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Repeated implantation of skeletal myoblast in a swine model of chronic myocardial infarction

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Aims Although transplantation of skeletal myoblast (SkM) in models of chronic myocardial infarction (MI) induces an improvement in cardiac function, the limited engraftment remains a major limitation. We analyse in a pre-clinical model whether the sequential transplantation of autologous SkM by percutaneous delivery was associated with increased cell engraftment and functional benefit.

Methods and results Chronically infarcted Goettingen minipigs ($n = 20$) were divided in four groups that received either media control or one, two, or three doses of SkM (mean of 329.6×10^6 cells per dose) at intervals of 6 weeks and were followed for a total of 7 months. At the time of sacrifice, cardiac function was significantly better in animals treated with SkM in comparison with the control group. A significantly greater increase in the ΔLVEF was detected in animals that received three doses vs. a single dose of SkM. A correlation between the total number of transplanted cells and the improvement in LVEF and ΔLVEF was found ($P < 0.05$). Skeletal myoblast transplant was associated with an increase in tissue vasculogenesis and decreased fibrosis (collagen vascular fraction) and these effects were greater in animals receiving three doses of cells.

Conclusion Repeated injection of SkM in a model of chronic MI is feasible and safe and induces a significant improvement in cardiac function.

Keywords Stem cells • Myoblast • Myocardial infarction • Animal model • Swine

Introduction

Since the initial report from Marelli *et al.*[1] in which skeletal myoblast (SkM) were transplanted in a dog model of myocardial infarction (MI), over 70 studies in small and large animal models of MI and several clinical trials in patients with chronic MI have demonstrated the feasibility of this approach (reviewed in 2). These studies have consistently shown that transplanted cells engraft and differentiate into multinucleated myotubes but not into cardiomyocytes[3] and although engraftment is indeed very limited with less than 10% of the cells being detected 72 h after transplantation,[4] functional improvement has been a consistent finding, at least in animal models.[5–8] Among the different parameters affecting the functional outcome after SkM transplant, the total cell dose has been demonstrated to play a significant role in the effect observed after transplantation.[9,10] Importantly, despite a dose–response relationship between the number of SkM transplanted and the functional benefit, the low degree of engraftment and lack of cardiac differentiation indicates that indirect mechanisms such as the release of paracrine growth factors are responsible for the functional improvement.[11]

Injection of SkM has with some exceptions been applied directly into the myocardium.[10,12–16] Although this system has the advantage of injecting the cells into the ischaemic area, from the clinical

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point of view it requires major surgery which is associated with significant morbidity. We have recently demonstrated that percutaneous delivery of SkM in a swine model of chronic MI induces a similar degree of functional improvement that direct surgical injection[17] and at the same time allows for sequential injection of cells with very limited morbidity. In this context, we decided to examine the long-term effect of sequential percutaneous endomyocardial delivery of autologous SkM in a chronic model of MI in swine.

Methods

Animal preparation and study design

Adult Goettingen pigs procured from our breeding centre were maintained in the animal facilities of CIFA (GLP accredited centre at the University of Navarra, Spain) in accordance with the *Guide For The Care And Use Of Laboratory Animals* published by US National Institutes of Health (NIH publication no. 85-23, revised 1996).[18] In each procedure, animals were pre-medicated with atropine i.m. (0.05 mg/kg) and a combination of ketamine (10 mg/kg) and azaperon (2 mg/kg). After sedation, animals received 3 mg/kg of etomidate i.v. and were endotracheally intubated and mechanically ventilated with supplemental oxygen. During surgery, anaesthesia was maintained with a combination of isofluorane and fentanyl (0.01 mg/kg/h i.v.). At the end of the procedure and after extubation, all animals received the non-steroidal analgesic ketoprofen (3 mg/kg/24 h i.m.) for 3 days as well as antibiotics (amoxicillin 7 mg/kg/24 h) for 5 days.

Myocardial infarction was provoked as previously described.[17] Briefly, an introducer sheath was placed by dissection in the left carotid artery and heparin (200 μ/kg) was intravenously infused. Under fluoroscopic guidance, a 8fr guiding catheter was positioned in the left coronary ostium and MI was induced by selectively delivering a vascular embolization coil (via a microcatheter advanced through the guiding catheter; 3 and 4 mm VortX coils (Boston scientific/target, Natick, MA, USA) to the intermediate branch or first or second marginal artery. Coronary occlusion occurred between 15 and 20 min after coil placement, as demonstrated by coronary angiography and ST segment changes in the electrocardiogram. Lidocain 2 mg/kg and advanced life support were used when needed. Following artery occlusion, the delivery catheter was removed, the carotid artery ligated, and the cut down site sutured.

A summary of the study design is depicted in *Figure 1*. After MI, Goettingen pigs were divided into four groups according to the number of myoblasts doses. Group 1 (control group) received three doses of culture medium while groups 2, 3, and 4 received, respectively, one dosage of myoblasts and two of media, two dosages of myoblasts and one of media, and three dosages of myoblasts. Animals included in the study were followed for 2 months and only those with an EF below 50% were finally included in the study. Implantation procedures were performed every 6 weeks and sacrifice 10 weeks after the last injection of myoblasts or media.

For media or cell transplantation, a percutaneous access through dissection of the femoral or carotid artery was performed and intramyocardially delivered under simultaneous fluoroscopic and echocardiographic guidance using the Myocath® catheter (Bioheart, FL, USA). Multiple injections (average of 20 per time point of 0.25–0.5 mL per injection) were performed in each animal in and around the infarct area.

Cell culture and labelling

Muscle biopsies (5–10 g) were obtained from the pig limb and cut into small pieces, stripped of connective tissue, minced and digested as previously described.[17] Cells were grown in Ham-F12 media (GIBCO-BRL, Spain) supplemented with 20% foetal calf serum, 10 ng/mL b-FGF (Sigma, Germany), and 1% penicillin/streptomycin (GIBCO-BRL, Spain). Seventy two hours before implantation, cells were labelled with 5 μg/mL BrdU. Assessment of myoblast content was performed as described.[19] At the time of harvest, cells were thoroughly washed to eliminate any source of foetal calf serum and BrdU and re-suspended in media at a concentration of 30–50 × 10^6 cells/mL. Myoblast purity was measured by flow cytometry after staining with antibodies against CD56 (BD PharMingen, San Diego, CA, USA).

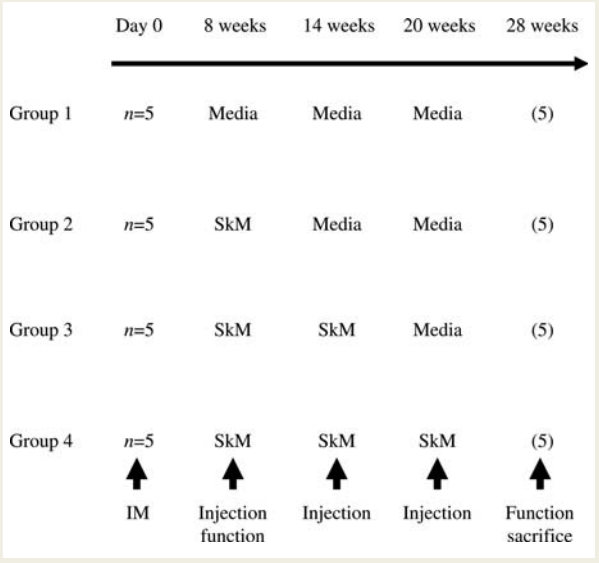

Figure 1 Study design. IM, myocardial infarction; SkM, skeletal myoblast.

Assessment of ventricular function and arrhythmias

Animals under general anaesthesia were placed in the left lateral decubitus position and transthoracic two-dimensional echocardiography was performed using a Sonos 4500 ultrasound system (Philips) and a 3 MHz linear array transducer. Left ventricular remodelling was assessed by measuring end-systolic and end-diastolic volumes and diameters, according to the American Society of Echocardiography and adjusted for animal weight at the time of analysis. Assessment of diastolic function was performed by mitral filling pulse Doppler and mitral annulus tisular Doppler. Left ventricular ejection fraction was determined in paraesternal short axis.[20] Echocardiogram was performed at baseline (before MI), 2 months after MI, before each implantation procedure, and at the time of sacrifice by two investigators blinded to the type of treatment. Major arrhythmic events (ventricular tachycardia and ventricular fibrillation) during procedures were registered in all groups.

Histological immunostaining and analysis

At the end of the experimental protocol, animals were sacrificed with pentobarbital and a saturated solution of potassium chloride and the heart excised. Explanted hearts were fixed in formalin and paraffin embedded for histological analysis. Location and extent of MI were visually assessed. Sampling of the tissues consisted of scar surrounded by a ring of viable myocardium. Formalin-fixed tissues were embedded

Table 1 Characteristics of the groups (mean and SD)

	Group 1	Group 2	Group 3	Group 4
N	5	5	5	5
Weight (baseline)	47.4 (16.7)	39.5 (8.5)	46.2 (8.8)	48.5 (11.8)
Weight (sacrificed)	75.1 (17.9)	88.2 (17.2)	97.6 (9.3)	96.7 (8.1)
Heart rate (baseline)	76.5 (11.2)	85.2 (17.3)	79.4 (9.84)	77.2 (10.3)
Weight of biopsy	NA	6.25 (2.02)	8.25 (1.36)	10.08 (4.89)
Cells injected (10^6)	NA	344 (146)	617 (113)	1246 (314)
Number of injections	60	59	58	58

in paraffin and 5 μm sections were stained with haematoxylin-eosin and Gallego Tricromic for qualitative assessment and with the appropriate antibodies for the detection of transplanted cells. For determination of cellular engraftment, immunostaining for BrDU was performed in serial slides from the different heart blocks. Counting was performed in some of the positive blocks and values extrapolated for all the positive ones. In each block, $BrDU^+$ cells were counted in three random fields per section and the average number of BrDU cells per field, multiplied by the total number of positive fields present in each slide. Counting was performed in serial slides from the first to the last slide with engrafted cells. Analysis was performed on 5 hearts per group. Also, the infarct area repopulated by the myoblasts was determined on the same sections, by quantification of the area occupied by the positive cells and the total infarcted area, with an interactive computerized analysis system (AxioVision 4.6, Zeiss, Germany).

Antibodies against BrdU (BrdU-DNAse kit, Amersham), fast myosin-heavy chain (MY-32 clone, 1/200, Zymed Germany), smooth muscle actin-PE (1/1000, Dakocytomation, Spain), and a biotinilated BSL-1 Lectin (1/100, Vector, Spain) were used. Primary antibodies were followed by EnVision System Labelled Polymer Horse Radish Peroxidase (DakoCytomation) and FITC labelled secondary antibody (for BrdU staining) and diaminobenzidine (DakoCytomation) as a chromogen (for Lectin staining).

Vasculogenesis (arterioles and arteries) was evaluated in paraffin-embedded sections by counting the size of areas occupied by smooth muscle coated vessels in the infarct myocardium using antibodies against smooth muscle actin for immunostaining. Vascular density was expressed as the percentage of area occupied by smooth muscle coated blood vessels per high power field (15 sections were examined per heart). Images were processed by Nikon Eclipse E800 microscope. Collagen content was determined in Sirius red stained slides as described previously,[17] measured with an interactive computerized image analysis system (Optimas 5.2 colour image analysis, Germany) and expressed as a percentage of the area of the field occupied by collagen [collagen volume fraction (CVF)]. A mean of 15 fields captured with X20 objective was calculated.

Statistical analysis

Statistical analysis was performed with the Windows SPSS 15.0 software package. Comparisons were performed using the paired and unpaired *t*-test when appropriate. Normality was demonstrated with the Shapiro–Wilk and Kolmogorov–Smirnov normality test. In case of non-normal distribution, Wilcoxon test was used. Comparisons for repeated measurements were performed with ANOVA or Friedman test followed by *pos hoc* analysis using Tuckey-b. Lineal regression analysis was performed using Pearson correlation coefficients. Descriptive analysis is presented as mean ($\pm$SD) for normally distributed values. Statistical significance was achieved if *P*-values were <0.05.

Results

Myocardial infarction was induced in a total of 30 animals of which 5 died due to complications during the procedure and 5 did not fulfil the criteria (EF$<$50%) 2 months after infarction. Thus, a total of 20 pigs were included in the study divided in four groups according to the number of doses of cells administered. Characteristics of the groups are shown in *Table 1*. The mean number of cells implanted per procedure was 329.6×10^6 (32.31) cells while the total number of cells received was 344×10^6 (73.2), 617×10^6 (50.9), and 1246×10^6 (119.1) in groups 2, 3, and 4, respectively (mean $\pm$ SD). The mean percentage of $CD56^+$ was 94.7 $\pm$ 4.9% with no differences among the different groups. The total volume of injection was 10 mL per procedure. Injection of the cells or media was not associated with any death while the implantation procedure was associated with ventricular arrhythmias in some cases (10% of the procedures) irrespective of whether the animals received cells or media.

Functional studies

Left ventricular ejection fraction at baseline was 73.33% (1.23) (mean $\pm$ SD) and decreased to 40.31% (1.69), 2 months after MI ($P < 0.001$). Pre-transplant functional values were equivalent in the different groups of animals (*Table 2*). At the time of sacrifice (7 moths post-MI), animals treated with SkM showed an increase in LVEF that was statistically significant in groups 3 and 4 (two or three injections of SkM) in comparison with their pre-transplant value (*Figure 2A*). When we compared the ΔLVEF among the different groups, we observed that animals receiving media alone showed a further derangement in LVEF while animals treated with SkM showed an improvement in LVEF that was statistically significantly in comparison with control animals. Furthermore, the ΔLVEF in animals receiving three doses of cells was significantly greater than in the animals that were treated with only one dosage of SkM suggesting a greater functional effect associated with the number of injections (*Figure 2B*).

A reduction in the LV end-systolic diameter was also observed in animals treated with SkM in comparison with pre-treatment value. These differences were significant for groups 3 and 4 and

Table 2 Echocardiographic studies (mean and SD)

	Pre-transplant	Sacrificed	*P*-value
LVEF (%)			
Group 1	40.6 (9.6)	37.7 (5.9)	0.880
Group 2	45.1 (6.1)	56.2 (4.6)	0.113
Group 3	41.3 (6.4)	56.4 (8.2)	0.02
Group 4	39.3 (6.5)	65.6 (5.9)	0.01
LVEDD (mm/g)			
Group 1	0.055 (0.002)	0.061 (0.001)	0.104
Group 2	0.058 (0.011)	0.052 (0.009)	0.078
Group 3	0.051 (0.009)	0.046 (0.001)	0.234
Group 4	0.054 (0.003)	0.043 (0.004)	0.123
LVESD (mm/g)			
Group 1	0.046 (0.001)	0.051 (0.001)	0.109
Group 2	0.043 (0.007)	0.036 (0.007)	0.073
Group 3	0.040 (0.006)	0.032 (0.003)	0.035
Group 4	0.044 (0.004)	0.022 (0.002)	0.030
E/A ratio			
Group 1	1.43 (0.1)	1.56 (0.1)	0.138
Group 2	1.36 (0.4)	0.96 (0.5)	0.144
Group 3	1.51 (0.3)	1.57 (0.2)	0.893
Group 4	1.51 (0.2)	1.29 (0.3)	0.063
E/E′ ratio			
Group 1	5.11 (0.7)	4.91 (0.6)	0.282
Group 2	5.63 (0.6)	4.65 (0.4)	0.109
Group 3	4.71 (0.6)	4.09 (0.5)	0.273
Group 4	4.69 (0.8)	3.99 (0.7)	0.089

did not reach a statistically significant difference in group 2 (*Table 2*), suggesting an improvement of the remodelling at the expense of the systolic function. In fact, other parameters that assess diastolic function such as E/A and E/E′ ratio did not show any significant differences.

Cell engraftment

Next, we analysed the differences in cell engraftment between the different groups of animals. As shown in *Figure 3B*, the number of engrafted cells was significantly higher in the groups of animals receiving two and three doses of cells vs. a single dose of SkM ($P < 0.05$), although the percentage of cells that engrafted was similar among the groups; 0.69% (0.08), 0.52% (0.07), and 0.58% (0.12) of the cells transplanted were found 7 months after transplant. These percentages of cell engraftment are in accordance with previous reports[4,21] demonstrating a very low engraftment only a few days after transplantation. Interestingly, there was a statistically significant correlation between the LVEF at the time of sacrifice and the number of cells transplanted ($P = 0.02$) and engrafted ($P = 0.002$) (*Figure 3*).

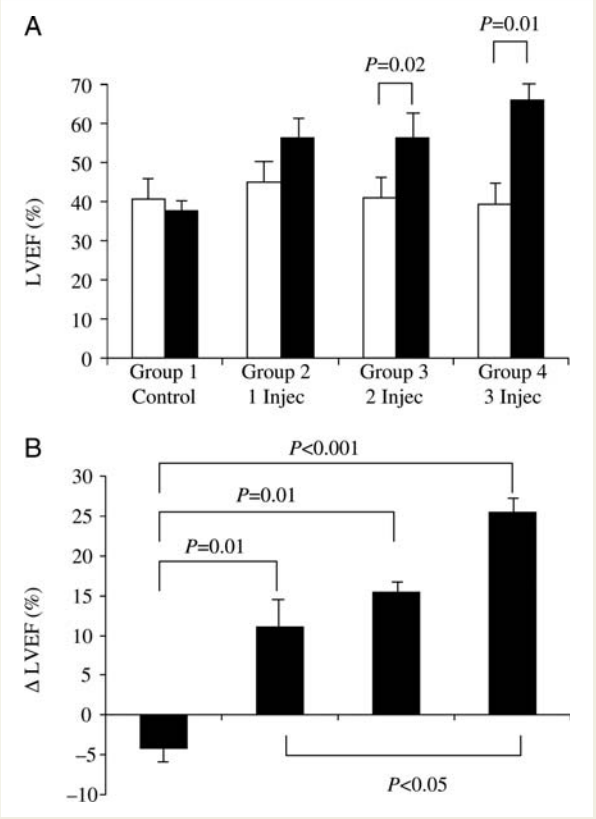

Figure 2 Echocardiographic assessment. (*A*) LVEF was assessed at baseline (white columns) and sacrificed (black columns) in each treatment group. (*B*) Percentage of change in LVEF between baseline and sacrificed in each treatment group. Results represent the mean ± SD.

In vivo differentiation of transplanted SkM was assessed by immunofluorescence. Among the BrdU positive cells, co-localization of smooth muscle-actin was demonstrated with labelled cells forming vessel-like structures suggesting the potential of SkM to differentiate into smooth muscle cells (*Figure 4*). None of the transplanted cells expressed markers of endothelial cells (lectin) (*Figure 4*) or cardiomyocytes (data not shown). The number of transplanted cells that contributed to the formation of new vessels (double positive for BrdU and SM-actin) was below 5%.

Repeated implantation of skeletal myoblast induces vasculogenesis and reduced fibrosis

Finally, we analysed the effect of autologous SkM transplantation on vasculogenesis and fibrosis in the area of the implant (infarct area). The area of SM-actin positive blood vessels in the infarct zone was significantly higher in animals transplanted with SkM myoblasts than in the control group (ANOVA $P < 0.001$), although the differences in vasculogenesis were not statistically significant among groups treated with SkM (*Figure 5*). Similarly, we compared the degree of fibrosis in the infarct zone. Quantitative analysis of hearts from animals receiving SkM demonstrated significantly reduced fibrosis in comparison with control animals (ANOVA

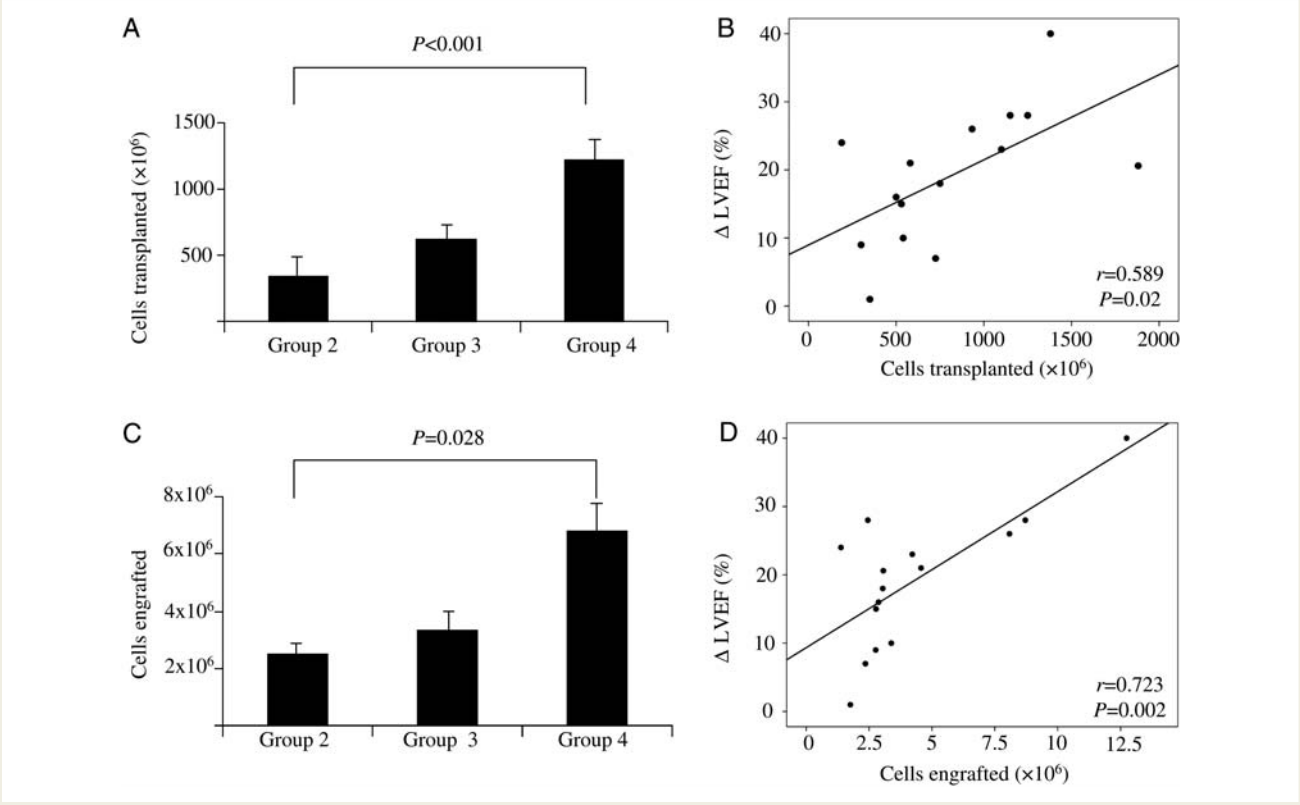

Figure 3 Cell engraftment. (*A*) Number of cells transplanted. (*B*) Correlation between the number of cells transplanted and the percentage of change in LVEF. (*C*) Number of cells engrafted at time of sacrifice. (*D*) Correlation between the number of cells engrafted and the percentage of change in LVEF. (A and C) Results represent the mean ± SD.

$P < 0.001$) which was significantly lower in animals receiving two and three doses of cells in comparison with animals that received only single dose of SkM $P < 0.02$. The CVF was 74.47% (5.7) in the control group and 71.06% (2.9), 52.32% (4.7), and 49.94% (4.7) in groups 2, 3, and 4, respectively (*Figure 6*). Finally, when we analysed the relation between LVEF and degree of fibrosis and vasculogenesis, we observed a negative correlation between LVEF and the total area occupied with collagen (CVF) ($r = -0.594$; $P = 0.02$) and a positive correlation between LVEF and the area occupied by smooth muscle-coated vessels ($r = 0.798$; $P < 0.0001$) (*Figures 5* and *6*) suggesting that SkM may contribute to improvement in cardiac function by decreasing fibrosis and increasing the vasculogenesis in the infarcted tissue.

Discussion

The concept underlying cell therapy represents the replacement of a pool of dead cardiomyocytes by a new pool of functional cells regardless of whether the graft derives from an endogenous reservoir of tissue-resident cardiac stem cells or an exogenous supply of cells acting as precursors or surrogates of cardiomyocytes.[22] The results obtained in pre-clinical models as well as clinical studies[23–27] and the difficulties to induce a true and meaningful cardiac differentiation have currently shifted this paradigm into the possibility that the use of (stem) cells in patients with cardiac diseases could improve cardiac function by mechanisms such as the release of growth factors that stimulate proliferation of host cells or modify the remodelling process alleviating the adverse effects of post-infarction dysfunction.[22,28] Regardless of the mechanisms, the low engraftment and survival of transplanted cells indicate that strategies aimed to improve engraftment could have a great impact in the efficacy of cell therapy.[21,29–31]

In our study, we hypothesized that sequential transplantation of SkM could improve the functional effect by allowing the delivery of a higher number of cells and thus, presumably increasing the engraftment and survival of cells. Our results in a representative pre-clinical model suggest that in fact administration of sequential doses of SkM may result in a modest but significant increase in the functional benefit. Although the total number of cells that remain in the heart was increased in the groups of animals receiving more doses of cells, the percentage of engrafted cells was not significantly modified (*Figure 3*). These results are in accordance with previous studies supporting a direct relationship between cell dose and functional benefit.[9,10] A similar study has recently been published in which 18×10^6 of SkM were transplanted in a rat model of chronic MI either as a single injection or divided in three doses at 2 week intervals and compared with animals treated with culture media.[32] The results suggested an increase in engraftment associated with the group of rats receiving sequential injections of cells and an improvement in cardiac function

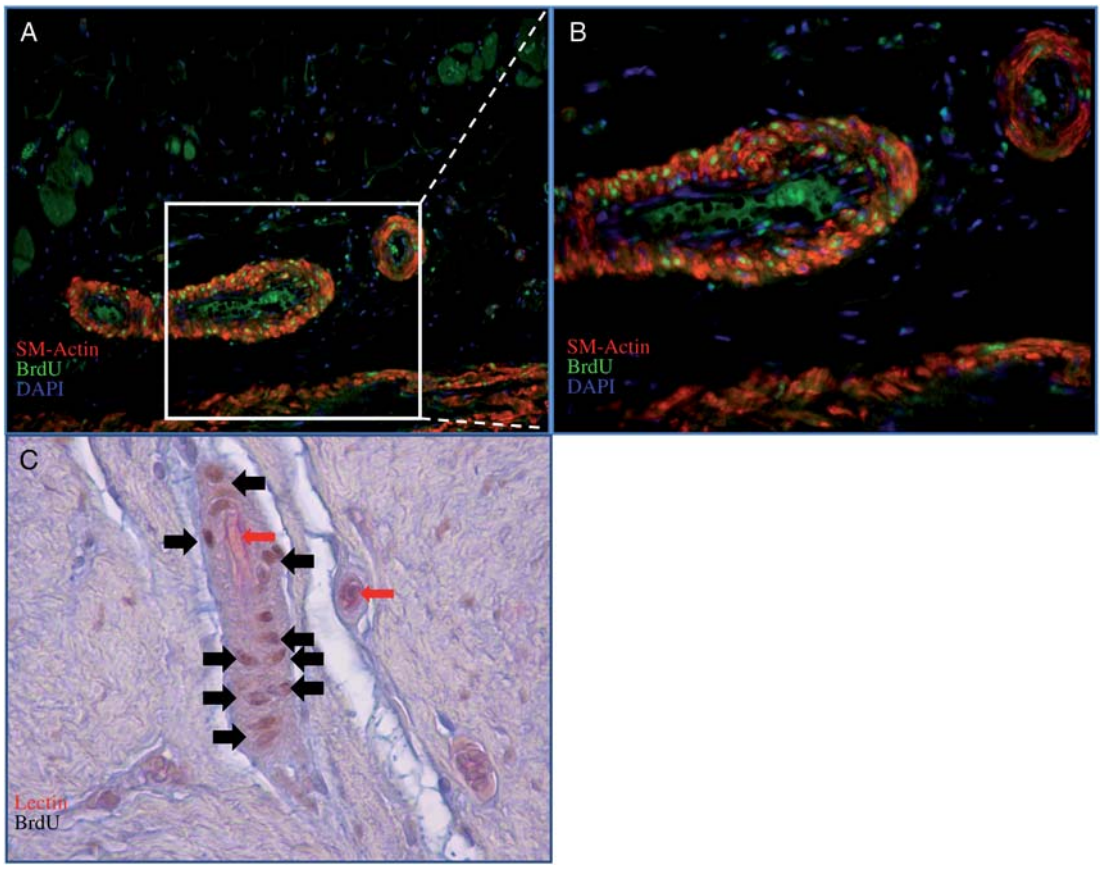

Figure 4 Myoblast detection in heart sections of animals transplanted with autologous skeletal myoblast 7 months after transplantation. (*A*) Immunoflourescence against BrdU labelled cells (green) and SM-actin (red). DAPI was used for nuclear staining (blue) (scale bar = 25 μm). Green auto-fluorescence within the vessel corresponds to unspecific labelling of erythrocytes. (*B*) Insert at higher magnification (scale bar = 12 μm). Double immunohistochemistry against BrdU positive cells (black arrows) and lectin positive cells (red arrows) (scale bar = 10 μm).

between control animals and cell-treated animals. However, no significant difference was found between animals treated with single dose or three doses of cells.[32] The use of a swine model as well as the approach employed to deliver the cells makes our study particularly relevant from the clinical perspective.

Being the goal of the study to determine the effect of sequential injection in comparison with a single cell dose, we did not include a group of animals treated with the same cell dose as a single bolus, which must be acknowledged as a potential caveat. Nevertheless, injection of large numbers of cells may predispose to the development of arrhythmias[33] or may require large volumes which may not be feasible in a damaged heart with a thin myocardium making the sequential delivery of more cells attractive from a clinical view. In fact, *in vitro* studies have shown that neonatal cardiomyocytes cocultured with SkM suffered a decrease of the conduction velocity together with arrhythmic contractions, in a cell dose-dependent manner.[33]

If direct contribution of cells is not responsible for the results obtained in cell therapy studies, but rather paracrine mechanisms are likely responsible then, a transient effect could be expected. In that sense, studies with long-term follow-up are particularly important. We followed the animals for over 7 months and observed a sustained benefit in heart function as well as a persistence of engrafted cells, albeit at very low level. Furthermore, the degree of engraftment was directly associated with the increase in LVEF which stresses the importance of strategies directed to improve engraftment at long term. In our previous study using the same model, a single injection of SkM was associated with a significant improvement in LVEF 3 months post-transplant.[17] However, as shown in *Figure 1*, the increase in LVEF after a single injection was not statistically significant in comparison with the pre-transplant value (*Figure 2*). These differences between both studies could be explained by the fact that in the current study, the follow-up was longer (7 months). A decrease in the functional effect after stem cell transplant with longer follow-up has been described also in clinical studies.[34,35] It is important to point out that the characteristics of our animal model (maximum adult weight does not surpass 80–90 kg) allowed for the long-term follow-up.

We have previously reported that the paracrine activity of the SkM is due to the production of angiogenic cytokines together with factors involved in the modulation of the composition of

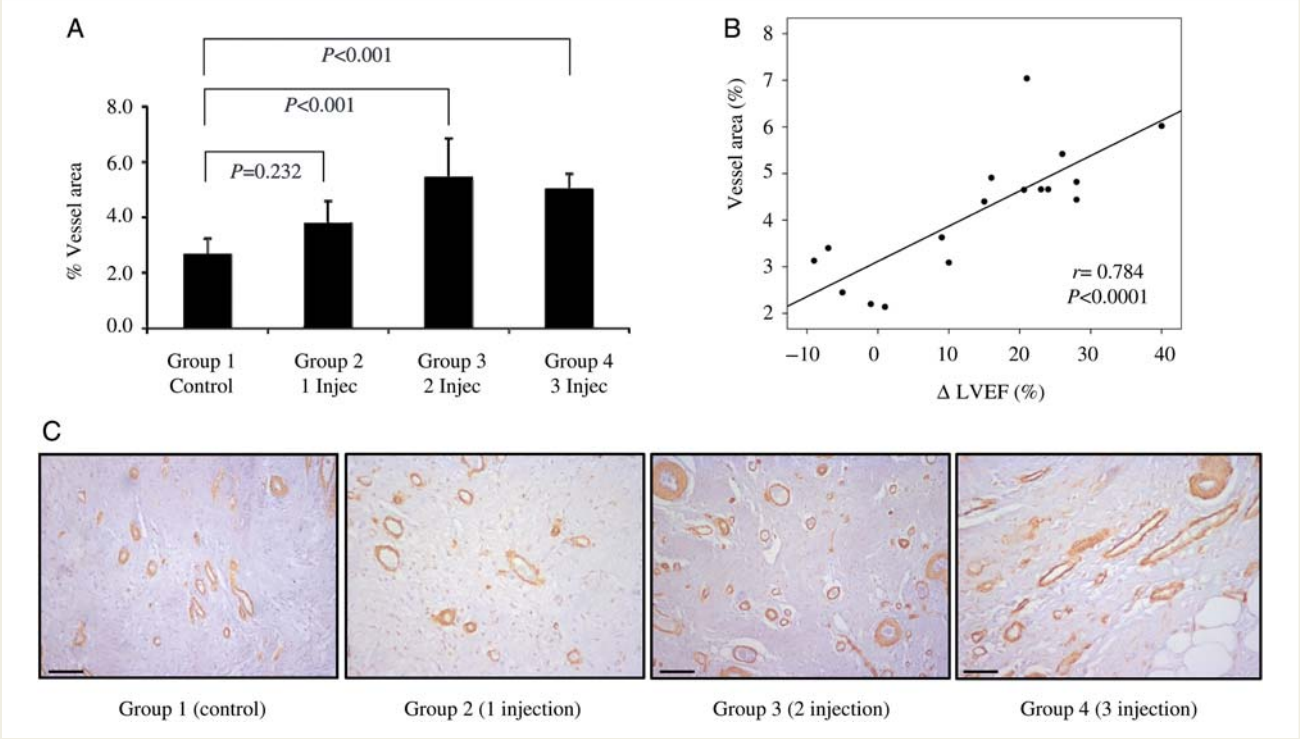

Figure 5 Skeletal myoblast transplant is associated with increased vasculogenesis. (*A*) Percentage of area occupied by smooth muscle coated blood vessels in infarct area. (*B*) Correlation between the areas occupied by smooth muscle coated vessels and the increase in LVEF in each group of animals. (*C*) Representative infarct area stained with antibodies against smooth muscle actin (brown) in sections of each group of treated animals (scale bar = 100 μm). Data in (A) represent the mean ± SD.

the extracellular matrix.[11] In this study, we examined indirectly in the pig model, the mechanisms responsible for the functional improvement. Changes in the remodelling process—mainly in the degree of fibrosis—and the increase in angio-vasculogenesis are likely players. As shown in *Figures 5* and *6*, the effect on vasculogenesis and reduced fibrosis was greater in animals receiving three doses of cells and the increase in LVEF was inversely correlated with CVF and directly correlated with the degree of vasculogenesis. The contribution of cells toward increased vasculogenesis and decrease in CVF is not specific of SkM as it has also been described for other type of cells consistent with the release of growth factors such as IGF, VEGF, HGF, or TGF-β[36–40] as well as matrix metalloproteinases or their inhibitors among others.[11] If that is the case, the sequential delivery of cells at adequate intervals could further stimulate tissue repair.

The presence of cells that co-express BrdU and SM-actin and contribute to the formation of vessels in the infarct tissue suggest that a percentage of them contribute directly to the formation of new vessels. Two experimental studies have reported that mouse skeletal muscle harboured a population of cells which, despite their skeletal muscle origin and different nomenclature (skeletal precursors of cardiomyocytes[41] and muscle-derived stem cells[42,43]), have the capacity to acquire some key phenotypic features of cardiac cells and vascular cells. In animal models of MI, these myoendothelial-cardiac progenitor cells yielded greater engraftment rates and better functional outcomes than unpurified SkMs.[41,44] We cannot rule out that the observed contribution to vascular phenotypes is due to a small percentage of these progenitors present in our cultures as could be suggested by the presence of the CD56 antigen in the transplanted cells. Further phenotypic analysis including CD34 and CD144 antigens could help identify different populations of progenitors, but the lack of reliable antibodies specific for pig cells impose difficulties to the analysis.

Finally, the potential for arrhythmias associated with SkM transplant remained an unsettled issue.[2,45] The results of the single randomized clinical trial with SkM in patients with chronic MI has shed some light suggesting that even though an increase in arrhythmogenic events may be observed early after transplant, the careful monitoring based on the use of ICD in every patient does not indicate that transplantation of SkM is associated with an increase in severe arrhythmias.[10] In our clinical phase I/II trial, the incidence of arrhythmias was not above what it is expected for these types of patients[14] and we did not find an increased incidence of arrhythmias in our previous animal study.[17] In the current study, we did not specifically address this issue but overall, size effects were equivalent in the different groups.

In conclusion, our results indicate that repeated injections of SkM are feasible in a pre-clinical model and provide a long-term functional improvement. The effects on tissue vasculogenesis and fibrosis further confirm previous findings that support the potential of SkM to improve cardiac function by modifying the process of tissue remodelling. Nevertheless, the correlation between the

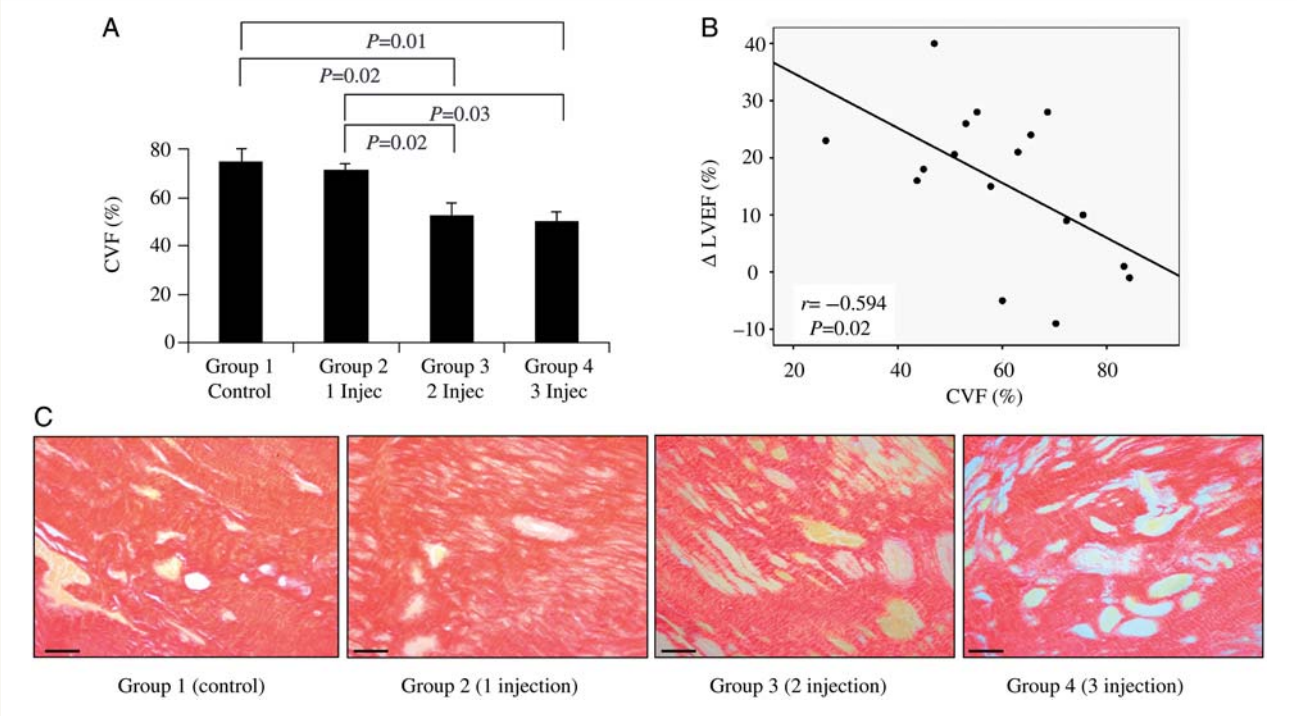

Figure 6 Transplantation of skeletal myoblast is associated with decreased fibrosis. (*A*) The area occupied by collagen [collagen volume fraction (CVF)] was assessed by picrosirius red staining in the infarcted tissue of the different groups of animals. Data represent the mean ± SD. (*B*) Correlation between the decreased in CVF and the increase in LVEF in each group of animals. (*C*) Representative picrosirius red-stained sections of infarct area in each group of treated animals. Collagen stains red and cardiac muscle stains yellow. Scale bar = 10 μm.

number of cells and the functional results suggest that this may be a useful approach in clinical trials that could be combined with other strategies aimed to increase cell engraftment and survival and eventually led us into true cardiac regeneration which should be the ultimate goal of cell therapy.

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