Fate of undifferentiated mouse embryonic stem cells within the rat heart: role of myocardial infarction and immune suppression

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Abstract

It has recently been suggested that the infarcted rat heart microenvironment could direct pluripotent mouse embryonic stem cells to differentiate into cardiomyocytes through an *in situ* paracrine action. To investigate whether the heart can function as a cardiogenic niche and confer an immune privilege to embryonic stem cells, we assessed the cardiac differentiation potential of undifferentiated mouse embryonic stem cells (mESC) injected into normal, acutely or chronically infarcted rat hearts. We found that mESC survival depended on immunosuppression both in normal and infarcted hearts. However, upon Cyclosporin A treatment, both normal and infarcted rat hearts failed to induce selective cardiac differentiation of implanted mESC. Instead, teratomas developed in normal and infarcted rat hearts 1 week and 4 weeks (50% and 100%, respectively) after cell injection. Tight control of ESC commitment into a specific cardiac lineage is mandatory to avoid the risk of uncontrolled growth and tumourigenesis following transplantation of highly plastic cells into a diseased myocardium.

Keywords: mouse embryonic stem cells • cell therapy • myocardial infarction • immunosuppression • rat

Introduction

Cardiovascular disease is a leading cause of morbidity and mortality worldwide. Heart-related injuries, such as myocardial infarction (MI), lead to an irreversible loss of cardiac cells followed by ventricular remodelling and finally by organ failure, in spite of aggressive pharmacotherapy and surgical procedures. Indeed, the limited mitotic capacity of cardiomyocytes cannot support myocardial self-renewal. To restore the function of a damaged heart, cell based therapies have been developed to supply the failing myocardial tissue with non-terminally differentiated cells capable of forming viable cardiomyocytes.

Among multiple candidate cell types isolated from cardiac or non-cardiac sources and possessing varying cardiogenic potential [1–3], embryonic stem cells (ESC) derived from the inner cell mass of the blastocyst rank highest with regard to their cardiogenic potential (reviewed in [4]). Due to their ability to indefinitely proliferate and to differentiate *in vitro* into functional cardiomyocytes, ESC represent an extensive reservoir for tissue regeneration. Successful transplantation of mESC-derived cardiomyocytes into healthy hearts was first demonstrated by L. Field's group [5]. Injected into injured hearts, mESC could repopulate significant

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regions of a dysfunctional myocardium, resulting in improved contractile function [6, 7].

Tissue microenvironment is thought to play a major role in cell differentiation towards specific phenotypes through the local release of growth factors and/or cytokines. For instance, differentiating mouse embryoid bodies (EBs) injected under the mouse skin or the renal capsule induced teratomas containing different cell types depending on the site of injection [8]. Engrafted into the spinal cord or brain of mice or rats, mESC differentiated towards neuronal cell types [9, 10], while under the kidney capsule they transformed into other cell types coexisting into a heterogeneous tissue [11]. Within the knee joint of severe-combined immunodeficiency (SCID) mESC formed tumours [12], whereas they differentiated into chondrocytes when injected into the rat joint [13]. Therefore, differences in the microenvironment into which cells are embedded are likely to influence the differentiation pathway.

Two recent papers reported that undifferentiated mESC injected in a rat model of MI, fully differentiated towards functional cardiomyocytes *in situ* without the need for immunosuppression and led to an improvement of heart function [14, 15]. The authors suggested that such an appropriate *in situ* cardiac differentiation of mESC across a xenogenic barrier takes place through a BMP/TGFβ-dependent paracrine signalling pathway provided by the heart.

In this study, we investigated on a larger scale whether the cardiac host microenvironment, in particular the one created by the growth factors and cytokines released during MI, could selectively promote ESC differentiation toward the cardiac phenotype and improve heart performance. We also addressed the question of whether the rat heart is immunotolerant toward undifferentiated mESC, allowing them to survive without the need of immunosuppression.

Materials and methods

Embryonic stem cell preparation for transplantation

We genetically modified the mouse ESC line CGR8 [16] to express as reporter gene either the enhanced green fluorescent protein (eGFP) fused with CD63 or the human anti-apoptotic gene Bcl2 (hBcl2), inserted into pcDNA3.1(-) vectors at EcoRI and HindIII restriction sites. The constructs were electroporated into CGR8 cells according to standard protocols in a Gene Pulser (BioRad Laboratories, Reinach, Switzerland) at 240 V, 500 μF. Expressed eGFP-CD63 fusion protein targets eGFP to the plasma membrane and intracellular endosomes (Fig. 1A) and prevents the dilution of the marker, thus enabling a better visualization of cells by immunohistochemistry, as opposed to diffuse cytoplasmic eGFP detection. The hBcl2-CGR8 clone confers a resistance to apoptosis when exposed to an unfavourable environment such as in the infarcted heart. Transfected EGFP-CD63 positive mESC were sorted by flow cytometry (FACStar+, Becton Dickinson, Allschwil, Switzerland), while stable clones carrying hBcl2 were selected by neomycin resistance after 10 days treatment with G418 (250 μg/ml, Gibco, Invitrogen, Basel, Switzerland). Wild-type (wt) D3

mESC were propagated on mitomycin-treated mouse embryonic fibroblasts, while stable CGR8 ESC clones were propagated in the presence of leukaemia inhibitory factor (LIF). EGFP and hBlc2 were identified by immunohistochemistry using anti-eGFP and anti-Blc2 antibodies, respectively (see below).

Cells were split by trypsinization every 2 days and propagated in BHK21 medium (Gibco) supplemented with pyruvate, non-essential amino acids, L-glutamin, β-mercaptoethanol, 10% fetal calf serum and conditioned medium obtained from pre-confluent 740 LIF-D cells expressing LIF. Immunoreactivity to antibodies against MHC class I or class II was assessed by staining undifferentiated mouse ESC colonies with anti-MHC-I (anti-mouse H-2D, dilution 1/100, Becton Dickinson, Allschwil, Switzerland) or anti-MHC-II (anti-OX6, anti rat RT1B class II, dilution 1/100, Serotec, Düsseldorf, Germany), and by appropriate secondary FITC-coupled antibodies. Images correspond to the middle slice of a deconvoluted stack of confocal images acquired by a Zeiss Meta confocal microscope.

Prior to engraftment, the cardiogenic potential of mESC clones was assessed *in vitro* by forming EBs and scoring the percentage containing efficiently beating cardiomyocytes, as previously described [16, 17]. On the transplantation day, undifferentiated mESC were harvested by trypsinization, washed and then suspended in serum-free culture medium at a concentration of 10^7/ml.

Rat model of MI

All procedures conformed to the Swiss and Cantonal Veterinary Office regulations. The experiments were performed on Male Sprague-Dawley rats (Charles Rivers Laboratories, L'Arbresle, France) with an initial body weight ranging from 300 to 350 g. After induction of anaesthesia with 4–5% isoflurane and tracheal ventilation at 70 cycles/min. (2.5–3.0 mL tidal volume, Harvard Apparatus Co, Inc., Les Ulis, France), animals were maintained under 1.5–2% isofluorane. ECG and respiration curve were monitored during the whole procedure. The heart was aseptically exposed by left intercostal thoracotomy *via* a lateral incision between the fourth and fifth ribs and the pericardium removed to access the left anterior descending artery and its branch under surgical microscope. Permanent ligation was performed with a 6–0 polypropylene suture passed with a tapered needle underneath the left anterior coronary artery, around 2 mm after the origin of the artery between the left atrium and the right pulmonary outflow tract [18]. The appearance of a clearly identifiable white area on the left ventricle corresponding to the unperfused tissue consistently resulted in large infarctions of comparable size (confirmed by *post-mortem* histological evaluation). After suture of the chest, the rats were allowed to recover. Mortality was <25% and it was never observed beyond 1 hr of the surgery.

To assess a possible differential outcome of ESC when transplanted either in an acutely infarcted myocardium (presence of inflammation at 1 week) or in a chronically infarcted myocardium (presence of scare tissue at 4 weeks), animals were divided into normal (n = 24) and myocardial infarcted rats (n = 42). The latter were subdivided into (*i*) acutely infarcted animals (1 week post-MI, n = 24), a time-point associated with extensive neutrophil/macrophage infiltration into the heart (positive anti-MHCII staining, not shown), and (*ii*) chronically infarcted rats (4 weeks post-MI, n = 18), presenting ventricular scar tissue accompanied by an extensive remodelling (enlarged LV chamber, thinning of the left ventricular wall and extensive fibrosis detectable by Landrum staining of collagen). To investigate whether the duration of ESC engraftment could matter on cell differentiation, survival and fate, animals were sacrificed 1 or 4 weeks after cell injection. For each experimental condition (1 and 4 weeks post-MI), we made three shame-injected animals (medium alone).
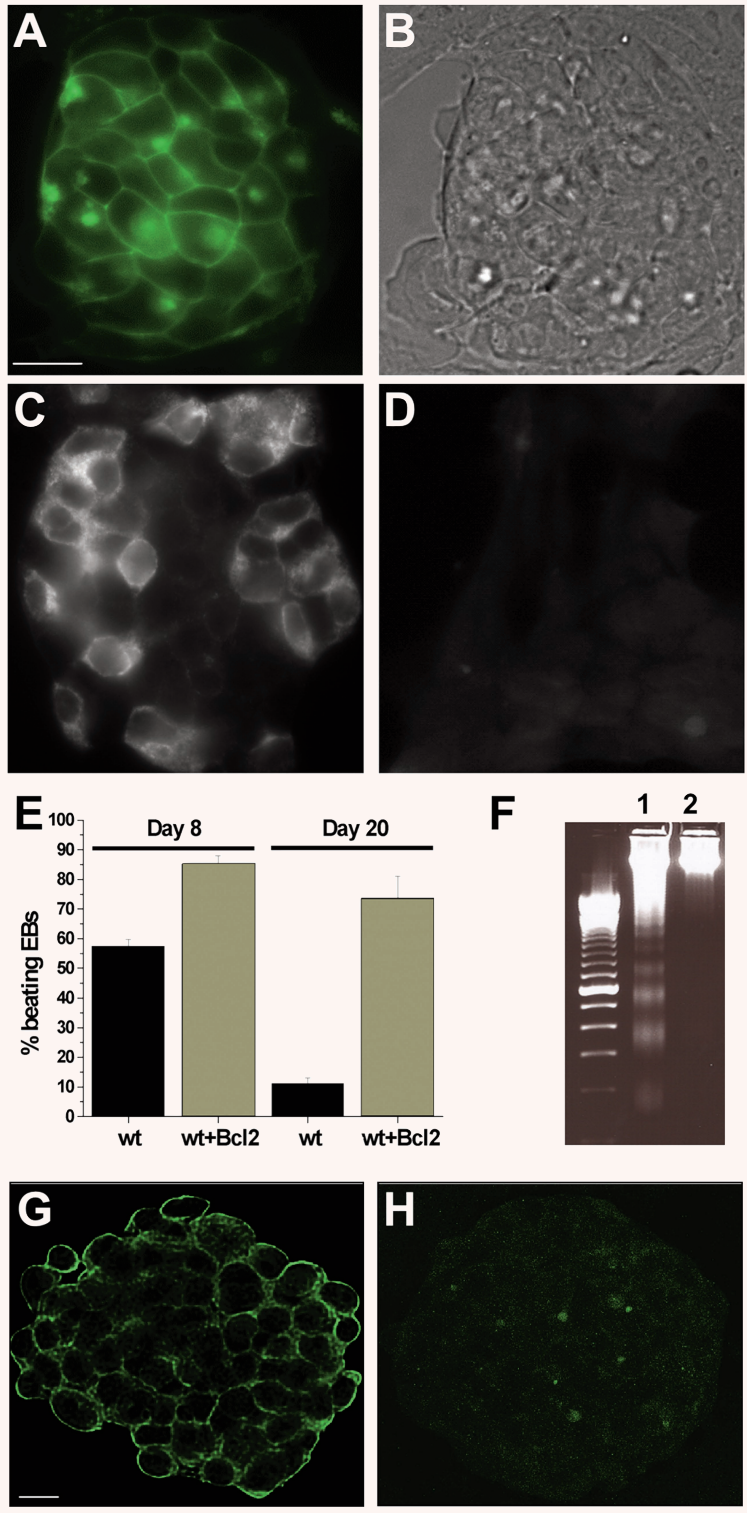
Fig. 1 Characterization of the ESC lines used for the transplantation experiments. (**A**) Expression of eGFP or CD63 transgene by CGR8 stable clones. Panel a: undifferentiated colony positive for eGFP fluorescence localizing to membranes and endosomes. (**B**) Phase contrast picture of the CGR8 colony. (**C**) Immunostaining of a CGR8 cell clone expressing the hBcl2 gene. (**D**) Untransfected cells do not react with the anti-hBcl2 antibody. (**E**) Comparison of the beating activity expressed as percentage of beating EBs measured in wt or hBcl2-CGR8 EBs at day 8 and day 20 of culture. In contrast to wild-type EBs, 20-day-old EBs derived from human Bcl-2 expressing cells maintained a high percentage of beating. (**F**) DNA laddering performed on wild-type (wt) or hBcl2-CGR8 cells (lane 1 and 2, respectively) treated with 10 μM hydrogen peroxide for 24 hrs. (**G**, **H**) Immunoreactivity of mESC to antibodies against MHC class I or class II. Undifferentiated ESC colonies were stained with anti-MHC-I (**G**), or anti-MHC-II (**H**). Images correspond to the middle slice of a deconvoluted stack of confocal images acquired by a Zeiss Meta confocal microscope.

To evaluate the putative immunotolerance toward mESC, two-third of the engrafted rats (16 out of 24 and 11 out of 18, respectively) received a treatment with Cyclosporin A (CsA, 50 mg/ml, Sandimmune, Novartis, Basel, Switzerland), administrated trough a miniosmotic pump (Alzet, ALZA Corporation, Charles River Laboratories, France) filled with 100 mg/2 ml CSA. The pumps were kept overnight in a 37°C incubator before subcutaneous implantation under the back skin. The pump release volume was 2.5 μl per hour corresponding to 6–9 mg/Kg/day during either 7 or 28 days [19].

Transplantation procedure

Rats were anaesthetized and the chest was opened to expose the left ventricular wall. In animals with MI, the infarcted area was visually identified on the basis of surface scar, wall motion abnormality and lack of vascularization (whitish tissue area). Four times 20 μl (2×10^5 cells per injection site) for a total of 0.8×10^6 cells were injected into the left ventricular anterior free wall around the scar border using a tuberculin syringe (29G). Control animals received serum-free culture medium (vehicle) using the same protocol. The chest was closed layer by layer with a 3–0 suture.

Evaluation of left ventricular function by echocardiography

Left ventricular function was assessed by transthoracic echocardiography before cell engraftment (in normal, acute or chronic MI) and before sacrifice, *i.e.* 1 or 4 weeks after transplantation. Rats were anaesthetized by isofluorane (first 4–5% than 1–2%), continuously supplied *via* a mask [20]. Three electrodes were applied on the paws to record the electrocardiographic tracing simultaneously with the cardiac image identifying the phase of a cardiac cycle. Echocardiograms were performed using a 12 MHz phased–array transducer (Philips-Hewlett-Packard, Gland, Switzerland), positioned on the left anterior side of the chest [21]. At first, longitudinal images of the heart were obtained, including the left ventricle, left atrium, the mitral valve and the aortic root, followed by the cross-sectional images from the left ventricular base to the left ventricular apical region. M-mode tracings were obtained below the mitral valve leaflets at the level of the papillary muscles [22]. All two-dimensional images, M-mode tracings and Doppler curves were recorded on videotape for later analysis. We calculated the fractional shortening (FS) as a measure of systolic function, according to the M-mode tracings obtained from the short-axis parasternal view: maximal LV end-diastolic diameter (LVEDD, measured at the time of the QRS deflection), minimal LV end-systolic diameter (LVESD, measured at the time of end-systole), FS (%) = [(LVEDD – LVESD)/LVEDD] × 100 [23]. All measurements were averaged for three consecutive cardiac cycles. The data were expressed as delta of parameters (see Table 1).

Histological and immunohistochemical study

One or four weeks after mESC implantation, the rats were sacrificed with intravenous injection of potassium chloride in order to stop cardiac ventricular contraction on diastole. Hearts were rapidly excised and the cardiac cavities were rinsed in phosphate-buffered saline (PBS) to remove blood and thrombi. The hearts were fixed with 10% formalin for 24 hrs, then embedded in paraffin, sectioned into 3 μm thickness slices and stained with haematoxylin and eosin. Slices were then serially rehydrated in 100%, 95% and 70% ethanol after deparaffinization with xylol. Endogenous per-

oxidase in samples was blocked with hydrogen peroxide, and antigens were retrieved on microwave for 15 min. in 10 mM citrate buffer, pH = 6.0. Non-specific binding was blocked with a 5% BSA/PBS solution for 15 min. Slices were stained at room temperature for 1 hr with one of the following antibodies: anti-hBcl2 (1:100, DAKO, M0887, Baar, Switzerland); anti-alpha-actinin (1:200, Sigma, clone EA-53); anti-PCNA (1:100, DAKO clone PC-10); rabbit polyclonal anti-GFP (1:400, Abcam, ab6556, Cambridge, UK); anti-sarcomeric myosin monoclonal antibody MF 20 (Developmental Studies Hybridoma Bank, University of Iowa). As muscle tissue were shown to often contain highly autofluorescent fibres [24, 25], we always detected eGFP by non-fluorescent immunohistochemistry.

Slices were rinsed with PBS and incubated for 30 min. at room temperature with one of the following affinity-purified biotinylated secondary antibodies (diluted 1:100 or 1:200 in 5% BSA/PBS): anti-rabbit IgG and rat adsorbed anti–mouse IgG (Vector Laboratories, Rectolab, Servion, Switzerland). Sliced were then incubated for 30 min. in avidin-biotin solution (Vectastin ABC Kit, Alexis Corp., Lausen, Switzerland) and the reaction products were visualized with 3,3′-Diaminobenzidine tetrahydrochloride Dehydrate and H_2O_2. After washing with distilled water, counterstaining was performed with haematoxylin for 1 min. The tissues were observed and photographed with a ZEISS Axioskop 2 Plus photomicroscope.

Statistics analysis

All values are presented as means ± S.E. Difference between two groups was compared by unpaired Mann-Whitney U-test. A level of *P* < 0.05 was considered as significant.

Results

Xenotransplantation of undifferentiated mouse ESC into the normal rat heart

To assess the potential of the rat heart to induce undifferentiated xenogenic mESC to differentiate into cardiomyocytes *in situ,* we studied the engraftment of four different mESC lines: wild-type feeder cell-independent CGR8, stable CGR8 clones expressing the eGFP-CD63 fusion protein (Fig. 1A and B) or the human Bcl2 protein (hBcl2, Fig. 1C and D), and the D3 feeder-dependent ESC line. Upon *in vitro* differentiation within embryoid bodies (EBs), we observed a comparable cardiac differentiation potential (around 60 % of beating EBs) using either wt or eGFP-CD63 CGR8 clones. Instead, hBcl2-expressing EBs showed an improved contractile activity (80–90%), both at day 8 and day 20 of culture (Fig. 1E). Moreover, mESC displayed higher resistance to apoptosis upon exposure to reactive oxygen species (Fig. 1F). Of note, our mESC revealed a positive staining for MHC class 1, but not class II (Fig. 1G and H).

We first tested whether mESC could engraft and survive in a healthy rat myocardium in the absence of immune suppression. We histologically examined hearts either 1 or 4 weeks after transplantation of undifferentiated eGFP-CD63 or hBcl-positive mESC (Fig. 2, *n* = 5 and *n* = 8, respectively). Healthy heart sections

Table 1 Left ventricular function in post-infarcted rat models

	MI = 1 week − CsA	MI = 1 week + CsA	*P* Δ (1 week ± CsA)	MI = 4 weeks + CsA	*P* Δ (1 *vs* 4 weeks +CsA)
N	4	8		3	
Heart rate (bpm)	−10.8 ± 44.9	29.0 ± 12.9	0.396	13.3 ± 3.3	0.837
LVEDD (mm)	−20.7 ± 14.8	−12.4 ± 7.3	0.497	8.5 ± 3.4	0.153
LVESD (mm)	−12.1 ± 8.5	−6.4 ± 5.0	0.610	1.7 ± 9.6	0.540
FS (%)	3.3 ± 1.9	−2.3 ± 4.4	1.000	3.3 ± 15.9	0.683

Effect of CsA treatment in acute myocardial infarction (MI = 1 week), 4 weeks after transplantation, and comparison between acute and chronic myocardial infarction, *i.e.* MI = 1 week *versus* MI = 4 weeks in the presence of CsA.
Parameters are expressed as difference (Δ) between values measured in infarcted hearts after and before transplantation (T), in the presence of CsA. Effect was evaluated 4 weeks after cell transplantation. LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; FS, fractional shortening. The formula is expressed as follows: (LVEDD $_{after\ T}$ − LVEDD $_{before\ T}$) and (LVESD $_{after\ T}$ − LVESD $_{before\ T}$), values are mean ± standard error (SE). bpm, beats/min.

stained with haematoxylin and eosin revealed the presence of granulation tissue (Fig. 2A and B) 1 week after engraftment (also containing MHC-II-positive cells, not shown), whereas this was no longer observed 4 weeks after implantation (Fig. 2C and D). At both time-points, no marker-positive mESC were identified at the site of injection, as illustrated by the absence of hBcl2 (0/13, Fig. 2D) or eGFP staining (not shown).

To address the question whether the absence of engrafted cells was possibly due to immune rejection, we repeated the experiment in rats treated with 5 mg/kg/day Cyclosporin A (+CsA). In contrast to what we observed in the absence of CsA (−CsA), one-third of the immunosupressed mESC-injected animals displayed compact tissue masses (1 out of 3 hearts) 1 week after implantation (Fig. 2E and F), later histologically confirmed as small teratomas at the injection site. At 4 weeks, all animals (*n* = 6) presented large teratomas located within the left ventricular wall and often exceeding the left ventricular wall thickness (Fig. 2G and H). These tumours were highly proliferative (strong PCNA staining, see Fig. 5G and H), and revealed heterogeneous staining for hBcl2 (Fig. 2F and H) or eGFP protein (data not shown). We obtained similar results after implantation of wild-type D3 or CGR8 mESC (not shown). We never observed teratomas outgrow within the chest or abdominal cavity. Although we did not systematically assess the migration of ESC in other organs, macroscopic analysis of liver did not reveal any gross abnormality or the presence of tumours, regardless of CsA treatment.

By macroscopic and histological examination we identified teratomas as tumours disrupting the muscle cytoarchitecture (Fig. 3A) and containing tissue originating from the endoderm (epithelium of pancreas and bronchi, Fig. 3B and C), the mesoderm (muscle and cartilage, Fig. 3D and E) and the ectoderm (skin,

Fig. 3F). However, we could not demonstrate a consistent enrichment for ESC-derived cardiac cells positive for eGFP or hBcl2 protein.

Engraftment of undifferentiated mESC into acutely or chronically infarcted rat myocardium (1 week *versus* 4 weeks MI)

We then investigated whether an acutely infarcted myocardial lesion, as opposed to a chronic and remodelled scar tissue, could specifically promote mESC differentiation toward a cardiac phenotype, possibly through the release of cytokines or growth factors occurring during tissue remodelling. Indeed, when compared to normal heart tissue (Fig. 4A, B and G), acutely infarcted ventricular walls showed granulation tissue at 1 week post-MI, detected by haematoxylin and eosin or using proliferation and cardiac markers (MHC) (Fig. 4C, D and H). Instead, granulation tissue with MHC-positive infiltrating cells (data not shown) was not observed at 4 weeks post-MI, both in sham-injected (*n* = 8, not shown) or ESC-injected hearts (Fig. 4E and F). This was also the case in normal ESC-injected hearts (Fig. 2C and D).

In parallel, we tested cell survival and fate in immunosupressed animals. Figure 5A–D shows that in CsA⁻ group, the acutely infarcted hearts injected with mESC and monitored 1 or 4 weeks later displayed only scar tissue, with no staining for proliferation marker PCNA (Fig. 5C). Teratomas or even eGFP-positive (Fig. 5D) or hBcl2-positive cells (not shown) were undetectable (*n* = 8). In contrast, we found highly proliferative ESC-derived tumours within the injected ventricular walls of CsA⁺ rats, strongly positive for the proliferation marker PCNA (Fig. 5G and H), and for eGFP (Fig. 5F), or hBcl2 (Fig. 2F and H). In acutely infarcted CsA⁺ rats (1 week MI) examined 1 week after injection, we found teratomas in 3 out of 5 hearts (60%),

Fig. 2 Fate of undifferentiated mouse ESC transplanted into a normal rat heart as a function of time and immune suppression treatment. (**A–C**) Haematoxylin and eosin (H & E) staining and (**D**) hBcl2 immunohistochemistry of heart sections 1 week and 4 weeks after cell transplantation in the absence of CsA. Magnification: A, C, D = ×10; B = ×50. (**E**, **G**) Haematoxylin and eosin staining and (**F**, **H**) hBcl2 immunohistochemistry of heart sections at 1 and 4 weeks after engraftment in the presence of CsA treatment. Teratomas are visible at 1 (**E**) and 4 weeks (**G**, **H**) under CsA treatment. Magnification: E, G, H = ×10; F = ×50. I = time of injection.
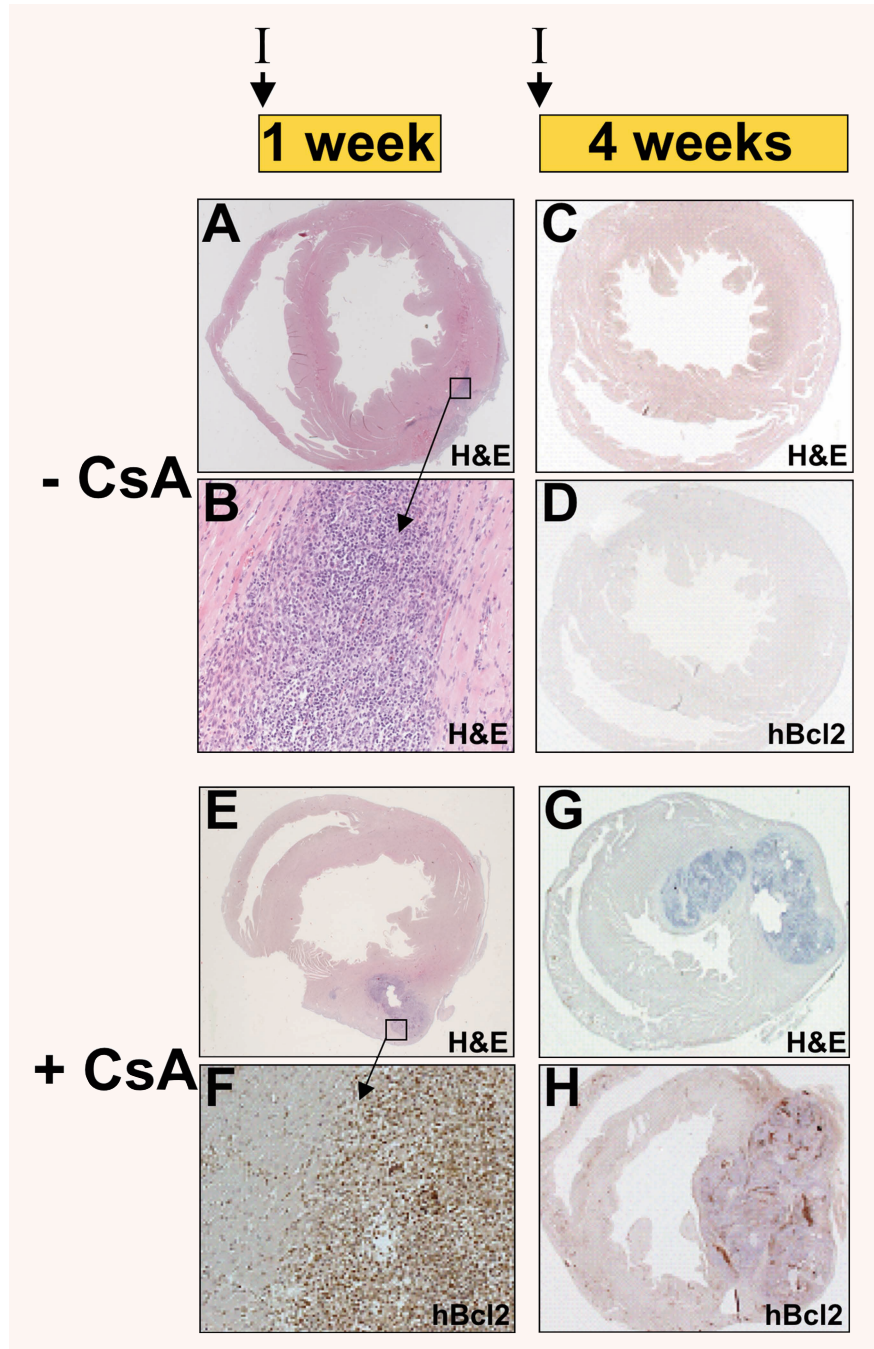
whereas 100% of rats sacrificed after 4 weeks presented highly proliferating teratomas (*n* = 13). Histological evaluation confirmed the presence of transmural scar tissue in a large portion of the left ventricular wall (*n* = 21), accompanied by a dilatation of the left ventricle (see also Fig. 4E). The tumours were positive for either eGFP (Fig. 6A and B) or hBcl2 (Fig. 6F), as well as PCNA-positive (Fig. 6G).

In summary, 4 weeks after implantation, undifferentiated mESC formed teratomas in either normal, acutely or chronically infarcted rat hearts only when the animals were immunosupressed.

Importantly, we preferentially detected eGFP-positive cardiomyocytes displaying aligned sarcomeres (Fig. 6C and D) within the tumoural mass of infarcted animals but also at the peripheral
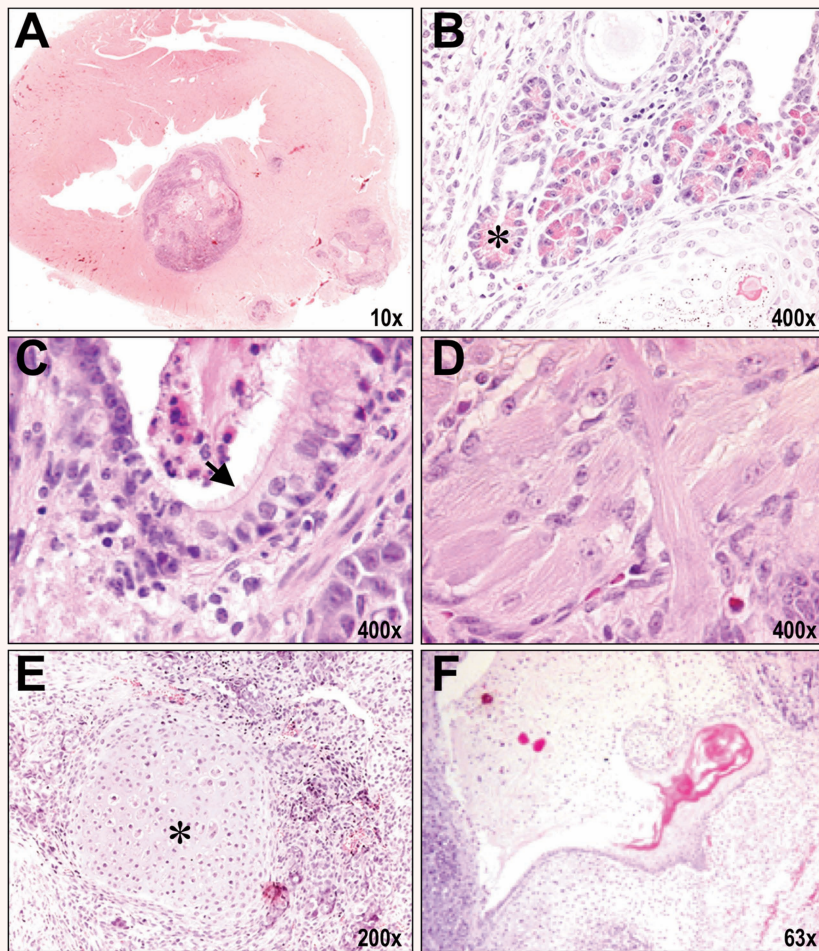
Fig. 3 Histology of teratomas masses, as visualized by haematoxylin and eosin staining (**A**), 4 weeks after transmyocardial implantation of undifferentiated ESC in the presence of CsA. A variety of mature tissues arranged in a disorganized fashion were observed, such as (**B**) pancreatic glands, (**C**) ciliated epithelium, (**D**) muscle fibres, (**E**) cartilage and (**F**) skin. Magnification: A = ×10; B–E = ×400 and F = ×63. Asterisks and arrows highlight the different tissues.

border of the tumours (positively stained for cardiac specific markers such as MHC/MLC (Fig. 6D) and α-actinin (not shown)). We therefore addressed the question of whether the inflammation-free environment of chronically infarcted left ventricles in CsA$^+$ rats could be more favourable to cardiogenic cell differentiation than normal or acutely infarcted left ventricles. Cardiac cells positive for hBcl2 and MHC (identified in consecutive sections) were also observed in chronically infarcted hearts (Fig. 6F and H), but there was no significant difference in the occurrence of cardiomyocytes in chronic compared to acute MI condition (Fig. 6B, D, F and H).

## Effect of time and immunosuppression on teratoma formation

As summarized in Figure 7, animals were tumour-free in the absence of CsA, while under immune suppression, 100% of the hearts presented ESC-derived teratomas 4 weeks after transplan-

tation. At earlier time-points, *i.e.* 1 week after engraftment, however, chronic MI led to teratoma formation at a lower frequency (20%) than in acute MI (60%, 1/5 *versus* 3/5, respectively). Teratoma occurrence in normal hearts was 33%.

Evaluation of cardiac function in xenotransplanted animals

To assess the influence of implanted mESC on rat heart structure and function, we assessed left ventricular size (left ventricular end-diastolic diameter [LVEDD]; left ventricular end systolic diameter [LVESD]; change in shape of LV cavity) and monitored functional cardiac parameters (heart rate and LV systolic function described as LV fractional shortening [FS]) with 2-D and M-mode echocardiography. The animals were evaluated before cell transplantation and at the end of the experiment (either 1 or 4 weeks later). The presence of a tumour growing towards or outwards

Fig. 4 Histomorphology and immuno-histochemical evaluation of rat heart sections. (**A**, **B**) Haematoxylin and eosin staining of normal rats, (**C**, **D**) acutely infarcted heart (1 week MI) and (**E**, **F**) chronically infarcted hearts (4 weeks MI). Magnification: A, C, E = ×10, B, D, F = ×400. (**G**, **H**) Immunohistochemical staining of different markers present in heart sections of normal (**G**) and acutely infarcted rats (1 week MI, H). Top insets illustrate macroscopic views of the explanted heart. The white triangles indicate the coronary ligature suture (either shame or true), while the dotted oval shows the infarction area (hypointense whitish zone). Granulation tissue and disruption of myocardial architecture is visible in 1-week MI hearts.
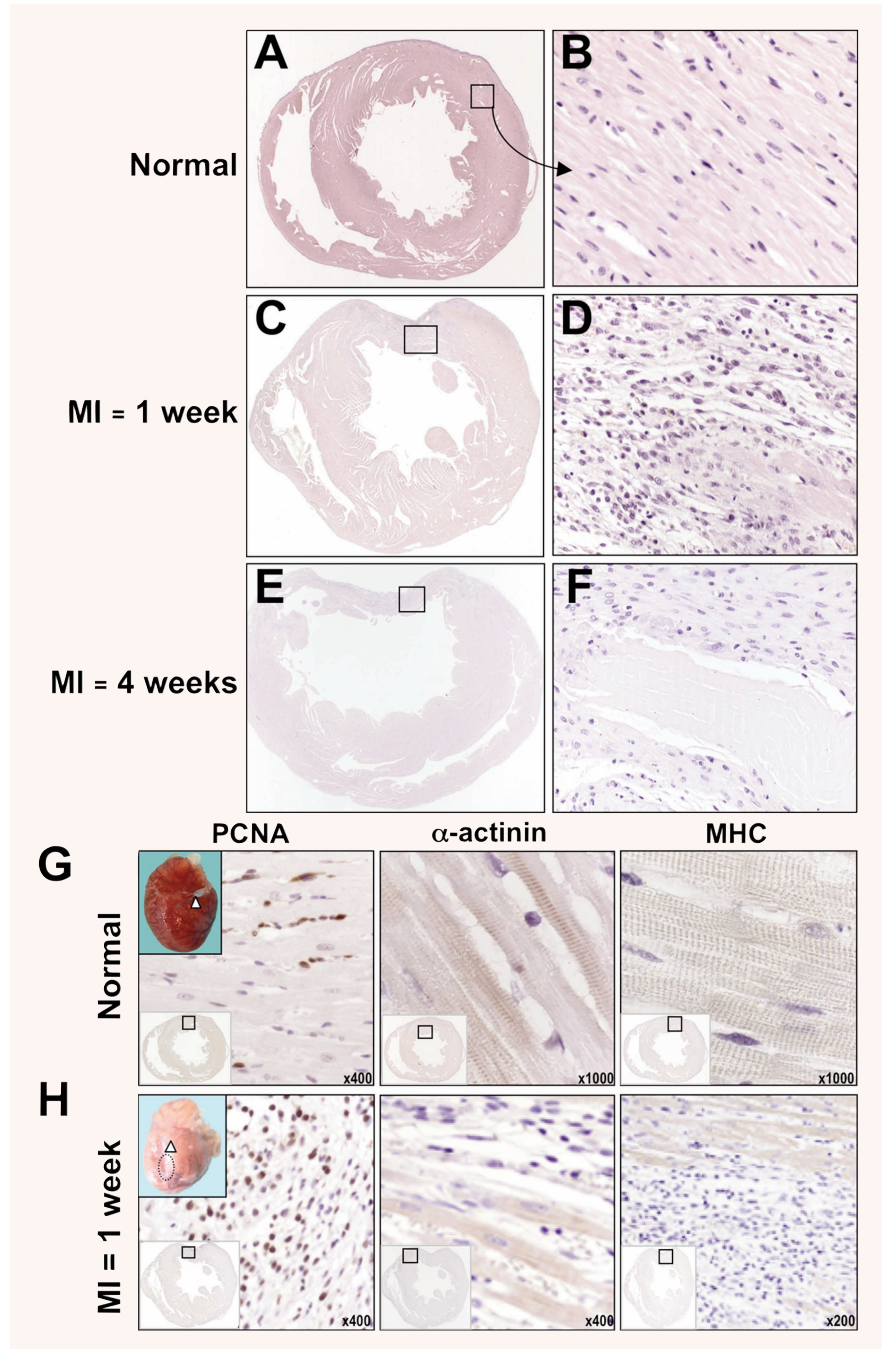
the LV chamber could be identified on both 2-D and M-mode echocardiographic images as a space occupying lesion, highly affecting LV wall motion and sometimes even global LV systolic function (not shown). Table 1 summarizes the effect of mESC implantation on cardiac function, expressed for each parameter as changes in the values measured before and after the cell injection. We did not observe any statistical difference in heart rate, LVEDD, LVESD and percentage of FS in acute MI, independently from the administration of CsA (Table 1, column 1 and 2). Whether mESC engrafted into acute or chronic MI (Table 1, column 2 and 4) under immunosuppression, there was no significant difference 4 weeks after implantation.
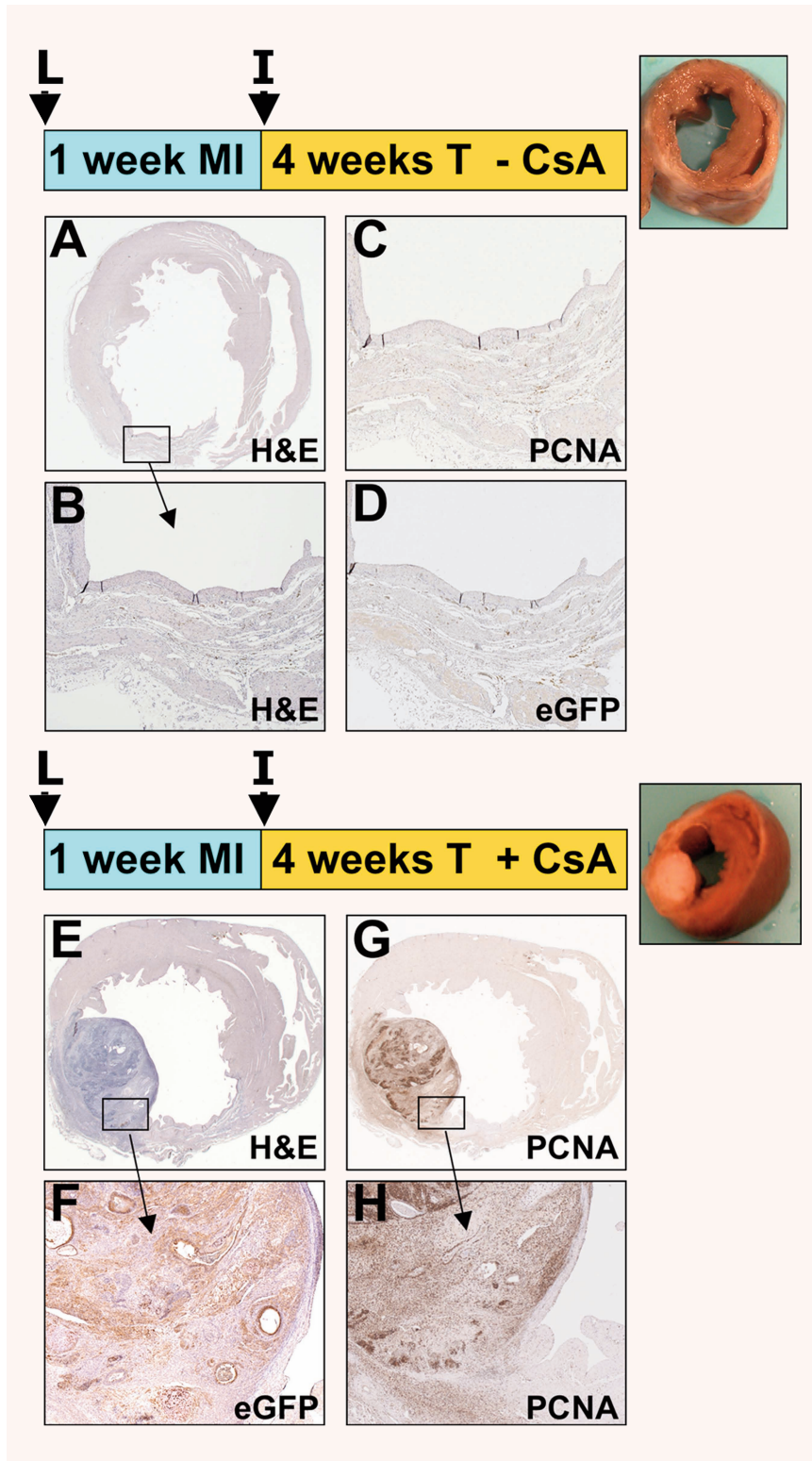
Fig. 5 Fate of undifferentiated eGFP-ESC 4 weeks after their transplantation (T) into acutely infarcted hearts (1 week MI), in the absence (**A–D**) or presence (**E–H**) of CsA treatment. Haematoxylin and eosin and immuno-histochemistry staining with: PCNA (**C** and **G**, **H**) and eGFP (**D** and **F**). In the absence of CsA, a fibrotic tissue devoid of transplanted mESC is visible (**A–D**). Magnification: A, E, G = ×10, C–D and F, H = ×50. L = coronary ligation; I = cell injection.

Fig. 6 Identification of ESC-derived cardiomyocytes into the teratoma masses in (**A**–**D**) 1 week or (**E**–**H**) 4 weeks MI hearts, both analysed 4 weeks after cell engraftment in the presence of CsA. EGFP-labelled ESC are shown for 1 week MI (**A**, **B**) while hBcl2-transduced ESC are illustrated for 4 weeks MI (**F**). Differentiating ESC positive for myosin heavy chains (MHC) were identified in both conditions (**C**, **D** and **H**). Tumours contained proliferating PCNA-positive cells (**G**). Magnification: A, C = ×20; B, D = ×400, E = 10×, F–H: 100×. L = coronary ligation; I = cell injection.
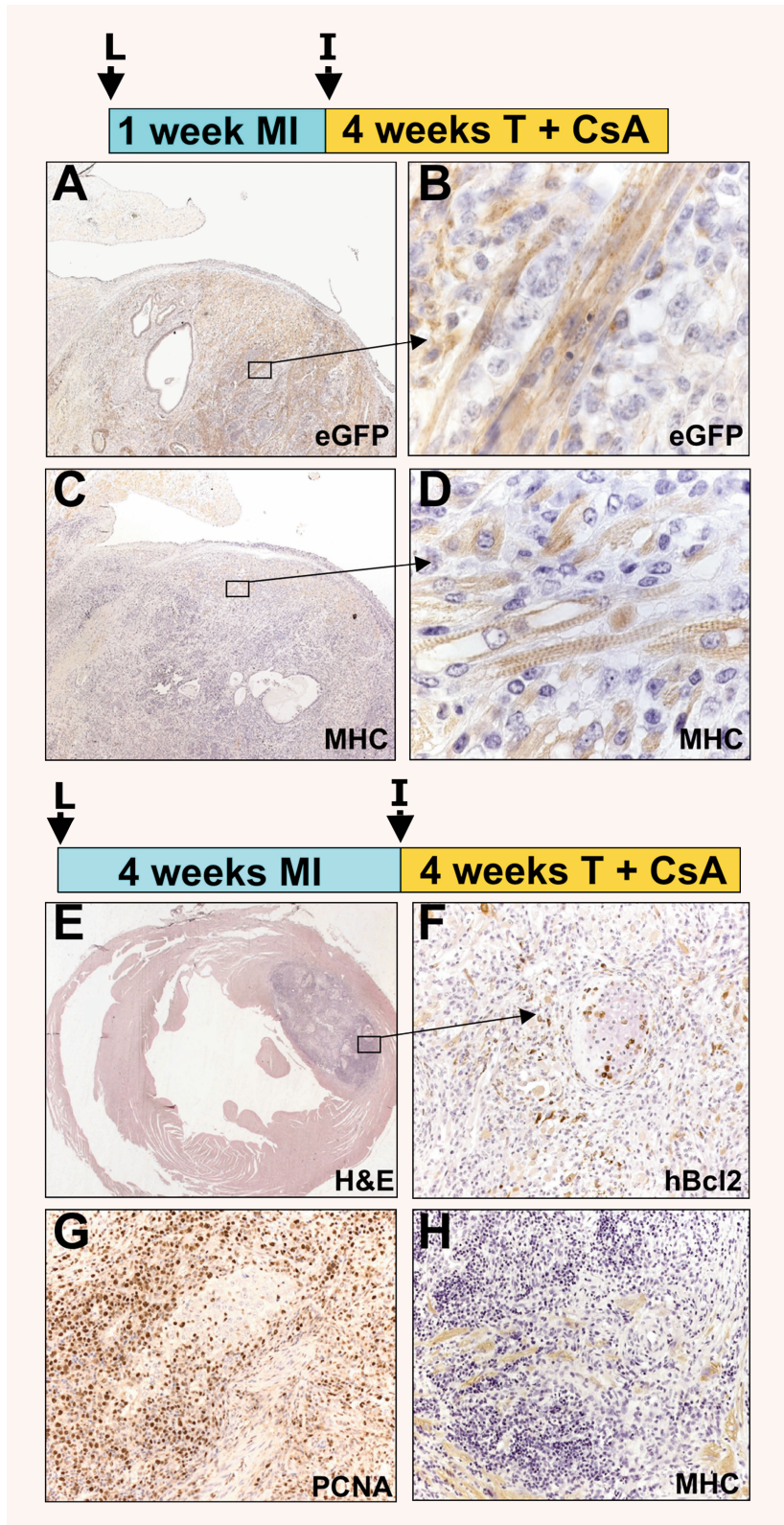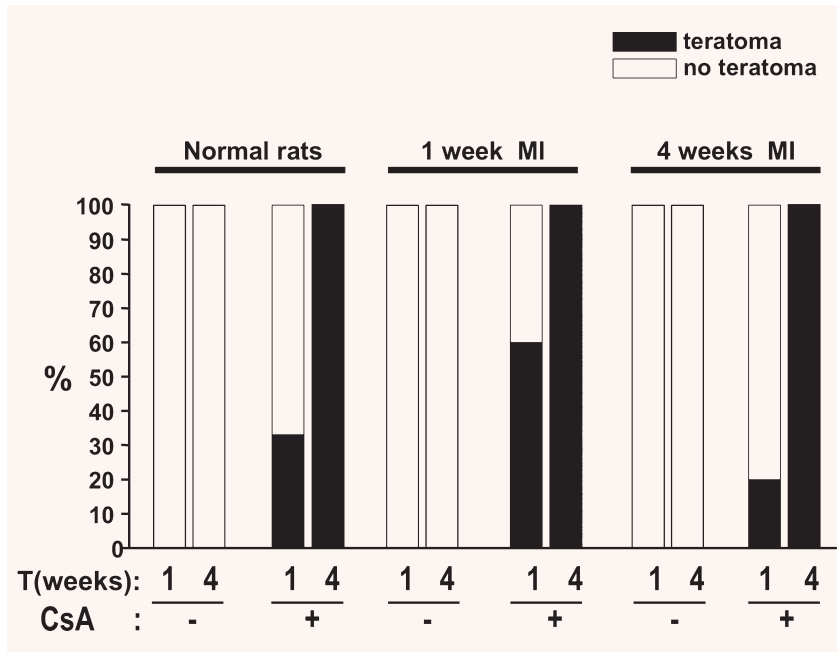
Fig. 7 Effect of time and immunosuppression on the occurrence of teratomas after transplantation of undifferentiated mESC. Normal rats were compared to post-infarcted rats (1 week MI and 4 weeks MI), and evaluated at 1 week and 4 weeks after implantation in presence or absence of CsA. In all cases, teratoma formation was dependent on the administration of CsA as well as on the duration of cell implantation, but not on the type of injected ESC, nor on the presence of a myocardial injury.

Discussion

We investigated whether a rat heart, either healthy or lesioned by MI, could tolerate and instruct undifferentiated mouse ESC to acquire a cardiac phenotype *in vivo,* thereby leading to an improved cardiac function. An important major finding of our study is that xenogenic undifferentiated mESC could not survive within the rat heart in the absence of immunosuppression, regardless of the type of mESC used, *i.e.* feeder-dependent or independent, wild-type or expressing eGFP-CD63, or even the hBcl2 gene, known to confer resistance to apoptosis in response to insults like reactive oxygen species. Although hBcl2 could predispose some cell types to neoplasia, increased expression of hBcl2 alone is not oncogenic *per se* [26], and chimeric or transgenic mice generated from hBcl2-expressing mESC are tumour-free and require additional hits before oncogenesis [27, 28].

Oncogenicity as well as complications due to immunosuppressive drugs, and, ultimately, immune rejection of engrafted ESC are major concerns for clinical transplantation. At present, the *in vivo* immunogenicity of ESC is still controversial and cellular immune responses to ESC and related potential mechanisms remain to be demonstrated.

The concept that ESC are immune-privileged or have reduced immunogenicity is supported by the several observations. First, mESC seem to express low or no levels of MHC class I [29–32]. Second, undifferentiated and differentiated ESC appeared resistant to killing by activated NK cells [30], as they lack costimulatory molecules and Nk lysis receptors [33, 34] (data not confirmed by [35]). Potential mechanisms include: (*i*) contact-independent

inhibition of CD4+ T-cell proliferation [34] *via* a TGFβ-mediated inhibition of dendritic cells [34, 36], (*ii*) local immunosuppressive cell–cell paracrine factors, *i.e.* Serpin-6 (serine protease inhibitor 6)-mediated resistance of mESC and EBs to CTL-mediated lysis [34, 36] and (*iii*) tolerance induction *via* Fas ligand activation of CD95 and inducted apoptosis in recipient T cells (lack of ESC engraftment in Fas-deficient mice) [29, 35]. Third, intraportal injection of undifferentiated rat ES cell-like cells into fully MHC-mismatched rats induced a state of tolerance, allowing the subsequent long-term acceptance of second-set transplanted cardiac allografts [29]. However, this was not confirmed upon *in utero* transplantation of mouse foetuses with MHC-mismatched ESC [37]. Forth, xenogenic undifferentiated mESC survived and differentiated into cardiac cells within immunocompetent rat or sheep hearts [14, 15, 38]. In contrast, this was not the case in immunocompromized or allogeneic mice in which intense immune responses with infiltration of T and B cells led to graft removal [39–41].

Whether chimerism does happen in a mouse-to-rat transplantation model, and whether injected mESC escape from the heart into the circulation, remains to be demonstrated. As our mESC were positive *in vitro* for MHC type 1 (but not for type II), we thus speculate that ESC are likely to be removed through the activation of the rat immune system, as in the case of allogenic setting [41]. Accordingly, as reported in a very recent publication by Robertson *et al.* [42], the expression of minor histocompatibility antigens was sufficient to provoke acute rejection of tissue differentiated from mESC. Despite the immunogenicity observed *in vivo*, they could establish a transplantation tolerance using minimal host conditioning with nondepleting monoclonal antibodies for the

T-cell coreceptors, CD4 and CD8. In our experiments, although we detected MHCII-positive cells in the infarcted tissue (not shown), leading us to infer a possible T-cell-mediated rejection, the mechanisms responsible of *in vivo* cell rejection in a xenogenic setting remain to be addressed. Moreover, given the avascularized character and therefore low oxygen conditions in the highly damaged and remodelled cardiac tissue, we would expect some degree of apoptosis according to the site of injection (infarcted *versus* peri-infarcted zone).

The environment provided by a lesioned or destroyed tissue clearly constitutes a critical parameter to be taken into account for an efficient cell-based tissue reconstruction. In this context, we tested whether the myocardium, considered as a non-proliferative tissue in which occurrence of tumours is uncommon, may work as an instructive tissue to ESC and are less likely to proliferate in an uncontrolled way [14, 15, 38]. Our data, however, did not confirm the ability of an acutely infarcted myocardium, as compared to a healthy or a scar tissue, to commit undifferentiated mESC towards the cardiac fate by providing instructive molecules, cytokines and/or cardiogenic growth factors such as TGFβ family members [43–46]. Instead, immunosupressed mESC-engrafted rats developed intracardiac teratomas enriched with cells of the three embryonic layers, thus alleviating concerns over the alleged potential side effects CsA might exert on cell signalling through the inhibition of the calcium-dependent phosphatase calcineurin [47]. Moreover, calcineurin signalling does not appear to be required for the initial events of heart formation since mouse embryos with targeted mutations of calcineurin subunits undergo normal heart tube formation and cardiomyocyte differentiation [48, 49]. In line with our data, Nussbaum *et al.* also showed that undifferentiated mESC consistently formed teratomas in infarcted hearts of nude or immunocompetent syngeneic mice, while they were rejected if allogeneic *via* an up-regulation of class I and II MHC [41], possibly mediated by IFN-γ, as observed *in vitro* [30].

Although puzzling, at this point we cannot exclude that in very specific xenogenic conditions, *i.e.* mouse to rat or mouse to sheep (as reported [14, 38]), and provided a BMP2-mediated pre-commitment of ESC *in vitro* [14, 38, 50] the heart may constitute a permissive and cardiac instructive niche especially in fibrotic areas known to secrete TGFβ [51–53]. A similar niche-induced tumour-free neuronal differentiation was observed when mESC were engrafted into rat, but not mouse brain [54]. However, our xenotransplantation experiments designed to closely reproduced conditions used by Befhar *et al.* could not confirm these observations. Of note, two recent papers by Puceat and collaborators reported the inability to reproduce xenogenic transplantation of mouse ESC into baboon [55], and achieved BMP2-treated hESC engraftment into rat hearts exclusively under immunosuppressive conditions [56], suggesting that the xenogenic mouse to baboon and human to rat transplantation is not permissive. Consistent with this is the work by Grinnemo *et al.* [57] who demonstrated that human ESC do not inhibit immune response during direct or indirect antigen presentation and they are acutely rejected in a xenogenic mouse heart setting.

Possible explanations for such discrepancies could reside in how and where cell injections were made in the myocardial tissue. Whereas we always injected the cells into the border area of the infarct zone in thoracotomized animals, the authors reached the left ventricle through the diaphragm or by thoracotomy. This may also affect the number and the position of cells that actually engraft. In contrast, we do not expect these discrepancies to be dictated by differences in the type of ESC used, since we obtained the same outcome with feeder-dependent and independent cells, as well as with gene-transfected or untransfected ESC. Moreover, a recent dose-dependence study did not support the hypothesis that low cell numbers would rather engraft and correctly differentiate instead of turning into teratomas [41]. Nevertheless, at this point we cannot formally exclude that a small number of cells may have survived rejection after injection into the immunocompetent animals. It is possible that some cells may have escaped detection either because (*i*) they are too few to be detected by IHC, and/or (*ii*) they have a silenced or weakly expressed transgene (*i.e.* GFP). However, as in our experiments, we did not see GFP or Bcl-2 silencing when mESC differentiated within tumours in the presence of CsA, therefore it seems unlikely that this would preferentially occur in the absence of immune suppression. Moreover, we could not detect cells positive for cardiac markers (MHC or alpha-actinin staining) in the scar tissue of infarcted hearts at 1 month after injection, a tissue mainly composed of collagen fibres (that we could identify by Landrum staining), thus indicating that the implanted cells did not preferentially commit to the cardiac lineage. Further, the lack of improvement of heart function indicates that, if residual transplanted cells had survived, they did not contribute, either actively or passively, to heart function.

In conclusion, characterization of the immunologic mechanisms of both tolerance to and rejection of ESC in relation with the host species will require further dedicated investigations, including strategies to eventually remove residual undifferentiated or non-cardiogenic cells devoted to heart regeneration. For safe ESC therapy of the heart, it is mandatory to ensure a secure commitment toward specific cardiac lineages, namely the cardiac ventricular myocyte, using cardiogenic factor-based protocols preventing the risk of tumour formation as well rejection, thus ensuring a safe therapeutic implementation in clinical practice.

Acknowledgements

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