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A comparison between percutaneous and surgical transplantation of autologous skeletal myoblasts in a swine model of chronic myocardial infarction☆

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Abstract

Objective: Our aim was to compare the efficacy of surgical versus percutaneous administration of skeletal myoblasts (SkM) in a swine model of chronic myocardial infarction and to determine the mechanism(s) involved in their beneficial effect.
Methods: Two months after induction of myocardial infarction (MI), Goettingen miniature pigs underwent autologous SkM transplant either by direct surgical injection ($n=6$) or percutaneous access and intramyocardial delivery under fluoroscopic and echocardiographic guidance ($n=6$). Control animals received media alone ($n=4$). Functional analysis was performed by 2D echocardiography. Myoblast engraftment, *in vivo* cell differentiation, vessel formation, fibrosis, and the ratio between collagen type I/III deposition were analyzed in the infarct (IA) and non-infarct area (NIA) by immunohistochemistry.
Results: Animals received a median of $407.55\pm115\text{x}10^6$ BrdU-labeled autologous SkM. Myoblast transplant was associated with a statistically significant increase in left ventricular ejection fraction ($p<0.01$), increased vasculogenesis and decreased fibrosis ($p<0.05$), and reduced collagen type I/III ratio in the IA and NIA areas as compared with control animals. No differences were found between groups receiving SkM by percutaneous or surgical access.
Conclusions: Our results indicate that increased vasculogenesis and changes in matrix remodeling with decreased fibrosis are associated with the beneficial effect of SkM transplant in chronic MI. The equivalent benefit observed from surgical and percutaneous delivery has important clinical implications.

Keywords: Skeletal myoblasts; Myocardial infarction; Vasculogenesis; Fibrosis; Cell therapy

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1. Introduction

Myocardial infarction (MI) is associated with loss and dysfunction of cardiomyocytes that leads to heart failure and death. The inability of resident cardiac stem cells to contribute significantly to repair lost cardiomyocytes has prompted the search for other sources of stem cells with the potential to differentiate into cardiac muscle (reviewed in [1]). The widest experience in cell therapy for cardiac regeneration has been obtained with SkM [2]. Studies in small [3–5] and large animal models [6,7] of myocardial infarction over the last 10 years have indicated that SkM engraft into the myocardium and differentiate into myotubes, contributing to the improvement in cardiac function. These preclinical studies have led to the development of the first clinical trials in patients with cardiac failure, which have further supported the suggestion that autologous myoblast transplantation in patients with chronic MI has a potential beneficial effect [8–11].

While most studies have used the surgical approach for transplantation of myoblast in association with CABG surgery [8,10,12], a recent report has demonstrated that SkM can be safely administered using a percutaneous access system with guided electromechanical catheter [11]. This approach allows better assessment of the effect derived from SkM transplant and also prevents the need for major surgery. However, it is unclear whether surgical and percutaneous delivery result in similar benefits in large animal models of MI and thus have the same potential for human application.

It is generally accepted that SkM have a very limited capacity, if any, to acquire characteristics of cardiac muscle [13–15], and the mechanism by which they contribute to cardiac function is currently unknown. Several studies have suggested that the release of cytokines [16] or immune mediated mechanisms [17] may be contributing factors. Furthermore, the significant degree of cell attrition observed after SkM transplant with less than 5% of the cells remaining one week after transplant suggests that a direct benefit derived from the cells is not the most likely explanation [18].

In our study we sought to understand the mechanism(s) involved in the improvement in cardiac function observed after SkM transplant, by examining their role in the process of vasculogenesis and fibrosis that occurs after MI. From a clinical perspective we analyzed whether percutaneous and surgical delivery of SkM is associated with a similar benefit in cardiac function.

2. Materials and methods

2.1. Animal preparation

In this study, adult Goettingen pigs (weighed 35–50 kg) procured from our breeding center were maintained in the animal facilities of CIFA (GLP accredited center 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) [19]. In each procedure, animals were premedicated 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 supplementary oxygen. During surgery, anesthesia was maintained with a combination of isofluorane and fentanyl (0.01 mg/kg/h i.v.). At the end of the procedure, 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.

2.2. Myocardial infarction

All the pigs received a single dose of Heparin (1.5 mg/kg) before the procedure commenced. Then an introducer sheath was placed in the left carotid artery. Under fluoroscopic guidance, a guiding catheter was positioned in the left coronary ostium and myocardial infarction was induced by selectively delivering (via a microcatheter advanced through the guiding catheter) a vascular embolization coil (3- and 4-mm Vortx coils, Boston Scientific/Target, Natick, MA, USA) to the intermediate branch of first or second marginal artery. Coronary occlusion occurred between 15 and 20 min after coil placement, as demonstrated by coronary angiography and ECG ST-segment changes. Following artery occlusion, the delivery catheter was removed, the carotid artery ligated, and the cut down site was closed with sutures.

2.3. Autologous myoblast transplantation

Eight weeks after the infarct, animals underwent transplantation of autologous myoblasts either by direct surgical intramyocardial injection with an ophthalmologic needle (Steriseal Ophthalmic cannula 23G, Maersk Medical Ltd. Redditch, B98 9NL GB) through a left intercostal incision ($n=6$), or by percutaneous access through the femoral artery ($n=6$) and intramyocardial delivery of multiple injections under simultaneous fluoroscopic and echocardiographic guidance using the Myocath® (Bioheart, Florida) injection catheter. A total of 11(9–12) injections of 0.25–0.5 mL was performed in each animal in and around the infarct area. Control animals received culture media without cells either by percutaneous access ($n=2$) or surgical delivery ($n=2$) with the same volume and number of injections.

2.4. Cell culture and labeling

Muscle biopsies from pigs were obtained from one leg of anesthetized animals and cut into small pieces (5–10 g) under strictly sterile conditions in the operating room. Muscle biopsy was stripped of connective tissue, minced and digested as previously described [10]. Cells were grown in 79% Ham-F12 medium (GIBCO-BRL) supplemented with

20% fetal calf serum, 10 ng/mL bFGF (SIGMA) and 1% penicillin/streptomycin (GIBCO-BRL). Seventy two hours before implantation, cells were labeled with 5 μg/mL BrdU. Assessment of myoblast content was performed as described [10]. At the time of harvest, cells were thoroughly washed to eliminate any source of fetal calf serum and re-suspended in medium at a concentration of $50–80 \times 10^6$ cells/mL.

2.5. Assessment of ventricular function and arrhythmias

Under general anesthesia, animals 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 remodeling was assessed by measuring end systolic and diastolic volumes and diameters, according to the American Society of Echocardiology. Left ventricular ejection fraction (LVEF) was determined according to Teichholz [20] in parasternal short axes. Echocardiographic studies were performed at baseline (before infarct), immediately before myoblast implantation and before sacrifice. All studies were performed by the same investigator blinded to the type of treatment. Measurements were made in three cycles and the mean value was obtained. All echocardiographic data were recorded at a similar heart rate. Reproducibility values within studies were 2.8±6.4 mL (CV 5.5%) for LV end-diastolic volume and 0.3±4.6 (CV 6.6%) for LVEF.

Animals underwent implantation of a Reveal Plus Implantable loop recorder (Medtronic, Minneapolis, MN) in the left chest region after myocardial infarction. Pre-implantation mapping was performed in the left upper parasternal region to optimize device sensing of *R* waves and minimize *T* wave amplitude. Whenever possible, *p* wave amplitude was optimized when an R/T ratio >3 could be obtained. The implantable loop recorder is a continuous electrocardiogram (ECG) monitor capable of recording 42 min of a single-lead ECG in compressed memory mode. For the purpose of this study, the memory was configured with 13 1-minute automatic activation bins. The gain and sensitivity of the device were assessed after implantation in the left and right lateral decubitus position to optimize automatic detection. The parameters for automatic detection were set to store events during a pause >3 s and a heart rate <40 or >160 beats/min. After device implantation, animals were seen for implantable loop recorder interrogation with a standard pacemaker programmer (Medtronic 9790C) at 1, 2, 4 and 8 weeks after infarction and at 1, 2, 4, 8 and 12 weeks after cells or media implantation.

2.6. Histological and immunohistochemistry analysis

At the end of the experimental protocol (3 months after myoblast implantation), animals were anesthetized with ketamine and euthanized with pentobarbital and a saturated solution of potassium chloride, and the heart excised. Explanted hearts were fixed in formalin for histological analysis. Locations of myocardial infarction were visually assessed. Tissue samples consisted of scar surrounded by a ring of viable myocardium. Formalin-fixed tissues were embedded in paraffin, and sections stained with hematoxylin–eosin and Gallego's trichrome method for qualitative assessment. Paraffin-embedded blocks were cut in 5-μm sections, deparaffinized and stained with the appropriate antibodies. The detection of transplanted cells was primarily based on the morphological analysis, the presence of BrdU positive signals and the presence of cells positive for fast myosin-heavy chain. Antibodies directed against fast myosin-heavy chain (MY-32 clone, Zymed), BrdU (BrdU-Dnase kit, Amersham), smooth muscle actin (DakoCytomation) and CD45 (Serotec) were used. MY-32 and CD45 were followed by EnVision System Labelled Polymer Horse Radish Peroxidase (DakoCytomation) and diaminobenzidine (DakoCytomation) as a chromogen. Slides were counterstained with Harris hematoxylin (Sigma). For smooth muscle actin EnVision System Labelled Polymer AP (DakoCytomation) was used, developed with new fucsin (Merck) and counterstained with methyl green. BS-I lectin (Vector) was used for staining of endothelial cells.

Vasculogenesis was evaluated in paraffin-embedded sections by counting the size of areas occupied by smooth muscle coated vessels in the infarct myocardium using smooth muscle actin antibodies for immunostaining. Vascular density was expressed as the 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.

Sirius red staining was used to identify interstitial collagen on slides as described previously [21]. Paraffin sections (5 μm) were stained with Sirius Red F3B, which specifically stains all types of fibrillar collagen. The sections were rehydrated and then stained for 90 min in 0.1% Sirius Red in saturated aqueous picric acid. Sections were then treated with absolute alcohol for one minute, dehydrated, and mounted. Sirius red staining was measured with an interactive computerized image analysis system (Optimas 5.2 color image analysis). A density slice of the sirius red positive staining area was produced and expressed as a percentage of the area of the field occupied by collagen (collagen volume fraction — CVF), and the mean of 15 fields captured with ×20 objective was calculated. Analysis of collagen type I and III was performed using a polarized filter on sections stained for collagen with Sirius Red. Collagen type I is characterized by a red/yellow and type III by green collagen fibrils [22]. The percentage of the infarcted area stained for collagen type I and III was calculated in 15 randomly selected fields captured with ×20 objective. The CVF and collagen type I/III ratio was analyzed separately in the infarct (IA) and non-infarct (NIA) area. NIA was considered to be the area with normal cardiomyocytes surrounding the scar tissue.

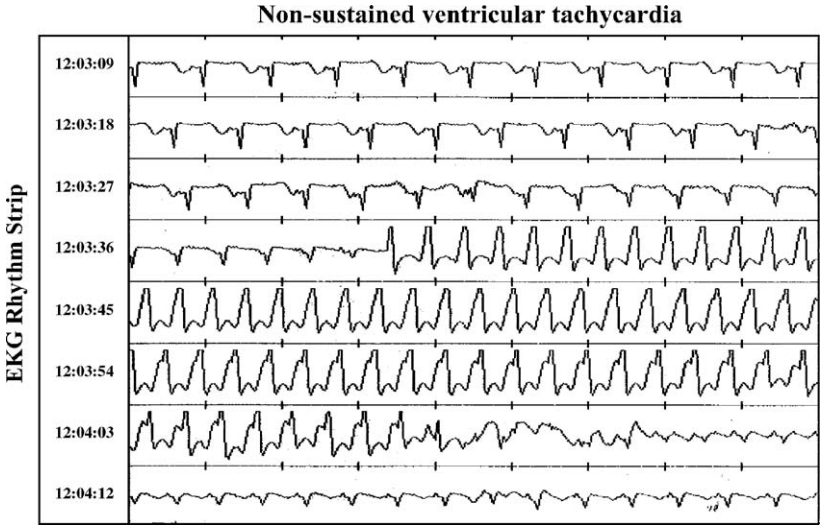

Fig. 1. Rhythm strip obtained with an implantable loop recorder during a non-sustained ventricular tachycardia 24 h after myoblast transplantation.

2.7. Statistical analysis

Statistical analysis was performed with the SPSS 12.0 for windows software package. Comparisons were performed using the paired and unpaired *t* test when appropriate, once normality had been demonstrated using the Shapiro–Wilk and Kolmogorov–Smirnov normality test. In cases of non-normal distribution, the Wilcoxon test was used. Comparisons for repeated measurements were performed with ANOVA or the Friedman test. Lineal regression analysis was performed using Pearson correlation coefficients. Descriptive analysis is presented as mean (SEM) for quantitative variables or median (IQR) for categorical variables. Statistical significance was achieved if *p* values were <0.05.

3. Results

Myocardial infarction was created in 19 pigs by intracoronary insertion of a coil; 3 animals were excluded because of lethal ventricular fibrillation at the time of the infarct. Thus the study group included 16 animals which were divided into groups including 4 control animals and 12 treated with myoblasts (6 by direct surgical injection and 6 by percutaneous access). All animals underwent myoblast transplantation without complications. There were no arrhythmias, ST-T changes, or Q waves associated with the procedure. One animal in the control group and one in the percutaneous group developed non-sustained ventricular tachycardia during the follow up period (Fig. 1), both within 24 h after myoblast or media implantation. No episodes of bradiarrhythmia were detected during follow up and no pericardial effusions were observed on 2D echocardiogram.

Myoblast cultures yielded an average of $407.55\pm115\times10^6$ cells (190 to 680×10^6 cells) with a mean (±SEM) of 92.4±8.2% of myogenic cells ($CD56^+$) of which 57±16% were labeled with BrdU. Animals in the 2

Table 1
Experimental model of MI in pigs: baseline data

	Control	Surgical	Percutaneous	*p*
N	4	6	6	NA
Weight	45.5 (5.5)	53.6 (8.2)	49.5 (5.6)	0.378
Heart rate	70.7 (5.4)	70.8 (10.2)	71.4 (8.5)	0.922
Weight of biopsy	NA	6.48 (0.9)	7.61 (1.7)	0.206
No. cells injected ($\times10^6$)	NA	434.60 (108.9)	385 (194.4)	0.625
CD56 (%)	NA	94 (6.7)	92.1 (9.7)	0.687
No. of injections	11 (1.1)	11 (1.6)	11 (0.9)	0.899

Table 2
Echocardiographic analysis in the different groups of animals

	Baseline	Pretransplant	3 months	*p*-value
LVEF				
Percutaneous	70.3 (5.7)	47.5 (8.7)	63.00 (8.1)	0.008
Surgical	74.2 (3.7)	49.0 (8.1)	64.4 (7.5)	0.009
Control	72.5 (6.8)	50.2 (10.4)	49.5 (9.1)	0.995
LVEDD				
Percutaneous	35 (3.1)	36.4 (2.1)	38.3 (5.7)	0.576
Surgical	33.3 (1.5)	37.7 (5.1)	33.2 (2.2)	0.325
Control	31.3 (5.1)	30.3 (11.4)	38.3 (6.1)	0.080
LVESD				
Percutaneous	20.7 (0.9)	27.6 (2.3)	25.6 (6.4)	0.652
Surgical	17.5 (1.6)	25.1 (3.7)	19.9 (2.6)	0.257
Control	19.2 (3.3)	26.6 (4.2)	27.8 (8.2)	0.302
LVEDV				
Percutaneous	45.5 (12.1)	55.9 (7.9)	57.9 (19.8)	0.428
Surgical	47.9 (13.4)	65.4 (20.4)	58.9 (16.7)	0.229
Control	41.8 (15.3)	43.7 (21.6)	64.7 (22.2)	0.045
LVESV				
Percutaneous	14.3 (1.5)	29.5 (6.5)	25.9 (12.9)	0.778
Surgical	13.8 (3.1)	21.1 (11.6)	19.3 (4.2)	0.558
Control	13.3 (4.9)	28.6 (4.4)	32.2 (18.4)	0.434

p: comparison between pre-transplant values and 3 months post-transplant. LVEF: left ventricular ejection fraction; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; LVEDD: left ventricular end-diastolic diameter; LVESD: left ventricular end-systolic diameter.

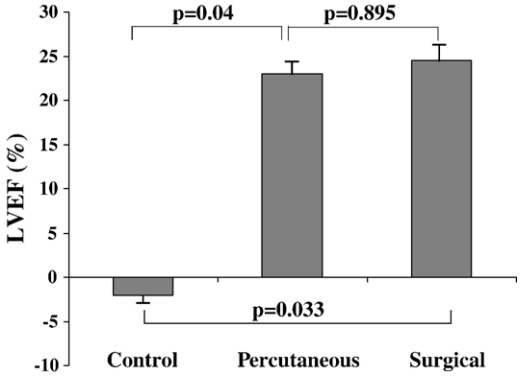

Fig. 2. Echocardiographic assessment of LVEF 3 months after SkM transplant. Numbers represent percentage of change 3 months after SkM transplant in comparison with pre-transplant values.

myoblast transplanted groups (surgical and percutaneous) received equivalent doses of cells (Table 1). Pigs were sacrificed 3 months after transplantation.

3.1. Functional assessment

Baseline data for ventricular function were not significantly different between groups (Table 2). An akinetic or dyskinetic area either in the lateral or posterolateral wall of the left ventricle was detected by echocardiography in every pig. There were no statistically significant differences in the functional analysis in the control animals regardless of whether percutaneous or surgical injection of control media was performed, so both groups were pooled together for comparison with myoblast transplanted animals. After myocardial infarction, there was a significant reduction ($p<0.05$) in the LVEF that was equivalent in the three groups (Table 2). Animals receiving myoblasts showed a statistically significant increase in the LVEF in comparison with controls, 3 months after transplantation ($p<0.05$) (Fig. 2), with no differences between animals receiving myoblasts by percutaneous or surgical injection (Fig. 2). Although there were differences between myoblasts treated animals and controls in the parameters of ventricular remodeling, they did not reach statistical significance (Table 2).

3.2. SkM engraftment and differentiation

Myoblasts were detected in 11 of 12 animals transplanted by morphology, BrdU staining and MY-32 labeling (Fig. 3). To determine the fate of transplanted cells, we performed double immunohistochemistry against BrdU and specific

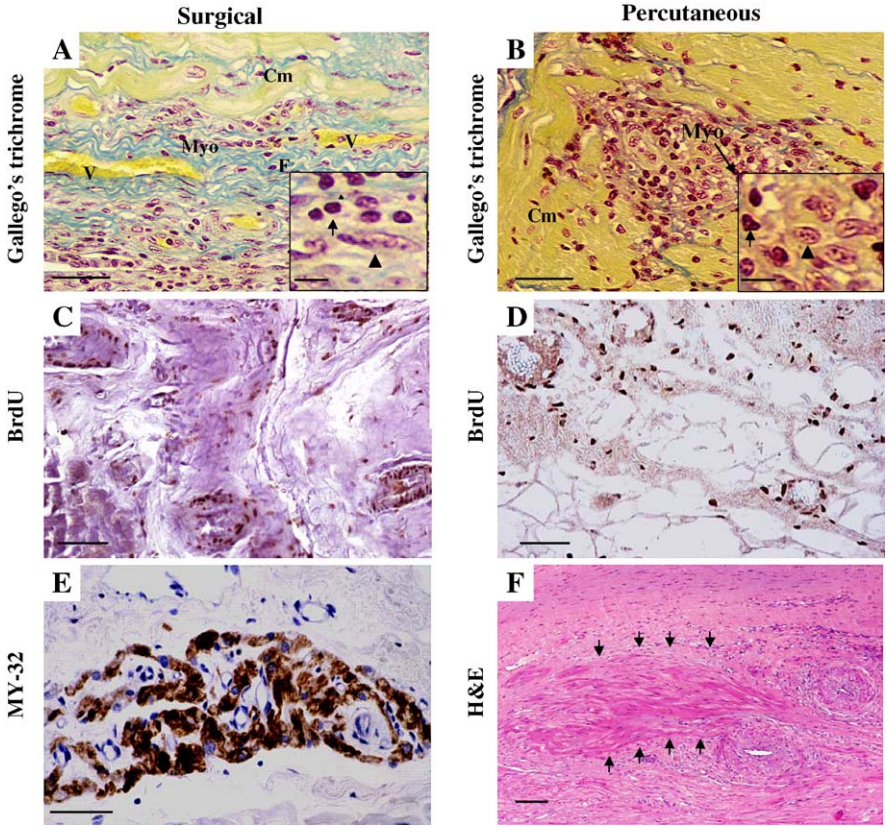

Fig. 3. Myoblast detection in heart sections of animals transplanted with autologous skeletal myoblast at 3 months after transplantation. A,B, Gallego's trichrome method. Cm: cardiomyocytes; Myo: skeletal myoblast; F: fibrosis; V: blood vessels. At higher magnification (insert, scale bar=10 μm) myoblast (arrowhead) and inflammatory cells (arrows) are observed (scale bar=50 μm). C,D, Immunohistochemistry against BrdU labeled cells. BrdU positive cells were detected 3 month after surgical and percutaneous transplant (scale bar=50 μm). E, Immunohistochemistry against myosin heavy chain (MY-32) (scale bar=50 μm). F, Alignment of skeletal myotubes (arrows) (scale bar=100 μm).

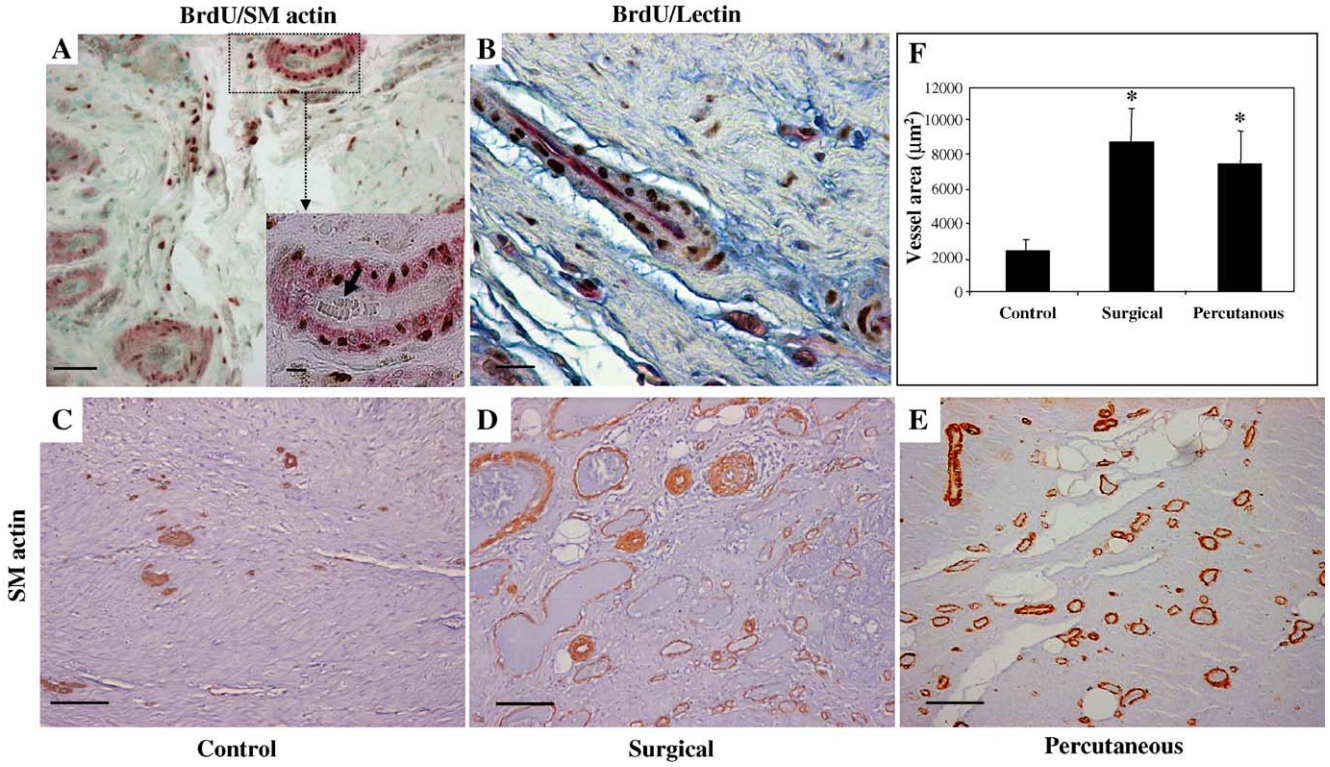

Fig. 4. SkM transplant is associated with increased vasculogenesis. A, Immunohistochemistry with antibodies against smooth muscle actin (pink) and BrdU (brown): double positive cells in blood vessels of animals treated with myoblast (scale bar=50 μm). Erythrocytes are observed inside the vessel (arrow). B, Immunohistochemistry with antibodies against BS-I lectin (endothelial cells) (pink) and BrdU (brown) (scale bar=100 μm). C,D,E, Representative infarct area stained with antibodies against smooth muscle actin in sections of control (C) surgical (D) and percutaneous (E) treated pigs. F, Area occupied by smooth muscle coated blood vessels in infarct area (μm^2/100.000 μm^2). Data are mean±SD, *$p<0.05$ between SkM treated animals and controls (scale bar=100 μm).

cell markers for cardiac and smooth muscle as well as endothelial markers. None of the BrdU labeled cells were stained with markers of cardiomyocytes (not shown). However, a significant number of BrdU positive cells co-localized with antibodies against smooth muscle actin (Fig. 4). These cells accumulate in areas with an increased number of blood vessels coated with smooth muscle, suggesting that some cells within the injected population had the capacity to differentiate into smooth muscle and contribute to the formation of new blood vessels (Fig. 4). The presence of

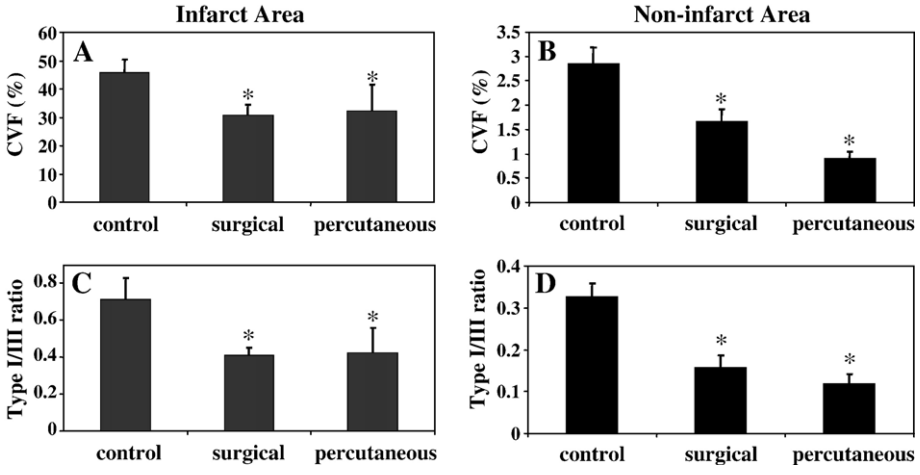

Fig. 5. Transplantation of SkM is associated with decreased fibrosis in IA and NIA. Fibrosis (A,B) and collagen Type I/III ratio (C,D) (assessed by picrosirius red staining) in infarct area (A,C) and peri-infarct area (B,D) of animals treated with SkM and controls. There was statistically significant less fibrosis in SkM treated pigs. Data are mean±SD, *$p<0.05$ between SkM treated animals and controls.

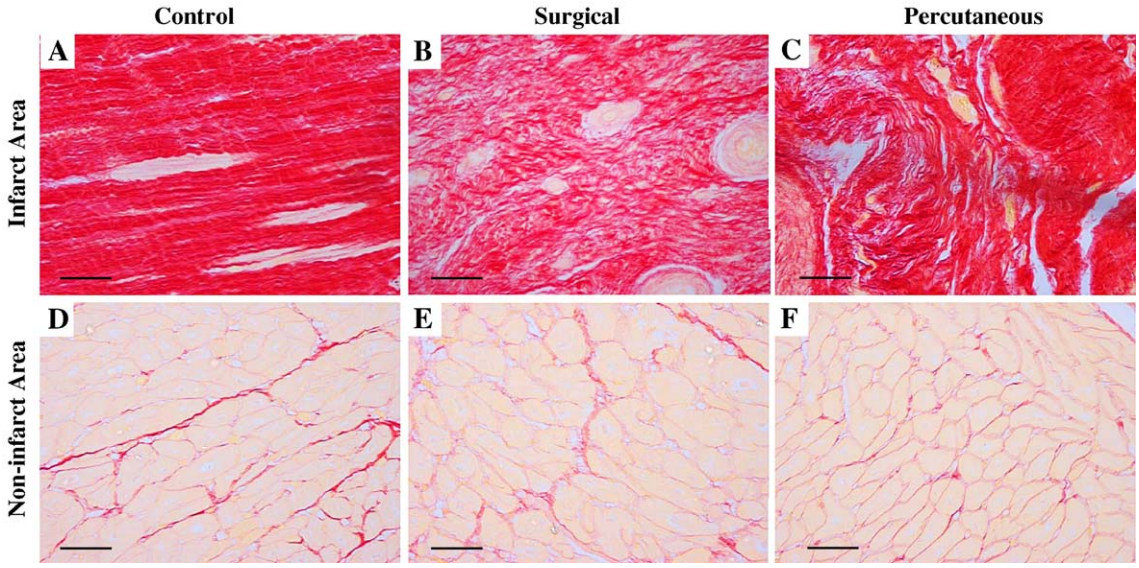

Fig. 6. Representative picrosirius red-stained sections of infarct area (A,B,C) and non-infarct area (D,E,F) show decreased fibrosis in SkM treated pigs (B,C,E,F) in comparison with control animals (A,D). Collagen stains red and cardiac muscle stains yellow. Scale bar=50 μm.

endothelial cells (BS-I lectin^{+}) surrounded with BrdU positive cells, as well as the presence of erythrocytes inside the vessels suggests the functionality of the blood vessels.

3.3. SkM induced vasculogenesis

To address potential mechanisms involved in the beneficial effect of SkM transplant, we analyzed the presence of vasculogenesis in animals receiving SkM in comparison with the control group. Arteriolar density was analyzed by measuring the number of smooth muscle actin positive vessels in the infarct area of each group of animals as well as the area occupied by smooth muscle coated blood vessels. As shown in Fig. 4, the area of SM-actin positive blood vessels was significantly higher in animals transplanted with myoblasts than in the control group ($p<0.01$), which suggests an increase in vasculogenesis. No differences were observed between the two groups of animals receiving SkM.

3.4. Fibrosis and matrix remodeling

We analyzed the degree of fibrosis in the infarct (IA) and non-infarct area (NIA) of both control and myoblast treated

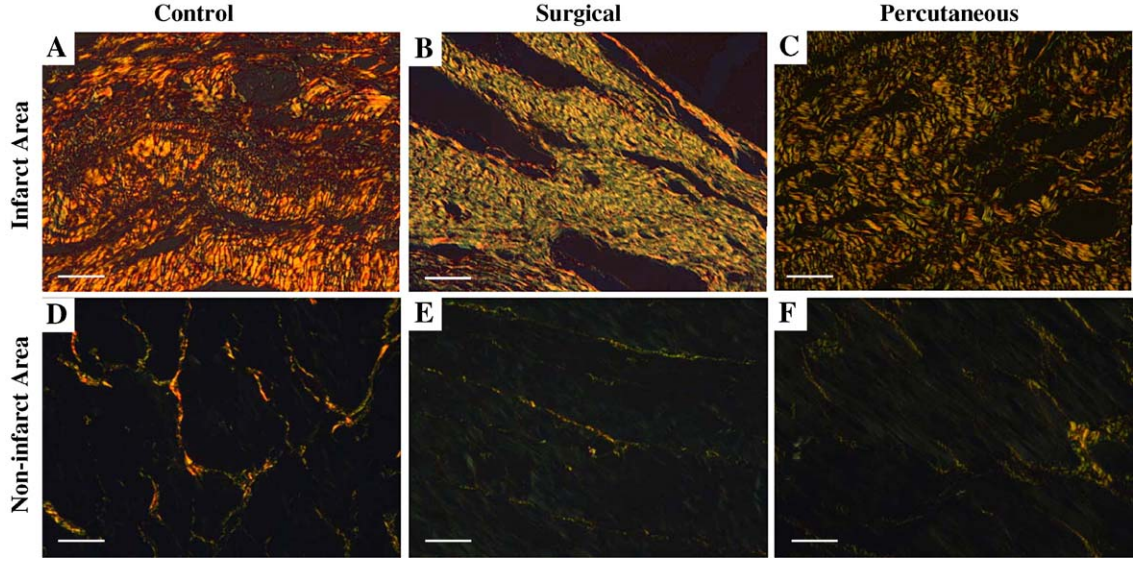

Fig. 7. Representative picrosirius red staining of infarct area (A,B,C) and non-infarct area (D,E,F) under polarized light shows a decreased collagen type I/III ratio in SkM treated pigs (B,C,E,F) versus control animals (A,D). Collagen type I stains red and collagen type III stains yellow. Note the decrease in total collagen as well as in collagen type I. Scale bar=50 μm.

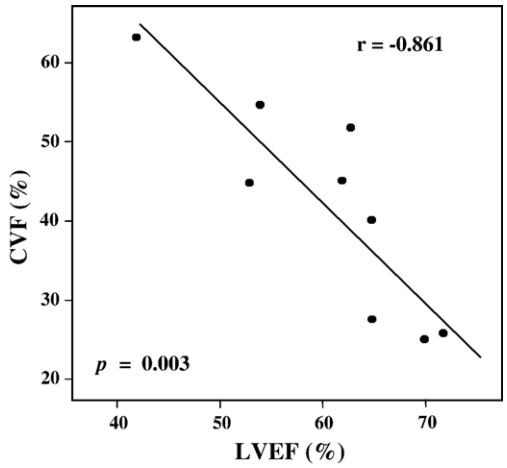

Fig. 8. Correlation between CVF and left ventricular function (LVEF) of the animals ($r=-0.861$; $p=0.003$) ($y=117.62-1.25x$).

animals. Quantitative analysis of hearts from animals receiving SkM demonstrated significantly reduced fibrosis in the infarct area in comparison with control animals, while no significant differences were observed in the amount of fibrosis between cell treated groups. The CVF was 45.9± 4.3% in the control group and 30.9±3.4% and 32.3±9.2% in animals receiving SkM by surgical or percutaneous delivery respectively ($p<0.05$) (Figs. 5 and 6).

It has been demonstrated that dilated cardiomyopathy and development of CHF are associated with an increase in the ratio of collagen type I/III [23], so we analyzed the effect of SkM transplant on the type of collagen in the IA. Transplantation of SkM was not only associated with reduced fibrosis but also with a decrease in collagen I/III ratio in animals treated with SkM (percutaneous injection 0.42±0.13, surgical injection 0.40±0.04) in comparison with control animals (0.71±0.11), which indicates that type I collagen deposition was preferentially reduced by transplantation of SkM (Figs. 5 and 7).

Similarly, there was a reduction in the total amount of fibrosis as well as in the collagen I/III ratio in the NIA of animals treated with SkM in comparison with controls. As expected, the degree of fibrosis in the NIA was massively reduced in comparison with the IA (Figs. 5–7).

Finally, when we analyzed the relation between LVEF and degree of fibrosis, we observed a negative correlation between the total area occupied with collagen (CVF) and LVEF ($r=-0.861$; $p=0.003$) (Fig. 8).

4. Discussion

Although previous studies have already established the potential of SkM to engraft and improve cardiac function in animal models of MI as well as in patients, most studies have used a direct intramyocardial injection of cells [3,6,24] and only one report including a small number of patients has used percutaneous endomyocardial [11,25] administration. While direct intramyocardial transplantation guarantees that cells are delivered to the target tissue [26], from a clinical perspective it has several limitations. Firstly, it requires major surgery which currently can only be justified if the patient is undergoing CABG. Second, as administration of cells is associated with bypass surgery, the benefit of SkM transplant can only be established by randomized studies. In this study we demonstrate that echocardiograph guided endomyocardial transplantation of SkM in a chronic model of MI results in an improvement in LVEF sustained for at least 3 months. The fact that both intramyocardial and endomyocardial administration of SkM were equivalent in terms of cardiac function, as well as the lack of side effects, supports the use of percutaneous administration of SkM in the clinical setting. Although 2D echocardiography provides a more accurate measurement of ventricle volumes, we decided to use the Teicholz method instead, due to the fact that using a 4-cavity apical axis is generally not possible in a swine model of MI. Further, as our model is based on the embolization of the circumflex or marginal artery which induces a lateral or posterolateral MI, the parasternal axis allows optimal visualization of the infarct in all cases without significantly altering the geometry of the left ventricle.

The mechanisms by which SkM transplant contributes to improve cardiac function are largely unknown. Some studies have suggested that skeletal myoblasts might differentiate into cardiomyocytes in vitro [14]. It is generally accepted, though, that myoblasts differentiate into skeletal muscle fibers which are functionally isolated from host cardiomyocytes [13,27], suggesting that transdifferentiation is very unlikely to be the mechanism underlying the functional benefit derived from SkM transplant [2]. Nevertheless, it has been recently demonstrated in a mouse model, that a minor population of muscle-derived stem cells can be isolated with the potential to differentiate in vitro and in vivo into “bona fide” cardiomyocytes which contribute to improved cardiac function [28].

The potential of skeletal myoblasts to increase angiogenesis in animal models of MI has previously been suggested [29]. While the release of chemokines such as SDF-1, able to recruit stem cells with angiogenic potential from the bone marrow, has been hypothesized in some studies [29], others suggest that release of angiogenic growth factors such as VEGF may underlie the angiogenic potential of SkM. Furthermore, a number of studies have used either direct delivery of VEGF or VEGF-expressing myoblast in order to increase angiogenesis in models of MI, with promising results [16,30,31]. However, a note of caution has been raised by a study indicating that upregulation of VEGF may result in deleterious effects including the formation of vascular tumors in the area of implantation [32]. The results of our study clearly support the role of SkM in the stimulation of neovascularization and vasculogenesis — rather than angiogenesis — as indicated by the increase in the area occupied by smooth muscle coated vessels. Interestingly, we found that a number of transplanted cells

acquire specific markers of smooth muscle while being incorporated into the blood vessel wall. Recent studies indicate that skeletal myoblasts express both VEGFR-1 and 2, and that VEGF regulates proliferation, migration and differentiation of satellite cells and skeletal myoblast, thus supporting our findings [33]. However, we cannot exclude contamination of the implanted population with smooth muscle cells, despite the high percentage of myoblast in the cell implant, nor can we rule out fusion between transplanted myoblast and smooth muscle cells.

Myoblasts also have an effect on the metabolism of extracellular matrix. Collagens I and III are essential components of the myocardium, maintaining its structural and functional integrity. Whereas collagen III forms a network storing kinetic energy as elastic recoil, collagen I is a stiff fibrillar protein providing tensile strength. Because of their different physical properties, the altered collagen I/III ratio may have an impact on the diastolic and systolic function of the heart. Significant associations between increased collagen deposition and elevated collagen I/III ratio have been observed in autopsy specimens of failing explanted hearts [23,34] and it has also been suggested that the accumulation of type I collagen may promote arrhythmias by electrical isolation of adjacent myocytes.

Development of fibrosis owing to the disruption of the equilibrium between the synthesis and degradation of collagen type I and III molecules, which results in an excessive accumulation of collagen type I and III fibers in the interstitium and the perivascular regions of the myocardium, is commonly observed in ischemic or hypertensive failing heart [35]. The observation that the development of fibrosis after myocardial infarction was attenuated in myoblast treated animals along with a decreased collagen type I/III ratio and the inverse correlation between LVEF and collagen deposition clearly suggests that SkM transplant is able to reverse ventricular remodeling, thus contributing to improved cardiac function. The fact that increased collagen type I synthesis has been observed in patients with hypertensive heart disease and heart failure [36] suggests that the decrease in collagen type I/III ratio in SkM treated animals could be due to a reduced synthesis of type I collagen, as has been described for patients with heart failure treated with β-receptor blockers [37], angiotensin II receptor antagonists [38] or even resynchronization therapy [39]. We cannot rule out the possibility that neovascularization in infarcted myocardium may contribute to decreased fibrosis by reducing the ischemic area. Similar findings were observed in rats where MSC transplantation decreased collagen gene expression, and this effect was more discernible for type I collagen [40].

Although our study was not specifically designed to analyze the potential of SkM transplant to induce arrhythmias, the use of implantable loop recorders allowed us to determine the spontaneous rate of ventricular arrhythmias during follow up. The two episodes of spontaneous ventricular tachycardia were detected in the early period after transplant, a finding which is consistent with recent studies [41]. It is possible that SkM transplant may increase the susceptibility to arrhythmia, as has been recently suggested using Programmed Electric Stimulation [42]. However, time from infarct to transplantation as well as baseline arrhythmia susceptibility in patients with chronic scar tissue may also increase the incidence of arrhythmias, regardless of cell implant.

We may conclude that 1) transplantation of SkM in a large animal model of chronic MI is associated with improvement in cardiac function; 2) importantly, percutaneous echocardiograph-guided endomyocardial delivery provides equal benefit in terms of cardiac function to direct intramyocardial injection; 3) increased neovascularization and vasculogenesis and decreased fibrosis provide some of the mechanisms involved in the improvement associated with SkM transplant in chronic MI.

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