Efficacy of epicardially delivered adipose stroma cell sheets in dilated cardiomyopathy

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Aims Few studies have assessed the effects of cell therapy in non-ischaemic cardiomyopathies which, however, contribute to a large number of cardiac failures. Assuming that such conditions are best suited for a global delivery of cells, we assessed the effects of epicardially delivered adipose tissue-derived stroma cell (ADSC) sheets in a mouse model of dilated cardiomyopathy based on cardiac-specific and tamoxifen-inducible invalidation of serum response factor.

Methods and results Three weeks after tamoxifen administration, the function of the left ventricle (LV) was assessed by echocardiography. Twenty-nine mice were then allocated to control (n = 9, non-transgenic), sham (n = 10, transgenic non-treated), and treated (n = 10, transgenic) groups. In the treated group, 3 × 10^6 allogeneic ADSCs were cultured for 2 days onto temperature-responsive polymers and the generated sheets were then transplanted over the surface of the heart. In 10 additional mice, the sheet was made of green fluorescent protein (GFP)-labelled ADSCs to track cell fate. Function, engraftment, and fibrosis were blindly assessed after 3 weeks. In the non-treated group, fractional shortening declined compared with baseline, whereas the sheet application resulted in its stabilization. This correlated with a lesser degree of LV remodelling, as LV end-diastolic and end-systolic diameters did not differ from baseline values. Many GFP^+ cells were identified in the epicardial graft and in the myocardium. Treated animals also displayed a reduced expression of the stress-induced atrial natriuretic factor and beta-myosin heavy chain genes. These protective effects were also accompanied by a reduction of myocardial fibrosis.

Conclusion These results strongly suggest the functional relevance of epicardially delivered cell-seeded biomaterials to non-ischaemic heart failure.

Keywords Cardiomyopathy • Cell therapy • Stem cells • Remodelling • Fibrosis

1. Introduction

Dilated cardiomyopathy is the most common type of non-ischaemic cardiomyopathy and is characterized by dilatation and contractile dysfunction of the left (LV) and right ventricles leading to heart failure.1 So far, few studies have assessed the effects of cell therapy in non-ischaemic cardiomyopathies which, however, contribute to a large number of cardiac failures. The problem is clinically relevant because patients who have exhausted conventional medical therapies can only be offered invasive options like heart transplantation or destination therapy-targeted implantation of an assist device.2 Thus, there is still a critical need for less invasive therapeutic strategies. In an effort to address this issue, we epicardially delivered adipose-derived stroma cell (ADSC) sheets in a mouse model of dilated cardiomyopathy.

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Indeed, cell retention in damaged hearts appears as one of the major roadblocks to successful myocardial regeneration in stem cell therapy. The low rate of engraftment and high mortality of transplanted cells are initially caused by a mechanical leakage of cells\(^1\) and subsequently worsened by an interplay of biological factors that include inflammation, ischaemia due to poor vascularization of the injected areas, and apoptosis subsequent to detachment of anchorage-dependent cells.\(^4\) The emerging fields of tissue engineering and biomaterials for delivery and support of transplanted cells have begun to provide potential solutions for this obstacle. We recently supported this concept by demonstrating that epicardial delivery of ADSC sheets results in greater post-infarction survival, better engraftment, and better preservation of LV geometry than direct intramyocardial injections.\(^3\) This result was in keeping with those previously reported by several other studies which, however, were all relevant to ischaemic cardiomyopathies.\(^6\)–\(^8\)

ADSCs were selected as the candidate cells because (i) they can be harvested and grown in a straightforward fashion and (ii) they secrete a wide array of angiogenic and anti-apoptotic factors,\(^9\) which likely account for their beneficial effects on perfusion and function in both small\(^5,10\) and large\(^11,12\) animal models of myocardial infarction. Given that this cell population has been mainly tested in models of ischaemic cardiomyopathy, the goal of the present study was to investigate the potential benefit of these cells in a mouse model of a non-ischaemic cardiomyopathy, focusing particularly on their engraftment as well as their potential to restore the functional capacity of the heart.

2. Methods

2.1 Animals

All procedures were approved by our institutional Ethics Committee and complied with the European legislation (Directive 2010/63/EU) on animal care. In this study, conditionally invalidated serum response factor (SRF) mice (MHC-MerCreMer/Sf/Sf) were used as recipients.\(^13\) C57BL/6j (Janvier, France) and green fluorescent protein (GFP) transgenic mice\(^14\) were used as donors. The Cre-mediated excision of floxed SRF alleles in the heart was induced by daily intraperitoneal tamoxifen (20 \(\mu\)g/g/day; Sigma-Aldrich, France) injections on 3 consecutive days (more details are available in Supplementary material online). After isolation, animals were sacrificed by cervical dislocation.

2.2 Experimental groups and cell sheet transplantation

Thirty-nine 3-month-old mice were used in this study: \(\alpha\)MHC-MerCreMer/Sf/Sf (\(n = 30\)) and Sf/Sf (\(n = 9\)). Tamoxifen was administrated to all groups. Three weeks after tamoxifen administration, the animals were first anaesthetized with isoflurane (Baxter, France) for a baseline echocardiography and then allocated to control (\(n = 9\), Sf/Sf mice), sham (\(n = 10\), \(\alpha\)MHC-MerCreMer/Sf/Sf mice), and treated (\(n = 20\), \(\alpha\)MHC-MerCreMer/Sf/Sf mice) groups. After baseline echocardiography, all animals underwent a left lateral thoracotomy after intraperitoneal ketamine (100 mg/kg; Merial, France)-xylazine (10 mg/kg; Bayer, Germany) anaesthesia and tracheal ventilation. Analgesia was performed for 2 days after surgery. The protein concentration was measured by the method of Bradford using bovine serum albumin as a standard. Cytokine antibody arrays were carried out according to the manufacturer’s instructions (RayBiotech, USA) using 200 \(\mu\)g of protein per sample. The intensity of the different spots was determined using a densitometric software (Multi Gauge, Fujifilm, Japan).

2.3 Assessment of LV function

Pre- and post-transplantation cardiac function was evaluated by transthoracic echocardiography (Sequoia 516, equipped with a 13 MHz phased-array linear 15L8 probe; Siemens, France) in animals sedated with 2% isoflurane. Two-dimensional parasternal long-axis views were used to measure the LV end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD) in a time-motion mode. LV fractional shortening (LVFS) was calculated as (LVEDD – LVESD) \times (100/LVEDD). All measurements were made online on freeze frames images in triplicate and averaged by an investigator blinded to the treatment group.

2.4 Isolation of ADSCs and preparation of cell sheets

ADSCs were isolated from both C57BL/6j and GFP\(^14\) mice from inguinal subcutaneous adipose tissues after intraperitoneal ketamine (100 mg/kg; Merial)-xylazine (10 mg/kg; Bayer) anaesthesia as described previously.\(^15\) Cells were cultured on 24 well plates (NalgeNunc International, USA) and kept at 37 \(^\circ\)C in an incubator for 2 days. Cell sheets were detached spontaneously at room temperature within 30 min, and then washed once with phosphate-buffered saline, thereby yielding a scaffold-free monolayered ADSC graft.

2.5 Characterization of ADSC sheets

2.5.1 Histological examination

ADSC sheets were fixed in 4% formalin overnight and embedded in paraffin. Five-\(\mu\)m-thick sections were stained with haematoxylin–eosin. ADSC sheets obtained from the GFP transgenic mice were also examined using epifluorescence microscopy for GFP expression. Images were taken with an inverted microscope (Leica DMIL, Leica, Germany) equipped with a digital camera (Qicam, Qimaging, Canada).

2.5.2 Cytokine measurement using cytokine antibody arrays

The protein concentration was measured by the method of Bradford using bovine serum albumin as a standard. Cytokine antibody arrays were carried out according to the manufacturer’s instructions (RayBiotech, USA) using 200 \(\mu\)g of protein per sample. The intensity of the different spots was determined using a densitometric software (Multi Gauge, Fujifilm, Japan).

2.5.3 Quantitative cytokine measurement using ELISA

Quantitative analysis of the hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1) secretion was measured using a mouse HGF and IGF-1 ELISA kit (RayBiotech) according to the manufacturer’s instructions. Quantities of cytokines were expressed as pg of cytokine per mg of protein.

2.6 Histological and immunohistochemical assessment

After the last echocardiographical assessment and sacrifice, hearts were removed and separated in two halves by a short-axis section through the mid-portion of the heart. The blocks were immediately fixed in Tissue-Tek (Sakura, USA) and frozen in liquid nitrogen-cooled isopentane until they were sliced into 8 \(\mu\)m thick cryosections using an ultramicrotome. A total of 120 sections per heart were generated in the control, sham, and treated hearts. General morphology, fibrosis, angiogenesis, engraftment, and grafted cell characterization were performed as described in detail in Supplementary material online.

2.7 Relative quantification of gene expression by real-time PCR

Total RNA was extracted from the heart muscle using Qiazol\(^\text{TM}\), metallic beads and Tissue Lyser-II (Qiagen, France) following the manufacturer’s instructions. The RNA quality for each sample was checked with Experion...

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RNA StdSens Analysis Kit and the Experion automated electrophoresis station (Bio-Rad, France) according to the manufacturer’s instructions. Only samples with an RNA quality indicator superior to 7 were used for further studies. Real-time PCR was performed using LightCycler® 480 (Roche Diagnostics, France) as described in detail in Supplementary material online.

2.8 Statistics
Data are summarized using the mean ± SD. Comparisons were performed across groups using analysis of variance or Kruskal–Wallis test with exact $P$-value when appropriate. Multiple comparisons were done using pairwise comparison with the Bonferroni correction for post hoc tests. Percentages of fibrosis and angiogenesis were compared between the groups using a mixed model analysis of variance considering the intra-mice correlation between measurements. For all analyses, a two-tailed $P$-value of $<0.05$ was considered as statistically significant. Analyses were conducted using SAS 9.3 (Statistical Analysis System, Cary, USA).

3. Results

3.1 Characterization of the ADSC sheets before delivery
The ADSC sheets detached spontaneously at room temperature, thereby yielding a scaffold-free monolayered ADSC graft (Figure 1A). As seen in Figure 1B, these sheets feature a ‘carpet’ of cells well connected with each other and surrounded by an abundant extracellular matrix (see arrowheads in Figure 1B). ADSC sheets featured the same morphological characteristic regardless of whether they were prepared from the GFP or C57BL/6j mice (Figure 1C). It should be noted that FACS analysis of ADSCs isolated from the GFP mice showed a heterogeneous population as only 50% of the cells expressed detectable levels of GFP (Figure 1C and D).

3.2 Engraftment and vascularization of ADSC sheets
Three weeks after their epicardial delivery, the ADSC sheets were macroscopically visualized on the surface of the heart (Figure 1E and F). For a closer analysis, 120 sections per heart were generated and some of these sections were stained with haematoxylin–eosin. As seen in Figure 1G and H, the majority (8 out of 10) of treated hearts displayed well-attached ADSC sheets on the LV wall. Furthermore, at this time point, numerous blood vessels were identified within the sheet area and in the underlying myocardium in close proximity to the sheet as evidenced by CD31 staining (Figure 1I and J). To visualize the grafted cells in the hearts, some of the ADSC sheets were made of GFP-labelled ADSCs. In those treated hearts, many GFP$^+$ cells were identified within the grafted sheet (Figure 1K) and also in the myocardium (Figure 1L) suggesting that some of these cells had migrated to the depth of the myocardial wall. Using a semi-quantitative score (0: no cells; 1: minimal amount of cells; 2: moderate amount of cells; 3: large amount of cells), we found that among the 10 ADSC-GFP sheet-treated hearts, 3 were in the 0 (no cells) category, 3 qualified for score 1 while 4 were in the 2–3 (moderate or large number of cells) range. Some of these GFP$^+$ cells expressed markers of the mesenchymal and endothelial lineages, demonstrated by their positive staining for vimentin and CD31, respectively. However, none of these GFP$^+$ cells co-expressed cardiomyocyte-specific markers such as alpha-actinin, vinculin, and Nkx2.5 (data not shown).

3.3 Functional efficacy of epicardially delivered ADSC sheets
To avoid the possible confounding effects of GFP expression on functional and mechanistic analysis, ADSC sheets used for assessing these endpoints were made of non-labelled ADSCs. Three weeks (baseline) and 6 weeks (sacrifice) after tamoxifen administration, the function of the LV was assessed by echocardiography. LVFS was significantly decreased in the mutant mice at baseline compared with control animals ($P = 0.009$). At sacrifice, in the non-treated group, fractional shortening had declined compared with baseline (26 ± 7 vs. 17 ± 5%, $P = 0.0005$), whereas in the sheet application resulted in its stabilization (22 ± 5 vs. 22 ± 4%, $P = 0.87$; Figure 2A). This correlated with a lesser degree of LV remodelling as LVEDD did not differ from baseline values (3.9 ± 0.3 vs. 4.1 ± 0.4 mm, $P = 0.33$), whereas it significantly increased in the non-treated group (3.9 ± 0.5 vs. 4.4 ± 0.6 mm, $P = 0.04$). As seen in Figure 2A, the changes in LVESD featured similar patterns.

The functional efficacy of epicardially delivered ADSC sheets was further supported by real-time PCR analysis, which demonstrated a reduced expression of the stress-induced beta-myosin heavy chain genes ($P = 0.005$), atrial natriuretic factor ($P = 0.009$), bone morphogenetic protein 4 ($P = 0.014$), and brain natriuretic peptide ($P = 0.082$) in the treated group compared with the non-treated one (Figure 2B).

3.4 Effects of epicardially delivered ADSC sheets on cardiac remodelling
To examine the impact of cell sheets on cardiac remodelling, we analysed cardiac fibrosis (Figure 3) in the non-GFP group only to again avoid the possible adverse effect of the label. The extent of fibrosis was first assessed by Sirius red staining. At sacrifice, all of the mutant mice presented extensive interstitial fibrosis. This result was also confirmed by immunostaining for vimentin, a marker of cardiac fibroblasts, which demonstrated a higher interstitial staining in mutant compared with the non-transgenic mice, as previously reported[16]. However, despite the presence of extensive interstitial fibrosis in mutant mice, the mean percentage area of fibrosis was reduced in treated compared with non-treated mutant animals (21 ± 5 vs. 15 ± 8%, $P = 0.02$; Figure 3A). Reduced fibrosis in the treated group in comparison with sham was also supported by real-time PCR, which demonstrated significant decreases in the ratio of collagen 1/collagen 3 ($P = 0.04$), bone morphogenetic protein 4 ($P = 0.014$), and brain natriuretic peptide ($P = 0.082$) treated compared with non-treated mice but the differences failed to reach a significant level.

Further assessment of the proliferative activity of cardiac fibroblasts was performed by co-immunostaining of Ki67, a cellular marker for proliferation, with vimentin (Figure 4). Proliferation of myocardial fibroblasts was a rare event in the control group (non-transgenic mice), whereas in the mutant mice the number of double-positive vimentin$^+$.Ki67$^+$ was increased by a factor of 5 (21.9 ± 5.5 vs. 4.1 ± 0.8 per mm², $P < 0.05$). However, the number of double-positive (Ki67$^+$ vimentin$^+$) cells in treated animals was significantly decreased compared with the sham group (Figure 4).

As seen in Figure 4, there was a considerable number of Ki67$^+$ cells, which did not express vimentin. To quantify and characterize this population, we performed additional immunostainings using resident stem cell, cardiomyocyte, or endothelial cell markers. None of the Ki67$^+$ cells were co-labelled with cardiomyocyte or resident stem cell
markers, whereas a majority of the Ki67+ cells is co-labelled with the endothelial cell marker CD31. However, no difference in the percentage of double-positive (Ki67+CD31+) cells was detected between the treated and non-treated groups (Figure 4). As we observed proliferative endothelial cells in both the treated and non-treated animals, we assessed the extent of angiogenesis. Our results demonstrate that the total capillary density within myocardial tissue was not significantly increased in treated compared with non-treated mice (3929 ± 441 vs. 3752 ± 656 per mm², P = 0.48).

To gain a mechanistic insight into the decrease of fibrosis in treated animals, we examined the cytokine expression profile of ADSC sheets using an antibody array. Our results demonstrated that the ADSC sheet, before delivery, expresses proangiogenic, anti-apoptotic, and/or anti-fibrotic cytokines such as vascular endothelial growth factor, stromal cell-derived factor 1 alpha, stem cell factor, basic fibroblast growth factor, IGF-1, and P-selectin (data not shown). Of note, two cytokines known to possess a powerful anti-fibrotic activity, i.e. IGF-1 and HGF were found to be expressed in large amounts in the sheet before implantation (848 ± 202 and 160 ± 34 pg/mg, respectively), as assessed by ELISA. However, 3 weeks after transplantation, the myocardial tissue levels of these two cytokines failed to show significant differences between the treated and non-treated mice (data not shown).

4. Discussion

The major finding of this study is that epicardial delivery of an ADSC sheet improves the functional outcomes of non-ischaemically diluted mouse hearts and that this benefit is associated with a significant reduction of fibrosis.

Non-ischaemic dilated cardiomyopathy largely contributes to the number of patients with heart failure.1 In contrast to ischaemic cardiomyopathies, which are amenable to a variety of pharmacological, electrical, and interventional treatments, the non-ischaemic forms of heart failure can only be treated by drugs and if the latter fail, the only remaining options entail heart transplantation or permanent implantation of an assist device, i.e. treatments which are both invasive and still fraught with several adverse effects.2 There is thus a real need for developing novel therapies for non-ischaemic cardiomyopathies and cell therapy might be one of them. Nevertheless, this treatment has primarily been tested in ischaemic models, although some previous studies using skeletal myoblasts,17,18 bone marrow-derived cells,19,20 and embryonic stem cells21,22 in drug- or genetically induced cardiomyopathies have already provided preliminary evidence for the functional efficacy of cell transplantation in these settings. The present study further supports these data by showing that ADSCs can stabilize the contractile function of SRF-deficient transgenic mice while reducing the extent of ventricular
dilation. SRF, an MADS-box transcription factor regulates the expression of genes involved in growth, proliferation, differentiation, and the actin cytoskeleton. An abnormal truncated form of SRF has been found in human failing hearts and the cleavage of SRF by caspase may promote heart failure. Conditional invalidation of SRF using the Cre/LoxP strategy in heart demonstrates that SRF is crucial for cardiac differentiation and maturation during embryogenesis and the postnatal period. In our study, we used a double transgenic mouse model which allows to induce the inactivation of SRF only in cardiomyocytes as tamoxifen-inducible Cre recombinase expression is under the control of the cardiomyocyte-specific alpha myosin heavy chain promoter. As early as 5 days after tamoxifen injection there is a marked decrease in SRF mRNA and protein levels; mice then exhibit a progressive impairment of cardiac function, with reduced energy flux to the myofibril and decreased contractility, ending up in dilated cardiomyopathy, heart failure, and death within 10 weeks. To the best of our knowledge, it is the first time that cell therapy is assessed in this model which features, among others, the advantage of a timely controlled onset of functional deterioration (once tamoxifen has been given), thereby allowing a standardized comparison of outcomes between treated and non-treated mutant animals.

Mechanistically, our data support the commonly accepted view that transplanted cells primarily act through paracrine signalling. Indeed, none of the grafted cells which could be identified co-expressed cardiac-specific markers, thereby ruling out any ‘regeneration’ of the diseased myocardium from the donor cells. Conversely, both the antibody array and ELISA data consistently demonstrated the expression of a wide spectrum of angiogenic and anti-fibrotic biomolecules released by the ADSC, in keeping with the previously published data. In addition, the quantification of HGF and IGF-1 using ELISA showed that ADSC sheets at the time of implantation expressed large amounts of IGF-1 and HGF, both known for their anti-fibrotic activity. Although the myocardial tissue levels of these cytokines did not differ 3 weeks after transplantation, their possible effect at an earlier time point is suggested by the reduced proliferation of fibroblasts seen in the sheet-treated group. It is thus tempting to speculate that altogether, these data account for the reduction in fibrosis which, in turn, might have favourably influenced the preservation of ventricular geometry and function.

Another type of cells which acts paracrinically is the skeletal myoblast. To determine whether skeletal myoblast sheets would have the same effect in our model of non-ischaemic cardiomyopathy, we assessed these constructs in an exploratory study and found that 3 weeks after the procedure, epicardially delivered skeletal myoblast sheets were functionally less effective than the ADSC ones, as demonstrated by a decrease in LVFS compared with baseline (23.5 ± 5.0 vs. 26.1 ± 4.8, n = 7). This result further highlights the therapeutic potential of
ADSC sheets in non-ischaemically dilated mouse hearts. Indeed, if one assumes that the grafted cells do not differentiate in new cardiomyocytes (unless cardiac-committed cells are used) but will primarily act as biofactories releasing cardioprotective mediators, the choice of mesenchymal stem cells is clinically appealing because these cells are easily scalable, feature a rich secretome, and are credited for some immune privilege which could then allow their temporary persistence in the grafted tissue after an allotransplantation. While mesenchymal stem cells can be harvested from different tissue sources, retrieval of cells from the adipose tissue may have the advantage of a lesser degree of invasiveness compared with iliac crest puncture for bone marrow collection. Indeed, several studies have now demonstrated the functional benefits of ADSCs in ischaemic small and large animal models and clinical trials are currently underway to test these cells in the context of both acute and chronic ischaemic heart diseases. However, even if the initial goal of myocardial regeneration by permanent structural integration of the grafted cells in the recipient heart tends now to shift towards a transient paracrine activation of endogenous pathways by these cells, it still remains mandatory that, for being efficacious, cells remain engrafted for a sufficient period of time. So far, however, the expected efficacy of cardiac cell therapy has been hampered by the low rate of sustained engraftment resulting from both massive mechanical leakage at the time of cell delivery and subsequent cell death because of the intermingled effects of ischaemia, inflammation, apoptosis, and eventually rejection. There is thus a critical need for improved methods of cell delivery and, from this standpoint, the use of the cell sheet technology features distinct advantages, which include the avoidance of cell damage induced by their pre-implantation proteolytic dissociation when they are injected by a conventional needle-based technique, maintenance of cell cohesiveness through their anchoring to a self-secreted extracellular matrix, an attendant increase in cell survival, the lack of foreign supporting materials, and the simplicity of their application provided one has a direct control over the surface of the heart. Previous studies have now established the superiority of these cell sheets over conventional needle-based injections in various animal models and with a wide variety of cell phenotypes and the global nature of the disease in non-ischaemic cardiomyopathies makes ‘wrapping’ of the heart by these sheets particularly sound. Our data support this view by showing that 3 weeks after the epicardial delivery of the sheets, cells could still be identified, both at the surface of the heart and to a lesser extent in the underlying myocardium, whereas, at this time point, most studies using injections already fail to show any residual cell engraftment. Even though cells making up the sheet ultimately disappear, this loss occurs gradually over time and it is thus sound to postulate that, in the present study, the cell sheet allowed the cells to persist long enough to release the factors that activated the host-associated signalling pathways and harnessed endogenous repair mechanisms accounting for the observed cardioprotective effects.

In conclusion, the present study supports the efficacy of an epicardial coverage by an ADSC-based sheet in a genetically induced model of non-ischaemic dilated cardiomyopathy. It is fair, however, to acknowledge that handling of these fragile sheets may not always be straightforward.
These data should thus be considered primarily as proof-of-concept that ‘wrapping’ diseased areas of the myocardium in non-ischaemic dilated cardiomyopathies may be an efficacious therapy even though it might be more user-friendly to replace the cell sheet by a more mechanically robust biocompatible scaffold, of which many are yet available. Likewise, alternate cells, particularly those featuring a force-generating capacity and appropriately patterned onto engineered constructs could also be considered. The clinical applicability of this approach might be facilitated by newly developed thoracoscopic approaches allowing access to the surface of the heart through minimally invasive procedures.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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**Figure 4** Characterization of proliferative interstitial cells in the treated mice myocardium. Three weeks after epicardial delivery of the ADSC sheet, the heart sections were co-stained with the antibodies against the cell-proliferation-associated antigen-Ki67 (red fluorescence) and either vimentin (upper panel, green fluorescence) or CD31 (lower panel, green fluorescence). Combined red and green fluorescence and DAPI-stained nuclei (blue) are shown in merged images. Arrows indicate co-stained cells; arrowheads indicate cells only labelled for Ki67. Ki67+ cells were counted manually in whole heart sections and the results are expressed as mean ± SD. Bar: 25 μm.


