

Direct myocardial implantation of human embryonic stem cells in a dog model of Duchenne cardiomyopathy reveals poor cell survival in dystrophic tissue

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Duchenne muscular dystrophy is characterized by progressive muscle weakness and early death resulting from dystrophin deficiency. Spontaneous canine muscular disorders are interesting settings to evaluate the relevance of innovative therapies in human using pre-clinical trials. One of them, muscular dystrophy in Golden Retrievers (GRMD dogs) provides a powerful animal model of Duchenne muscular dystrophy, mimicking the human cardiac phenotype. They develop mild fibrosis which then degenerates into calcified fibrosis and later with age ventricular dysfunctions. Our goal was to test whether such a cardiac environment was proper for human embryonic stem cell (hESC)-derived cardiac progenitor engraftment and differentiation. For that purpose we have grafted young GRMD dogs with cardiac progenitors derived from hESC, using direct epicardial implantation under immunosuppression. Our main result is that dystrophic tissue is not suitable for direct epicardial hESC injection.

Keywords: Duchenne Muscular Dystrophy, Human Embryonic Stem Cells, Golden Retriever Muscular Dystrophy, Transplantation

Introduction

Duchenne muscular dystrophy (DMD), the most common X-linked disorder (affecting 1 in 3,500 male births), typically results in death as a result of respiratory or cardiac failure by age of 30 [1]. Loss of dystrophin leads to disruption of the dystrophin glycoprotein complex (DGC) in the sarcolemmal membrane connecting the cytoskeleton and contractile apparatus of muscle cells to the extracellular matrix and basement membrane [2, 3]. The onset of the disease occurs between 2 and 5 years of age, and most patients die from respiratory or cardiac failure [4, 5]. The closest animal model for human DMD is the golden retriever muscular dystrophy dog (GRMD), which has a splice acceptor site mutation in intron 6, causing a frameshift due to deletion of exon 7 from the mature mRNA [6]. This mutation results in the absence of the muscle protein dystrophin [7]. GRMD dogs exhibit fibrosis which then degenerates into calcified fibrosis [8, 9].

Until recently, the myocardium has been viewed as a terminally differentiated organ without potential for regeneration [for review, 10]. However, the emergence of stem cell technology and the opportunity for myocardial repair provide the foundation for a regenerative approach to the therapy of cardiovascular diseases [11]. Because of indefinite proliferation *in vitro*, human embryonic stem cells (hESC) provide a reservoir for extensive tissue regen-

eration [12] and in contrast to many adult stem cell types for whom this capacity is controversial, hESCs have unquestioned cardiomyogenic potential [13, 14, 15]. Human ESCs can also be differentiated into noncardiac cell types present in the myocardium (e.g., endothelial cells), suggesting that hESCs might eventually be useful in repopulating all myocardial tissue elements, not just cardiomyocytes. Moreover, hESCs can be isolated and maintained by well-established protocols, and they are tremendously scaleable [16, 17, 18].

Embryonic stem cell therapy demonstrates benefit in ischemic heart disease [11, 19] and genetic cardiomyopathies [20] but whether DMD in a cardiac environment is proper for human embryonic stem cell engraftment and differentiation remained to be tested. In this study conducted in young GRMD dogs and using direct epicardial implantation under immunosuppression, we report that transplanted hES cells do not survive the injections, certainly due to inadequate host environment.

Methods

All experimentations were performed in strict accordance with the recommendations of the European Ethical Committee (EEC) (86/609 EEC), the French National Ethical Committee (87/848) and by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) for the care and use of laboratory animals.

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Cells preparation and injection

α -actin-GFP hESC lines generation

To obtain a stable cell line expressing GFP under the α -actin promoter, the human ES -HUES24- cell line (Drs C. Cowan, D. Melton, Harvard, USA), was transduced with a lentivirus carrying the green fluorescent protein (GFP) under the control of the α -actin promoter at the concentration of 40 MOI for 48h. The culture medium was then removed and replaced by serum-free DMEM supplemented with bFGF (basic Fibroblast Growth factor, 10ng/ml, Invitrogen, France), and changed every day.

Culture and cardiac commitment of hESC

HUES-24 cell line was cultured on Mouse Embryonic Fibroblasts (MEF) prepared from E14 mouse embryos using DMEM-F12 medium supplemented with 0.1 mM β -mercaptoethanol, 1% non essential amino acids, 20% KSR and 10ng/ml FGF2 (Invitrogen, France). Medium was changed every day. Cell colonies were dissociated into single cells or cell clusters every 4-5 days using trypsin. A similar enzymatic digestion was used prior to cell transplantation in GRMD dogs.

In order to induce cardiac commitment HUES-24 cells were treated for 4 days with 10ng/ml BMP2 in the presence of 1mM SU5402, a FGF receptor inhibitor [21].

HEK and human myoblasts culture

HEK293 (Human Embryonic Kidney, ATCG, France) cells were cultured using MEM α medium (Invitrogen) supplemented with 10% FCS (Foetal Calf Serum, HyClone, Logan, UT, USA), 1% Non Essential Amino Acid (Invitrogen).

Human Myoblasts (Mub2B3 cell line, Bioalternative, [22]) cells were cultured on gelatin-coated plates (0.1%) (Sigma, France) using 65% MEM α medium (Invitrogen) and 25% 199 Medium (Invitrogen) supplemented with 1% GlutaMAX™ I, with L-Alanyl-L-Glutamin, 10% FCS (Foetal Calf Serum, HyClone, Logan, UT, USA), 1% Non Essential Amino Acid (Invitrogen). On the day of use, 0.1% Insulin (10 μ g/ml, Invitrogen), 0.1% of EGF (epidermal growth factor, Invitrogen) and 0.02% of bFGF (basic Fibroblast Growth Factor, Invitrogen) were added.

Cell proliferation assay

hESC, HEK and myoblasts were cultured for 1 to 3 days and their proliferation was evaluated by cell counting using Malassez cell chamber.

Animals and Surgical procedures

On their arrival into the unit, the animals (GRMD; n=8, aged between 6 and 12 months and one normal dog, age 26

months) had a clinical examination, a biochemical, hematologic and coproscopic assessments, as well as thoracic radiographies. Clinical observation did not show any sign of cardiopathy in the GRMD dogs on the day of cell transplantation. At this age, there is also no echocardiographic alteration in left ventricular function [23]. Before beginning, all the procedures on the animals were validated by a local ethical committee.

Cyclosporine A (20 mg/kg/day) and prednisolone (2 mg/kg/day) starting 21 days before surgery were used as immunosuppression regimen.

On the day of surgery, anaesthesia was induced by propofol and maintained by isoflurane. Vital parameters were controlled by continuous monitoring of oxymetry, capnography, body temperature (which was stabilized by a warm water circulation carpet). After thoracotomy, the pericardium was opened. The sites of injections were delimited with Prolene 4/0 and the injections were carried out. At the end of the injections, the chest was closed. Analgesia was ensured by immediate post-operative administration of morphine and by delayed transdermic buprenorphin delivery.

Cells injection

On the day of injection, 20x10⁶ hESC cardiac committed cells, HEK cells and Human Myoblasts were resuspended in serum-free DMEM and injected into 2 separate regions (10 μ l of a solution with 2x10⁶ cells/50 μ l injected over multiple sites, using a 27G needle, inside each region at a rate of 10 μ l/10 min) through epicardial injections.

Histopathology

At two weeks (3 dogs), one month (3 dogs) and three months (2 dogs) after myocardial injection, dogs were euthanized after general anaesthesia, respectively. Transverse sections of the hearts were immediately embedded in OTC (Tissutec) and frozen at -180°C nitrogen. Ten μ m sections were cut on an ultramicrotome (LM 1850, Leica). The cryosections were fixed with 4% PFA at room temperature for 20 min, then washed with PBS, and incubated for 1 h at room temperature with a saturating solution consisting of 5% normal goat serum, 5% normal horse serum in PBS-0.6% Triton X-100 (Sigma, France). Slices were incubated overnight at 4°C with the following primary antibodies used at 1:400 anti-human nuclei mouse monoclonal (HNA, Chemicon France), anti-GFP (Chemicon), anti-CD4 (Rat anti-dog CD4, AbDSerotec, Düsseldorf, Germany), anti-CD8 (Rat anti-dog CD4, AbDSerotec, Düsseldorf, Germany). After three washes with PBS, the slides were incubated with a 1:500 dilution of fluorescent-conjugated secondary antibodies for 1h at room temperature. Secondary antibodies used were: Alexa-Fluor 488 goat anti-rabbit IgG and Alexa-Fluor 555 goat anti-mouse IgG (Molecular Probes, France).

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The specificity of all secondary antibodies was tested by incubation of only the secondary antibody without a primary antibody incubation. All secondary antibodies were negative for non-specific staining in our immunostaining conditions. After three washes the slices were incubated with 1/10000 DAPI and then were mounted by using Fluoromount-G (CliniSciences, France). The slides were observed under an epifluorescence microscope (Zeiss Imager Z1), and images processed using the Axio-vision software.

To evaluate the HEK proliferation after transplantation, heart sections were stained with haematoxylin and eosin, some of the sections were stained with Sirius Red or Von Kossa stain for the determination of interstitial collagen and calcification density respectively. The slides were observed under a transmitted light microscope (Axioplan2, Zeiss) and images processed using the Cool-Snap Software. Measurement of collagen density was performed using the Histolab software. Briefly, fibrotic area was measured as collagen density above the background noise represented by the collagen density measured in the normal dog and reported to the total slice area.

Real-Time Quantitative PCR by SYBR Green Detection

Total RNA was extracted from hESC and heart slices using Quiagen kit and TRIZOL Reagent (Invitrogen, France) respectively, according to the manufacturer's protocol. RNA (1 µg) was reverse-transcribed using SuperScript II RNase H-Reverse Transcriptase (Invitrogen, France). cDNA was used as a template for gene expression analysis of human cardiac markers. Real-time quantitative PCR was performed using a Light Cycler with SYBR Green I master (Roche Diagnostic, France).

The following primer pairs were used: Human-Is1: forward 5'-CGC-GTG-CGG-ACT-GTG-CTG-AAC and reverse: 5'-TTG-GGC-TGC-TGC-TGC-TGG-AGT; Human-Tbx6 forward: 5'-AGG-CCC-GCT-ACT-TGT-TTC-TTC-TGG and reverse: 5'-TGG-CTG-CAT-AGT-TGG-GTG-GCT-CTC; Human- α -actin forward: 5'-CAC-TGA-AGC-CCC-GCT-GAA-CG and reverse: 5'-TCG-CCA-GAA-TCC-AGA-ACA-ATG-C; human-Oct-4 forward: 5'-CTT-GCT-GCA-GAA-GTG-GGT-GGA-

GGA-A-3' and reverse: 5'-CTG-CAG-TGT-GGG-TTT-CGG-GCA-3'; human-Myocyte enhancer factor 2c (Mef2c) forward: 5'-CGC-ATG-AGA-GCC-GGA-CAA-ACT-3' and reverse: 5'-TGG-CTG-GAC-ACT-GGG-ATG-GAG-3'; human Ubiquitin C (UbC) forward 5'-ATT-TGG-GTC-GCG-GTT-CTT-G-3' and reverse 5'-TGC-CTT-GAC-ATT-CTC-GAT-GGT-3'; Amplification PCR reaction was performed with an initial denaturation step at 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec, and extension at 72°C for 15 sec. Quantification of gene expression was based on the DeltaCt Method and normalized on human UbC. Human UbC was used as it is specific to human gene and did not cross with dog's tissues. Melting curve analysis was used to determine the specificity of PCR products, which was confirmed using gel electrophoresis run.

Statistical analyses

Statistical analysis was performed using ANOVA and a Student's test. For time course of cell differentiation statistical analysis was performed using ANOVA and Dunnett's test. A value of $p \leq 0.05$ was considered statistically significant.

Results

Confirmation of hESC quality and cardiac commitment

As hESC are sensitive to culture conditions and could develop abnormal karyotype, their statuses were checked and no abnormalities were shown (Figure 1a). The HUES-24 cell line, after being cultured in vitro in culture, showed expected hESC morphology (Figure 1b). hESC were treated for 4 days with 10ng/ml human recombinant BMP2 in the presence of the FGF-R inhibitor SU5402. Immunofluorescence analysis showed a down-regulation of all the stemness marker cell analysed, *i.e.* Oct4 and Nanog in the BMP2-hESC treated (Figure 1c) compared to untreated cells. Gene induction was also tested using RT-qPCR. Figure 1d shows that Oct-4, was down regulated and that both mesodermal (*i.e.*, Tbx6, Is1) and cardiac (Mef2c, α -actin) genes were induced by BMP2 in HUES-24 cells.

Figure 1

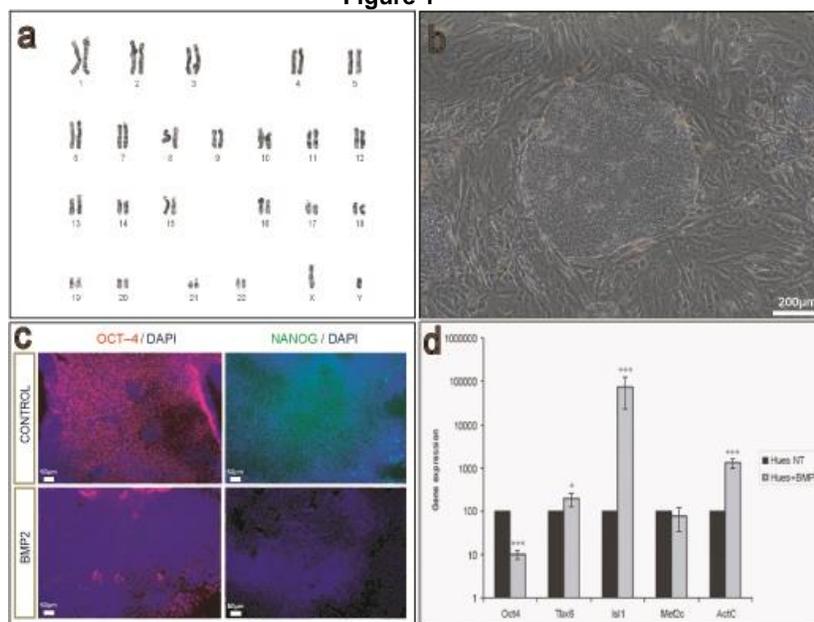


Figure 1: Analysis of undifferentiated hES and cardiac committed cells. a) Karyotype analysis of hESC lines HUES-24 using the G-band method. b) Microscopic image showing undifferentiated colony morphology of hESC line HUES-24. c) Immunofluorescent labeling of differentiating hES cells demonstrated decreased expression of pluripotent oct-4 and nanog in the hESC treated 4 days with BMP2. d) Percent gene expression, compared to hESC at day 0, was estimated by RT-qPCR and expressed as fold stimulation in gene expression (Hues + BMP2, grey bars) when compared to untreated hES cells (Hues NT, black bars). hESC. Data are normalised to β -tubulin expression and expressed as means \pm SEM (n=3). * $p \leq 0.05$ and *** $p \leq 0.001$ (Dunnett's test after ANOVA).

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hES-Cardiac-committed cells transplantation

20x10⁶ cardiac committed cells were injected into two regions within the left ventricle after a thoracotomy and through epicardial injections (Figure 2a, b) in young GRMD dogs (age less than 6 months old). Cell viability was tested before and after passage through injection's needle and 30% cell death was measured. Macroscopic analysis of injected cardiac tissues, disclosed the presence of histological abnormalities (fibrosis and calcification). Eight dogs were included in this study. Three died after 15 days due to bronchopneumonia, three after one month and two were sacrificed 3 months after hESC injections. GRMD were very difficult to maintain because of their severe phenotypes [24]. Analysis of sections from injected cardiac tissues were analysed by RT-qPCR and reveal the presence of human cells in all dogs analysed (figure 2c).

Figure 2

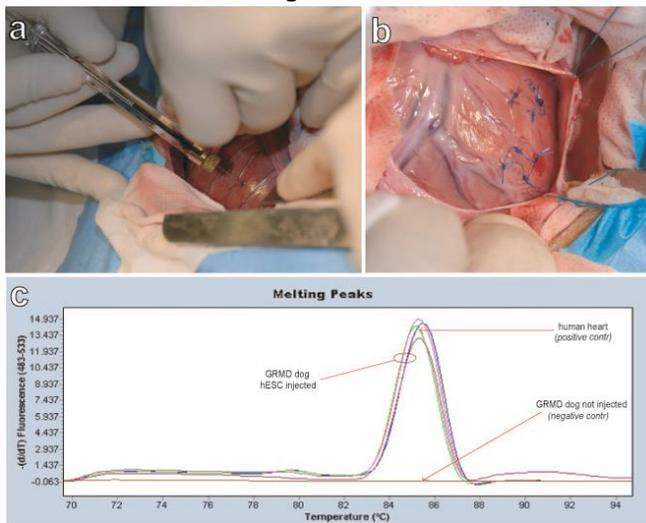


Figure 2: Transplantation and analysis of cardiac-committed cells. a, b) BMP2-treated hESC were transplanted using a 27G needle into GRMD hearts. c) Their fate was examined 15 days, one or two months later by real time PCR of human-ubiquitin (hUbC) mRNA following reverse transcription of mRNA extracted from myocardial sections. The figure shows the profile of the melting curves of amplicons. Human heart RNA was used as a positive control, and non injected dog heart as negative control.

To further define the phenotype of hESC derived cardiomyocytes, sections were immunostained with anti-HNA and anti-GFP antibodies. These experiments failed to reveal the presence of human cardiac-committed cells in the transplanted areas (Supplementary Figure 1). These results show very poor survival of injected cells.

Survival and immune reaction.

In order to evaluate the cells surviving after grafting, we injected two other cell lines in parallel to the hESC in a normal dog. The first one was a human myoblasts cell line (Mub2B3, [22]) and the second one was an HEK cell line. In culture, the main differences between the cells were that human myoblasts did not proliferate and that were slow proliferative cells compared to HEK cells (Supplementary Figure 2). RT-qPCR analysis of transplanted heart show very low expression of a human housekeeping gene, ubiquitin C (an human specific gene which is not detected in non injected dog's tissue) in both hESC and human myoblasts compared to HEK cells (Figure 3).

Figure 3

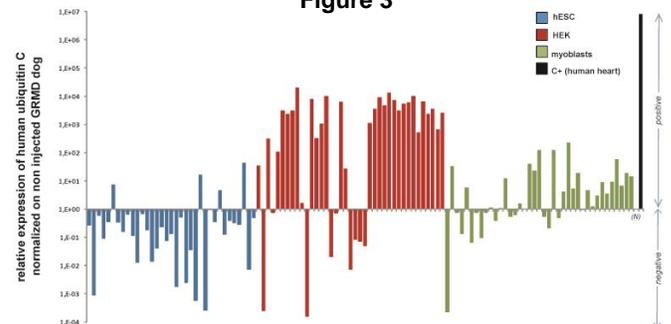


Figure 3: Gene expression analysis of different transplanted cells. Gene expression was estimated by real-time PCR and expressed as relative human ubiquitin C expression. Each group of injected cell (hESC, HEK or human myoblasts) is represented under a brace. (N) represent non injected hESC. Each bar represents one sample. Bars higher than 1 are considered as positive expression of transplanted cells, bars below than 1 are considered as negative expression of transplanted cells.

We looked for a possible immune reaction that could explain the poor cell survival observed in the transplanted hearts. Sections from injected and non-injected areas were stained with antibodies against CD4 and CD8. We saw a very mild staining in the injected areas for both type of lymphocytes (Figure 4a,b) compared to the non-injected area where we could not detect any staining. The absence of staining in non

Figure 4

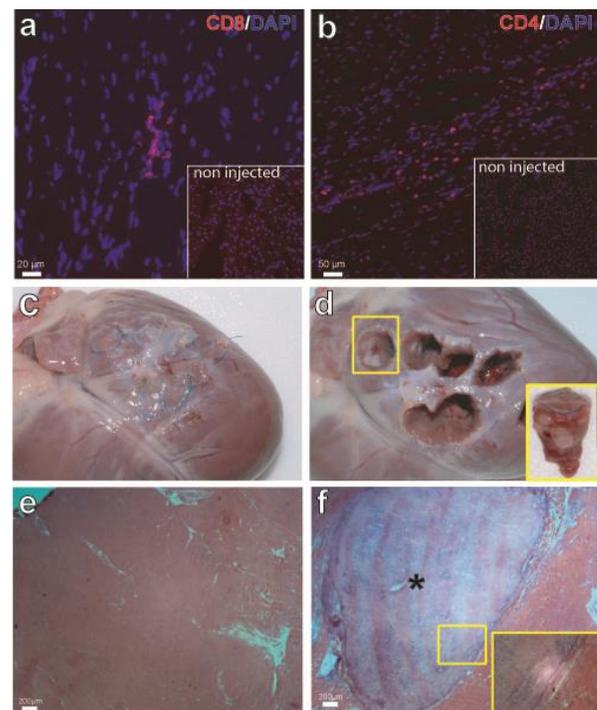


Figure 4: Immune reaction and HEK proliferation in dogs' hearts. a) Observation of few CD8-positive (inserted small panel show negative staining in non injected dog), and b) CD4-positive cells in the injected area (inserted small panel show negative staining in non injected dog). c) macroscopic view of GRMD heart dog 30 days after HEK cell implantation, d) dissection of injected area showing the HEK proliferation in the cardiac parenchyma. e) Hematoxylin and eosin staining of heart of non injected (f) and HEK injected area.

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injected dogs was in relation with the immunosuppressive therapy of dogs before cell transplantations. In order to assess that this poor hESC survival could not be due only to an immune reaction, we injected high proliferative cells, the HEK cells, into the apical region of the heart. One month after implantation we found the development of massive tumors, showing that, not only the cells survive but also proliferate (Figure 4c-f).

GRMD dogs show severe and heterogeneous fibrosis and calcification

The phenotypes of the cardiac tissue of 8 GRMD, aged between 4 and 12 months, and one normal dog, aged 26 months, were evaluated using histopathological examinations. The examined areas were non-injected areas and were located in between the injected ones. None of the dogs in the present study showed clinical signs of heart failure at the time of injection. Portions of the left ventricular wall were examined macroscopically and with histopathological stainings. Macroscopic view show large fibrotic bands visible in the entire heart and collection of wall

Figure 5

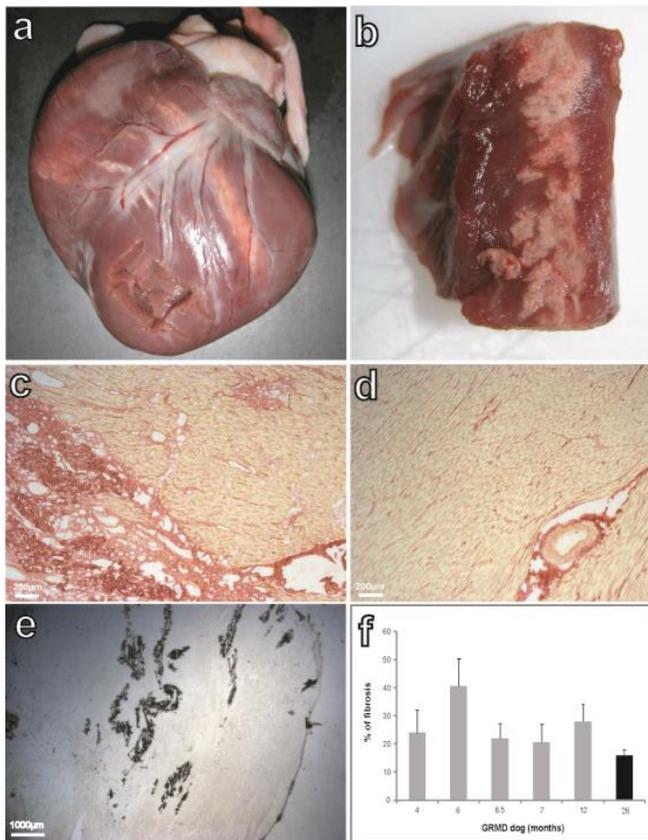


Figure 5: Histology of GRMD hearts. a, b) macroscopic observation show large fat and fibrotic tissue bands, distributed heterogeneously over the left ventricle. c) Silivius red staining for histopathological evaluation of fibrosis in one GRMD heart (6 months old). d) and one normal dog. e) Von Kossa staining for calcification evaluation in GRMD dog (6 months old). f) Fibrosis quantification in GRMD dogs (grey bars) and one normal dog (black bar).

pieces show clearly fibrosis inside the ventricular wall (Figure 5a,b). Histopathological abnormalities (fibrosis and calcification) were detected in the GRMD cardiac tissues as early as 4 months (Figure 5c,d and e) and were not correlated with age (Figure 5f).

Discussion

The present study is the first cell therapy using hES-derived cardiac committed cells conducted in GRMD dogs. This study show poor cell survival probably due to inadequate fibrotic and calcified host tissue environment.

Cardiomyopathies result from complex interactions between the genetic background of an individual and the imposed environmental stress [25]. Although decoding of disease-causing mechanisms and cardioprotective molecular circuits has provided a real opportunity for development of predictive strategies using emerging genomic information [26], management of heritable cardiomyopathies remains largely palliative in scope, limited to treatment of symptoms. Despite advances in pharmacotherapy, and promising gene therapy using the exon skipping strategy for DMD [27], prognosis is poor overall, mandating consideration of new modalities capable of targeting the root cause of disease [28]. More recently, the discovery of cardiac resident stem cell has opened the field to the possibility that these cells could acquire the distinct cell lineages of the myocardium and actively contribute to heart homeostasis, yet functional exhaustion and aging of progenitor cells in patients with heart failure limit the available autologous stem cell pools, contributing to disease [29, 30]. Diseases with a genetic origin potentially preclude the full effectiveness of an autologous reparative approach [31], warranting consideration of nonself sources as alternative therapeutic approaches like hESC.

However, as shown in the present study, a consistent finding of cell therapy studies is the very low rate of sustained cell engraftment, which can drop as low as 1% of the initial number of donor cells a few weeks after transplantation [32]. Associated with a very non-friendly environment (like fibrosis and calcification), cells that have a slow proliferative course, like hESC and myoblasts, did not survive injection and did not colonise the host myocardium. This low rate of engraftment has been shown to be initially caused by a mechanical leakage of cells [33, 34, 35] and this is probably subsequently worsened by a poor vascularisation and calcification of the host myocardium. Indeed, it has been shown that when peripheral blood mononuclear cells were delivered into the myocardium, most of the cells were distributed to extracardiac organs shortly after delivery [36]. Moreover, it has been shown that cells needed the environment of the diseased myocardium (*i.e.*, scar) enriched with growth factors and this is sufficient to drive primed hESC toward a cardiac fate [37]. We have shown in this study that GRMD dogs develop very severe heart fibrosis and calcification as early as four month old, probably impeding the survival of transplanted cells. Valentine and colleagues have reported that GRMD dogs at 6.5 months of age had acute severe lesions with focal myocardial mineralization associated macrophages and giant cells in the left ventricular papillary muscle and left ventricular wall [38]. Moreover, GRMD dogs at 12 months of age or older demonstrated prominent myocardial fibrosis in more widespread lesions [39].

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The recognition of these contributing factors provides a rationale for research of transplantation alternatives like embedding cells into three-dimensional biodegradable scaffolds that might better preserve cell survival and proliferation compared with proteolysis followed by injection of isolated cells. During the last decade, many studies using cardiac cell therapy have demonstrated that the outcome of the procedure was not only dependent on the cell type but also on the methods used for transferring cells and ensuring their optimal engraftment [40]. Furthermore, the epicardial delivery of scaffolds, rather than cell suspensions, might avoid the drawbacks associated with multiple needle-based injections, which include (i) a washout of cells through channel leakage and the vascular system, (ii) an inhomogeneous distribution of cells, and (iii) the disruption of the extracellular matrix and the subsequent loss of signals that modulate cell survival, differentiation, and patterning [32, 40]. Moreover, some undifferentiated cells can still be present and further transplantation studies will require sorting of cardiac committed cells. Recently it has been shown that the treatment used in our study was generating early population of cardiovascular progenitors, characterized by expression of OCT4, stage-specific embryonic antigen 1 (SSEA-1), and mesoderm posterior 1 (MESP1), derived from human pluripotent stem cells [19]. This progenitor population was multipotential and able to generate cardiomyocytes as well as smooth muscle and endothelial cells. When transplanted into the infarcted myocardium of immunosuppressed nonhuman primates, an SSEA-1+ progenitor population derived from Rhesus embryonic stem cells differentiated into ventricular myocytes and reconstituted 20% of the scar tissue [19].

Conclusion

This study is the first to investigate the injection of hESC-derived cardiac committed cells in a well recognized animal model of DMD, *i.e.* the GRMD dogs. The present results importantly emphasize the fact that host environment is a crucial parameter to take into account when a heart cell therapy strategy is considered. Moreover, this study underlines the necessity to develop different novel approaches for cardiac cell therapy.

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Supplementary Information

Supplementary figures are linked to the online version of the article.

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