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Cardiac Cell Therapy: Overexpression of Connexin43 in Skeletal Myoblasts and Prevention of Ventricular Arrhythmias

Sarah Fernandes[1,2], Harold V.M. van Rijen[3], Virginie Forest[1,2], Stéphane Evain[1,2,4], Anne-Laure Leblond[1,2], Jean Mérot[1,2,5], Flavien Charpentier[1,2,5], Jacques M.T. de Bakker[3,6], Patricia Lemarchand[1,2,4,*].

[1] INSERM, UMR915, l'institut du thorax, IFR26, Nantes, France

[2] Université de Nantes, *UFR Médecine,* Nantes, France

[3] Department of Medical Physiology, University Medical Center Utrecht, Utrecht, The Netherlands

[4] CHU Nantes, l'institut du thorax, Nantes, France

[5] CNRS, ERL3147, Nantes, France

[6] Department of Experimental Cardiology, Academic Medical Center, Amsterdam, The Netherlands

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Short title: Cardiac cell and Cx43-gene therapy for arrhythmias

*Correspondence should be addressed to P.L. (patricia.lemarchand@univ-nantes.fr)

INSERM UMR915, l'institut du thorax, Faculté de Médecine, 1 rue Gaston Veil, F-44035 Nantes cedex 1, France

Tel: (33) 2 40 41 29 91 Fax: (33) 2 40 41 29 50

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ABSTRACT

Cell-based therapies have great potential for the treatment of cardiovascular diseases. Recently, using a transgenic mouse model Roell *et al.* reported that cardiac engraftment of connexin43-overexpressing myoblasts *in vivo* prevents post-infarct arrhythmia, a common cause of death in patients following heart attack [*Nature* 2007; 450: 819-24]. We carried out a similar study but in a clinically relevant context via transplantation of autologous connexin43-overexpressing myoblasts in infarcted rats. Seven days after coronary ligation, rats were randomized into 3 groups: a Control group injected with myoblasts, a Null group injected with myoblasts transduced with an empty lentivirus vector (Null), and a Cx43 group injected with myoblasts transduced with a lentivirus vector encoding connexin43. In contrast to Roell's report, arrhythmia occurrence was not statistically different between groups (58%, 64% and 48% for the Control (n=12), Null (n= 14) and Cx43 (n=23) -groups, respectively, p=0.92). Using *ex vivo* intramural monophasic action potential recordings synchronous electrical activity was observed between connexin43-overexpressing myoblasts and host cardiomyocytes, whereas such synchrony did not occur in the Null-transduced group. This suggests that *ex vivo* connexin43 gene transfer and expression in myoblasts improved intercellular electrical coupling between myoblasts and cardiomyocytes. However, in our model such electrical coupling was not sufficient to decrease arrhythmia induction. Therefore, we would suggest a note of caution on the use of combined Cx43 gene and cell therapy to prevent post-infarct arrhythmias in heart failure patients.

Keywords: cell therapy, gene therapy, arrhythmia, connexin43, myoblast

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INTRODUCTION

The hypothesis behind cell-based therapy for cardiac injury is that adding healthy cells to injured myocardium increases the rate of recovery and, in so doing, improves cardiac function and prevents life-threatening arrhythmias, the major cause of sudden death in heart failure patients. Yet, to date, success with cell therapies has been limited, and under some conditions, such therapy results in arrhythmias, a documented risk of skeletal muscle myoblast delivery into the heart[1]. The exact mechanism of these arrhythmias is unknown, but it has been suggested that they result from a lack of electrical coupling between the skeletal myoblasts and the host cardiomyocytes[2].

Electrical coupling between ventricular cardiomyocytes is very efficient in healthy myocardium, and depends mainly on Connexin43 expression (Cx43, the primary ventricular gap junction protein). Interestingly, proliferating myoblasts express Cx43 but down-regulate Cx43 expression progressively upon fusion, mature skeletal myofiber (myotube) formation and further differentiation. Several preclinical and clinical studies have shown that once injected into the heart, myoblasts differentiate into myotubes, and thus, are not coupled to neighboring cardiomyocytes[3,4]. Interestingly, transplanted myotubes are able to contract spontaneously occasionally, but these contractions do not spread to neighboring cardiomyocytes[2]. *In vitro* and *ex vivo* studies have shown that a mixture of myotubes and cardiomyocytes without sufficient functional gap junctions results in slower conduction velocities and greater tissue heterogeneity[5,6]. Such heterogeneity predisposes to wave breaks and reentry, both key elements for inducing ventricular arrhythmias[7].

Recently, in a well-designed study using an *in vivo* infarcted mouse model, Roell *et al.* showed that cardiac transplantation of myoblasts from transgenic mice overexpressing

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connexin43 (Cx43, the main cardiac gap junction protein) not only eliminates myoblast pro-arrhythmogenic effect but also provides potent protection against ventricular arrhythmias[8]. They concluded that an increase in intercellular coupling by cell-based therapy may be an effective therapy to prevent post-infarction ventricular arrhythmias[8].

In a previous study[9], we transplanted autologous myoblasts or autologous bone marrow cells into infarcted heart of Wistar rats. Like Roell *et al*, using *in vivo* programmed electrical stimulation (PES), we showed that transplantation of myoblasts but not of bone marrow mononuclear cells increases arrhythmia induction. As a follow up, the purpose of this new study was to evaluate arrhythmogenicity after autologous cell therapy and Cx43 *ex vivo* gene transfer. This combination of cells and genes represents a clinically relevant and pragmatic approach to Roell's hypothesis. Despite electrical coupling between transplanted cells and host cardiomyocytes (as demonstrated by Roell and confirmed in our study), we did not observe any reduction in post-infarct arrhythmias.

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MATERIALS AND METHODS

Experimental model

All animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Autologous myoblasts were injected into the infarcted area of the myocardium of Wistar rats 7 days after coronary ligation. As previously described, intramyocardial injections of a total of 10.10^6 autologous myoblasts were performed under direct observation via left thoracotomy[9]. Myoblast primary cultures were sourced from tibialis anterior muscles of male Wistar rats as previously described[9,10].

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Lentivirus vector construction and production

A self-inactivating HIV-derived gene-transfer plasmid (pHR'-CMV-Cx43-W-sin18; Figure Ia) containing the cDNA for rat Cx43 downstream of the cytomegalovirus (CMV) promoter elements was kindly provided by Pr P. Meda (University of Geneva, Switzerland). As controls we used a lentivirus vector containing the same expression cassette but without the Cx43 cDNA (Null) or a lentivirus vector containing the same expression cassette and the Green Fluorescent Protein (GFP) cDNA. Lentivirus vector production was performed by the LentiVirus Production Unit (LVPU, Geneva, Switzerland).

Lentivirus vector transduction

Transduction was carried out by adding lentivirus vector to myoblast primary culture 24 hr after cell isolation (40 transducing units (TUs) /cell). Transduced cells were

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cultured *in vitro* for 6 days before intramyocardial transplantation. Non-transduced myoblasts and Null-transduced myoblasts served as controls.

FACS analyses

Quantification of myoblasts and of lentivirus vector transduction efficacy in primary culture was performed using desmin (a specific marker for muscle cells) and GFP expression, respectively, in flow cytometry analyses. A mouse anti-human desmin antibody (D33, Dako-Cytomation, Denmark), and a second fluorescent antibody (alexa red anti mouse IgG; Molecular Probes) were used to detect desmin. For all GFP analyses thresholds were chosen using a cell sample from the same primary culture that has not been transduced with GFP-lentivirus and that did not undergo desmin immunolabeling. Analyzes were performed using a FACSCalibur instrument (BD Biosciences, San Jose CA, CellQuestPro software).

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RNA isolation

Total RNA was isolated from myoblasts and from myocardial tissue injected with myoblasts, using a RNeasy Mini kit (QIAGEN) and a RNeasy fibrous tissue Mini kit (QIAGEN), respectively. DNase treatment was performed after each RNA extraction to eliminate genomic DNA (RNase free DNase set; QIAGEN). Absence of RNA degradation was verified by capillary electrophoresis on a 2100 Bioanalyser (Agilent).

Real time RT-PCR

First-strand cDNA was synthesized from 2 µg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) and was preamplified using TaqMan®PreAmp Master Mix Kit (Applied Biosystems). On-line PCR was performed

with the following primers: desmin (Rn00574732_m1), and Cx43 (Rn01433957_m1). Fluorogenic TaqMan probes were labeled on the 5'-end with the fluorescent reporter dye 6-carboxyfluorescein (FAM®, Applera), and on the 3'-end with non-fluorescent quencher (Applied Biosystems). Data were collected with instrument spectral compensations by the Applied Biosystems SDS 2.3 software and analyzed using the threshold cycle (C_T) relative quantification method. Fluorescence levels were normalized to the hypoxanthine guanine phosphoribosyl transferase (HPRT, Rn01527838_g1), used as reference gene. Specific mRNA quantifications were performed in duplicate. Absence of DNA contamination in RNA samples was verified by performing real time PCR on RNA samples that were not reverse transcribed. All data were averaged and then used for the $2^{-\Delta CT}$ calculation. $2^{-\Delta CT}$ corresponds to the ratio of each gene expression *versus* HPRT.

Immunolabeling

Serial cryosections (10µm) were performed 2 weeks after myoblast transplantation. A mouse monoclonal antibody against the fast skeletal myosin heavy chain (clone My32, NCL-MHCf, Novocastra) and a rabbit polyclonal antibody against Cx43 (Zymed Laboratories, USA) were used for identification of differentiated myotubes and Cx43, respectively.

Ex vivo intramural electrophysiologic recordings

Animals were sacrificed 2 weeks after autologous myoblast transplantation by pentobarbital injection (100mg/kg ip; Pentobarbital sodique®, Cerva Santé Animale). After heparin injection (3750 UI/kg ip; Héparine Choay), hearts were harvested for Langendorff perfusion at 37°C with a Krebs modified solution (NaCl, 118.3 mM;

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KCl, 3.8 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM; KH_2PO_4, 1.2 mM; glucose, 11.1 mM; $CaCl_2$, 1.25 mM), saturated with carbogen (O_2 95% and CO_2 5%). Monophasic action potentials (MAPs) were recorded during sinus rhythm (250ms) at different sites of the myocardium (in the healthy myocardium, in the infarct border zone and in the transplanted area of the infarct). These different sites were probed serially with a single MAP sharp, tungsten needle-electrode that was isolated except at the tip, as previously described [11]. Recordings in the tibialis muscle were performed *in situ* from a nerve/tibialis muscle preparation. The nerve was stimulated and the same MAP-electode was inserted in the tibialis. Trains of 1 ms stimuli (S1-S1 250ms) were applied to the nerve and the MAPs were recorded. Because the nerve was stimulated, no pacing artifacts were present. Because the tip was in the extracellular space, it also recorded extracellular potentials [12].

In vivo programmed electrical stimulation

Ventricular electrical instability related to cell transplantation was evaluated in all groups using the PES procedure, as described previously[9]. Briefly, an epicardial electrode was tied to the viable left ventricular myocardium during surgery for coronary ligation. For PES stimulation, animals were sedated with etomidate (8 mg/kg ip; Hypnomidate®, Janssen-Cilag) and pentobarbital (40 mg/kg ip). The distal tip of the epicardial electrode was externalized to be used as the negative lead. Another electrode was placed on the thorax to be used as the positive lead, allowing unipolar stimulation (UHS 20, Biotronik). Surface six-lead ECGs were recorded for monitoring and later analyses. Standard criteria were used for interval measurements (RR, PR, QRS and QT). For further comparison between groups, QT interval were corrected using bot Fredericia and Bazett fomulas (QTc(F) = QT/ (RR/150)1/3and QTc(F) = QT/

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(RR/150)1/2 respectively; Table I). Standard clinical PES protocols were used, including single, double and triple extrastimuli applied under spontaneous rhythm or following a train of 9 stimuli at 100-ms drive cycle length. The coupling interval of the last extrastimulus was decreased to the ventricular effective refractory period (VERP). Protocols were interrupted if sustained ventricular tachycardia (VT) was induced. Sustained VT was defined as fast ventricular rhythm of 15 or more beats, according to the Lambeth Conventions[13].

Data Analyses

Data were expressed as mean±SEM and frequencies (expressed as percentages). Statistical analyses were performed using MedCalc 9.1 software. Real time RT-PCR data and cell count data were assessed using the Student t-test. Occurrences of sustained VT were compared with Cox's model and were analyzed as failure time data (rats without event were considered as censored). The assumption of proportional hazards between groups was confirmed, and the group was the unique covariate selected in the Cox's model. Overall mortality between groups was compared using Fisher's exact test. ECG parameters (P, RR, PR, QRS, QT and QTc and VERP values) were assessed by a linear mixed model with random slope and intercept in the control, Null, and Cx43 groups. The fixed effects were the group and the time. Interaction between group and time was tested but not included in the model (not significant). The power of the study was 0.40 for all statistical analysis. A p-value <0.05 was considered significant.

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RESULTS

In vitro Cx43 overexpression

A lentivirus vector was used to overexpress Cx43 in rat myoblast primary cultures *ex vivo*, prior to autologous intramyocardial injection (Figure 1a). As controls we used lentivirus vectors containing an empty expression cassette (Null) or the Green Fluorescent Protein (GFP) cDNA. Efficacy of lentivirus transduction was evaluated *in vitro* using flow cytometry analyses for both GFP and desmin after GFP lentivirus vector transduction. GFP was expressed by 50% of the desmin positive cells, suggesting that 50% myoblasts expressed the transgene before transplantation (Figure 1b). The transduction rate of non-myoblast contaminating cells (i.e. GFP+ desmin – cells) was 16.3±4.1% (Figure 1b). Seven days after Cx43 or Null lentivirus transduction, cell counts and desmin expression levels were similar in both Null- and Cx43-transduced myoblasts, whereas Cx43 expression level was 2.5 fold higher in Cx43- than in Null-transduced myoblasts ($p<0.05$), showing that Cx43 overexpression did not alter myoblast expansion (Figure 2a).

To evaluate exogenous Cx43 expression due to Cx43 lentivirus vector transduction, we used gene expression quantification of the post-transcriptional regulatory element Woodchuck hepatitis virus (Wpre), that is located within the expression cassette of the lentivirus vector in 3’ of the Cx43 cDNA and proximal to the polyadenylation signal (Figure 1a). Wpre gene expression was detected only in Cx43-transduced myoblasts 6 days after Cx43- and Null-transduction (Figure 2b). In Cx43-transduced myoblasts, total Cx43 gene expression level correlated with Wpre expression level (R^2=0.8710; Figure 2b). Finally, *in vitro* time-course studies demonstrated that Cx43 expression remained at least 2.5 fold higher in Cx43-transduced myoblasts than in Null-transduced myoblasts ($p\leq0.05$, Figure 2c) for at least 35 days after Cx43 transduction.

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Moreover, in Cx43-transduced myoblasts, Wpre gene expression remained stable. In view of these results, Wpre was used as a marker to detect exogenous Cx43 expression *in vivo* after intramyocardial myoblast transplantation.

In vivo Cx43 overexpression

Seven days after coronary ligation, rats were randomized into 3 groups: a Control group injected with autologous myoblasts, a Null group injected with autologous myoblasts transduced with the Null lentivirus vector, and a Cx43 group injected with autologous myoblasts transduced with the lentivirus vector encoding Cx43.

Using real time RT-PCR, Wpre gene expression was detected in 9/9 hearts injected with Cx43-tranduced myoblasts up to 35 days after their *in vivo* injection (Figure 3a), suggesting that Cx43-transduced muscle cells overexpressed Cx43 *in vivo*. Two weeks after Cx43-transduced myoblast transplantation, Cx43 protein was detected in cryosections of infarcted myocardium in cells expressing fast skeletal myosin heavy chain (Figures 3b-h), suggesting that *ex vivo* Cx43 lentivirus vector transduction lead to *in vivo* Cx43 protein expression in differentiated myotubes.

Electrophysiological analyses

PES was performed at 1, 2, 3 and 4 weeks after intramyocardial myoblast transplantation. No differences between groups were observed in ECG parameters prior to the first PES procedure (week 1). Neither standard ECG measurements nor VERP at 100-ms pacing cycle length were significantly altered by the repeated PES procedures (Table I), suggesting that lentivirus vector transduction (in Null group) or Cx43 overexpression (in Cx43 group) in transplanted myoblasts did not modify ECG parameters. Overall the percentage of rats that underwent at least one arrhythmia event

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during one of the PES were similar between groups (58%, 64% and 48% of animals in the Control (n=12), Null (n=14) or Cx43 (n=23) group, respectively, Cox's model, p=0.92, Figure 4a). Additionally, the percentage of newly inducible rats did not differ among groups (Figure 4b). In each group, ECG parameters of rats that underwent sustained VT during PES did not differ from those that did not show sustained VT (not illustrated). Mortality was similar in Control, Null or Cx43 groups (42%, 50%, 43%, respectively, p=0.87).

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***Ex vivo* measurements of intramural monophasic action potentials (MAPs)**

To evaluate electrical coupling between Cx43-overexpressing myoblasts and host cardiomyocytes, *ex vivo* intramural monophasic action potential (MAP) recordings were performed 14 days after myoblast injection in Langendorff-perfused hearts, using a tungsten electrode that recorded both local MAP and remote electrical activity. For each rat, MAPs were recorded during sinus rhythm at 7 different sites (1 located in the right ventricle and 6 located in the left ventricle). Control included recordings from rat tibialis muscle (Figure 5a) and from healthy non-infarcted myocardium (Figure 5b). A total of 16 rats were evaluated, 6 in the Cx43 and in the Null groups, and 4 in the Control group. Recordings in the infarct solely showed the remote signal of healthy myocardium in both Null and Cx43 groups in 9/16 animals (Figures 5c,d). In 5/16 rats monophasic action potentials (MAPs) were recorded with average duration of 52.9±4.6ms, while in 2/16 rats significantly shorter MAPs were recorded of 2.8±1.9ms ($p<0.001$, Figures 5e,f, and Figures 5h,i (asterisks)). These short MAPs were similar to those recorded in rat tibialis muscle (MAP duration 3.5ms, Figure 5a,g), while the longer MAPs recorded in the infarct compared well to MAPs recorded in healthy cardiac muscle (MAP duration 55.1±2.4ms, p=0.96, n=16). No such short MAPs were

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recorded in the non-transplanted area of the heart, strongly suggesting that the short MAPs reflected electrical activity of transplanted skeletal muscle cells, while the longer MAPs reflected electrical activity of host cardiomyocytes.

In myocardium transplanted with Null-transduced myoblasts, skeletal muscle cell MAPs and myocardial electrograms were not synchronized (Figures 5e,h), suggesting that skeletal muscle cells were not activated in synchrony with host cardiomyocytes. In contrast, in left ventricle from the Cx43 group, skeletal muscle cells exhibited electrical activity in synchrony with surrounding healthy myocardium (Figures 5f,i). Multiple registrations of 2 seconds of myoblast spikes were performed from the same location in these rats. In the Null group, the mean interval for myoblast spikes was 545 ms (range 541 – 549 ms) and for myocyte spikes was 224 ms (range 214 – 233). In the Cx43 group, the mean interval for myoblast spikes was 225 ms. Myoblast spikes were synchronous with myocyte activity. These results suggest that *ex vivo* Cx43 gene transfer and expression in myoblasts enhanced electrical coupling and frequency entrainment of skeletal muscle cells and host cardiomyocytes.

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DISCUSSION

In this study, using an *in vivo* model of cardiac cell and gene therapy with autologous myoblasts (in which arrhythmic risk related to myoblast transplantation has been previously evaluated[9]), *ex vivo* Cx43 gene transfer prior to intramyocardial transplantation enhances Cx43 expression and *in vivo* electrical coupling between transplanted myoblasts and host cardiomyocytes. However, in our model, improved electrical coupling was not sufficient to significantly decrease arrhythmogenicity related to myoblast transplantation.

Several *in vitro* studies showed that in myoblast/cardiomyocyte coculture models, lentiviral-mediated overexpression of Cx43 in myoblasts was sufficient to induce gap junction formation between both cell types[5,14-16]. Although we did not demonstrate gap junction formation in the present study, the functionality of these gap junctions was demonstrated by others, using western blot analyses and *in vitro* dye transfer techniques[5,15,16]. In our study, gap junction formation and functionality were suggested *in vivo* by electrical coupling between skeletal muscle cells and cardiomyocytes that occurred only in myocardium injected with Cx43-transduced myoblasts. Cx43 overexpression in myoblasts and gap junction formation have also been successfully obtained using retrovirus[15] or adenovirus[17] vectors. In contrast with our present study, increased cell death was observed *in vivo* after Cx43 adenovirus vector transduction and cell transplantation[17]. This was clearly linked to very high vector transduction rate, as usually obtained with adenovirus vectors[18] in contrast to retrovirus or lentivirus vectors. In our study, 50% myoblasts were transduced by the lentivirus vectors, and Cx43 expression increased only 2.5-fold as compared to baseline levels, a level compatible with studies using similar gene transfer conditions[15]. In one study, no Cx43 overexpression was observed after cell

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transplantation of Cx43 retrovirus vector-transduced myoblasts, an observation that was linked to promoter silencing[15]. In our study, this was clearly not the case since Wpre, a regulatory element within the expression cassette, was expressed *in vivo* more than 8 weeks after cell transplantation. In summary, although each vector type lead *in vitro* to significant Cx43 overexpression and gap junction formation, lentivirus vector transduction offered long *in vivo* expression without deleterious effects.

An *in vitro* study showed an increase in electrical coupling between cardiomyocytes and myoblasts transduced with a lentivirus vector encoding Cx43 associated to a reduction (but not elimination) of myoblast arrhythmogenicity[5], an hypothesis that needed to be evaluated *in vivo*. Both Roell's study and ours provided evidence of *in vivo* host cardiomyocyte and transplanted myoblast electrical coupling when myoblasts overexpressed Cx43. In this regard, our results confirm the feasibility of *ex vivo* gene transfer to modify *in vivo* electrophysiological properties of injected cells[19]. The rat number in which myoblast MAPs were observed in the heart was very low, most probably because MAP recordings were performed each time using one single electrode and that this electrode recorded only local electrical activity. Therefore the chance that the electrode was placed in close proximity with myoblasts was low, as myoblast number was also probably low. Furthermore, myoblast spikes might have been hidden in the upstroke of myocyte MAPs. Some myocyte MAPs in the Cx43 group showed fractionated upstrokes (not shown), suggesting that myoblast spikes caused fractionation of the upstroke of these myocyte MAPs. Nevertheless, fractionated upstrokes were not counted, since there was no final proof that these deflections were indeed myoblast spikes. MAP recordings were performed at a pacing cycle length of 250 ms. This cycle length was chosen because this is the rat normal spontaneous sinus cycle length. Evaluating the level of electrical coupling between

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transduced myoblasts and cardiomyocytes *in vivo* was not possible by changing pacing rate. Conduction is determined by 3 parameters (excitability, cell-to-cell coupling and myocardial architecture), and it is not possible to determine the contribution of a single parameter by changing stimulation frequency. In addition, in remodeled infarcted myocardium, all three parameters are changed. If conduction delay increased between myoblasts and cardiomyocytes at a higher stimulation frequency it is unclear whether this would be due to an inadequate coupling between host and donor cells, a reduced coupling between cardiomyocytes, impaired sodium current of the cardiomyocytes or the changed myocardial architecture.

In contrast to Roell's study, electrical coupling was not sufficient to significantly decrease arrhythmogenicity related to myoblast transplantation in a clinically relevant model combining gene and autologous cell therapy. This result highlights the differences between the *in vivo* study by Roell *et al* and ours, including the animal model and the level of Cx43 overexpression in transplanted cells. Although the type of injury may have an impact on arrhythmia triggering, arrhythmias associated to cell therapy were evaluated in different models of myocardial injury (cryolesion[8], ischemia/reperfusion[20] or even pharmacological models[21]. This suggests that myoblast-induced arrhythmias are not dependent from the myocardial infarction model. As in Roell's study, we did not evaluate spontaneous ventricular tachyarrhythmias, because their frequency is low in rodents[9]. Although in Roell's study VT frequency was 100% in the myocardial infarction model, VT frequency was 60% in ours (a result similar to that of our previous study, confirming the reproducibility of our model), probably because we counted only VT>15 beats. Although we did not measure the extent of myocardial infarction in the present study, histological analysis of the same model in our previous study showed a reproducible

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scar of 25+3% of the left ventricle 7 weeks after coronary ligation[9], an infarct size comparable to that in other studies using the same animal model[22]. Finally, as mentioned above in our study only 50% transduced myoblasts expressed Cx43, inducing an increase of the overall Cx43 expression only 2.5 fold as compared to baseline levels. Because Roell *et al.* used myoblasts from a genetically modified mouse overexpressing Cx43, both percentage of Cx43-overexpressing myoblasts and Cx43 expression level may have been significantly higher, favoring extensive electrical coupling between myoblasts and cardiomyocytes. Importantly, only frequency but not waveform entrainment (typical for low coupling between cells with different intrinsic action potential waveforms) was observed in our study, suggesting that electrical coupling was too low to significantly affect the electrical stability of the heart. It has also been suggested that the occurrence of arrhythmias depends on cell distribution within the infarcted area, an hypothesis that was not confirmed in a recent study on rabbits[23]. Notably, in the transgenic mouse study, electrical stability occurred even in animals whose stem cell grafts were physically isolated from the native myocardium. Finally, because myoblasts do not transdifferentiate into cardiomyocytes and because their action potential duration remains significantly shorter than host cardiomyocytes[24], an electrical coupling between both cell types might induce locally heterogeneous distribution of action potential duration, another risk factor for arrhythmia[1]. Although this potential adverse effect has not been detected *in vitro* or in rodent models, because early preclinical studies did not reveal the tendency of myoblasts to induce life-threatening arrhythmias such hypothesis needs to be evaluated in larger animal models.

Our study has some limitations. First, we did not correlate the injected-cell number with arrhythmia inducibility, and Cx43 overexpression might have increased *in vivo*

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cell engraftment and/or proliferation as compared to engraftment of Null-transduced cells. Although a dose-dependence between cell engraftment rate and arrhythmia might be expected, results of a recent randomized double-blind clinical trial did not support a dose-response increase in arrhythmic episodes[25]. In our study, we did not evaluate the level of myoblast contamination with smooth muscle cells nor their level of viral infection with Cx43 transgene. In humans and in rats, myoblast primary culture from muscle samples does not lead to pure myoblast preparations[10, 3, 26], the culture being contaminated mostly with fibroblasts. In Roell's study, myofibroblasts induced arrhythmias, suggesting that if myofibroblasts contaminated myoblast culture, they may induce arrhythmia when injected *in vivo*. Therefore we cannot rule out the contribution to arrhythmia triggering of transduced or untransduced contaminating cells with Cx43 lentivirus vector. In Roell's study, Cx43 was expressed under the control of a promoter specific for myotubes. Therefore contaminating smooth muscle cells did not overexpress Cx43 in Roell's study. Nevertheless, there was a significant decrease in arrhythmias following injection of Cx43-overexpressing myoblasts, suggesting that contaminating cells within myoblast preparations did not play a major role in arrhythmias.

Finally, improvement of the heart function after myoblast transplantation was not studied. Myoblast transplantation in the failing heart has been initially motivated by the hope that transplanted cells would actively improve systolic contraction. It has been shown that myoblasts could organize in fibers with contractile capacity, with the right orientation such that their synchronous contraction would increase the heart contraction strength. Without electrical coupling, skeletal muscle cells are incapable of contributing to contraction. Myoblasts have been shown to improve heart function (possibly by paracrine effect), and one important question would be to evaluate if an

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enhanced electrical coupling (leading to synchronous contraction) improves efficacy of myoblast therapy[24].

Although Cx43 expression level in Cx43 lentivirus vector-transduced myoblasts was compatible with *in vitro* studies using similar gene transfer conditions[5], it may be ineffective to restore full electromechanical coupling in an injured heart, as expression of other junction proteins such as N-cadherin may also be necessary[27]. Therefore, based on our present electrophysiological study we would suggest a note of caution on the use of combined gene and cell therapy to prevent post-infarct arrhythmias. Current technologic limitations to gene therapy, including low gene transduction and low foreign gene expression may explain these results. In this regard, further studies to improve gene therapy vectors will be rewarding.

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Acknowledgements

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Tables

Table I: ECG parameters and VERP values

Time	Groups	ECG parameters (ms)							
		RR	P	PR	QRS	QT	*QTc (B)*	*QTc (F)*	VERP
Week 1	Control (n=14)	154±3.9	19±0.6	48±0.7	21±0.9	84±3.9	*83±2.4*	*84±25*	69±3.2
	Null (n=12)	158±3.6	19±0.5	50±1.6	21±0.7	83±2.8	*81±2.2*	*82±2.2*	65±3.5
	Cx43 (n=23)	156±2.9	18±0.3	48±1.5	22±1.2	84±1.7	*83±1.2*	*84±1.3*	59±1.7
Week 2	Control (n=12)	151±4.0	20±0.7	49±0.9	21±0.8	86±3.0	*85±2.3*	*86±2.3*	59±2.9
	Null (n=12)	158±4.4	19±0.5	50±1.9	21±0.5	86±2.9	*84±2.5*	*85±2.6*	65±5.6
	Cx43 (n=21)	150±2.3	18±0.3	48±1.0	20±0.6	80±1.5	*80±1.8*	*80±1.7*	62±3.5
Week 3	Control (n=9)	150±2.6	18±0.6	47±0.9	21±0.8	84±2.1	*84±1.6*	*84±1.7*	68±3.4
	Null (n=9)	152±3.4	18±0.5	48±1.3	21±0.5	84±1.9	*83±1.6*	*84±1.6*	59±4.6
	Cx43 (n=17)	149±2.8	18±0.4	48±1.0	21±0.7	80±2.2	*80±2.3*	*80±2.2*	60±2.4
Week 4	Control (n=7)	152±2.2	19±0.5	49±1.7	19±1.3	85±2.3	*86±2.2*	*86±2.3*	61±2.1
	Null (n=7)	157±4.6	20±0.4	50±1.5	22±0.7	84±1.8	*83±1.5*	*83±1.5*	52±3.6
	Cx43 (n=13)	149±3.9	18±0.5	48±1.1	21±0.8	83±1.6	*83±1.2*	*83±1.3*	61±2.4

ECG measurements were performed under sinus rhythm. Ventricular Effective Refractory Period was measured at a basic pacing cycle length (BCL) of 100ms at week 1, 2, 3 and 4 after myoblast transplantation. Abbreviations: P, P wave duration; RR, PR, QRS, QT, QTc(B) and QTc(F): RR, PR, QRS, QT intervals, QT interval corrected with Bazett formula (B) or Fredericia formula (F) respectively; VERP, Ventricular effective refractory period. All measurements were performed on lead I under general anesthesia. Results are expressed as mean±SEM.

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Figure legends

Figure 1: Cx43 transgene expression in myoblasts.

(a) The Cx43 lentivirus vector contained an expression cassette including the rat Cx43 cDNA under the control of the cytomegalovirus (CMV) promoter, followed by the post-transcriptional regulatory element of the woodchuck hepatitis virus (Wpre). (b) Evaluation of lentivirus vector transduction in myoblast primary culture 6 days after GFP-lentivirus vector transduction, by flow cytometry analyses: cells from primary culture that have not been transduced with GFP lentivirus and that have not been labeled for desmin were used as controls to design thresholds (non-transduced cells/Des-; top left panel). The same thresholds were further used for FACS analyses of non-transduced cells/Des+ cells (non-transduced cells with desmin immunostaining; top right panel), of GFP-transduced/Des- cells (GFP-transduced cells with no immunostaining; bottom left panel) and of GFP-transduced/Des+ cells (GFP-transduced cells with desmin immunostaining; bottom right panel).

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Figure 2: Characterization of myoblast primary cultures after lentivirus vector transduction.

(a) Cell numbers (left panel) and desmin or Cx43 mRNA levels (right panel) in Null- and Cx43-transduced myoblasts (n=10 and n=9, respectively), 6 days after lentivirus vector transduction. Gene expression levels were measured using TaqMan real time RT-PCR. Desmin and Cx43 gene expression levels were corrected by HPRT gene expression levels. (b) Correlation between Cx43 and Wpre mRNA expression levels in Cx43 transduced myoblasts, 6 days after lentivirus vector transduction (n=9). (c) Cx43

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gene expression from day 14 to day 35 post-transduction in Null- (dotted line) and Cx43- transduced myoblasts (solid line, n=3 for both groups). * indicate p<0.05.

Figure 3: *In vivo* Cx43 and Wpre gene expression, following *ex vivo* lentivirus vector transduction and intramyocardial transplantation.

(a) Wpre expression in myocardium transplanted with Cx43 transduced myoblasts. (b-h) Section of left ventricle transplanted with Cx43-transduced myoblasts, in fluorescent microscopy (b to e) and in confocal microscopy (f to h), using immunolabeling against the fast skeletal myosin heavy chain (b and f) and Cx43 (c and g). Cell nuclei were labeled with DAPI (d). (e) Superposition of the b-d panels. (h) Superposition of the f and g panels. Cryosections were performed within the infarcted area, 14 days after myoblast transplantation.

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Figure 4: Ventricular hyperexcitability of the myocardium after myoblast transplantation. Rats with myocardial infarction underwent in vivo Programmed Electrical Stimulation (PES) procedures at 1, 2, 3 and 4 weeks after myoblast transplantation. (a) Percentage of Control, Null and Cx43 rats with at least one episode of sustained ventricular tachycardia (VT) during one of the PES procedures (p=0.92, Cox's model). (b) Percentage of rats with first episode of sustained VT between week 1 and 4 after myoblast transplantation.

Figure 5: Ex vivo intramural electrophysiological recordings. Recordings were performed using a sharp, tungsten needle electrode that recorded both local Monophasic Action Potentials (MAPs) and remote electrograms. (a) MAPs from rat tibialis anterior muscle (paced at 250-ms intervals); stars indicate fast spikes of 5 to

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10ms length, typical for skeletal muscle. (b) MAPs from healthy myocardium in the left ventricular free wall. Diamonds indicate typical rat cardiac MAPs of 80ms duration. (c,d) Electrograms within the infarcted myocardium area (triangles), 14 days after intramyocardial transplantation of Null-transduced myoblasts (c) or of Cx43-transduced myoblasts (d). The MAP-needle only recorded the electrograms of remote ventricular activity, as indicated by the triangles. (e,f) MAPs and electrograms from the same infarcted regions as in c and d, but in the transplanted area. Asterisks and triangles indicate MAPs from skeletal muscle cells and electrograms from remote non-infarcted myocardium, respectively. Note the synchrony between MAPs from skeletal muscle cells (asterisks) and ventricular electrograms (triangles) in the Cx43 group. The extracellular complex (triangle) in tracing f was remote. The small deflection prior to the MAP signal (arrows) suggests that myocardial activation preceded myoblast activation, which suggests, but does not prove, that myocytes drove the myoblasts. (g, h, i) enlargement of the recordings a, e and f, respectively. (j) higher enlargement of the recording f/i.

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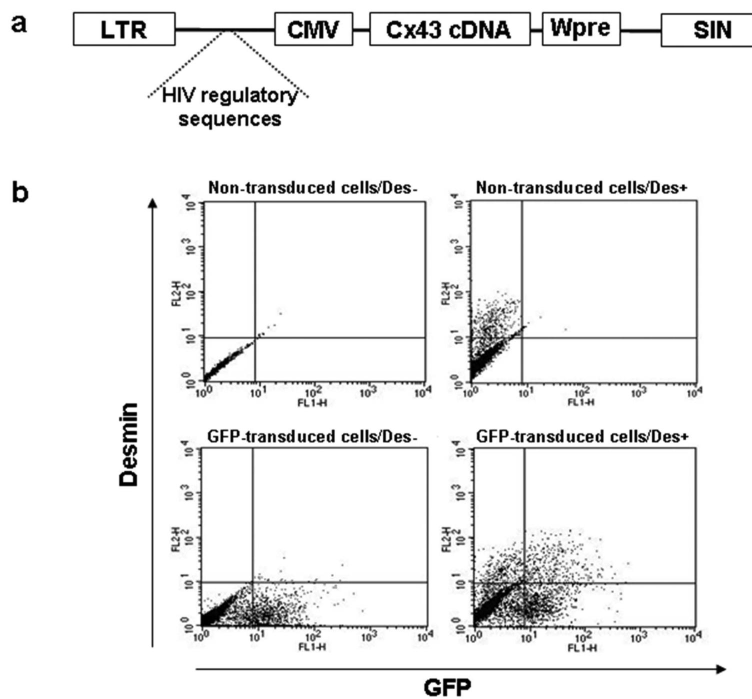
Figure 1



Cx43 transgene expression in myoblasts.
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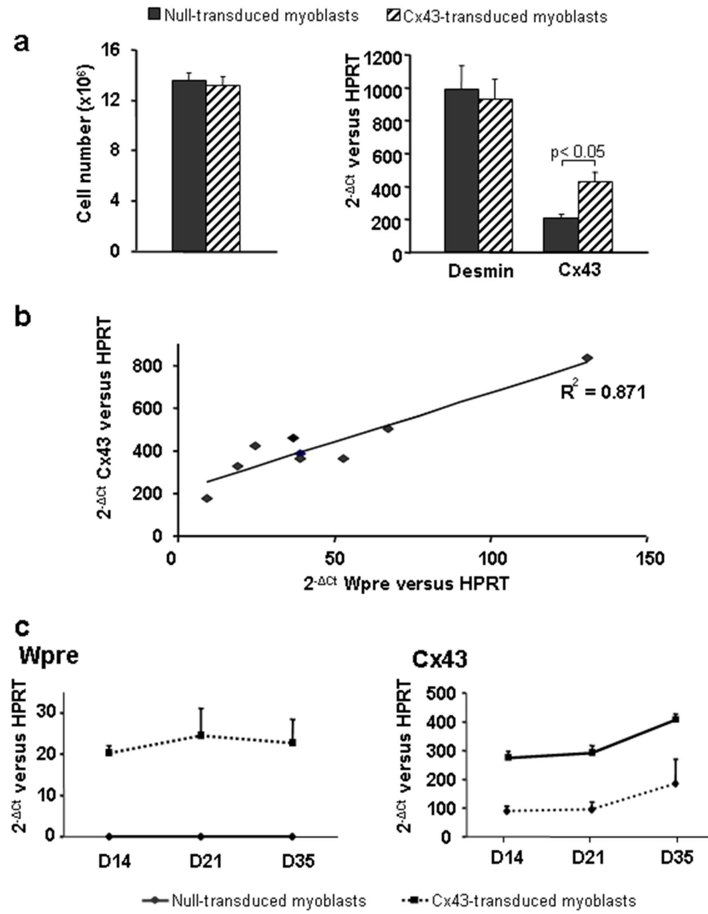

Characterization of myoblast primary cultures after lentivirus vector transduction.
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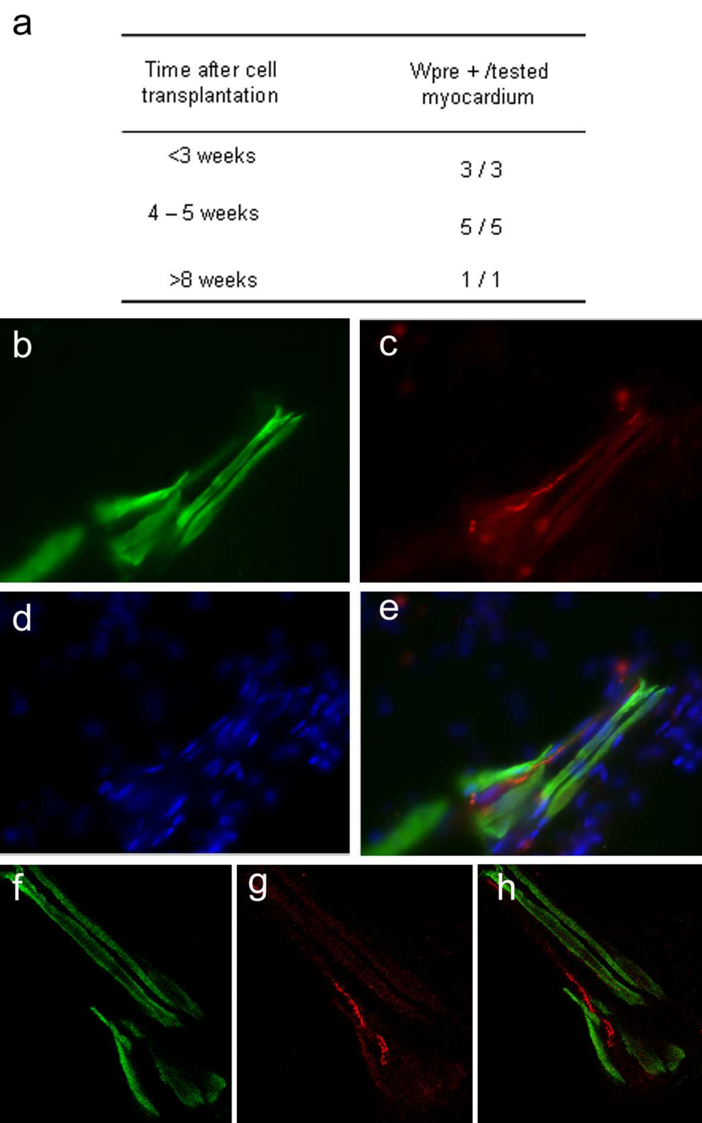
Figure 3



Time after cell transplantation	Wpre + /tested myocardium
<3 weeks	3 / 3
4 – 5 weeks	5 / 5
>8 weeks	1 / 1

Figure 3: In vivo Cx43 and Wpre gene expression, following ex vivo lentivirus vector transduction and intramyocardial transplantation.
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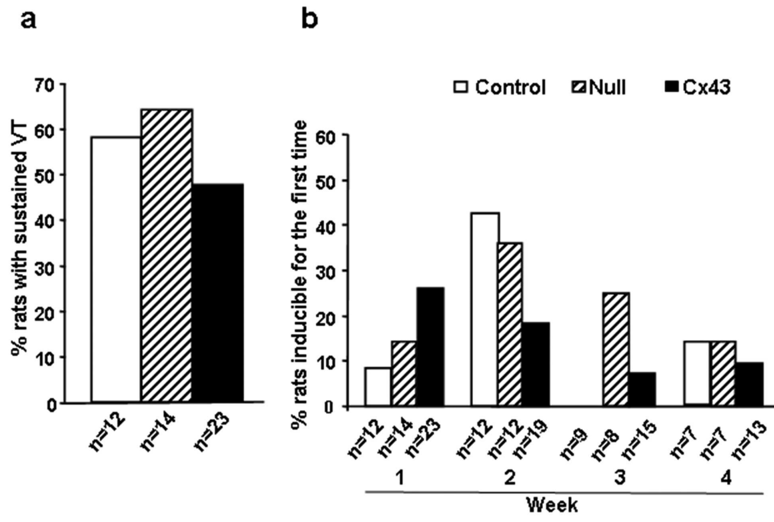

Ventricular hyperexcitability of the myocardium after myoblast transplantation.
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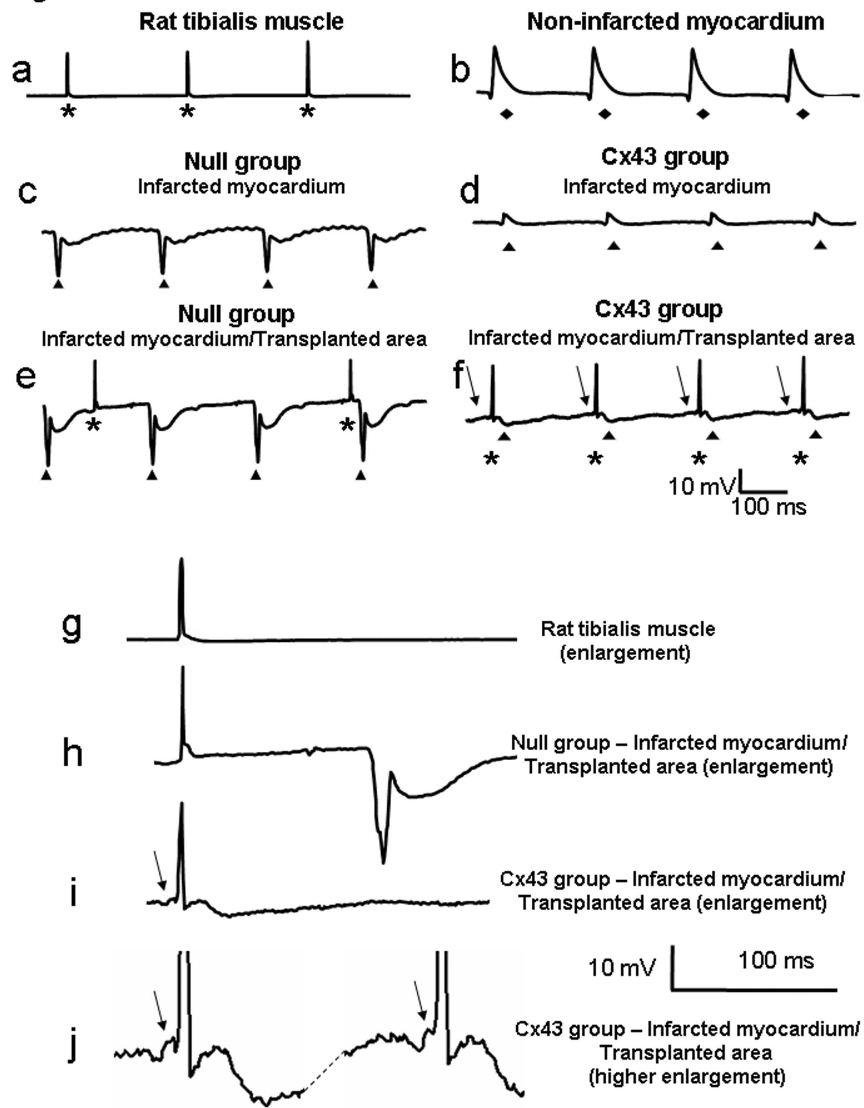

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