Ablation of connexin43 in uterine smooth muscle cells of the mouse causes delayed parturition

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Summary

Gap junctions are characteristically increased in the myometrium during term and preterm delivery and are thought to be essential for the development of uterine contractions during labour. Expression of connexin43 (Cx43), the major myometrial gap junction protein, is increased during delivery. We have generated a mouse mutant (*Cx43$^{fl/fl}$:SM-CreER^{T2}*), in which the coding region of *Cx43* can be specifically deleted in smooth muscle cells at any given time point by application of tamoxifen. By this approach, we were able to study long-term effects on myometrial functions that are necessary for parturition as well as gap junction intercellular communication in primary myometrial cell cultures. We found a prolongation of the pregnancy in 82% of tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice as well as decreased dye coupling in cultured primary myocytes of these animals. Other parturition-specific parameters such as the regulation of oxytocin receptor, prostaglandin F receptor or progesterone remained unchanged. Our results indicate the important function of Cx43 during parturition in the living animal and suggest further strategies to investigate the role of connexins in uterine contractility in transgenic mice.

Key words: Gap junction, Cre/loxP, Transgenic mice

Introduction

During pregnancy, the uterus is relatively quiescent until the onset of contractile activity in association with labour. The cascade of events precipitating labour remains unclear but it is proposed that the myometrium becomes primed to contract before the initiation of labour. This is caused by activation of a series of genes, that encode a number of contraction-associated proteins (Challis and Lye, 1994), including the oxytocin receptor, the prostaglandin F receptor and the gap junction protein connexin43 (Cx43) (Imamura et al., 2000; Palliser et al., 2004; Lye, 1994).

Gap junctions are specialized conduits between eukaryotic cells that allow direct intercellular communication via gap-junctional plaques which are aggregates of single intercellular channels (Kumar and Gilula, 1996). Each channel consists of two hemi-channels (termed connexons) one of which is composed of six connexin (Cx) subunit proteins. Generally, gap junction channels allow the passive intercellular diffusion of molecules up to 1000 Da, which can be nutrients, waste products, metabolites, second messengers or ions, thereby facilitating electrical and metabolic communication between coupled cells (Willecke et al., 2002). So far, 20 connexin genes have been described in mouse and 21 in the human genome (Söhl et al., 2004).

In uterine tissue, four different connexins have been described: Cx26, Cx40, Cx43, and Cx45 that are differently regulated during pregnancy. Expression levels of Cx26 are highest during late pregnancy but decrease to low levels before the onset of labour (Orsino et al., 1996). Cx40 was found in human myometrial muscle cells at term (Kilarski et al., 1998; Kilarski et al., 2001) but there is no evidence that its expression is regulated during pregnancy. In humans and other mammals, Cx43 gap junctions are scarce in the myometrium of the non-pregnant uterus but increase in size and abundance with parturition (Chow and Lye, 1994; Orsino et al., 1996, Ou et al., 1997; Kilarski et al., 1998; Kilarski et al., 2001). By contrast, Cx45 channels are present in the non-pregnant and early pregnant myometrium but are decreased before term (Albrecht et al., 1996). While this may imply that gap junction formation is sufficient to ensure labour and delivery of the fetus, there have been no reports providing definitive documentation that labour and delivery may occur in the absence of myometrial gap junctions.

The purpose of this study was to determine whether Cx43-containing gap junctions are required to coordinate synchronous contractions at the end of pregnancy, thereby allowing for an increase in myometrial cell coupling. Since *Cx43*-deficient mice die shortly after birth (Reaume et al., 1995), it was necessary to circumvent this postnatal lethality. In order to investigate the role of Cx43 specifically in smooth

muscle cells (SMC), we crossed a mouse line that carries a 'floxed' *Cx43*-coding region, i.e. flanked by loxP recognition sites for the Cre recombinase (*Cx43*^{fl}) (Theis et al., 2001; Eckardt et al., 2004) with mice harbouring a tamoxifen-inducible *Cre* transgene under control of the smooth-muscle-cell-specific *SM22α* promoter (*SM-CreER*^{T2}) (Kühbandner et al., 2000). Cre-mediated deletion led to a replacement of the *Cx43*-coding region by a *lacZ* reporter gene. Pregnant tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} mice were studied during pregnancy and at term. In order to investigate whether ablation of *Cx43* in SMCs changes pregnancy-related processes, we analysed: (1) dye coupling of cultured SMCs; (2) the time of parturition; (3) expression levels of other contraction-associated protein genes, and the transcription factor Fos; and (4) the progesterone status of mice with SM-CreER^{T2}-mediated deletion and of control littermates.

Results

Smooth muscle cell-specific deletion of *Cx43*

We generated transgenic *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} and *Cx43*$^{2lox/2lox}$*:SM-CreER*^{T2} mice to bypass postnatal lethality of *Cx43*-null animals and to study the role of Cx43 gap junctions during pregnancy and parturition. Since we found no differences between animals harbouring the *Cx43*^{fl} alleles or not carrying the floxed neomycin cassette (*Cx43*^{2lox}), we refer to both groups as *Cx43*^{fl} mice. As shown in Fig. 1A, Cre-mediated deletion of the floxed *Cx43*-coding region resulted in expression of the *NLS-lacZ* reporter gene under control of the *Cx43*-specific promoter (Theis et al., 2001; Eckardt et al., 2004). The Cre recombinase used in this study is a fusion protein of Cre and the tamoxifen-responsive estrogen receptor (ER^{T2}; Fig. 1B) regulated by the smooth-muscle-cell-specific *SM22α* promoter (Kühbandner et al., 2000). Only after application of tamoxifen, the HSP90-bound SM-CreER^{T2} fusion protein changed its conformation, separated from HSP90, translocated to the nucleus and mediated the deletion of the *Cx43*-coding region. To monitor the expression profile of *Cx43* by means of the *lacZ* reporter gene, we used *Cx43*$^{del/+}$ mice (i.e. in which one floxed *Cx43* allele had been deleted).

Upon SM-CreER^{T2}-mediated deletion, lacZ staining was detected in 38±6% (P=0.005) of all Cx43-expressing myometrial cells, probably depending on the quality of tamoxifen induction (Fig. 2B) compared with the virtually completely lacZ-positive uterine tissue of *Cx43*$^{del/+}$ mice (98±2%, P=0.0002; Fig. 2C). Furthermore, *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} animals, that were not treated with tamoxifen never showed expression of the reporter gene (Fig. 2A).

Immunofluorescence analysis corroborated the lacZ findings and revealed a slight reduction of Cx43 immunoreactivity in tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} myometrium in contrast to control tissue (Fig. 2E compared with 2D). Since Cx43 is expressed in myometrial cells other than SMCs, the remaining subset of Cx43-positive cells might represent SM-actin-negative fibroblasts, vessel-associated endothelial cells, or mast cells (Reynolds and Redmer, 1999; Yeh et al., 1997; Oviedo-Orta and Howard, 2004). Still, the majority of Cx43 protein seems to be present in myometrial smooth muscle cells of tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} mice.

Immunostaining (Fig. 3) and western blot analyses (Fig. 4) of uterine tissue revealed no changes in the expression pattern of Cx26 (P=0.42), Cx40 (P=0.85) and Cx45 (P=0.73) upon

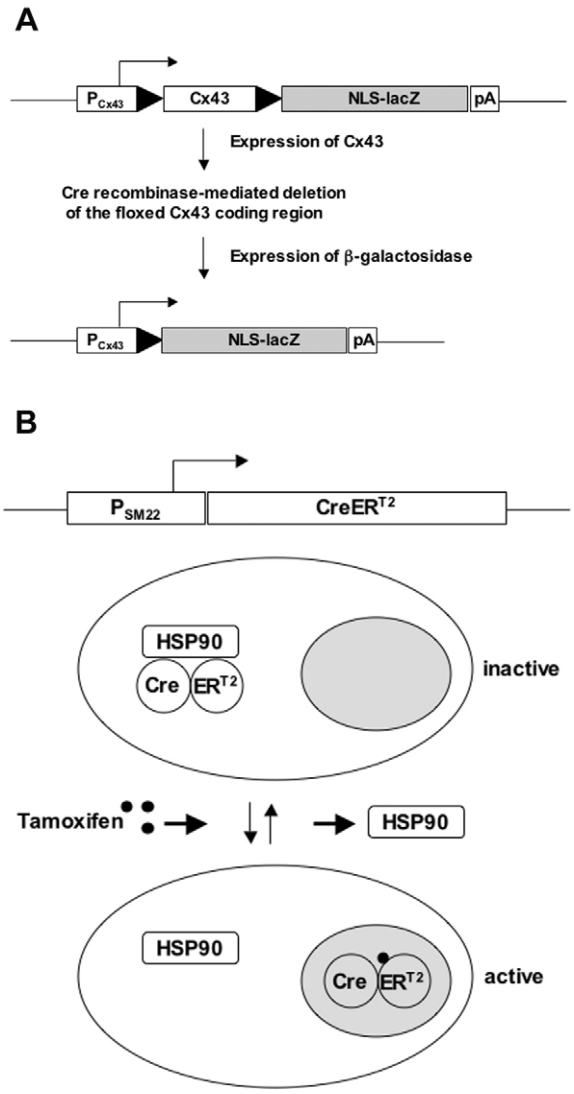

Fig. 1. *LacZ* activation upon conditional *Cx43* deletion. (A) A silent *lacZ* reporter gene (*NLS-lacZ*) was integrated into the floxed *Cx43* allele. After Cre/loxP-mediated rearrangement of the *Cx43*-coding region, indicated by loxP sites (triangles), the *lacZ* gene is activated and the β-galactosidase protein is expressed. (B) The CreER^{T2} fusion protein is specifically transcribed under control of the *SM22α* promoter in smooth muscle cells. In the cytosol, CreER^{T2} is associated with the heat shock protein 90 (HSP 90). After application of the ligand (tamoxifen) this complex dissociates and *CreER*^{T2} is translocated into the nucleus, where deletion of the floxed coding region occurs.

SM-CreER^{T2}-mediated deletion of the *Cx43*-coding region compared with controls in three independent experiments. In contrast to human tissue (Kilarski et al., 1998; Kilarski et al., 2001) only very weak Cx40 immunosignals could be detected in mouse myometrial SMCs, whereas abundant expression was found in blood vessels (Fig. 3B,C).

Loss of Cx43 in primary smooth muscle cells causes reduction of intercellular dye transfer

Thirty intercellular injections of Lucifer Yellow were

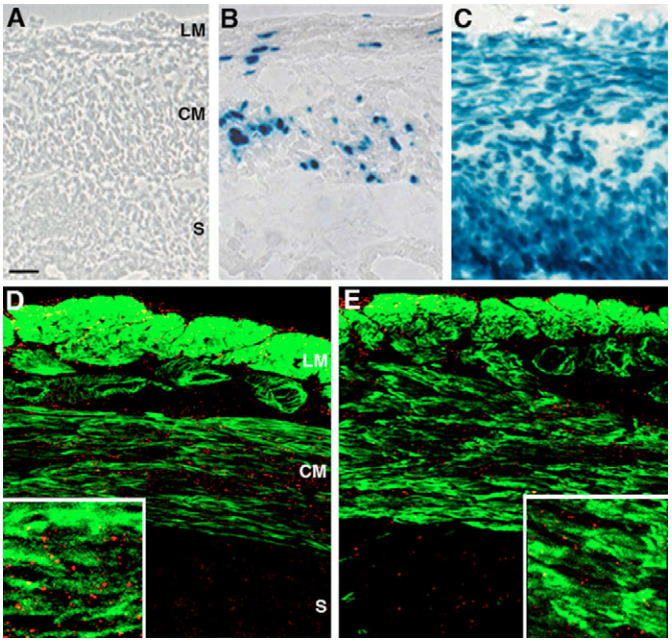

Fig. 2. LacZ staining and immunofluorescence analyses of the uterus of different genotypes. (A-C) LacZ staining of untreated (A), tamoxifen-treated (B) *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} and *Cx43*$^{del/+}$ (C) uterine tissue was performed according to standard protocols. (D,E) Cryosections of untreated (D), tamoxifen-treated (E) *Cx43*$^{fl/fl}$*: SM-CreER*^{T2} uterus were analysed by immunofluorescence using antibodies to Cx43 (red) and smooth muscle actin (green). Inserts represent magnified images. CM, circular muscle layer; LM, longitudinal muscle layer; S, stroma. Bar, 50 μm.

performed in primary cultures of untreated and tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} myocytes. Coupling of control cultures varied from 0 to 4 cells with a mean of 2.07 cells. Dye transfer in Cx43-ablated SMCs was markedly decreased to a mean of 0.5 cells (coupling of 0-2 cells; P=0.03). Representative examples of microinjection and coupling are shown in Fig. 5A-D. Only tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} myocytes that still express low levels of Cx43 protein allowed the transfer of Lucifer Yellow to a neighboring cell (Fig. 5E) whereas non-expressors never showed dye coupling (Fig. 5F).

A decrease by 65±3% of Cx43 protein in cultured primary SMCs of tamoxifen-treated compared with untreated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} mice was found by western blot analyses in three independent experiments (P=0.04). Immunofluorescence analyses of cultured SMCs corroborated these findings (Fig. 6A,B). By contrast, immunoblot analyses using Cx26 (P=0.84), Cx40 (P=1.00) and Cx45 (P=0.93) antibodies revealed no changes in the protein amount of the corresponding connexins (Fig. 6C).

The ablation of Cx43 in myometrial SMCs impairs parturition

To assess the time of delivery in pregnant untreated and tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} mice, delivery of the first pup was monitored. As shown in Fig. 7A, 89% (16 out of 18) of untreated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} females delivered between 4 and 8 a.m. on day 19.5. Only 11% (2 out of 18) gave

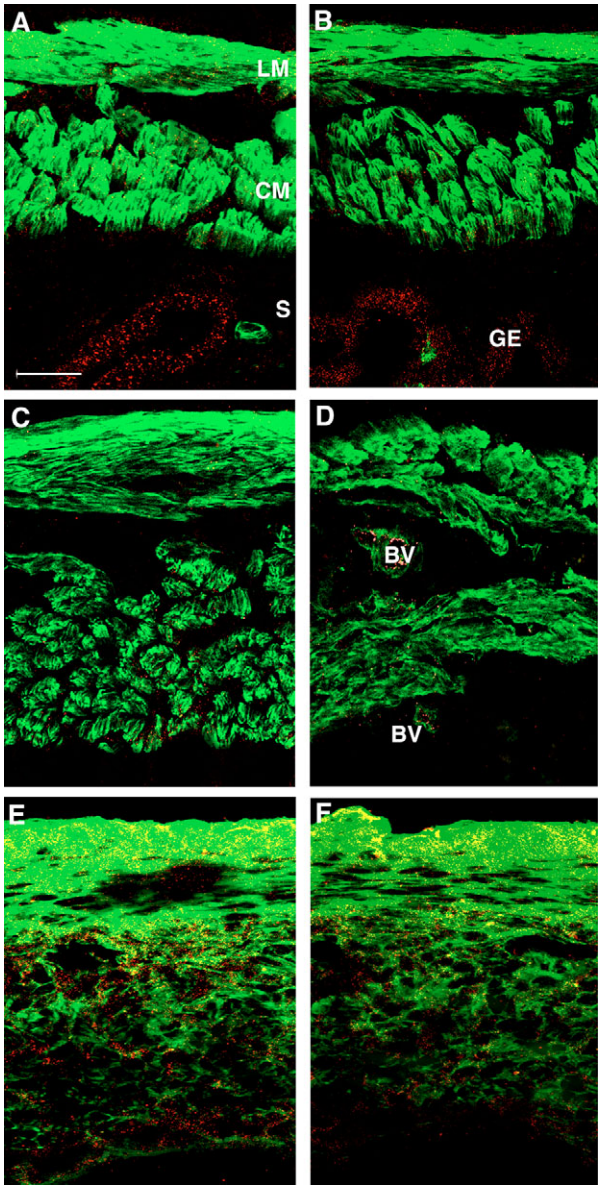

Fig. 3. Immunofluorescence analyses of untreated (A,C,E) and tamoxifen-treated (B,D,F) *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} uterus. (A,B) Cryosections of uterine tissue were analysed using antibodies to Cx26 (red) and smooth muscle actin (green). Most of the signals were detected in the glandular epithelium (GE). (C,D) Immunofluorescence of Cx40 (red) and smooth muscle actin (green) in uterine cryosections revealed only sparse signals in myometrial SMCs but abundant staining of blood vessels (BV). (E,F) Staining of uterine tissue using antibodies to Cx45 (red) and smooth muscle actin (green) revealed extensive expression in myometrial smooth muscle cells. The colocalization of connexin and smooth muscle actin signals is visualized in yellow. BV, Blood vessel; CM, circular muscle layer; GE, glandular epithelium; LM, longitudinal muscle layer; S, stroma. Bar, 100 μm.

birth between 8 and 12 a.m. By contrast, 82% (14 out of 17) pregnant tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} mice delivered after 8 a.m. Six of these late-delivering animals gave birth to the first, in most cases dead and already partially

degraded pup on day 20 to 22 (Fig. 7B). Normal parturition was observed in 18% (3 out of 17) of tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice. Pregnant homozygously floxed *Cx43^{fl}* females that did not carry the *SM-CreER^{T2}* allele were also examined. Only three out of 16 (19%) tamoxifen-treated and one out of 18 (6%) untreated Cx43$^{fl/fl}$ mice showed a delay in parturition. All *Cx43$^{del/+}$* females (n=6) investigated, that carry only one copy of the *Cx43* gene, gave birth before 8 a.m. Thus, the delay in parturition cannot be attributed to the administration of tamoxifen but to the reduction of Cx43 protein expression in tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice.

The expression levels of selected contraction-associated protein genes, Fos and progesterone are unaffected

Analysis of myometrial RNA by quantitative real-time PCR of day 16 and term pregnant mice revealed no significant changes in expression levels of the contraction-associated protein genes, oxytocin receptor (d16, P=0.98; d19, P=0.09), prostaglandin receptor (d16, P=0.39; d19, P=0.85), and Fos (d16, P=0.14; d19, P=0.87) between tamoxifen-treated and untreated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice (Fig. 8).

In the same mice, the concentration of circulating progesterone was evaluated by radio-immuno analysis. Levels of circulating progesterone were significantly decreased from day 16 to day 19 in untreated and tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice (P=0.0003 and P=0.0004, respectively). Therefore, tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice were indistinguishable from untreated controls with regard to progesterone concentration on day 16 (P=0.13) and 19 of gestation (P=0.93).

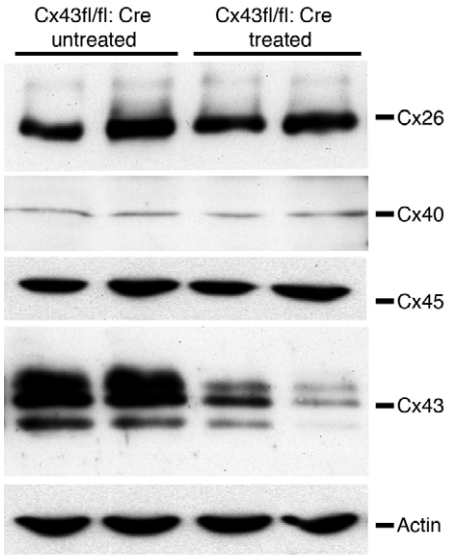

Fig. 4. Immunoblot analyses of uterine tissue homogenates of two untreated and tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice. Membranes were probed with primary antibodies to Cx26, Cx40, Cx45 and Cx43. Normalization to β-actin levels revealed that the amount of Cx43 protein was reduced by 69±5% in tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* animals, whereas the levels of other connexin proteins investigated remained unchanged (n=3).

Discussion

The current notion on the role of gap junctions in the onset of labour during the birth process is limited to the hypothesis that intercellular coupling mediated by gap junction channels might be necessary to coordinate synchronous myometrial contractions during term and preterm birth (Chow and Lye, 1994; Balducci et al., 1993). Here we present the first study to define the in vivo requirement for Cx43-containing gap junctions in the myometrium of pregnant mice and to demonstrate the association between the Cx43 expression and the onset of labour. Moreover, using a conditional deficient mouse we provide evidence that Cx43 plays a major role during parturition.

By lacZ staining, immunofluorescence and western blot analyses we have shown that upon tamoxifen treatment, the deletion of the *Cx43*-coding region takes place in 30-40% of *Cx43$^{fl/fl}$:SM-CreER^{T2}* uterine SMCs causing a loss of Cx43 protein of more than 60% in these cells. This leads to a decrease in intercellular dye coupling and an impairment of

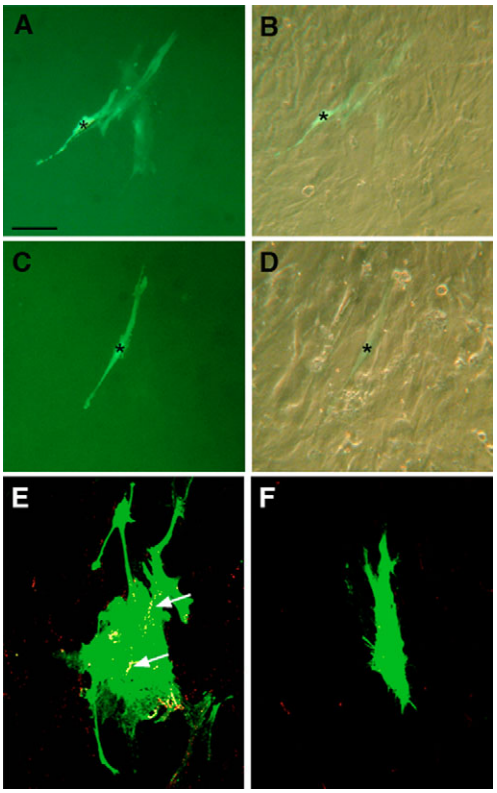

Fig. 5. Representative images of microinjections in confluent cultures of myometrial cells with the gap junction permeable tracer Lucifer Yellow (A,C) and phase-contrast images of the same injection (B,D). Dye transfer shown in cultured untreated (A,B) and tamoxifen-treated (C,D) *Cx43$^{fl/fl}$:SM-CreER^{T2}* SMCs 5 minutes after Lucifer Yellow microinjection into a single cell (asterisks). The restricted transfer in tamoxifen-treated cells contrasts to the basal transfer in untreated cells. (E,F) Microinjected cells (green) of tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice were fixed and stained using antibodies to Cx43 (red). Dye transfer was only present in Cx43-expressing cells (white arrows in E) but absent in non-expressors (F). The overlay of Cx43 and Lucifer Yellow dye is visualized in yellow. Bar, 50 μm (A-D); 20 μm (E,F).

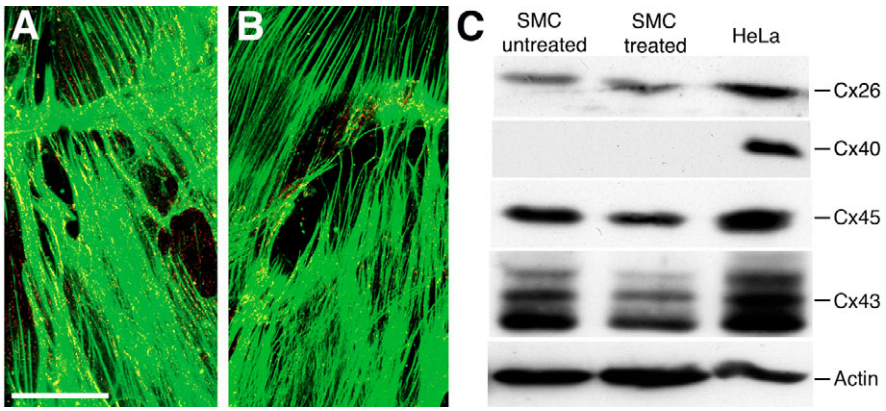

Fig. 6. (A,B) Immunofluorescence of Cx43 (red) and smooth muscle actin (green) in cultured myometrial cells from untreated (A) and tamoxifen-treated (B) *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} mice. Decrease in immunoreactive Cx43 in tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} cultured SMCs is shown in contrast to untreated control cells. The colocalization of Cx43 and smooth muscle actin bundles is shown in yellow. (C) Immunoblot analyses of protein extracts (100 μg) of untreated and tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} SMC cultures using antibodies to Cx26, Cx40, Cx45 or Cx43. Normalization to β-actin levels revealed a reduction of Cx43 protein by 65±3%, levels of other connexin proteins investigated remained unchanged. Bar, 50 μm.

parturition in mice with smooth-muscle-cell-specific deletion of *Cx43*.

Our data indicate that extensive, although not complete, loss of the native levels of Cx43 is detrimental to proper function of SMCs in vitro and in vivo, particularly with regard to their main physiological functions: to mediate intercellular coupling and coordinate uterine contractions. Similar results were found by other groups, where a knockdown and therefore partial ablation of connexin proteins is sufficient to cause alterations of physiological functions like cardiac conduction velocity and rhythmogenesis, insulin production or wound healing (van Rijen et al., 2004; Le Gurun et al., 2003; Kretz et al., 2004). Since a complete deletion of the *Cx43*-coding DNA in tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} mice can be achieved in SMCs of the gastrointestinal system (B.D., unpublished observations), it is likely that the *Cx43* gene locus of myometrial SMCs is less accessible than in other visceral organs, possibly owing to the tight hormonal regulation of connexin expression in the uterus. Furthermore, the inducible Cre activity is dependent on the level of expression (Feil et al., 1996; Zhang et al., 1998) and the absence of recombination in subpopulations of Cre-expressing cells (Schwenk et al., 1998; Zhang et al., 1998). Alternatively, insufficient accessibility of the inducer to the tissue has been discussed to explain incomplete Cre-mediated deletion (Seibler et al., 2003; Guo et al., 2002).

Possible side effects of the *Cx43* deletion in SMCs other than myometrial, e.g. vascular smooth muscle, can largely be excluded because no ablation of the *Cx43*-coding region was detected in SMCs of uterine vessels (data not shown) which is probably due to a limited expression of the SM-CreER^{T2} recombinase in smaller vessels (S.F. and R.F., unpublished observations). The mild phenotypic abnormalities found in the gut of these animals are unlikely to influence parturition and will be reported elsewhere. Therefore, the delay in delivery is presumably not attributable to abnormalities in other muscular tissues.

Oxytocin (OT) and prostaglandin are uterotonic agents. During late gestation, OT receptors (OTR) and prostaglandin F receptors (FP) are significantly induced in the myometrium in many mammalian species (Challis and Lye, 1994; Zingg et al., 1995; Imamura et al., 2000; Al-Matubsi et al., 2001; Arosh

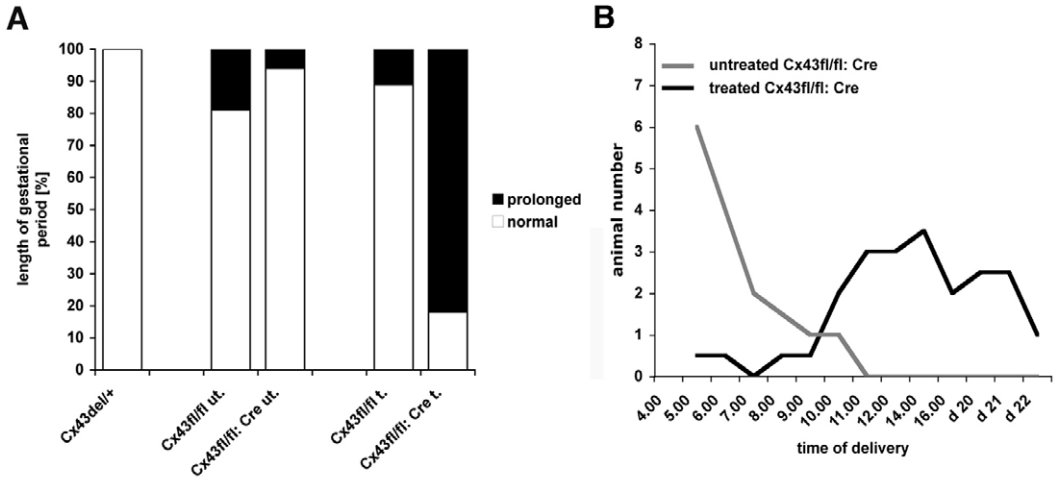

Fig. 7. Effect of *Cx43* deletion on the length of gestation. Pregnant mice were monitored for delivery of the first pup. Delivery until 8 a.m. was considered normal, delivery after 9 a.m. was classified as a prolonged birth process. (A) Percentages of normal and prolonged parturition of *Cx43*$^{del/+}$ and untreated (ut.) as well as tamoxifen-treated (t.) *Cx43*$^{fl/fl}$ and *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} mice. (B) Untreated and tamoxifen-treated *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} animals are shown as the moving average of animals delivering at certain time points between day 19 and day 22.

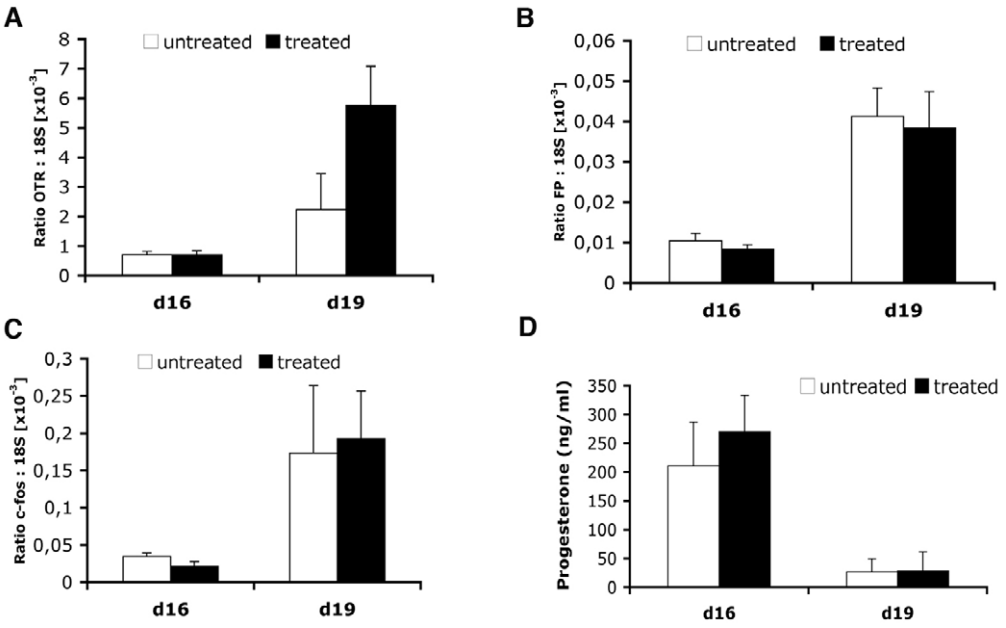

Fig. 8. Relative OTR (A), FP (B) and Fos (C) mRNA levels during pregnancy and labour as well as changes in myometrial plasma progesterone (D). Samples were taken from mice at 10-10.30 a.m. on day 16 and during labour. Data are expressed as mean ± s.e.m. (n=5).

et al., 2004). In rats, the gestational profiles of the expressed Fos gene showed significantly higher transcript levels only during labour (Piersanti and Lye, 1995; Mitchell and Lye, 2002). In virtually all species, progesterone (P_4) increases throughout pregnancy (Graham and Clarke, 1997) and decreases dramatically on the day of labour (Pepe and Rothchild, 1974; Lye et al., 1993; Hendrix et al., 1995).

Our data showed that the expression levels of the oxytocin receptor, the prostaglandin F receptor, and Fos as well as progesterone levels were similar in tamoxifen-treated and untreated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice. Therefore, the phenotypic abnormalities described cannot be attributed to hormonal changes caused by the anti-estrogen tamoxifen but are most likely provoked by the decreased Cx43-mediated intercellular communication.

Less than 20% of pregnant tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice delivered normally. In these animals it is likely that the tamoxifen-mediated recombination efficiency was too low to cause any phenotypic changes. Furthermore, delayed parturition was never observed in *Cx43$^{del/+}$* mice where only one allele expressed the Cx43 protein. As reported by van Rijen et al. (van Rijen et al., 2004), it is likely that only a decrease of Cx43 protein below the heterozygous level can cause physiological alterations. In the myometrium, the decrease in Cx43 protein of tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice may be sufficient to partially uncouple the muscular syncytium, creating smaller interconnected units of cells unable to allow the coordinated contraction of the complete uterine muscle during birth.

As a phenotypic effect of decreased Cx43 protein levels, tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice displayed delayed parturition but nonetheless delivery occurred. Therefore, other connexin isoforms may compensate for the function of Cx43 gap junctions. As described by different groups (Orsino et al., 1996; Kilarski et al., 1998; Kilarski et al., 2001; Albrecht et al., 1996) Cx26, Cx40, and Cx45 are expressed in addition to Cx43 in the uterus. Furthermore, Kilarski et al. (Kilarski et al., 2001) demonstrated, that in human myometrium Cx43, Cx45, and Cx40 are present together within the same gap-junctional plaque. Since no regulatory changes in the expression pattern of these connexin isoforms could be detected in tamoxifen-treated *Cx43$^{fl/fl}$:SM-CreER^{T2}* mice, compared with untreated controls, the present amounts of connexin protein may be sufficient to compensate for the partial loss of Cx43-mediated intercellular communication. On the other hand, the remaining Cx43 protein level may be adequate to allow for the delayed birth of living pups in most cases and may therefore be independent of compensation by other connexin channels.

Cx43 is increased in preterm labour (Balducci et al., 1993; Cook et al., 2000), the most important contributor to neonatal mortality and morbidity, and a condition that is increasing in occurrence (Bibby and Stewart, 2004; Barros et al., 2005). Our data show that Cx43 may represent a target for the therapeutic control of myometrial contractility and the prevention or delay of preterm labour as has been previously suggested (Balducci et al., 1993, Cook et al., 2000).

In conclusion, we have studied the impact of a reduced expression of Cx43 on the timing of labour using a mouse mutant in which the coding region of *Cx43* can be deleted at any given time point by application of tamoxifen. Our analysis of the SMC-specific ablation of *Cx43* offers, for the first time, insights into the role of this connexin in the myometrium of the living animal. Our data show that induced, SM-CreER^{T2}-mediated, conditional deletion of the *Cx43*-coding region in the myometrium significantly prolongs the birth process but is insufficient to completely inhibit delivery. It is likely that the expression of other connexin isoforms near term contributes to the initiation and progression of labour or that other pathways operate to ensure eventual delivery even in the absence of gap-junctional intercellular communication. In order to evaluate the role of connexins other than Cx43 at term, and possibly in preterm labour, double connexin-deficient mice with defects in SMCs should be analysed.

Materials and Methods

Animals

Cx43$^{fl/fl}$, *Cx43*$^{del/+}$ (Theis et al., 2001), *Cx43*^{2lox} (Eckardt et al., 2004) and *SM-CreER*^{T2}(ki) (Kühbandner et al., 2000) mice were maintained under a 12:12 hour light:dark cycle with food and water available ad libitum. Genotyping was performed by PCR amplification as previously described (Theis et al., 2001; Eckardt et al., 2004; Kühbandner et al., 2000).

Day 0.5 of pregnancy was defined when a vaginal plug was found in the morning. Females were separated from males on this day and housed individually until term. In our breeding colony, parturition (e.g. delivery of the first pup) occurred between 4 a.m. and 8 a.m. on day 19.5 for 98% of the mice. All experimental designs and procedures were in accordance with the guidelines of German law for animal welfare and with prior permission by local governmental authorities.

Preparation and administration of tamoxifen

A 10 mg/ml tamoxifen stock solution was prepared by suspending 100 mg tamoxifen-free base (Sigma, Taufkirchen, Germany) in 0.5 ml of ethanol followed by the addition of 9.5 ml peanut oil. The tamoxifen stock solution containing 0.1 mg tamoxifen in 100 μl was stored at –20°C for up to 4 weeks and thawed at 37°C before use. To achieve conditional deletion of *Cx43* in smooth muscle cells, six-week-old *Cx43*$^{fl/fl}$*:SM-CreER*^{T2} mice as well as controls were i.p. injected with 100 μl tamoxifen stock solution (1 mg tamoxifen) for five consecutive days and sacrificed 7 days after the last injection to analyse recombination (Kühbandner et al., 2000). To study the effects of Cx43 ablation during birth, tamoxifen-treated females were mated 1 week after the last injection.

Indirect immunofluorescence and detection of β-galactosidase activity

In order to show the abundance of gap junction plaques formed by Cx43, uterine tissue as well as cultured primary cells were subjected to indirect immunofluorescence analyses. The β-galactosidase activity was monitored to detect recombination at the cellular level.

Fresh uterine tissue was frozen in OCT (Tissue Tec, Sakura, Zoeterwoude, The Netherlands), sectioned at 10 μm with a cryostat (HM 500 OM, Microm, Heidelberg, Germany), and overlaid on SuperFrost glass slides (Menzel Gläser, Braunschweig, Germany). After fixation (10 minutes in 4% PFA), the sections/primary myometrial SMCs were blocked in a solution containing 5% normal goat serum (PAA, Pasching, Austria), 1% BSA (Sigma) and 0.1% Triton X-100 (Serva, Heidelberg, Germany) at room temperature for 60 minutes followed by incubation at 4°C overnight with primary rabbit polyclonal antibodies raised in our laboratory against amino acid residues 359-381 of the Cx43 C-terminal region (C. Schlieker, PhD Thesis, University of Bonn, 2000; diluted 1:700). The other primary antibodies we used were rabbit polyclonal Cx26 (Zymed, San Francisco, CA; diluted 1:300), Cx40 (BioTrend, Köln, Germany; diluted 1:150), Cx45 (U. Janssen-Bienhold, Oldenburg, Germany; diluted 1:700) and a FITC-conjugated mouse monoclonal smooth muscle actin specific antibody (Sigma; diluted 1:400 in the blocking solution). After three 5-minute washes with PBS, the slides/glass coverslips were incubated with Cy3-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) diluted 1:800 in the blocking solution at room temperature for 1 hour in the dark. The slides/glass coverslips were then washed three times with PBS and mounted with one drop of mounting media (Permafluor; Beckmann-Coulter, Marseille, France). The slides/glass coverslips were examined under a laser-scanning confocal microscope (LSM 510; Zeiss, Oberkochen, Germany). Adjacent sections/cultured cells incubated with blocking solution in the absence of the primary antibodies were used as negative controls.

For X-Gal staining, sections were fixed for 5 minutes at room temperature in PBS containing 0.2% glutaraldehyde, washed twice in PBS, and incubated in X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside, Sigma) staining solution [1 mg/ml X-Gal, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ in PBS, pH 7.4] overnight at 37°C. The slides were then washed three times with PBS, dried, and mounted with one drop of mounting media (Entellan; Merck, Darmstadt, Germany). In order to calculate the deletion efficiency of the Cx43-coding region, nuclei were visualized with Hoechst 33258 dye (Sigma; 1:1000) following X-Gal staining. LacZ-positive and -negative nuclei were counted in five randomly chosen areas of three different specimens per genotype at 100× magnification (Axiophot; Zeiss).

Isolation and culture of primary myometrial smooth muscle cells

Primary cultures of enriched uterine smooth muscle cells were generated as previously described (Shynlova et al., 2002). Briefly, myocytes were prepared from mouse uterus by enzymatic dispersion and centrifuged (200 ***g*** for 15 minutes). The cell pellet was resuspended in sterile Dulbecco's modified Eagle's medium, pH 7.35 (Gibco, Karlsruhe, Germany) without Phenol Red, supplemented with 10% FBS (Biochrom, Berlin, Germany), 25 mM HEPES buffer, 100 U/ml penicillin-streptomycin and 2.5 μg/ml amphotericin B (all from Sigma). To enrich for uterine myocytes, the freshly isolated cell mixture was subjected to a differential attachment procedure (Kasten, 1975). SMCs were plated on 6 cm culture plates (Falcon, Erembodegem, Belgium) and on 10 mm glass coverslips at a density of 5×10^6 cells per plate and 2×10^6 per coverslip. The cells were grown to confluence in Phenol-Red-free DMEM supplemented with 10% FBS, 25 mM HEPES, 100 U/ml of penicillin-streptomycin and 2.5 μg/ml amphotericin B. All experiments were carried out on day 4 of culture.

Intracellular dye injection

Glass micropipettes were pulled from capillary glass (Hilgenberg Glas, Malsfeld, Germany) with a horizontal pipette puller (PD-5; Narishige, Tokyo, Japan) and backfilled with tracer solution. Cells were rinsed with PBS and medium was changed before iontophoretical injection (Iontophoresis Programmer model 160; World Precision Instruments, New Haven, CT) of Lucifer Yellow (Sigma). Dye coupling was examined using an inverse microscope (IM35; Zeiss) with fluorescence equipment (HBO 100, filter set 09; Zeiss). During injection, the cell culture dishes were kept on a heated block at 37°C. Lucifer Yellow as 4% (wt/vol solution) in 1 M LiCl was injected for 2-3 seconds using negative current of 20 nA. Five minutes after Lucifer Yellow injection, cell-to-cell coupling was quantified by counting the number of fluorescent cells adjacent to the injected cell. Thirty injections were performed with each culture before cells were fixed and immunostained as described above. Images were recorded directly using a digital camera (Power Shot; Canon, Tokyo, Japan) or, after immunostaining, using a laser-scanning confocal microscope (LSM 510; Zeiss).

Immunoblot analyses

The protein concentration of primary myometrial cultures and freshly isolated myometrial tissue were determined using the bicinchoninic acid protein determination kit (Sigma) according to the manufacturer's instructions. Equal protein amounts were separated by SDS-PAGE (Laemmli, 1970) at 25 mA per gel and electroblotted for 2 hours at 100 V at 4°C onto nitrocellulose membranes (Hybond, 0.45 μm; Amersham Biosciences, Little Chalfont, United Kingdom). Blots were incubated with rabbit polyclonal Cx43 (C. Schlieker, PhD Thesis, University of Bonn, Germany, 2000; 1:1500), Cx26 (Zymed; 1:500), Cx40 (BioTrend; 1:500) and Cx45 antibodies (U. Janssen-Bienhold, Oldenburg, Germany; 1:3000) overnight at 4°C and immunoreactive proteins were visualized using species-specific horseradish peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany, 1:40,000) and an enhanced chemiluminescence (ECL) reagent (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL) as recommended by the manufacturer. ECL blots were developed on x-ray film (SuperRX; Fujifilm, Tokyo, Japan). Standardization was performed using mouse monoclonal β-actin (1:500, Sigma) antibodies.

Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from the frozen tissues using TRIZOL (Gibco BRL, Burlington, ON) according to the manufacturer's instructions. RNA samples were column purified using RNeasy Mini Kit (Qiagen, Mississauga, ON), and treated with 2.5 μl DNase I (2.73 Kunitz unit/μl, Qiagen) to remove genomic DNA contamination**.** Reverse transcription (RT) and real-time PCR were performed to detect the mRNA expression of oxytocin receptor (OTR), prostaglandin receptor (FP) and Fos in mouse myometrium. Two μg of total RNA was primed with random hexamers to synthesize single-stranded cDNAs in a total reaction volume of 100 μl using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The thermal cycling parameters of RT were modified according to the Applied Biosystems manual. Hexamer incubation at 25°C for 10 minutes and RT at 42°C for 30 minutes was followed by reverse transcriptase inactivation at 95°C for 5 minutes. Twenty μg of cDNA from the previous step were subjected to real-time PCR using specific sets of primers in a total reaction volume of 25 μl (Applied Biosystems). All primers were designed according to sequences available from GenBank (http://www2.ncbi.nlm.nih.gov/) and synthesized by ACGT (Toronto, ON). Specific forward and reverse primers were designed using Primer Express software, version 2.0.0 (Applied Biosystems), as follows: OTR mRNA, 5′-CTC-GCGCCTCTTCTTTTTCAT-3′ (sense primer) and 5′-CCCATAGAAGCGGA-AGGTGAT-3′ (antisense primer) (GenBank accession number NM_012871); FP mRNA, 5′-TCGCAAACACAACCTGCCA-3′ (sense primer) and 5′-GGCTGT-TCGATAAGATCCCCA-3′ (antisense primer) (NM_008966); Fos mRNA, 5′-TGT-TTCCGGCATCATCTAGGC-3′ (sense primer) and 5′-AAGGAATTGCTGTGCA-GAGGC-3′ (antisense primer) (V00727); 18S, 5′-GCGAAAGCATTTGCCAA-GAA-3′ (sense primer) and 5′-GGCATCGTTTATGGTCGGAAC-3′ (antisense primer) (V01270).

RT-PCR was performed in an optical 96-well plate with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), using the SYBR Green detection chemistry. The run protocol was as follows: initial denaturation stage at 95°C for 10 minutes, 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute. After PCR, a dissociation curve was constructed by increasing the temperature from 65°C to 95°C for detection of PCR product specificity. In addition, a no-template control (H_2O control) was analysed for possible contamination in the master mix. A cycle threshold (*Ct*) value was recorded for each sample. PCR reactions were set up in triplicates and the mean of the three *Ct* values was calculated. Relative quantitation of gene expression served to compare differences of gene expression across gestation. An arithmetic formula from the comparative *Ct* method (see ABI

User Bulletin #2) was applied to the raw *Ct* values to extract relative gene expression data. The mRNA level from each sample was normalized to ribosomal 18S rRNA. Validation experiments were performed to ensure that the PCR efficiencies for the target genes and 18S rRNA gene were approximately equal.

Hormone measurement

Blood was collected into heparinized tubes (Sarstedt), centrifuged, and plasma was stored at –70°C for later hormone analysis. Plasma concentrations of progesterone were measured in individual serum samples from day 16 and term pregnant animals using a human RIA kit (Coat-A-Count; DPC, Los Angeles, CA) according to the manufacturer's instructions.

Statistics

Results are expressed as means ± s.e.m. Statistical significance was assessed by Student's *t*-test for paired and unpaired data. A P value less than 0.05 was considered to be significant.

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