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Differentiation *in vivo* of cardiac committed Human embryonic stem cells in post-myocardial infarcted rats.

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Running Title: Human ES cells in postmyocardial infarction

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Abstract

Human embryonic stem (HES) cells can give rise to cardiomyocytes *in vitro*. However whether undifferentiated HES cells also feature a myocardial regenerative capacity after *in vivo* engraftment has not been established yet.

We compared two HES cell lines (HUES-1 & I6) that were specified towards a cardiac lineage by exposure to bone morphogenetic protein (BMP2) and SU5402, a FGF receptor inhibitor. Real time PCR revealed that the cardiogenic inductive factor turned on expression of mesodermal and cardiac genes (Tbx6, Isl1, FoxH1,Nkx2.5, Mef2c, and α-actin).

Thirty immunosuppressed rats underwent coronary artery ligation and, 2 weeks later, were randomized and received in-scar injections of either culture medium (controls) or BMP2 ($\pm$SU5402)-treated HES cells. After 2 months, human cells were detected by anti-human lamin immunostaining and their cardiomyocytic differentiation was evidenced by their expression of cardiac markers by RT-PCR and immunofluorescence using an anti-β myosin antibody. No teratoma was observed in hearts or any other organ of the body.

The ability of cardiac-specified HES cells to differentiate along the cardiomyogenic pathway following transplantation into infarcted myocardium raises the hope that these cells might become effective candidates for myocardial regeneration.

Introduction

Heart failure is becoming a predominant disease and a leading cause of death in most of developed countries. Regardless of the origin of myocardial failure (i.e, ischemic or genetic), the clinical symptoms result mainly from the death of cardiomyocytes replaced by a fibrotic and non contractile tissue. Pharmacological approaches to cure or relieve heart failure have been facing limitations. Because of a limited regeneration capability of the heart [1] and a shortage in donors for heart transplantation, an external source of cells has been envisioned as a therapeutic solution to bring a gain in function to diseased myocardium. For the last few years, hematopoietic stem cells had raised many hopes as a potential autologous cell source to repair diseased myocardium. However, the enthusiasm generated by the early non-controlled phase I studies has been dampened by the more recent recognition that out of four randomised controlled trials entailing intracoronary infusions of bone marrow-derived cells shortly after myocardial infarction, three failed to meet their primary end point, i.e., an improvement in left ventricular ejection fraction [2-5]. Combined with basic studies disproving the cardiogenic potential of these cells [6] [7], the outcome of these trials demonstrates that these cells do not really regenerate the diseased myocardium and it is unlikely that their paracrine effects may be sufficient for restoring function of extensively scarred myocardium [8]. The same limitations apply to skeletal myoblasts [9].Thus, these findings call for another stem cell source to achieve myocardial regeneration. Among various other cell types, embryonic stem (ES) cells [10-12] or ES cell-derived cardiomyocytes [13,14] have turned out to be the most promising for replacing scar fibrosis by new contractile elements. However, the number of cells required to regenerate a post-infarcted human myocardium (i.e., several hundreds of million) is too high to be reasonably achieved by *in vitro* engineering of ES cell-derived cardiomyocytes. For the last few years, we and others have shown that proliferative mouse ES cells engrafted in a diseased myocardium further differentiate into functional cardiomyocytes

following *in-vitro* commitment using the cardiogenic morphogen BMP2 [10-12]. Cardiac-specified cells then complete their differentiation in response to the local cues present in the scar and do not generate any kind of tumors. Thus, although Human ES cells do not share with mouse ES cells the same molecular mechanisms of self-renewal or capabilities of spontaneous differentiation [15], we designed this study to assess whether Human ES cells could be specified toward a cardiac lineage *in vitro* and subsequently differentiate into cardiomyocytes *in situ* following their transplantation in infarcted myocardium.

Herein, we bring the proof of concept that Human ES cells can also be directed toward a cardiogenic fate using the morphogen BMP2. Furthermore, the cells do differentiate into cardiomyocytes following engraftment into the myocardial scar without any sign of hyperproliferation. These data open the path for the use of early cardiac progenitors, which retain the capability to proliferate and repopulate the postinfarction scar.

Materials and Methods

Real-Time Quantitative PCR by SYBR Green Detection

RNA was extracted from HES cells or slices of rat myocardium using a Quiagen kit. One µg of RNA was reverse-transcribed using the Mu-MLV reverse transcriptase (Invitrogen, Cergy, France) and oligo(16)dT.

Real-time quantitative PCR was performed using a Light Cycler (Roche Diagnostic) or a Chromo4 thermal cycler (Biorad). Amplification was carried out as recommended by the manufacturers. Twelve or Twenty two µl reaction mixture contained 10 or 20 µl of Roche or Abgene SYBR Green I mix respectively (including Taq DNA polymerase, reaction buffer, deoxynucleoside trisphosphate mix, and SYBR Green I dye, 3 mM $MgCl_2$), 0.25 µM concentration of appropriate primer and 2 µl of cDNA. The amplification programme included the initial denaturation step at 95°C for 15 or 8 min, and 40 cycles of denaturation at 95°C for 10s, annealing at 65 °C for 8s (Light cycler) or 20s (Chromo4), and extension at 72°C for 8 or 30s. The temperature transition rate was 20 (Light Cycler) or 4 (Biorad) °C/s. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was acquired by heating the product at 20 or 4°C/s to 95°C, cooling it at 20 or 4°C/s to 70°C, keeping it at 70°C for 20 s, and then slowly heating it at 20 or 4°C/s to 95°C. Fluorescence was measured through the slow heating phase. Melting curves were used to determine the specificity of PCR products, which were confirmed using conventional gel electrophoresis. Data were analysed according to Pfafll et al [16]. Primers specific for human genes are described in table 1.

Culture and cardiac commitment of Human Embryonic stem cells

HUES-1 and I6 cell lines were cultured on Mouse Embryonic Fibroblasts (MEF) prepared from E14 mouse embryos using KO-DMEM medium supplemented with mercaptoethanol, glutamine, non essential amino acids, 15% KOSR and 10 or 5 ng/ml FGF2 respectively. Medium was changed every day. Cell colonies were dissociated into single cells or cell clusters every 4-5 days using trypsin (HUES-1) or collagenase (I6), respectively. A similar enzymatic digestion was used prior to cell transplantation in infarcted rats.

HES cells were treated for 48 hrs with 10 ng/ml BMP2 in the presence or absence of 1 μM SU5402, a FGF receptor inhibitor, in low KOSR (5%) containing KO-DMEM. Embryoïd bodies were generated after trypsinisation (HUES-1) or collagenase (I6) dissociation of HES cell colonies and cell aggregation in low attachment dishes (Nunc) in DMEM, 10% foetal calf serum.

Myocardial infarction model

Myocardial infarction was induced in female Wistar (mean weight of 250 g) by ligation of the left coronary artery. Rats were operated on under general anaesthesia with isoflurane (Baxter), 3% at induction and 2% for maintenance. After tracheal intubation, mechanical ventilation (Alphalab, Minerve) was set at a rate of 70/min and with an 0.2 ml average insufflate volume. Analgesia was performed with a 10mg/kg subcutaneous injection of ketoprofen (Merial).

The heart was exposed through a left thoracotomy and the left coronary artery was permanently snared between the pulmonary artery trunk and the left atrial appendage.

Rats randomization and myocardial cell injection

On the 15th day following infarction, the rats were reoperated on by median sternotomy and randomized to receive injections of BMP2-treated HUES-1 cells (3×10^6 HUES-1 cells, n= 11

rats) in suspension of single cells, BMP2-treated I6 ES cells (3 10^6 I6 cells, n= 11 rats) in suspension of small cell clusters or control medium (n= 9 rats). Additional animals (n= 5 rats) received in-scar injections of 3 10^6 HUES-1 cells that had been exposed to both BMP2 and SU5402. We selected HUES-1 cell line for the latter experimental situation since this is the one which is not already committed to the mesoderm. One rat of each group (HUES-1 cell- and I6 cell- transplanted) died within 48 hrs after cell injection.

Immunosuppressive therapy, consisting in one daily 10mg/kg subcutaneous shot of cyclosporine A, was started on the same day and continued until sacrifice.

Histopathology

Myocardial sections were stained with eosin and hematoxylin using a standard protocol.

Two months after myocardial injection, rats were euthanized after general anaesthesia. Transverse-cut rat hearts were immediately fixed in OTC (Tissutec) and frozen at -180°C nitrogen. Eight μm sections were cut on an ultramicrotome (LM 1850,Leica).

Potential tumor growth was assessed with 8 μm standardized sections stained with hematoxylin and eosin.

Immunofluorescence of myocardial cryosections were performed after paraformaldhehyde fixation and permeabilisation using Triton X-100 with an anti-human ventricular β myosin heavy chain (MHC) (Chemicon) , anti-human lamin A/C (Novacastra) anti-atrial natriuretic peptide (ANP, Abgent) and anti-Connexin 43 (Cx43) (SIGMA) antibodies. The proteins were revealed using alexa-conjugated antibodies. Sections were observed in confocal microscopy (ZEISS LSM-510 meta).

In addition, a whole-body autopsy of each transplanted rat, including brain, lungs, liver, spleen, pancreas, kidneys, periaortic lymph nodes, thymus, spine and ovaries, was systematically performed for the detection of a tumor.

Results

Phenotype of undifferentiated I6 and HUES-1 cell lines

We used both HUES-1 and I6 HES cell lines to test their cardiogenic potential *in vitro* and *in vivo*. Indeed, a real-time PCR amplification of a few mesodermal and cardiac genes in both cell lines showed that the I6 cell line featured a higher basal expression of both mesodermal (Tbx6, SRF, Mesp1, brachyury) and early cardiac (Isl1, Mef2c,Tbx20) genes. GATA4 was weakly expressed in I6 but not in HUES-1 cells. Nkx2.5 was barely detected in either I6 or HUES-1 cell lines. Oct-4 level was not significantly different between both cell lines (Fig. 1).

Cardiac commitment of HES cells

Both I6 and HUES-1 Human ES cells were treated for 48hrs with 10 ng/ml human recombinant BMP2. Gene induction was tested using real time Q-PCR. Figure 2 shows that both mesodermal (i.e., SRF, Tbx6, FoxH1, Isl1) and cardiac (Mef2c, Nkx2.5, α-actin) genes were induced by the morphogen in HUES-1 cells. This effect was further enhanced by 3-10 folds when BMP2 was added in the presence of the FGF-R inhibitor SU5402. No significant difference was observed in the extent of the BMP2 cardiogenic response between both cell lines (Fig. 2A) although the total number of copies of each gene expressed following BMP2 induction was much higher (i.e. 10-15 fold) in I6 than in HUES-1 cell line (data not shown).

To test whether BMP2-induced HES cell commitment was translated into a process of cardiac differentiation and to envision the differentiation scenario that might take place *in vivo*, control or BMP2-challenged HUES-1 cells were allowed to aggregate to form embryoid bodies (EBs). Gene expression was then monitored in day 2 and day 5 EBs. BMP2 effect was observed at day 2 (i.e. two fold induction in gene expression) and became prominent at day 5 (Fig. 2B). At that stage of development, expression of both mesodermal and cardiac genes was dramatically increased by 3 to 10 folds (Fig. 2). In contrast, Oct-4 was downregulated

and almost absent in EBs generated from BMP2-treated ES cells. Similar results were obtained using I6 cell line.

Engraftment of cardiac committed cells in post-infarcted rat heart

Two months after coronary artery ligation, Human α-actin mRNAs were identified in transplanted hearts but not in those injected with the control medium (Fig. 3). In contrast, we could not detect any mRNA encoding Oct-4, Pax6 (an early ectodermal marker) or α-foeto protein, an early endodermal marker (data not shown).

Immunostaining with an anti-ANP and anti-human lamin antibodies revealed the presence of lamin-positive human ES cell derived-cardiomyocytes (Fig. 4A)

To further define the phenotype of ES cell-derived cardiomyocytes, sections were immunostained with an anti- human β-MHC antibody. These experiments revealed the presence of differentiated cardiomyocytes (Fig. 4B) in 40 % and 71% of cryosections examined from HUES-1- and I6-engrafted hearts, respectively and in 85% of sections examined from rat grafted with HUES-1 cells treated with *both* BMP2 and SU5402. The cell engraftment was however limited. BMP2 treated HUES-1, I6 and BMP2/SU5402 HUES-1 treated ES cell-derived cardiomyocytes colonized 2.4±0.3, 3.1±0.4 and 3,6±0.3% of the scar (n=10) (Fig. 4C), respectively. Careful examination of these sections further indicated that these cardiomyocytes were still at a foetal stage demonstrated by a shorter sarcomeric length (1.6±0.1μm) compared to 2 ±0.1μm in adult rat (Fig. 4D)

Eosin-hematoxylin stained sections did not show any sign of inflammation or cell hyperproliferation two months posttransplantation (Fig. 5). Likewise, whole-body autopsies failed to disclose any tumor in peripheral organs.

Discussion

Our study reveals that HES cells are capable to differentiate into cardiomyocytes without formation of teratomas after commitment toward a cardiac lineage using the cardiogenic factor BMP2. While BMP2 was shown to improve late cardiac differentiation of already differentiating cells [17], our study reports the strong instructive action of the morphogen on undifferentiated HES cells.

BMP2 is a potent mesodermal and cardiogenic instructor when used at low concentration. Its cardiogenic potential is a well conserved property throughout the evolution. Dpp, the drosophila homolog of BMP2, favours formation of the mesoderm including the heart [18]. Similar effects have been observed in zebrafish [19], Xenopus [20,21] and chicken [22]. Our data obtained in two separate cell lines uncovered that BMP2 function is conserved in human species. While I6 cells were more prone to give rise to a mesodermal lineage (Fig.1), maximal BMP2 response was not significantly different from the one observed with HUES-1 cells although maximal extent of gene expression was higher in I6 than in HUES-1 cells. Used alone, in a defined (KOSR) medium, BMP2 effect was weak while its instructive action was dramatically enhanced by addition of the FGF receptor inhibitor, SU5402. Indeed, Human ES cells are grown on feeder cells which secrete many factors including FGF2 which is known to antagonize the BMP2 signaling pathway. SU5402 could act through at least two mechanisms to unmask the BMP2 transcriptional effect. First, FGF2 phosphorylates smad2/3, thereby preventing the BMP2 signaling co–factor from translocating into the nucleus and thus to exert its transcriptional action [23]. Second, FGF2 is also known to act as a paracrine factor on both MEF and HES cells to regulate expression of Cerberus, a nodal and BMP antagonist enriched in HES cells [24]. Finally, it might be that SU5402 blocks self-renewal of cells and favours non-specific differentiation which is further directed to the mesoderm by BMP2. By blocking

all or either one of these pathways, the FGF inhibitor is required to unravel the BMP2 transcriptional response of HES cells.

In keeping with previous observations made in hearts transplanted with mouse [10,11,25], and human [26] ES cells, no hyperproliferation (teratoma) was observed in any of the rats injected with cardiac-committed HES cells. As intramyocardial injections in a beating heart are also known to cause leakage of a substantial proportion of cells [27], it is also noteworthy that we failed to document any extra-cardiac tumor. In fact, it was known for a long time that grafts of embryonic tissue also loose the capacity to form tumors very early after differentiation [28] when they acquire control of their proliferation by extracellular signal regulated kinases. It is thus not surprising that a similar scenario takes place after cardiac commitment of HES cells. As such, our findings are not in contradiction with the previous observation [11] that injection of HES cells into a *normal* immunocompetent myocardium results in teratoma formation since the latter results primarily suggest that such an environment is unlikely to provide enough cardiogenic factors required for differentiation of ES cells. Of note, the rather reassuring safety data yielded by our experiments were obtained despite the lack of pretransplantation sorting targeted at eliminating non specified cells. This suggests that the environment of the diseased myocardium (i.e., scar) enriched in growth factors is sufficient to drive primed ES cells toward a cardiac fate [25]. In a clinical perspective, however, such a selection step remains a major goal.

So far, two studies have assessed the effects of intramyocardial transplantation of HES cells. Both have entailed the use of embryoid body-derived cardiomyocytes into either *normal* myocardium [26] or *acutely* infarcted myocardium [29]. To make the protocol more clinically relevant, we selected a delayed timing of in-scar transplantation that tends to mimick the clinical scenario of heart failure and injected cardiac-specified but not yet fully differentiated monolayer-cultured cardiac progenitors. Altogether, the engraftment patterns seen after 2

months support the advantage of this cardiac commitment process before transplantation into the target scar where local signals are then expected to drive the fate of the graft further down the cardiomyocytic differentiation pathway.

We should however point out that the phenotype of HES cell-derived cardiomyocytes *in situ* was rather close to a foetal one. Indeed the cells still expressed β-MHC and ANP, two known markers of early stage of cardiac differentiation. The short length of the sarcomere is still characteristic of a foetal myocyte. Several reasons could account for this immature phenotype. HES cells may require a longer time (more than two months) to fully differentiate. Alternatively, the paracrine environment of the infarction scar may not provide the factors (some FGFs, Neuregulin, retinoic acid, BMP10…) [30] or signals taking place in embryogenesis to ensure a full differentiation process.

Another interesting observation is that I6 cells gave rise to larger engraftment areas than HUES-1 cells. Although both cell lines respond with the same efficiency to BMP2, I6 cells feature a higher basal expression of mesodermal cardiac genes (Fig 1). This indicates that the cell line is already committed to the mesoderm, which is likely to account for the better cardiogenic potential *in vivo*. Of note, HUES-1 cells pretreated with BMP2 together with SU5402 also featured a better engraftment than HUES-1 challenged by BMP2 alone. This further emphasizes that the stage of specification is crucial to ensure a proper differentiation of ES cells *in situ*. The finding that the rates of scar repopulation by the grafted cells was overall low probably reflects a combination of initially insufficient cell dosing, extracardiac cell leakage at the time of injections and possible death of retained cells. Clearly, optimisation of the functional benefits of ES cells transplantation will require that each of these issues be thoughtfully addressed.

Finally, and in contrast to what has been reported in our previous studies using mouse ES cells [10,31], we could not detect Cx43 mRNA or protein in HES cell-derived

cardiomyocytes. HES-cell derived differentiated cardiomyocytes [13] did not either express Cx43 when transplanted in injured left ventricle while they did express it when co-cultured with neonatal rat cardiomyocytes [32]. The reason for this discrepancy with mouse ES cells or the ex-vivo situation is still unclear and might involve line-specific differences in the cardiogenic potential, a still early stage of cell development, a level of expression below the threshold of detection by immunostaining, a mistargeting of the protein or inhibitory signals coming from the fibrotic scar of infarcted rat myocardium to which HES cells might be highly sensitive. Finally, Human ESC were transplanted into rat hearts and that some of the cues required for the full differentiation of the cardiac-specified cells into Cx43-expressing cardiomyocytes may have been missing. This issue is under investigation in the laboratory.

Expression of Cx43 remains, however, critical to establish unequivocally as a true cardiac regeneration implies that the donor-derived cardiomyocytes can establish gap junction-supported electromechanical connexions with those of the host. The formation of such a syncytium allowing graft-host synchronized beats which is critical for enhancement of contractility has not yet been achieved with adult cells, whether myogenic [33]or bone marrow-derived [34]. The demonstration that HES cells could fill this unmet need would likely be a major step for rationalizing their use in situations where patient outcomes are critically dependent on the replenishment of a new pool of contractile cells.

Altogether, our findings bring a proof of concept of the feasibility of cardiac commitment of Human ES cells. Although many challenges remain, our data are promising as to the safe use of Human ES cells in clinics.

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Figure 1: Comparison of mesodermal and cardiac gene expression in HUES-1 and I6 cells. RNA was extracted and reverse transcribed from undifferentiated HUES-1 (passages 22-25) and I6 cell lines (passages 27-32) cultured for at least 5 passages after thaw-out on MEF prior to stimulation with BMP2. Differentiated colonies were cut out the plate before RNA extraction. Gene expression was estimated by real-time PCR and expressed as a ratio between expression in I6 and expression in HUES-1. Data are normalised to β-tubulin expression and expressed as means ±SEM (n=3). * statistically significant (p≤0.01)

Figure 2: (A) HUES-1 and I6 cells were treated for 48 hrs with 10 ng/ml BMP2 in the presence or absence or FGF inhibitor SU5402 and RNA extracted and reverse transcribed. Gene expression was monitored by real-time quantitative PCR. Results are expressed as fold stimulation in gene expression when compared to untreated ES cells. **(B)** HUES-1 cells treated or not with BMP2 and SU5402 were allowed to aggregate to form embryoïd bodies (EB). EBs were kept in suspension for 5 days before RNA extraction and real time PCR. Data are normalised to β-tubulin expression and expressed as means ±SEM (n=3-5). * statistically significant (p≤0.01)
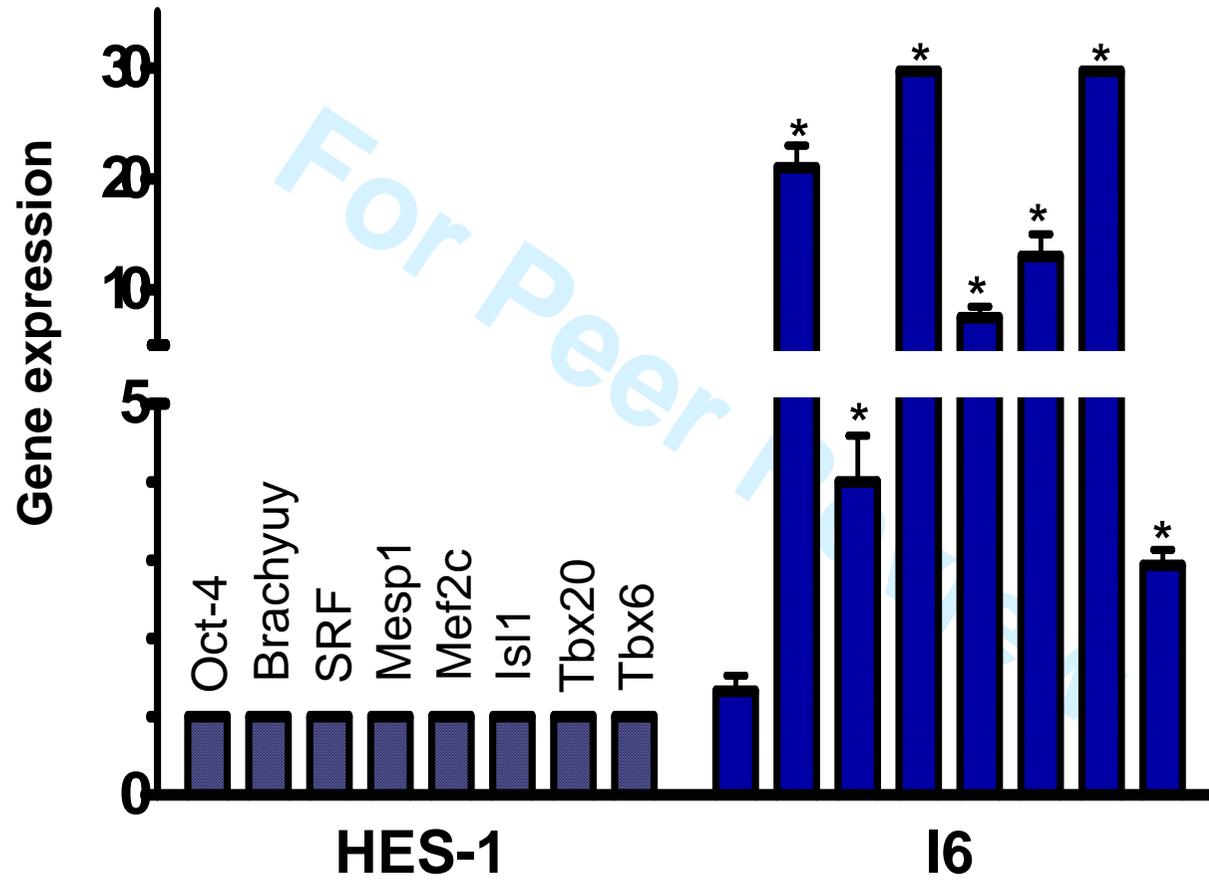
Figure 3: BMP2-treated HES cells were engrafted into postmyocardial infarcted rats and their fate was examined two months later by real time PCR of α-actin mRNA following reverse transcription of mRNA extracted from myocardial sections. The figure shows both the profile of the melting curves of amplicons and the amplicons on gel. Human RNA was used as a positive control. * statistically significant (p≤0.01)

Figure 4: Immunostaining of cryosections from HES cell-engrafted myocardium using an anti−human lamin antibody (A) (note that the anti-lamin antibody stained HES-derived cells within the scar area but not the surrounding endogenous rat cells) and an anti-human β myosin antibody (B). The antibody did not recognize the adult rat endogenous α-MHC (left panel B) while it bound human β-MHC. Images were acquired in confocal microscopy.(C) quantification (in %) of the human β-MHC positive regions in the scars of myocardium engrafted with BMP2- or BMP2 with SU5402-treated HUES-1 or BMP2-treated I6 cell lines: * statistically significant ($p \leq 0.025$). The area of β-MHC positive area within the scar was calculated using the threshold function of Metamorph software (D) a transversal section stained by the anti-β myosin antibody revealed some sarcomeric structures. The size was calculated using the scaling system of the ZEISS software driving the confocal microscope

Figure 5: Eosin-Hematoxylin stained section from HES cell-engrafted myocardium. The scar area does not show any sign of cell infiltration of cell proliferation

TABLE 1: PCR primer sequences

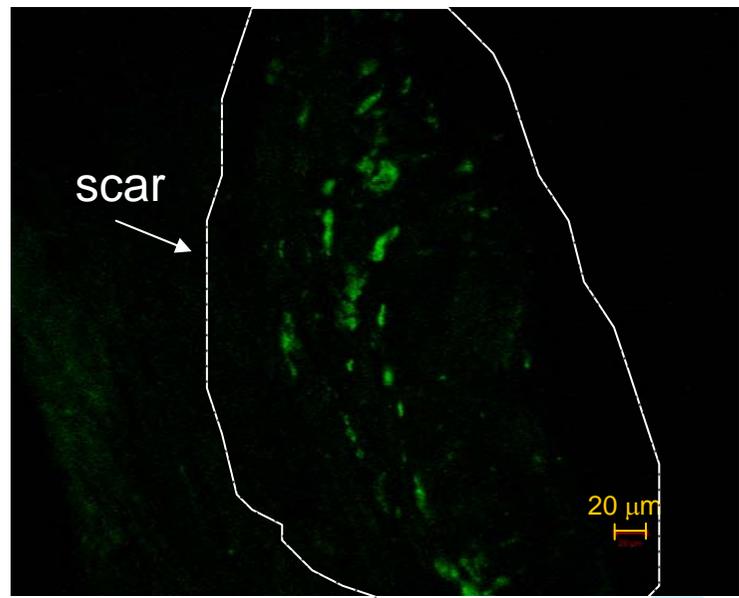| Genes	Forward	Reverse
β-tubulin	CCGGACAGTGTGGCAACCAGATCGG	TGGCCAAAAGGACCTGAGCGAACGG
Nkx2.5	CATTTACCCGGGAGCCTACG	GCTTTCCGTCGCCGCCGTGCGCGTG
Mef2c	AGATACCCACAACACACCACGCGCC	ATCCTTCAGAGAGTCGCATGC
SRF	CTCCGCCCCGCTCAGACCCCACCACAGA	AGGTAGTTGGTGATGGGGAAGGA
α-actin	CTATGTCGCCCTGGATTTTGAGAA	TGAGGGAAGGTGGTTTGGAAGAC
Oct-4	ACGACCATCTGCCGCTTTGAG	GCCTCTCACTCGGTTCTGAT
Tbx6	AGGCCCGCTACTTGTTTCTTCTGG	TGGCTGCATAGTTGGGTGGCTCTC
Isl1	CATCGAGTGTTTCCGCTGTGTAG	GTGGTCTTCTCCGGCTGCTTGTGG
FoxH1	GCCCCTGCCCACGCTGTCTA	GGTACCTCTTCTTCCTCCTCTT
Brachyury	CGGAACAATTCTCCAACCTATT	GTACTGGCTGTCCACGATGTCT-
Mesp1	CTCGTCTCGTCCCCAGACT	AGCGTGCGCATGCGCAGTT
Tbx20	CTGAGCCACTGATCCCCACCAC	CTCAGGATCCACCCCCGAAAAG
Gata4	GGTTCCCAGGCCTCTTGCAATGCGG	AGTGGCATTGCTGGAGTTACCGCTG
Pax6	GCCAGCAACACACCTAGTCA	TGTGAGGGCTGTGTCTGTTC
α-FP	ACTGCAATTGAGAAACCCACTGGAGATG	CGATGCTGGAGTGGGCTTTTTGTGT
Cx43	TACCATGCGACCAGTGGTGCGC	GAATTCTGGTTATCATCGGGGAA

Fig1

A

Fig2A

Fig2B

Fig3

A scar

20 μm

B HUES-1 transplanted myocardium

I6-transplanted myocardium

Control myocardium

C Scar recolonisation

HUES-1 BMP2   HUES-1 BMP2/SU5402   I6 BMP2

D 2μm

Fig 4 ABCD

Fig5