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 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 myofibre (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 neighbouring cardiomy-
ocytes [3, 4]. Interestingly, transplanted myotubes are able to con-
tract spontaneously occasionally, but these contractions do not
spread to neighbouring cardiomyocytes [2]. In vitro and ex vivo
studies have shown that a mixture of myotubes and cardiomy-
ocytes without sufficient functional gap junctions results in slower
conduction velocities and greater tissue heterogeneity [5, 6]. Such
heterogeneity predisposes to wave breaks and re-entry, 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 connexin43
(Cx43, the main cardiac gap junction protein) not only eliminates
myoblast pro-arrhythogenic 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 elec-
trical 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 trans-
planted cells and host cardiomyocytes (as demonstrated by Roell
and confirmed in our study), we did not observe any reduction in
post-infarct arrhythmias.

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
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].

Lentivirus vector construction and production

A self-inactivating HIV-derived gene-transfer plasmid (pHR-CMV-Cx43-W-
sin18; Fig. 1a) containing the cDNA for rat Cx43 downstream of the
cytomegalovirus (CMV) promoter elements was kindly provided by
Professor P. Meda (University of Geneva, Switzerland). As controls we
used a lentivirus vector containing the same expression cassette but with-
out the Cx43 cDNA (null) or a lentivirus vector containing the same expres-
ion 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 pri-
mary culture 24 hrs after cell isolation (40 transducing units [TUs] /cell).
Transduced cells were 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 mus-
cle cells) and GFP expression, respectively, in flow cytometry analyses. A
mouse anti-human desmin antibody (D33, Dako-Cytomation, Glostrup,
Denmark), and a second fluorescent antibody (Alexa red antimouse IgG;
Molecular Probes, Invitrogen, Carlsbad, CA, USA) 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 immunolabelling. Analyses
were performed using a FACScalibur instrument (BD Biosciences, San
Jose CA, USA; CellQuestPro software).

RNA isolation

Total RNA was isolated from myoblasts and from myocardial tissue injected
with myoblasts, using a RNeasy Mini kit (QIAGEN, Valencia, CA, USA) 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 cap-
illary electrophoresis on a 2100 Bioanalyzer (Agilent, Massy, France).

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 pre-amplified
using TaqMan®PreAmp Master Mix Kit (Applied Biosystems, Foster City,
CA, USA). Online PCR was performed with the following primers: desmin
(Rn00574732_m1) and Cx43 (Rn01433957_m1). Fluorogenic TaqMan
probes were labelled on the 5’-end with the fluorescent reporter dye 6-car-
boxyfluorescein (FAM®), Applera, Foster City, CA, USA), 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 analysed using the threshold cycle (Ct) relative quantifi-
cation 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

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performing real time PCR on RNA samples that were not reverse transcribed. All data were averaged and then used for the $2^{-\Delta\Delta CT}$ calculation. $2^{-\Delta\Delta CT}$ corresponds to the ratio of each gene expression versus HPRT.

**Immunolabelling**

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

**Ex vivo intramural electrophysiological recordings**

Animals were killed 2 weeks after autologous myoblast transplantation by pentobarbital injection (100 mg/kgip; Pentobarbital sodique™, Cerva Santé Animale). After heparin injection (3750 UI/kgip; Héparine Choay), hearts were harvested for Langendorff perfusion at 37°C with a Krebs modified solution (NaCl, 118.3 mM; KCl, 3.8 mM; MgSO4, 1.2 mM; NaHCO3, 25 mM; KH2PO4, 1.2 mM; glucose, 11.1 mM; CaCl2, 1.25 mM), saturated with carbogen (O2 95% and CO2 5%).

Monophasic action potentials (MAPs) were recorded during sinus rhythm (250 ms) 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-electrode was inserted in the tibialis. Trains of 1 ms stimuli (S1–S1 250 ms) were applied to the nerve and the MAPs were recorded. Because the nerve was stimulated, no pacing artefacts 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/kgip; 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, Rungis, France). 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 Both Fredericia and Bazett formulae ($\text{QTc(F)} = \text{QT}/(\text{RR}/150)^{1/3}$ and $\text{QTc(F)} = \text{QT}/(\text{RR}/150)^{1/2}$, respectively; Table 1). 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...
Table 1 ECG parameters and VERP values

| Time   | Groups                    | RR (ms) | P (ms) | PR (ms) | QRS (ms) | QT (ms) | QTc (B) (ms) | QTc (F) (ms) | VERP (ms) |
|--------|---------------------------|---------|--------|---------|----------|---------|-------------|-------------|-----------|
|        | 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 |
| Week 1 |                           |         |        |         |          |         |             |             |           |
|        | 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 ± 1.2 | 83 ± 1.3 | 83 ± 1.3 | 61 ± 2.4 |

ECG measurements were performed under sinus rhythm. VERP was measured at a basic pacing cycle length (BCL) of 100 ms 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 Frederic formula (F), respectively; VERP, ventricular effective refractory period. All measurements were performed on lead I under general anaesthesia. Results are expressed as mean ± S.E.M.

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 ± S.E.M. and frequencies (expressed as percentages). 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 analysed 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.

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 (Fig. 1a). As controls we used lentivirus vectors containing an empty expression cassette (null) or the 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 (Fig. 1b). The transduction rate of non-myoblast contaminating cells (i.e. GFP+/desmin− cells) was 16.3 ± 4.1% (Fig. 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 (Fig. 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 (Fig. 1a). Wpre gene expression was detected only in Cx43-transduced myoblasts 6 days after Cx43- and null-transduction (Fig. 2b). In Cx43-transduced myoblasts, total Cx43 gene expression level correlated with Wpre expression level (R² = 0.8710; Fig. 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 ≤ 0.05, Fig. 2c) for at least 35 days after Cx43 transduction. 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 three 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-transduced myoblasts up to 35 days after their *in vivo* injection (Fig. 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 (Fig. 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 1), suggesting that lentivirus vector transduction (in null group) or Cx43 overexpression (in Cx43 group) in transplanted myoblasts did not modify ECG parameters.
the percentage of rats that underwent at least one arrhythmia event 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, Fig. 4a). Additionally, the percentage of newly inducible rats did not differ among groups (Fig. 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).

Ex vivo measurements of intramural monophasic action potentials

To evaluate electrical coupling between Cx43-overexpressing myoblasts and host cardiomyocytes, ex vivo intramural 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 seven different sites (one located in the right ventricle and six located in the left ventricle). Control included recordings from rat tibialis muscle (Fig. 5a) and from healthy non-infarcted myocardium (Fig. 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 (Fig. 5c and d). In 5/16 rats MAPs were recorded with average duration of 52.9 ± 4.6 ms, while in 2/16 rats significantly shorter MAPs were recorded of 2.8 ± 1.9 ms (P < 0.001, Fig. 5e,f, h and i [asterisks]). These short MAPs were similar to those recorded in rat tibialis muscle (MAP duration 3.5 ms, Fig. 5a and g), while the longer MAPs recorded in the infarct compared well to MAPs recorded in healthy cardiac muscle (MAP duration 55.1 ± 2.4 ms, P = 0.96, n = 16). No such short MAPs were 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 (Fig. 5e and 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 (Fig. 5f and i). Multiple registrations of 2 sec. 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.

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,
Fig. 4 Ventricular hyperexcitability of the myocardium after myoblast transplantation. Rats with myocardial infarction underwent in vivo programmed electrical stimulation 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.

Fig. 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 10 ms length, typical for skeletal muscle. (b) MAPs from healthy myocardium in the left ventricular free wall. Diamonds indicate typical rat cardiac MAPs of 80 ms 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) and (i).

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 \textit{in vivo} by electrical coupling between skeletal muscle cells and cardiomyocytes that occurred only in myocardium injected with Cx43-transduced myoblasts. Cx43 overexpressions 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 \textit{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 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 because \textit{Wpre}, a regulatory element within the expression cassette, was expressed \textit{in vivo} more than 8 weeks after cell transplantation. In summary, although each vector type lead \textit{in vitro} to significant Cx43 overexpression and gap junction formation, lentivirus vector transduction offered long \textit{in vivo} expression without deleterious effects.

An \textit{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 \textit{in vivo}. Both Roell’s study and ours provided evidence of \textit{in vivo} host cardiomyocyte and transplanted myoblast electrical coupling when myoblasts overexpressed Cx43. In this regard, our results confirm the feasibility of \textit{ex vivo} gene transfer to modify \textit{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, because 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 transduced myoblasts and cardiomyocytes \textit{in vivo} was not possible by changing pacing rate. Conduction is determined by three 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 remodelled 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 \textit{in vivo} study by Roell \textit{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 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 \textit{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, favouring 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, a 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 \textit{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 overex-
pression might have increased in vivo 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 [3, 10, 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 fibres 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 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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