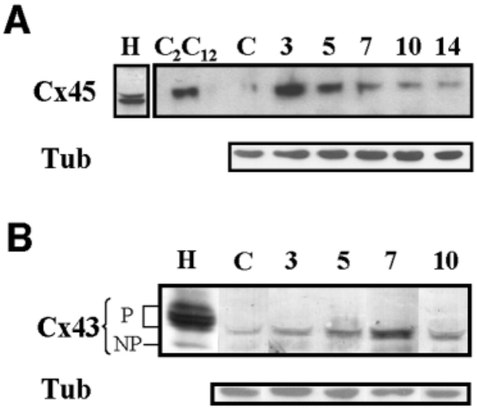

Fig. 2. Cx45 and Cx43 are expressed in tibialis anterior muscles (TA) and up-regulated during skeletal muscle regeneration. (A) Western blot analysis of Cx45 present in homogenate samples (100 μg protein) of TAs at different days post $BaCl_2$ injection (PI) (3, 5, 7, 10 and 14) and in HeLa Cx45 transfectants (H) and control (C) muscles. The different patterns of reactive bands in HeLa and C_2C_{12} cells are due to the differential posttranscriptional modifications of Cx45, which will be described elsewhere. (B) Western blot analysis of Cx43 in aliquots of heart homogenates (H, used as positive control) and of TAs (100 μg) under control conditions and at days 3, 5, 7 or 10 post $BaCl_2$ injection (PI). The non-phosphorylated (NP) and phosphorylated (P) forms of Cx43 are indicated. Tub: α-tubulin levels measured in stripped blots.

Cx43 (*Cx43*$^{del/+}$) (Theis et al., 2001) mice were then used for further experiments (see Materials and Methods). *Cx45*$^{+/-}$ and *Cx43*$^{del/+}$ adult mice expressed cytoplasmic or nuclear β-galactosidase, respectively, under the transcriptional control of the corresponding promoter. In cross sections of TAs from these mice, expression of the corresponding connexin gene, as revealed by β-galactosidase expression, was evident before and after $BaCl_2$ injection (Fig. 3A and Fig. 4A). In all the analyzed TA sections obtained from wild-type animals before and after $BaCl_2$ induced injury no X-gal staining was observed (not shown). To identify the cell types that expressed Cx45 and Cx43 in normal and regenerating skeletal muscle, co-localization of cell-type-specific markers and X-gal staining was studied. We focused our investigation on time periods during which the expression of Cx43 and Cx45 or the presence of a particular cell type was most abundant.

In cross sections of normal (control) and regenerating TAs [after 3, 5 (not shown) and 7 days PI] obtained from *Cx45*$^{+/-}$ mice, X-gal staining was present (Fig. 3A). Numerous cells showed X-gal staining at day 3 PI (Fig. 3A, empty arrowheads). At 5 days PI numerous myofibers were X-gal positive (not shown), and at 7 days PI, X-gal staining was found between fibers (Fig. 3A, empty arrowheads), and within the myofibers (Fig. 3A, arrows).

In control TAs Cx45 was possibly expressed in endothelial cells, as marked by von Willebrand factor (VWF) staining of microvessels (Fig. 3B, upper panels). Further, in large arterioles several X-gal-stained cells were observed without an apparent co-localization with VWF staining (Fig. 3B, middle panels). In contrast, at 3 and 5 days PI, Cx45 did co-localize with VWF staining (Fig. 3B, bottom panels), as well as with CD14 (a macrophage marker) (Fig. 3C), the myogenic marker desmin (not shown) and myogenin (only detected at day 5 PI) (Fig. 3D) in numerous small mononucleated cells. Moreover, at 7 days PI Cx45 co-localized with VWF positive cells (not shown).

In normal TAs from *Cx43*$^{del/+}$ mice, X-gal-stained nuclei were observed in cells closely attached to myofibers (Fig. 4Ab, empty arrowhead), in the myofiber (Fig. 4Ab.1, arrows), and X-gal staining co-localized with the endothelial cell marker VWF (Fig. 4B, upper pair panels) and the satellite cell marker M-cadherin (Fig. 4Ab, inset). At 3 days PI, Cx43 expression was observed in several cells (Fig. 4Ac,d). At this time, Cx43 co-localized with the myogenic marker desmin (not shown), VWF (Fig. 4B, second pair of panels) and CD14 (a macrophage marker) (Fig. 4B, third pair of panels) in numerous small mononucleated cells. At 5 days PI, Cx43 X-gal staining co-localized with myogenin (Fig. 4B, bottom panels), CD14, desmin and VWF immunoreactivity (not shown). Moreover, numerous myofibers were also X-gal positive at 5 days PI (not shown). At 7 days PI, cells found between fibers (Fig. 4Af, empty arrowheads), newly formed myofibers (Fig. 4Af, arrows) and VWF-positive cells (not shown) were X-gal positive.

Further efforts to quantify the number of each cell type exhibiting co-localization of X-gal staining with a corresponding cell marker were not performed because the X-gal staining interfered with the weak fluorescence of the immunostaining present in the same cell compartment and thus resulted in an underestimation of co-localization. Interestingly, TAs from *Cx45*$^{+/-}$ and *Cx43*$^{del/+}$ mice showed full fiber regeneration at 7 days PI (Fig. 3A and Fig. 4Ae,f), suggesting that a single copy of either Cx45 or Cx43 suffices for normal regeneration.

Primary cultures of myoblasts from newborn mice express Cx43 and Cx45

Cultures enriched in satellite cell-derived myoblasts were prepared from wild-type and transgenic newborn mice. In wild-type cells following 24 (not shown) and 48 hours of differentiation, Cx43 immunoreactivity was diffusely distributed in the cytoplasm and was localized to bright puncta at cell-cell appositions (Fig. 5A). Relative levels and patterns of

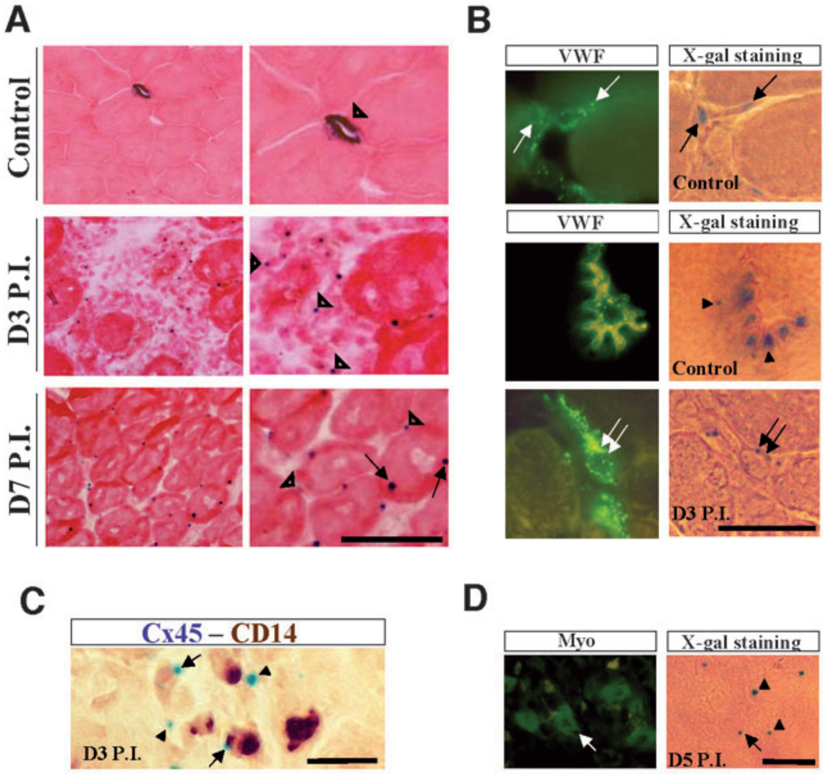

Fig. 3. Cx45 gene expression in regenerating tibialis anterior muscles (TA). (A) Eosin stained TA cross sections of control *Cx45*$^{+/-}$ mice showed X-gal reactivity in microvessels (empty arrowhead). At day 3, numerous cells showed cytoplasmic X-gal positive dots (open arrowheads). At 7 days PI X-gal positive dots were found within (arrows) and between (open arrowheads) fibers. (B-D) Co-localization of X-gal staining with specific cell markers in regenerating TAs from *Cx45*$^{+/-}$ mice. Arrows indicate sites of co-localization; both arrow and arrowheads indicate positive X-gal staining. These panels show co-localization of von Willebrand factor (VWF) with X-gal staining under control conditions and at D3 PI (B), with CD14 at D3 PI (C) and with myogenin (Myo) at D5 PI (D). Scale bars: In A, 100 μm (left column) and 50 μm (right column); in B, 45 μm (upper panels), 30 μm (middle panels) and 50 μm (bottom panels); (C,D) 20 μm.

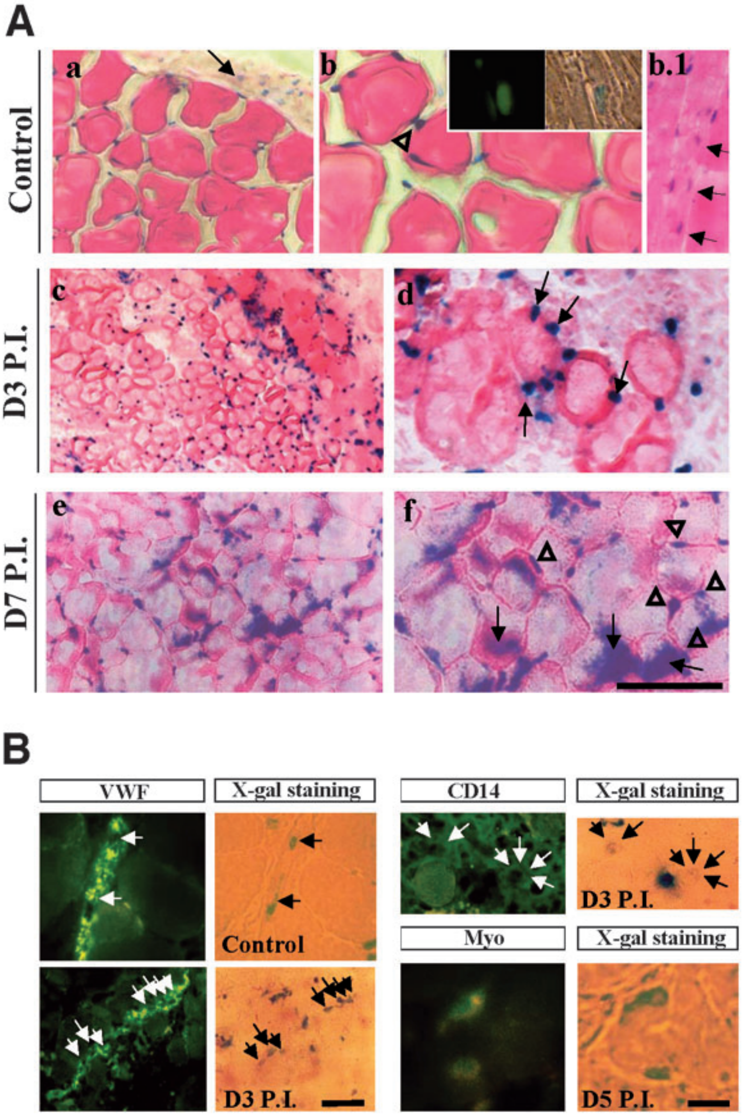

Fig. 4. Cx43 gene expression in regenerating TA. (A) Cross sections of TAs from $Cx43^{del/+}$ mice under control conditions and at different days PI (3 or 7 PI) show X-gal-stained cells. (a,b) Under control conditions, cells of the epymisum (a, arrow), myofibers (b.1, arrows) and cells closely associated with myofibers (b, open arrowhead) were X-gal positive. Inset in b shows co-localization of M-cadherin (fluorescence, left panel) and X-gal staining (phase contrast, right panel). (c,d) At 3 days PI (D3 PI) cells adhered (d, arrows) or close to necrotic fibers showed X-gal stained nucleus. (e,f) At day 7 (D7 PI), numerous myofibers (f, arrows) and cells located between myofibers (f, open arrowheads) showed X-gal reactivity. Scale bar: 160 µm (a); 85 µm (b); 100 µm (b.1); 270 µm (c); 70 µm (d); 90 µm (e); 50 µm (f and inset in b). (B) Co-localization of specific cell markers and X-gal staining in normal and regenerating TA of $Cx43^{del/+}$ mice. (Left panels) Co-localization of von Willebrand factor (VWF) in control and at 3 days PI (D3 PI); (top right panels) CD14 reactive cells with X-gal staining at D3 PI; (bottom right panels) co-localization of myogenin (Myo) reactive cells with X-gal staining at 5 days PI (D5 PI). Arrows indicate sites of co-localization. Scale bar in left pair of panels: 50 µm (top), 60 µm (bottom). Scale bar in right pair of panels: 30 µm (top), 20 µm (bottom).

phosphorylation of Cx43 determined by western blot analyses were similar at all times (0, 24 and 48 hours) tested during myoblasts differentiation (Fig. 5B). Furthermore, the incidence of dye coupling studied with Lucifer yellow (LY) was ~80% (n=45) at 24 and 48 hours of differentiation and the coupling index (see Materials and Methods) was 4.6±2.2 and 5.5±3.8 at 24 and 48 hours, respectively (not shown). In primary cultures of myoblasts from $Cx43^{del/+}$ and $Cx45^{+/–}$ mice, expression of Cx43 and Cx45 was also demonstrated by X-gal staining at 24 and 48 hours of differentiation (not shown).

Induced deletion of Cx43 coding DNA inhibits myogenesis in primary cultures of satellite cell-derived myoblasts

To understand the role of Cx43 in the absence of putative factors that might be present in vivo, we studied myogenesis in satellite cell-derived myoblasts obtained from $Cx43^{Cre\text{-}ER(T)/fl}$ mice. For a full description of the inducible Cre-mediated Cx43 deletion system see Eckardt et al. (Eckardt et al., 2004). In these cells, the absence of Cx43 expression was demonstrated by western blot analyses (Fig. 6A), X-gal staining (not shown) and immunofluorescence (n=3) (Fig. 6B). Moreover, gap junctional communication determined by the presence of dye coupling was strongly inhibited in cultured cells obtained from $Cx43^{CreER(T)/fl}$ mice following 4-OH-tamoxifen (4OHT)-treatment (15%; n=2 experiments) when compared to cultured 4OHT-treated cells from $Cx43^{fl/fl}$ mice (85%, n=2 experiments) that served as controls (Fig. 6C). Furthermore, the expression of myogenin and MyoD was drastically reduced in 4OHT-treated $Cx43^{Cre\text{-}ER(T)/fl}$ myoblasts (n=3) (Fig. 6A). Levels of α-tubulin, a protein unrelated to Cx43, were similar in both 4OHT-treated $Cx43^{fl/fl}$ and 4OHT-treated $Cx43^{Cre\text{-}ER(T)/fl}$ myoblasts, indicating that the induced Cre-mediated deletion of Cx43 was specific (n=3) (Fig. 6A). In addition, 4OHT-treated myoblasts of $Cx43^{Cre\text{-}ER(T)/fl}$ mice also showed a strong reduction in cell fusion (Fig. 6D). Accordingly, at day 4 of differentiation, the activity of creatine kinase (CK), a marker of differentiation, whose activity is proportional to its levels of expression (Chamberlain et al., 1985), was significantly reduced in 4OHT-treated myoblasts of $Cx43^{Cre\text{-}ER(T)/fl}$ mice as compared to 4OHT-treated cells of $Cx43^{fl/fl}$ mice (Fig. 6E).

In addition, the expression of myogenin was evaluated in primary cultures of myoblasts obtained from general Cx43-deficient mouse embryos (E18-20). In $Cx43^{del/del}$ myoblasts, Cx43 was not detected after 24 hours of differentiation either by immunofluorescence or western blot analyses (not shown). Protein levels of myogenin in cells of Cx43-deficient mice were reduced to about 50% as compared to that observed in $Cx43^{del/+}$ and wild-type mice (not shown).

Induced deletion of Cx43 expression in adult mice delays skeletal muscle regeneration

Is Cx43 playing any physiological role during adult skeletal muscle regeneration? To address this key question, the role of Cx43 during regeneration of adult skeletal muscles was studied

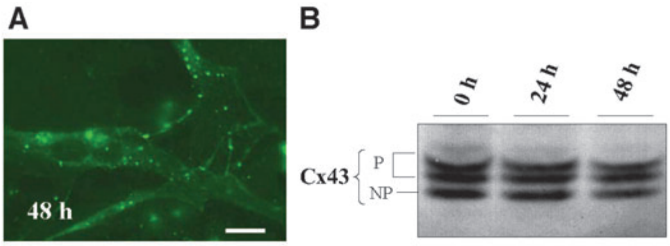

Fig. 5. Primary cultures of myoblasts express Cx43. (A) Primary cultures of satellite cell-derived myoblasts maintained for 48 hours in differentiation medium were fixed with ethanol and Cx43 was detected by immunofluorescence. Scale bar: 20 µm. (B) Western blot analysis of Cx43 in aliquots (100 µg of protein) of myoblast homogenates obtained at 0, 24 and 48 hours of differentiation. The non-phosphorylated (NP) and phosphorylated (P) forms of Cx43 are indicated.

in TAs from *Cx43*$^{fl/fl}$, *Mx-cre* mice, in which the interferon-induced Mx-cre system (Kühn et al., 1995) was used for inducible deletion of the floxed Cx43. An inducible deletion was necessary as Cx43-deficient mice die soon after birth (Reaume et al., 1995). In *Cx43*$^{fl/fl}$, *Mx-cre* animals, deletion of the Cx43 coding region was induced with pI-pC (see Materials and Methods). Animals treated with pI-pC, to induce the generation of interferon, will be referred to hereafter as pI-pC-treated mice.

To verify Cx43 deletion in *Cx43*$^{fl/fl}$, *Mx-cre* but not in *Cx43*$^{fl/fl}$ mice, these animals were both treated with pI-pC and Cx43 levels were compared in TAs by immunoblotting at different times after BaCl$_2$ injection. A transient up-regulation of Cx43 was observed in TAs obtained from *Cx43*$^{fl/fl}$ mice (Fig. 7A). Only in TAs from *Cx43*$^{fl/fl}$, *Mx-cre* mice was Cx43 drastically reduced at all times examined (Fig. 7A).

In order to study the functional roles of Cx43 in skeletal muscle regeneration, cross sections of TAs from both transgenic mice (*Cx43*$^{fl/fl}$ and *Cx43*$^{fl/fl}$, *Mx-cre*) were counterstained with Hematoxylin and Eosin. Complete fiber regeneration was evident at 7 days PI in TAs from pI-pC-treated *Cx43*$^{fl/fl}$ mice (Fig. 7B, upper left panel), and untreated *Cx43*$^{fl/fl}$ (not shown) and *Cx43*$^{fl/fl}$, *Mx-cre* mice (not shown). Nevertheless, regeneration was drastically delayed in TAs from pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice (Fig. 7B, D7-D14 PI). At 7 days PI, TAs from mice with the induced Cx43 deletion still showed extensive damaged areas. Complete regeneration was evident only at 14 days PI. Interferon-induced Cre-mediated deletion of Cx43, identified by X-gal staining, was observed in myogenin-, desmin-, CD14- and VWF-immunoreactive cells in TAs from *Cx43*$^{fl/fl}$, *Mx-cre*, (Fig. 7D) but not of pI-pC-treated *Cx43*$^{fl/fl}$ mice (Fig. 7C). Therefore, specific deletion of Cx43 was only observed in cells of *Cx43*$^{fl/fl}$, *Mx-cre* animals treated with pI-pC (Fig. 7C).

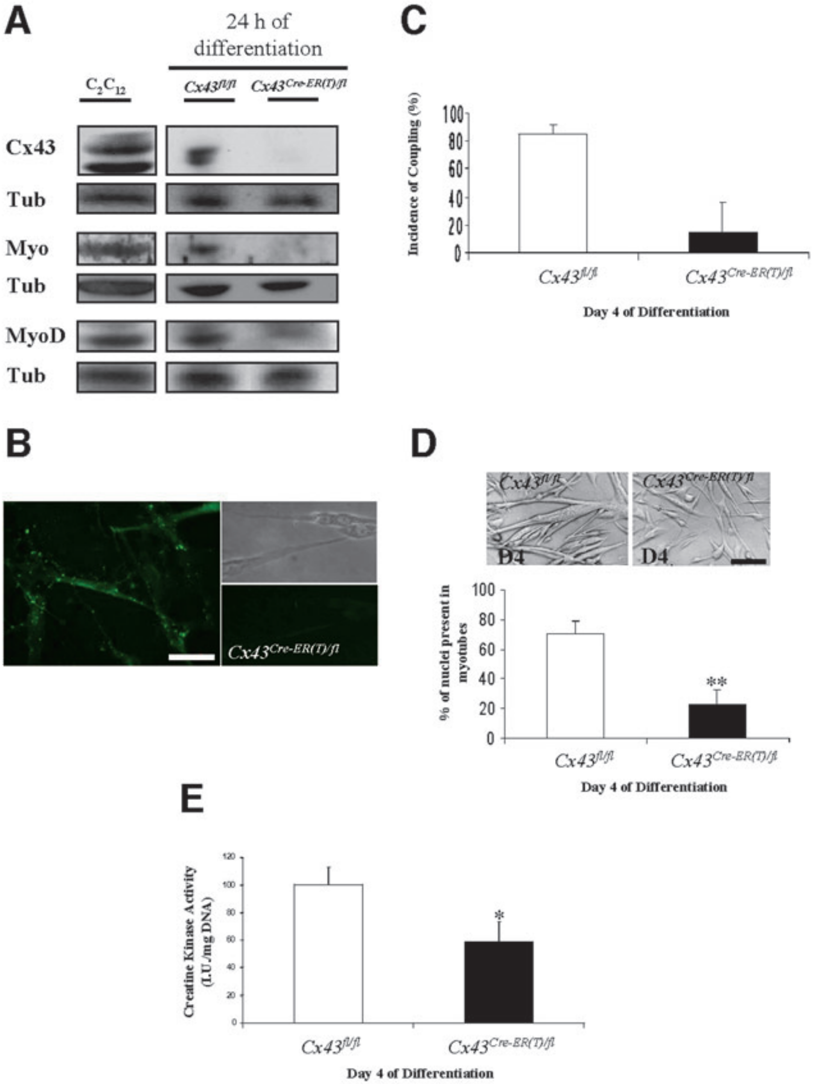

Fig. 6. Induced ablation of Cx43 expression inhibits myogenesis in primary cultures of myoblasts. (A) Western blot analysis of Cx43, myogenin (Myo), MyoD and α-tubulin (Tub) in homogenates (100 µg) of *Cx43*$^{fl/fl}$ or *Cx43*$^{Cre-ER(T)/fl}$ satellite cell-derived myoblasts treated with 4-OH-tamoxifen every 24 hours for 5 days followed by 24 hours of differentiation. (B) At 24 hours of differentiation, positive Cx43 immunolabeling was detected in control *Cx43*$^{fl/fl}$ myoblasts (left) but not in 4-OH-tamoxifen-treated cells of *Cx43*$^{Cre-ER(T)/fl}$ mice (right; upper panel shows phase contrast view). Scale bar: 40 µm. (C) Bar chart of the incidence of coupling (%) of 4-OH-tamoxifen-treated myoblasts of *Cx43*$^{Cre-ER(T)/fl}$ or *Cx43*$^{fl/fl}$ mice after 24 hours of differentiation. (D) Bar chart showing the percentage of the average number of nuclei present in myotubes at day 4 of differentiation in 4-OH-tamoxifen-treated myoblasts of *Cx43*$^{Cre-ER(T)/fl}$ mice as compared to 4-OH-tamoxifen-treated myoblasts of *Cx43*$^{fl/fl}$ mice (**$P<0.01$ vertical bars indicate the standard deviation (s.d.), $n=4$ independent experiments). Upper images are phase-contrast views at day 4 of differentiation (D4) of 4-OH-tamoxifen-treated *Cx43*$^{fl/fl}$ or *Cx43*$^{Cre-ER(T)/fl}$ myoblasts. Scale bar: 40 µm. (E) Bar chart showing the relative creatine kinase activity of cultured myoblasts at day 4 of differentiation. 4-OH-tamoxifen-treated cells were from *Cx43*$^{Cre-ER(T)/fl}$ or *Cx43*$^{fl/fl}$ mice. (*$P<0.05$ vertical bars indicate s.d., $n=3$).

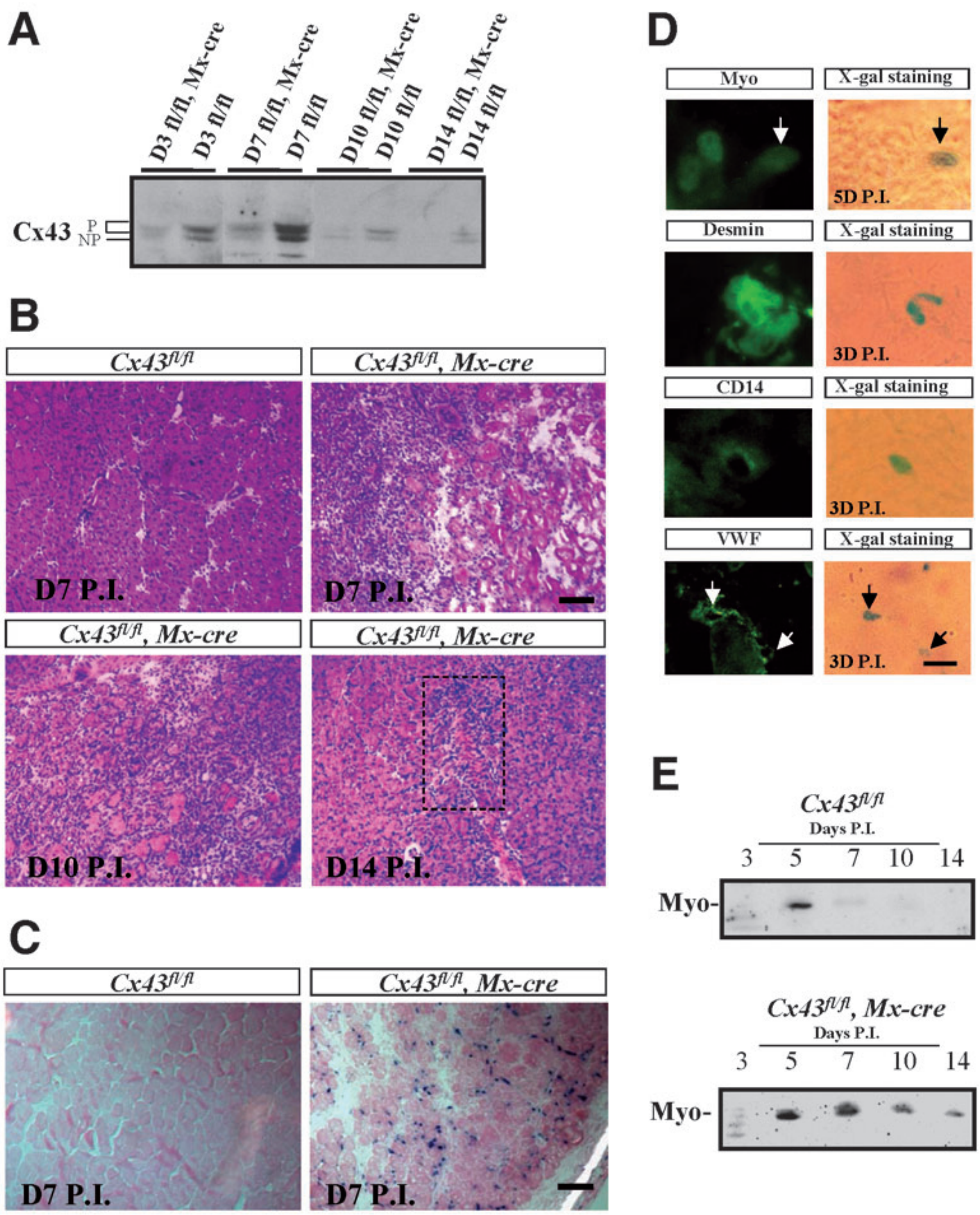

Fig. 7. Cell-specific deletion of Cx43 delays TA regeneration. (A) Levels of Cx43 were measured by western blot analysis in TA homogenates (100 µg of protein) obtained at day (D) 3, 7, 10 or 14 of regeneration after BaCl$_2$ treatment. Muscles were from pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice (fl/fl, Mx-cre) and pI-pC-treated *Cx43*$^{fl/fl}$ mice (fl/fl). The non-phosphorylated (NP) and phosphorylated (P) forms of Cx43 are indicated. (B) Hematoxylin and Eosin-stained cross sections of TAs from pI-pC-treated *Cx43*$^{fl/fl}$ and pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice after 7, 10 or 14 days post BaCl$_2$ administration (D7 PI, D10 PI, or D14 PI, respectively). A small group of damaged cells is present in the 14 days PI (D14 PI) sample, (indicated by a dashed rectangle). Scale bar: 100 µm. (C) At 7 days PI (D7 PI), X-gal stained nuclei were observed in sections of TAs from pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice but not in TAs from pI-pC-treated *Cx43*$^{fl/fl}$ mice. Scale bar: 100 µm. (D) Co-localization of specific cell markers and X-gal staining in regenerating TAs from pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice. These panels showed co-localization of X-gal staining with myogenin (Myo) at D5 PI and desmin, CD14 or von Willebrand factor (VWF) at D3 PI. Arrows indicate co-localization sites. Scale bar: 12 µm for Myo, Desmin and CD14 panels and 20 µm for VWF panel. (E) Relative levels of myogenin (Myo) were measured by western blot analysis in samples (100 µg of protein) of TA homogeneates obtained from pI-pC-treated *Cx43*$^{fl/fl}$ or pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice at different days (3, 5, 7, 10, and 14) PI.

To study whether the absence of Cx43 affects gene expression during regeneration, levels of myogenin were measured in regenerating TAs from pI-pC-treated *Cx43*$^{fl/fl}$ and pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice. In TAs from pI-pC-treated *Cx43*$^{fl/fl}$ mice, myogenin was detected mainly at 5 days PI (Fig. 7E) similar to wild-type mice (Fig. 1B). However, myogenin was detected between 5 and 14 days PI in TAs from pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice, being almost undetectable a day 3 PI (n=3) (Fig. 7E). Thus, Cx43 is required for TA regeneration to follow the normal time course. In BaCl$_2$-injected TAs from *Cx43*$^{fl/fl}$, *Mx-cre* animals (not treated with pI-pC), the time course of fiber formation and myogenin expression were the same as that found in TAs from wild-type mice (not shown), indicating that deletion of floxed Cx43 was not induced by basal interferon levels or by a possible increase in interferon levels caused by BaCl$_2$-induced muscle damage. Although regeneration was delayed in TA sections from pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice, at 3 and 5 days PI the number of CD14-positive macrophages present in TA cross sections from wild-type mice was similar to that found in TA sections from pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice [at 3 days PI: 16.0±3.5 vs. 15.0±4.9, respectively (P>0.05); at 5 days PI: 7.0±2.8 vs. 7.0±3.1, respectively (P>0.05); numbers are the mean CD14-positive cells/250 µm^2]. We did not evaluate later times, when there may be a difference. In contrast to what was observed after Cx43 deletion, BaCl$_2$-treated TAs from Cx40$^{-/-}$ mice showed a normal time course of regeneration when analyzed histologically and by immunoblotting of myogenin (not shown).

We tested whether the lack of Cx43 is compensated for by higher expression levels of Cx45 during regeneration of TA. Immunoblots of Cx45 revealed similar level of Cx45 in regenerating TA muscles of *Cx43*$^{fl/fl}$ and in pI-pC-treated *Cx43*$^{fl/fl}$, *Mx-cre* mice after 3 and 5 days PI, but levels of Cx45 stayed high for a longer period of time (not shown), suggesting that the onset and offset of its expression might not be regulated by Cx43 expression but rather by other factors present during regeneration. Hence, a possible compensatory role of Cx45 in the TA of Cx43 inducible-deleted mice can be ruled out.

Discussion

Although a role for gap junctions during myogenesis had been previously suggested (reviewed by Constantin and Cronier, 2000) their functional importance during regeneration of skeletal muscle was hitherto largely unknown. Furthermore, a detailed analysis of the expression of connexin isoforms in different muscle cell types had yet to be carried out. In this study, we identified Cx43 as a key element of skeletal muscle regeneration as well as in vitro differentiation. Moreover, we analyzed the expression of Cx45 and Cx43 in myogenic cells, macrophages and endothelial cells during skeletal muscle regeneration. We propose that Cx43 gap junction channels provide intercellular signaling pathways required for the normal timing of skeletal muscle ontogeny and regeneration. Our results with Cx43 inducible deletion in transgenic mice demonstrate for the first time the importance of Cx43 during skeletal muscle development and regeneration.

Expression of connexins in resting and regenerating TAs

Our data show that Cx45 and Cx43, but not Cx40, are present in normal adult skeletal muscle and that both are transiently up-regulated in muscle cells during regeneration. Up-regulation of at least Cx43 mRNA has recently been described by analyses of the complete transcriptome of cardiotoxin-injured skeletal muscle (Bakay et al., 2002). During muscle development, gap junctions between myofibers, and between myofibers and myoblasts have been identified at the ultrastructural level (Rash and Staehelin, 1974; Kalderon et al., 1977; Duxson et al., 1989). It is also known that gap junction structures and electrical coupling are lost 1 to 2 weeks before all muscle fibers develop the adult pattern of single innervation (Brown et al., 1976; Dennis et al., 1981). However, the expression of connexin genes in cells of adult skeletal muscles has not been reported until now. In normal TAs from adult mice, Cx43 gene expression was observed within myofibers and satellite cells, suggesting that Cx43-containing gap junction channels might mediate intercellular communication between resting adult myofibers and satellite cells under yet undefined conditions.

Our findings of Cx43 gene expression in different cell types of regenerating TA, including macrophages, satellite cells, myoblasts and endothelial cells are consistent with the increase in Cx43 mRNA observed in cardiotoxin-treated skeletal muscle (Bakay et al., 2002). We also found that myoblasts, satellite cells and infiltrated macrophages express Cx45. Although cultured monocytes/macrophages treated with proinflammatory agents are known to express Cx43 (Eugenín et al., 2003), our in vivo observation of Cx45 and Cx43 gene expression by macrophages should be interpreted cautiously, since these cells might have acquired β-galactosidase expressed by other cells from regenerating TAs by phagocytosis. Our results confirm previously reported patterns of connexin expression by endothelial cells of most vascular territories (Little et al., 1995; Yeh et al., 1998; Kumai et al., 2000). In normal and regenerating TAs, Cx45 was not observed in endothelial cells of large vessels, in agreement with previous reports by Krüger and coworkers (Krüger et al., 2000). However, Cx45 expression appears to be localized to endothelial cells of small vessels of normal TAs and to endothelial cells presumably undergoing angiogenesis in $BaCl_2$-treated TAs. Nevertheless, we did not rule out that Cx45 was expressed by pericytes showing close physical interactions with endothelial cells.

In regenerating TAs, desmin and Cx45 or Cx43 were co-localized. During skeletal muscle regeneration, desmin is expressed by descendants of activated satellite cells, called myogenic precursor cells (MPCs) committed to the skeletal muscle lineage (Kaufmann and Foster, 1988; Kaufmann et al., 1991; Allen et al., 1991; Yablonka-Reuveni et al., 1999), differentiating myoblasts and myofibres (Hill et al., 1986). In addition, Cx45 or Cx43 expression was co-localized with myogenin, coded by the master gene for terminal differentiation. Thus, gap junction channels formed in whole or in part by these connexins may fulfill functionally important roles at different stages of skeletal muscle differentiation from commitment to terminal differentiation.

Connexin expression and role of Cx43 in skeletal muscle differentiation

We describe the expression of both Cx45 and Cx43 during differentiation in cultured satellite cell-derived myoblasts. Since gap junctional communication is thought to coordinate numerous cell functions in diverse tissues (Simon and Goodenogh, 1998), it is likely that the reduced cell-cell communication observed in Cx43-deficient myoblasts may have numerous negative consequences similar to those that have been shown to occur during osteogenesis (Lecanda et al., 2000). Accordingly, in cultured cells we found a drastic reduction in MyoD and myogenin expression, as well as a reduction in the activity of creatine kinase and cell fusion. The reduced expression of MyoD suggests that Cx43 may play a functional role in cell commitment to a skeletal muscle lineage. In addition, the decreased expression of myogenin, creatine kinase activity and the decrease in cell fusion found in myoblasts after induced deletion of Cx43, suggests a role of Cx43 in myoblast terminal differentiation. The less pronounced impairment in the differentiation of myoblasts from general Cx43-deficient embryos ($Cx43^{del/del}$) as compared to myoblasts with Cx43-induced deletion might be explained by developmental compensatory mechanisms in the general deletion.

Specific and inducible deletion of Cx43 delays TA regeneration

It has been reported that skeletal muscle regeneration is attenuated in constitutive leukemia inhibitory factor null mice ($LIF^{-/-}$) (Kurek et al., 1997) and greatly delayed in plasminogen-deficient mice ($Plg^{-/-}$) (Suelves et al., 2002), myocyte nuclear factor-deficient mice ($MNF^{-/-}$) (Garry et al., 2000), $FoxK1^{-/-}$ (Hawke et al., 2003), $Pop1^{-/-}$ (Andrée et al., 2002) and $Slug^{-/-}$ (Zhao et al., 2002) mice. Similarly, we report here that specific deletion of Cx43 delays regeneration by >7 days. This effect manifests as decreased myofiber formation and a long lasting reduction in myogenin expression indicating that Cx43 is an essential protein for the normal time course of skeletal muscle regeneration. The timing for the onset of myogenin expression observed in regenerating TAs from mice with induced deletion of Cx43 was normal, but in cultured myoblasts with induced Cx43 deletion (4-OH-tamoxifen treated $Cx43^{Cre\text{-}ER(T)/fl}$) it was delayed, suggesting that multiple mechanisms control myogenin expression in vivo not all of which may be present in vitro. Thus, the in vitro experiments uncovered a role for Cx43 in the normal onset of myogenin expression.

We observed that deletion of floxed Cx43 by interferon-driven Cre expression also occurred in CD14 immunoreactive cells. Macrophages probably play a beneficial role in injured muscle. In support of this, the invasion of macrophages into injured tissue coincides with tissue repair (Hopkinson-Wolley et al., 1994; St Pierre and Tibdall, 1994) where these cells phagocytose tissue debris. Moreover, macrophages induce apoptosis in neutrophils that may attenuate muscle damage (Meszaros et al., 2000), as well as secrete, and respond to, factors that promote tissue repair (Merly et al., 1999; Cantini et al., 2002). We found a similar number of infiltrated macrophages in $BaCl_2$-damaged TAs from wild-type and Cx43-deficient mice, suggesting that the reduced myogenic response observed in Cx43$^-$deficient mice was not due to a reduced number of macrophages/monocytes. Nevertheless, an effect of the known gap junction-dependent secretory activity of monocyte/macrophages (Eugenín et al., 2003) cannot be ruled out. Similarly, the deletion of floxed Cx43 in VWF immunoreactive cells might have affected the endothelial secretion of factors known to induce satellite cell activation during skeletal muscle regeneration (Hawke and Garry, 2001).

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