Towards a clinical use of human embryonic stem cell-derived cardiac progenitors: a translational experience

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Aim

There is now compelling evidence that cells committed to a cardiac lineage are most effective for improving the function of infarcted hearts. This has been confirmed by our pre-clinical studies entailing transplantation of human embryonic stem cell (hESC)-derived cardiac progenitors in rat and non-human primate models of myocardial infarction. These data have paved the way for a translational programme aimed at a phase I clinical trial.

Methods and results

The main steps of this programme have included (i) the expansion of a clone of pluripotent hESC to generate a master cell bank under good manufacturing practice conditions (GMP); (ii) a growth factor-induced cardiac specification; (iii) the purification of committed cells by immunomagnetic sorting to yield a stage-specific embryonic antigen (SSEA)-1-positive cell population strongly expressing the early cardiac transcription factor IsI-1; (iv) the incorporation of these cells into a fibrin scaffold; (v) a safety assessment focused on the loss of teratoma-forming cells by in vitro (transcriptomics) and in vivo (cell injections in immunodeficient mice) measurements; (vi) an extensive cytogenetic and viral testing; and (vii) the characterization of the final cell product and its release criteria. The data collected throughout this process have led to approval by the French regulatory authorities for a first-in-man clinical trial of transplantation of these SSEA-1+ progenitors in patients with severely impaired cardiac function.

Conclusion

Although several facets of this manufacturing process still need to be improved, these data may yet provide a useful platform for the production of hESC-derived cardiac progenitor cells under safe and cost-effective GMP conditions.

Keywords

Stem cells • Cell therapy • Tissue engineering • Heart failure • Myocardial infarction

Translational Perspective

This paper describes the multi-step translational approach that has resulted in the generation of clinical-grade human embryonic stem cell-derived cardiac progenitor cells for transplantation in patients with severe ischaemic heart failure.
Introduction

Stem cell therapy has now emerged as one potential option for the treatment of severe heart failure. A major lesson drawn from the first wave of clinical trials has been that regardless of whether the grafted cells generated a new myocardial tissue or acted by harnessing endogenous repair pathways the most effective were likely those committed towards a cardiac lineage. In this setting, encouraging results have been reported with c-kit+ cardiac stem cells, cardiospheres-derived cells, and mesenchymal stem cells (MSC) exposed to a cardiopoietic cocktail prior to transplantation. In line with this reasoning, cardiac progenitor cells derived from pluripotent stem cells look equally appealing candidates, regardless of whether pluripotency is an intrinsic property of the cells, as for embryonic stem cells (ESC), or has been induced following reprogramming of somatic cells (hESC). As for embryonic stem cells, pluripotency over several passages, optimize the culture conditions with regard to cell engraftment and functional outcome. Shift from bench scale methods to a cell-based medicinal product was initiated by reverting the medium to a clinical-grade medium-essential medium Eagle (MEM) solution with daily changes of the medium.

Methods

Set-up of cell banks

The hESC used in this programme have been derived from the 16 cell line, generously provided by J. Itskovitz and M. Amit (Technion Institute, Haifa, Israel). Four cryovials were transferred to the Cell Therapy Unit of Hôpital Saint-Louis (Paris, France) where they were expanded, from passage (P) 23–35 to assess their growth kinetics, check for their sustained pluripotency over several passages, optimize the culture conditions, and train the cell production staff. Six P35 were then transferred to a biotechnology company (MAbgenè, Ales, France), GMP certified by the French Regulatory Agency (Agence Nationale de Sécurité des Médicaments et produits de santé, ANSM), for constitution of the cell banks in class A rooms under GMP conditions, using qualified reagents and raw materials. Two P35 cryovials were thawed and cells were passaged twice after 6 and 4 days of expansion, respectively. Five days after the second passage, an average of 600 million cells were cryopreserved (P38) in 60 cryotubes in liquid nitrogen, thereby yielding the master cell bank (MCB, P38) which, for this six-patient phase I trial, will also be used as the working cell bank. One cryovial from the MCB was then thawed (equivalent to P39) and underwent two additional passages under the same culture conditions followed by a cryopreservation/thawing step and two additional passages to give rise to the late-production cell bank (LCB, P44). Throughout the expansion process, cells were visually inspected daily for the morphology of colonies and aliquots were sampled and tested by assessed by quantitative real-time polymerase chain reaction (qPCR) for checking maintenance of pluripotency-associated genes (Nanog, Sox2, Octamer-binding transcription factor 4 [Oct-4]). Extensive microbiological testing and cytogenetic studies were also carried out, as described below, on cells from both the MCB and LCB.

Upon completion of the two cell banks, cryovials were shipped back to Hôpital Saint-Louis for the next production steps, i.e. specification and cell sorting.

Cell specification

Still pluripotent stem cells contained in a single cryotube from the MCB (P38) were thawed (equivalent to P39) and expanded twice, 1 week apart, in Nutristem™ (Supplementary materials online). Two days following the last passage (P41), commitment towards a mesodermal-cardiac lineage was initiated by reverting the medium to a clinical-grade α-Minimum Essential Medium Eagle (MEM) solution with daily changes of the medium. BMP-2 and SU-5402, onto the same clinical-grade feeder fibroblastic line with daily changes of the medium.

Cell purification

Assuming at least 50% of the BMP-2/SU-5402-exposed cells would not respond to these differentiation factors and might thus feature an unpredictable degree of residual pluripotency, the elimination of this unwanted cell population was critical with regard to clinical safety. Among several potential markers, we had previously identified the stage-specific embryonic antigen (SSEA)-1 (or CD15) as one of those expressed at the surface of hESC that have engaged into a differentiation pathway. Selection was thus based on immunomagnetic sorting using a microbead-coupled anti-SSEA-1 antibody (Miltenyi, Teterow, Germany). During the multiple pre-clinical runs, the positive fraction of cells expressing SSEA-1 was systematically assessed by flow cytometry for viability (using 7-aminoactinomycin D (7AAD) as a marker) and expression of SSEA-1 to determine the purity rate of the collected yield; the fractions were also assessed by qPCR for checking the knock-down of pluripotency genes and the expression of some relevant cardiac markers (Isl-1, Mef2c). In addition, a more global gene expression analysis (Supplementary materials online) was performed on 16 undifferentiated cells, SSEA-1-positive progenitor cells, and SSEA-1-negative cells, derived from three different runs, using the AffymetrixGeneAtlas™ system coupled to the new Human Genome U219 array (BioAlternatives, Gency, France).

Microbiological-viral testing

Because the 16 cell line and the derived progenitor cell population are of human origin and were exposed to murine fibroblasts (during the early stages of derivation), foetal calf serum, and porcine trypsin throughout the expansion and specification processes, potential contamination by adventitious viruses from these different species was extensively looked for (Supplementary materials online). Viral testing was also performed on the hybridoma from which the anti-SSEA-1 antibody had been generously provided by J. Itskovitz and M. Amit (Technion Institute, Haifa, Israel). Four cryovials were transferred to the Cell Therapy Unit of Hôpital Saint-Louis for the next production steps, i.e. specification and cell sorting.
been derived (courtesy gift from Miltenyi) as well as on the final microbead-conjugated antibody used for cell sorting. The anti-SSEA-1 antibody to be used for the clinical trial cell sorting is derived from the same batch as the one tested for adventitious viral contamination.

Cytogenetic studies
Both conventional (standard karyotype, resolution 5–10 mbp) and molecular [oligonucleotide-based-array comparative genomic hybridization (array-CGH 105 K Agilent with a mean resolution of 50 Kb) and fluorescent in situ hybridization (FISH))] cytogenetic techniques were performed at different stages (Supplementary materials online). Following preliminary tests aimed at ensuring that the cell line was genetically stable over time, undifferentiated I6 cells used for the constitution of the cell banks were karyotyped at P36, P38 (MCB) and P44 (LCB). Fluorescent in situ hybridization focusing on chromosomes 12, 17, and 20, known to be at higher risk of aberrations during ESC cultures10 was performed at the same time points and array-CGH was also performed at P40. SSEA-1-positive progenitors derived from the MCB and LCB also underwent a combinatorial assessment (karyotype, FISH, and array-CGH).

Manufacture of the fibrin patch
One of the challenges of this protocol has been the development and validation of a method for manufacturing a biocompatible and easy-to-handle patch onto which the cells could be seeded prior to implantation onto the outer surface of the infarcted myocardium. The final choice of fibrin as the scaffold material is discussed below. To formulate this patch, 10 million SSEA-1-positive progenitor cells were first added to 1.1 mL of a solution made of fibrinogen (20 mg/mL) and clinical-grade α-MEM medium (Macopharma) in a 20 cm² agarose-coated Petri dish. Four units of thrombin (diluted in 1.1 mL of the culture medium) were then added under the form of evenly distributed 25-μL droplets and gentle agitation of the Petri dish induced polymerization of the gel, which occurred in 5–10 min (at 37°C). Cell viability and persistence of the right phenotype, as assessed by TUNEL, caspase-3, and Isl-1 immunostaining, were used as read-outs for validating the patch composition and its biocompatibility with the progenitor cells.

Release criteria
Primarily focused on the characteristics and quality attributes of the cells immediately after sorting, i.e. before their inclusion into the fibrin patch, the release criteria include sterility (aerobic and anaerobic BactAlert technique, bioMérieux, Craponne, France), a viability rate (as assessed by flow cytometry using 7-AAD as a marker) >90%, a total number of cells >15 million (as determined on a Malassez cell, of which 10 million are intended to be delivered to the patient and the remaining 5 million are used for controls), a percentage of SSEA-1-positive cells >95% and a qPCR-based expression of Nanog, Sox 2, and Isl-1 <0.1%, <0.1%, and >5% respectively, (expressed as fold changes relative to the undifferentiated I6 population). In addition, a double labelling of SSEA-1 and CD29, taken as a marker for fibroblasts, is performed by flow cytometry to estimate the percentage of residual contaminating CD29-positive feeder cells. Additional, although more limited, tests are also performed on the final product, i.e. the cell-loaded patch whose release is based on adequacy of handling characteristics, morphology (lack of retraction), and documentation of the actual presence of the cells (visualized on an inverted microscope). Sterility tests on the culture medium (BactAlert including the testing for endotoxins (LAL test) are also carried out. However, because of the time required for their final read-out, these tests are not part of the release criteria and will be used for information and action, if necessary. The whole process, from the reception of the undifferentiated I6 ESC to the quality controls preceding the cell-loaded patch release, is illustrated in Figure 1.

Pre-clinical studies
Most of the pre-clinical work has been previously published and, in total, has included >350 rats, >50 immunodeficient mice and 32 non-human primates. Briefly, initial rat and non-human primate experiments, primarily designed as proof-of-concept studies, have established (i) the ability of BMP-2/SU5402-treated hESC to engraft, differentiate into cardiomyocytes and improve LV function without causing teratomas;11,12 (ii) the additional benefit of providing a trophic supply under the form of co-transplanted MSC;13 (iii) the superiority of epicardial patch delivery over intramyocardial injections5,11 and the long-term benefits of the patch-based approach,12 including in a non-ischaemic cardiomyopathy model;13 and (iv) the relevance of the scaffold-based approach to ESC-derived SSEA-1-positive progenitor cells.14 However, more specific investigations were deemed mandatory to specifically test the safety, efficacy, and surgical applicability of the final cell product generated by a process strictly similar to the one planned for the clinical application.

Safety has thus been assessed in 54 RAG2-α-γC-/- CS-/- lymphoid mice, deprived from T lymphocytes, B lymphocytes, and Natural Killer cells.15 These mice were injected subcutaneously with undifferentiated ESC (from the H9 and I6 human ESC lines), SSEA-1-positive progenitor cells alone or spiked with different proportions (2, 5, 10%) of undifferentiated I6 cells and exclusively SSEA-1-negative cells. All cells were mixed with 30% matrigel to enhance the development of teratomas.15 Outcomes were assessed after a follow-up ranging from 2 to 7 months and entailed the search for a teratoma at the injection site and in all the organs (heart, lungs, kidneys, liver, spleen, and brain) by a pathologist blinded to the treatment group. A biodistribution study was also performed with the search for human cells in the above-mentioned organs by qPCR (limit of detection: 6 cells).

Efficacy has been assessed in a nude rat model (Charles River, L’Arbresle, France) of myocardial infarction created by a permanent coronary artery ligation. This last study was primarily designed as confirmatory to specifically assess the functional benefits of the cell-loaded fibrin patch prepared with exactly the same materials and according to exactly the same procedures as those planned for the clinical trial, thereby allowing a final validation of the whole process. Four to eight weeks after the infarct, only animals with a LV ejection fraction (EF) ≤50% (n = 53) have been randomized to a sham-operated group (thoracotomy with placement of a few stitches similar to what is done for securing the fibrin patch, n = 18), a control group receiving a cell-free 1 cm² fibrin patch delivered onto the infarct area (n = 19) and a treated group (n = 20) receiving a 1 cm² fibrin patch loaded with 700,000 SSEA-1-positive progenitor cells prepared under conditions exactly similar to those of the clinical protocol, except for the down-scaling of volumes of fibrinogen- and thrombin-containing media (to 150 and 150 μL, respectively). Functional indices have been assessed echocardiographically at baseline (before transplantation) and 2 months thereafter, a time point by which 51 animals had survived (18, 15, and 18 in the sham, cell-free fibrin patch, and cell-loaded fibrin patch groups, respectively). Within each group, LV function variables between baseline and 2 months were compared using paired Student’s tests. Across groups, the change of LV function variables between repeated assessments (baseline, 1 and 2 months) was analysed by using a mixed analysis of variance model. For all analyses, a two-tailed P-value ≤0.05 was considered statistically significant and data are summarized using mean (95% CI) values. Analyses were conducted using SAS 9.3 (Statistical Analysis System, Cary, NC, USA). Both data collection and subsequent statistical analyses have been performed in a blinded fashion. All pre-clinical studies were
conducted in accordance with the European regulation on the use of experimental animals.

Finally, the surgical applicability of the cell-seeded fibrin patch has been tested in four sheep in which a myocardial infarction had been created 3–9 months earlier (by surgical ligation of obtuse marginal branches or percutaneous release of endocoronary coils). The objective here was to repeat the surgical procedure planned in patients, and more particularly, to check that the fibrin patch was easy to handle and could be sutured to the epicardium of the infarct area and then covered by a pericardial flap in a straightforward fashion.

**Results**

**Cell characterization**

Undifferentiated hESC of the I6 line grew steadily over a 2-week period of cell expansion, thereby allowing to generate 600 million cells for cryostorage as MCB. Throughout the expansion process (Supplementary material online, Figure S1), cell pluripotency was maintained, as demonstrated by the persisting expression of Nanog, Sox2, and Oct-4 assessed by qPCR. The BMP-2/SU-5402 specification protocol expectedly down-regulated these genes (primarily Nanog and Sox2) on the sorted SSEA-1-expressing progenitor cells, compared with undifferentiated I6 cells, as assessed by Affymetrix microarray analysis (Figure 2). MYC, which plays a key role in oncogenic networks, was also down-regulated. Conversely, the BMP-2-committed progenitors up-regulated genes that mark cardiac progenitor cells (predominantly Isl-1, but also GATA6, HAND1, EOMES) as well as more developmentally advanced cardiomyocytes (MYLK7, MYLK 9, tropomyosin1, MYH9, HAPLN1). At the completion of the specification process (and before any sorting), the percentage of cells that had responded to BMP-2/SU5402 signalling and expressed SSEA-1 averaged 44.2 ± 5.9% (mean ± SD).

At the selected concentrations of fibrinogen and thrombin, robust fibrin patches with good handling characteristics could be reproducibly generated. Four hours after sorting, a time point selected to match the timing by which the patch would be effectively delivered onto the heart, <10% of the SSEA-1-positive scaffold-embedded cells were found apoptotic, as shown by TUNEL staining and caspase-3 expression, while markers suggestive of a cardiomyogenic lineage like Isl-1 and Mef2c were clearly expressed (Supplementary material online, Figure S2).

Based on the transcriptomic analysis, the SSEA-1-negative cell fraction was also characterized by a switch-off of most pluripotency genes with a concomitant mix of mesodermal and endodermal
Following optimization of the sorting/selection procedure, the purity rate of the final cell population, as assessed by the percentage of SSEA-1-positive cells determined by flow cytometry in 27 pre-clinical runs, ranged from 95.3 to 98.8% (mean: 97.3 ± 1.2%). CD29-bright fibroblasts (used as feeders during the amplification and specification phases) accounted for an average of 2% of the final cell yield. To further confirm that the SSEA-1-positive progenitor cells had lost an uncontrollable proliferation potential, they were replated onto fibroblasts; in contrast with undifferentiated I6 cells that rapidly grew as confluent colonies, such a pattern was never seen with any of the two fractions of the cells after induction and sorting.

There was no microbial contamination of the cells throughout the entire culture process as both undifferentiated I6 cells from the cell banks and SSEA-1-positive progenitor cells derived from these banks were found to be free from adventitious agents. Murine retroviruses were unsurprisingly found in the hybridoma clone from which the anti-SSEA-1 antibody was derived but they were no longer detected in the final microbead-conjugated product. Likewise, cytogenetic analyses failed to show any abnormality in the pluripotent cells or in their differentiated derivatives, including at the latest passage studied (P44). All the identified copy number variations corresponded with polymorphisms commonly reported in the normal human genome as described in Toronto Database of Genomic Variants (http://projects.tcag.ca/cgi-bin/variation/gbrowse/hg18/). Thus, array-CGH allowed to eliminate either addition of tumour-promoting regions or deletions of tumour-suppressing regions.

Pre-clinical studies

To specifically assess the safety of the final cell product intended for clinical use, 54 severely immunocompromised mice were subcutaneously injected with the test cells. Teratomas were then expectedly observed in some of the mice injected either with undifferentiated hESC, or with SSEA-1-positive progenitor cells intentionally ‘contaminated’ with 5% undifferentiated I6 cells, but not in any of the mice injected with 500 000 to 900 000 SSEA-1-positive progenitor cells (follow-up: 3–7 months). Injection of the exclusive SSEA-1-negative population generated under clinical-grade conditions also failed to be teratogenic (Supplementary material online, Results and Table S2). Additional biodistribution studies were equally reassuring as qPCR did not detect human cells in any of the off-target remote organs (heart, lungs, kidneys, liver, spleen, and brain).

Efficacy was assessed echocardiographically in the rat model of myocardial infarction (Figure 3). Baseline LVEFs [mean (95% CI)] determined shortly before transplantation did not differ between sham [47.46% (45.28;49.63)], control [47.68% (45.96;49.40)], and treated [47.37% (44.97;49.77)] rats. After 2 months, LVEF decreased in the sham group [46.64% (44.12;49.17)]. In contrast, it slightly increased in control hearts [49.25% (46.75;51.74); P = 0.048 vs. sham] but the best recovery of function was yielded by hearts...
receiving the cell-loaded fibrin patch (51.43% (47.67; 55.19); \( P = 0.004 \) vs. the corresponding baseline value and \( P = 0.004 \) vs. the sham group). This group also achieved the greatest attenuation of adverse ventricular remodelling, when compared with sham-operated rats and those receiving a cell-free fibrin patch (Supplementary material online, Results).

The surgical applicability of the progenitor cell-loaded fibrin patch was successfully confirmed in the four studied sheep in which the patch could be expeditiously sutured onto the epicardium of the infarct area and then covered by a free pericardial flap (Figure 4). Of note, this flap consistently looked macroscopically viable at the time of sacrifice, 7–10 days later.

### Discussion

#### Selection and amplification

Human ESC lines are heterogeneous with regard to their differentiation capabilities. At the completion of a screening process, we selected the I6 line because of its favourable growth kinetics, high cardiogenic potential (reflected by the beating clusters seen when the I6 cells were allowed to aggregate as embryoid bodies) and permission from the institutional owner of the line (Technion Institute, Pr J. Itskovitz) to use it clinically. Although cells could be expeditiously amplified when cultured as 2D static monolayers in fibroblast-seeded flasks, a larger scale production of pluripotent ESC and their derivatives would require a more cost-effective expansion system such as those based on defined xeno-free and feeder-free culture conditions in automated stirred tank bioreactors.

#### Cell specification

Methods to drive pluripotent cells towards a cardiac fate basically entail the in vitro duplication of the key signalling pathways involved in embryonic cardiogenesis. Following M. Pucéat’s protocol, we have thus used BMP-2, which is involved in mesodermal differentiation (and is attractive, from a translational perspective, because of its availability as one component of the clinically approved medicinal product InductOs). The addition of a FGF inhibitor (SU-5402) was found to enhance the efficiency of the commitment step, in keeping with the observation that FGF co-operates with the Nodal/Activin pathway to maintain pluripotency. On average, 44% of the unspecified I6 hESC responded to our BMP-2/FGF inhibitor differentiation protocol by engaging into the mesendodermal lineage pathway. However, it is likely that this rate could be further increased by an additional modulation of the physical microenvironment of the cells; thus, ongoing studies in the laboratory now aim at empowering lineage-specific differentiation by exploiting mechano-transduction pathways through the control of matrix composition, micro/nano surface topography and elasticity.

#### Cell purification

The development of an efficient purification procedure was clearly a critical step to eliminate teratoma-forming cells. We selected to rely on the immunomagnetic recognition of surface markers, and among those previously described, selected SSEA-1. Transition from pluripotency to differentiation is associated with changes in glycosylation of cell surface proteins and it thus looked sound to use an antibody that recognizes LewisX (or, at least, one of its epitopes) which is a member of the glycan family expressed early upon differentiation. The transcriptomic analysis of the SSEA-1-positive cells demonstrated that they strongly expressed, among others, Isl-1 which is one of the earliest transcription factors that marks a progenitor of the three main lineages (cardiomyocytes, endothelial cells, and smooth muscle cells) comprising heart tissue. This finding was expectedly mirrored by the down regulation of pluripotency genes, except for Oct-4 which is consistent with the fact that BMP-2-induced up-regulation of this transcription factors contributes to the cardiogenic fate of the cells. However, immunomagnetic sorting has limitations, which legitimates to investigate alternate purification strategies. They may include a negative-type of selection targeting...
markers of still pluripotent stem cells such as SSEA-5 or lectins, or the selective elimination of residual undifferentiated cells by metabolic interventions, cytotoxic antibodies, or genotoxic drugs.

Safety data
The safety of the final cell product was based on two major findings: genetic stability and lack of teratogenicity. To screen genetic abnormalities that may occur during hESC cultures, we implemented a combinatorial approach associating (i) a karyotypic analysis which is mandatory to detect unbalanced rearrangements but also balanced rearrangements like translocation or inversion which escape array-CGH, (ii) array-CGH which fails to identify weak cellular mosaics or hyperploidies while these patterns can be detected by the karyotypic analysis of a sufficient number of cells, and (iii) FISH which allowed to exclude trisomy of chromosomes 12 and 17 by using specific centromeric probes as well as a 20q11.21 duplication of the long arm of chromosome 20, even at the state of weak mosaic, owing to the use of bacterial artificial chromosome-type locus-specific ADN probes. However, although chromosomal aberrations have been linked to the oncogenic potential of hESC, the absence of cytogenetic abnormalities is not an absolute safeguard against the development of teratomas. From this standpoint, reassuring data were provided by (i) the high purity rate of the final preparation (97.3 ± 1.2%), (ii) the finding that the contaminating SSEA-1-negative cells are no longer equivalent to fully undifferentiated cells (Supplementary material online, Figure S3), and (iii) the lack of teratoma observed in severely immunodeficient mice following subcutaneous matrigel-mixed injections of either the SSEA-1-positive population (given at a dose 100 times higher than that equivalent to the clinically planned dose) or of the negative fraction which, when administered, alone, at a dose (500 000) similar to the maximal one to which a patient could be exposed (5% of the projected 10 million total load with a minimal purity rate required for batch release set at 95%) also failed to be teratogenic. The latter experiments (injection of exclusively negative SSEA-1 cells) were explicitly required by the regulatory Agency before approval whereas the delayed down-regulation of Oct-4 in the SSEA-1+ cell population did not raise specific safety issues as long as this population had consistently been shown not to be teratogenic.

Cell scaffolding
Our selection of fibrin as the cell vehicle was built upon previous studies showing the benefits of the epicardial deposit of a cell-laden scaffold over intra-myocardial injections. Fibrin has a long-standing safety record as a sealant used in various surgical disciplines and features several advantages including biocompatibility, tunable mechanical properties, favourable biodegradation kinetics, and lack of toxicity. Indeed, in the rat myocardial infarction model, acellular fibrin scaffolds yet demonstrated some functional benefits compared with sham-operated rats. However, an incremental advantage was further observed when SSEA-1-positive progenitor cells were included in the fibrin scaffolds as this group yielded the best recovery of contractile function and the least degree of adverse remodelling compared with both sham-operated animals and controls receiving the cell-free fibrin patch. These results are in line with those reported with similar fibrin-based biomatrices incorporating mouse ESC-derived BMP-2-treated cardiac progenitor cells or hESC-derived vascular cells in rat and porcine models of myocardial infarction, respectively. For the planned clinical applications, the cell-loaded patch will be covered by a pericardial flap intended to provide a supportive environment to the underlying cellular graft, as shown with the omentum. As reported above, the sheep experiments have established the feasibility of this ‘sandwiching’ of the cell-loaded patch between the epicardium of the diseased area and an overlying free pericardial flap.

Cell developmental stage and mechanism of action
Several studies have documented the protective effects of hESC-derived cardiomyocytes on infarcted myocardium but the optimal developmental stage at which these cells should be grafted still remains elusive. Our approach has focused on early progenitor cells, with the premise that their plasticity could make them better responsive to locally present cardioinstructive cues and this hypothesis tends to be supported by the cardiomyogenic differentiation of these cells in the non-human primate infarction model of allogeneic transplantation. At the opposite, one could argue that a greater contribution to the contractile activity of the heart might be achieved with more fully mature and already beating cardiomyocytes. Head-to-head comparative studies are thus clearly required to select the optimal differentiation stage for transplantation.

Regardless of this stage, the mechanism by which ESC-derived cardiac-committed cells affect heart repair still remains elusive. Although ESC-derived cardiomyocytes are contractile force-generating cells which may induce some remuscularization, the increasingly prevailing hypothesis is that they might rather act paracrinally by harnessing endogenous repair mechanisms. This hypothesis is largely based on the consistent finding that the number of transplanted cells which survive in the myocardium is too small to account for their commonly reported functional benefits. This remains true with the patch approach where cells tend to remain clustered in the subepicardial space and physically insulated from the host myocardium. We thus clearly acknowledge that our data do not allow to claim that the transplanted progenitor cell population gave rise to newly differentiated contractile and electromechanically coupled bona fide cardiomyocytes. Conversely, these cells may paracrinally induce a host-derived cardiomyogenesis during their initial post-procedural residence in the transplanted tissue is mechanistically more plausible, as suggested by the ability of irradiated (and thus non-proliferating) ESC grafted into infarct areas in monkeys to trigger the proliferation of host cardiomyocytes in the peri-scar area. Several cell types have been shown to release biomolecules, possibly cargoes by exosomes, which might activate self-repair mechanisms but, based on head-to-head studies, the most effective seem to be those whose phenotype most closely matches that of the target tissue. This provides a robust rationale for the use of cardiac lineage-committed progenitors, such as those derived from pluripotent stem cells. In this context, it is yet difficult to predict to what extent induced pluripotent stem (iPS) cells may compete with ESC. It is noteworthy, however, that except for the initial iPS-specific phase of somatic cell reprogramming, the roadmap described in this paper would be fully applicable to the re-differentiation of reprogrammed iPS cells towards a cardiac fate.
In conclusion, this academia-driven translational programme has
to the generation of ESC-derived mesodermal/cardiac progeni-
tors intended for clinical use in patients meeting the following in-
clusion criteria: a severe LV systolic dysfunction with LVEF \( \leq 35\% \), an
history of myocardial infarction (older than 6 months), the presence
of symptoms reflected by a New York Heart Association (NYHA)
Class III or IV despite optimal standard of care, the previous implant-
ation of an automatic internal defibrillator associated, whenever
indicated, to ventricular resynchronization and an indication for a
conventional cardiac surgical procedure (coronary artery bypass
grafting or mitral valve repair/replacement). Several facets of this
protocol can and should definitely be improved. The outcomes of
the upcoming clinical trial based on this translational programme
might provide useful data for fueling a reverse bed-to-bench
pathway leading to optimize the safety and efficacy of these hESC-
derived cardiac-committed cellular therapeutics.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

**Authors’ contributions**

P.H.M., M.D., A.H., J.L.: conception and design, collection and assem-
by of data, data analysis and interpretation, manuscript writing, and
final approval of manuscript; V.V., J.L.: development of cell expansion,
specification, and purification procedures and final approval of manu-
script; J.R.F., J.H.T.: provision and validation of regulatory guidelines
and specification, and purification procedures and final approval of manu-
script; A.B., V.B., Y.F., O.A.: implementation of pre-clinical studies and final approval of manuscript; L.T., G.T.:
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vivo safety studies and final approval of manuscript; O.D.: provision
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