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A purified population of multipotent cardiovascular progenitors derived from primate pluripotent stem cells engrafts in postmyocardial infarcted nonhuman primates

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Cell therapy holds promise for tissue regeneration, including in individuals with advanced heart failure. However, treatment of heart disease with bone marrow cells and skeletal muscle progenitors has had only marginal positive benefits in clinical trials, perhaps because adult stem cells have limited plasticity. The identification, among human pluripotent stem cells, of early cardiovascular cell progenitors required for the development of the first cardiac lineage would shed light on human cardiogenesis and might pave the way for cell therapy for cardiac degenerative diseases. Here, we report the isolation of an 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 treated with the cardiogenic morphogen BMP2. This progenitor population was multipotent 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. Notably, primates transplanted with an unpurified population of cardiac-committed cells, which included SSEA-1- cells, developed teratomas in the scar tissue, whereas those transplanted with purified SSEA-1+ cells did not. We therefore believe that the SSEA-1+ progenitors that we have described here have the potential to be used in cardiac regenerative medicine.

Introduction

The embryonic human heart starts beating at around 20 days of gestation and is the target of many genetic and congenital diseases likely originating from the early and complex formation of the myocardium. Over the past decades, cardiac progenitors have been tracked in embryos and appeared as a very early lineage, determined in the primitive streak of chicks (1) as well as mammals (2), when epiblast cells are incorporated within the posterior primitive streak, around day 6 in mice and 16 in humans. Ethical reasons limit the possibility to study such an early stage of development in human embryos. Embryonic stem cells (ESCs) have been long recognized to provide a powerful model to study the early steps of cardiac specification under normal or pathological conditions. Furthermore, ESC-derived cardiomyocytes are a potential cell source for cardiac regeneration. Indeed, cell therapy is a potentially new option for patients with advanced heart failure. However, the clinical trials of bone marrow cell (3) or skeletal myoblast (MAGIC) transplantation (4) have only yielded marginal results. This has been attributed to the limited plasticity of adult stem cells, which precludes their differentiation into functionally integrated cardiomyocytes. Thus, the best substitutes for the missing host cardiomyocytes appear highly plastic cells that can recapitulate cardiomyogenesis. From this standpoint, cardiac stem cells are conceptually attractive but the uncertainties regarding their persistence in adulthood (5) cast serious doubts over their potential therapeutic usefulness. In contrast, pluripotent ESCs emerge as an appealing alternative, but so far their potential use in humans has been hampered by major safety concerns, in that, if undifferentiated and injected in immune depressed mice, they generate teratomas. There is also the possibility that a progenitor cell could stop in the course of differentiation and proliferate in an uncontrolled manner in vivo, as reported with neuronal progenitors grafted in rat brain (6). Thus, definitively committed,
early cardiac progenitors could be the best clinical cell source in regenerative cardiology. Several groups have transplanted human ESC-derived (HUESC-derived) cardiomyocytes in postmyocardial infarcted rats (7–9). However, as previously discussed (10), fully differentiated cardiomyocytes have a limited number of divisions, and an important number of cells would be required to replace those lost as a result of disease. Thus, a better option is to use cardiac progenitors still capable of dividing while differentiating.

Using mouse ESCs and HUESCs, several cardiac progenitors have been isolated, searching for a common progenitor at the origin of the whole heart (11). Among them, ISL1+ multipotent progenitors arising in vitro from around E8 of differentiation in mouse give rise to CD31+ SCA-1+ smooth muscle cells, has been identified from a mouse and later an human ESC line, following sequential treatment for 14 days with a cocktail of growth factors, including activin, BMP4, FGF2, and VEGF (14, 15). Despite these previous findings, the quest for HUESC- or induced pluripotent stem (iPS) cell-derived early progenitors at the origin of both the first and secondary cardiac lineages still remains a relevant issue both for basic developmental biology and clinical application. Here, we have isolated, sorted, and characterized such a cell population and we report the feasibility of allograft of primate ESC-derived enriched cardiovascular progenitors in a nonhuman primate model of myocardial infarction.

Results
Cardiogenic commitment of pluripotent stem cells and generation and sorting of a large and early population of cardiovascular progenitors. To drive HUESCs and iPS cells toward a cardiac lineage, we designed experiments to recapitulate the early embryonic developmental pathways from the epiblast stage leading to the precardiac mesoderm lineages. BMP2 together with Wnt3a constitutes a potent combination of factors to induce the mesoderm (16, 17). We thus used the cardiogenic morphogen BMP2 in a defined serum-free medium and in the presence of the FGF receptor inhibitor SU5402 (18). Such a protocol allowed us to combine the action of BMP2 with that of the autocrine Wnt3a ligand, whose expression was strongly induced by BMP2 (9 ± 1-fold increase in mRNA expression). The action of secreted Wnt3a was further demonstrated by adding the Wnt inhibitor, dickkopf homolog 1, in the presence of BMP2. Indeed, this inhibitor significantly decreased the effect of BMP2 on cardiac gene expression (19) and dramatically reduced the number of cells that lost their pluripotency and, in turn, expressed the stage-specific embryonic antigen 1 (SSEA-1) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI40120DS1), an index of differentiation of HUESCs as well as of the human blastocyst (20). As SSEA-1 is one of the earliest markers of HUESC differentiation and loss of pluripotency, and as it is expressed following 4-day treatment of cells with the cardiogenic morphogen BMP2, we reasoned that it might be used to exclude the cells that were not responsive to the morphogen. Indeed, cell sorting using an anti–SSEA-1 antibody allowed for separating cells expressing mRNAs and proteins encoding mesodermal and cardiac markers (21).

We substantially extended this finding and characterized the genetic and epigenetic profiles of BMP2- and WNT3A-induced SSEA-1+ sorted cells as well as their phenotypic fate when engrafted into diseased hearts.

Flow cytometry revealed that 40% (iPS cells) to 53% ± 10% (HUESCs, n = 15) of BMP2-treated cells stained positive for SSEA-1 (Figure 1A and Table 1). Sorting out the BMP2-induced SSEA-1+ cell population using an antibody coupled to magnetic beads, revealed that these cells still expressed a high level of OCT4 mRNA (i.e., above the one required to maintain stem cell pluripotency), an index of their cardiac specification (22), but had lost expression of NANOG, CRIPTO, or LEFTY, master stem cell pluripotency genes, not expressed in somatic cells. In contrast, the pluripotent stem cell SSEA-1+ population retained expression of these markers (Figure 1B). The BMP2-induced SSEA-1+ cell population could be sorted out from 5 separate HUESC lines, the Rhesus ORMES cell line, and the iPS cell line with different efficiencies (Table 1).

Table 1
Yield of SSEA-1+ cell sorting using anti–SSEA-1–conjugated magnetic beads

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BMP-2–induced SSEA-1 cells (%)</th>
</tr>
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<tbody>
<tr>
<td>HUES-24</td>
<td>49 ± 9 (n = 20)</td>
</tr>
<tr>
<td>HUES-26</td>
<td>20 ± 5 (n = 3)</td>
</tr>
<tr>
<td>HUES-9</td>
<td>53 ± 5 (n = 5)</td>
</tr>
<tr>
<td>I6</td>
<td>49 ± 6 (n = 7)</td>
</tr>
<tr>
<td>H9</td>
<td>60 ± 8 (n = 5)</td>
</tr>
<tr>
<td>IPS</td>
<td>30 ± 5 (n = 4)</td>
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Gene transcription is tuned by the epigenetic status of stem cells (i.e., the histone code). In ESCs, promoters of transcription factors specific for early cell lineages, feature bivalent domains, harboring both epigenetic marks, the activating methylation of lysine 4 of histone 3 (H3K4) and the repressive methylation of lysine 27 (H3K27) (28). Thus, to further characterize the BMP2-induced SSEA-1+ cell population, we designed an epigenetic strategy targeting early transcription factors (Figure 1, D and E). Using ChIP assays, combined with real-time PCR, we looked at both activating and repressive epigenetic marks on the OCT4 promoter and on 5 mesodermal and cardiac promoters. We found that the OCT4 promoter was associated with an increase in methylation of H3K4 and was occupied by the polymerase II (PolII), 2 epigenetic marks indicating a transcriptionally active chromatin. TBX6, ISL1, MEF2C, NKX2.5, and cardiac α-actin (ACTC1) promoters shared a common epigenetic status, with a 30- to 2,000-fold increase (NKX2.5) in PolII occupancy. These data were further confirmed in ChIP experiments using an anti-PolII phosphorylated on serine 5, a better mark of activated bivalent promoters (29) (Figure 1E).

Ten-fold enrichment in H3K4 methylation on cardiac promoters was observed in SSEA-1+ compared with SSEA-1- cells (Figure 1D), while the H3K27 methylation mark did not significantly change on the same promoters. Interestingly, the promoters of 2 pluripotency genes, SOX2 and NANOG, were not any more occupied by serine 5–phosphorylated RNA PolIII in SSEA-1+ cells (Figure 1E). The epigenetic status of the PAX4 promoter, an endodermal gene, was not changed in SSEA-1+ compared with SSEA-1- cells.

Early cardiovascular progenitors feature a unique microRNA signature. Besides gene transcription, expression of proteins defining a specific cell fate is also regulated at the posttranscriptional level by small noncoding RNAs (miRNAs, miRs), and profiles of miRNAs often reflect a cell-specific signature. We thus used a genomic wide approach (30) to compare the profile of miRNAs in BMP2-induced SSEA-1+ cells and remaining SSEA-1- or undifferentiated HUESCs. This broad approach showed that SSEA-1+ cells gained expression of specific miRNAs, belonging to the cluster miR-17-92. These include miR-20a and miR-20b, known to be turned on when ESCs acquire a cardiac fate (31), and miR-17-92, whose downregulation causes a cardiac defect. Furthermore, miR-106, whose deletion concomitant to the one of miR-17-92 results in severe cardiac defects (32), was also upregulated in SSEA-1+ cells. miR-513, another miRNA not expressed in undifferentiated ESCs, has still unknown functions that were highly upregulated in SSEA-1+ cells; its function is currently under investigation in the laboratory. The gain in expression of these miRNAs was marked in SSEA-1+ cells when compared with the one in SSEA-1- cells and became even more prominent when compared with the profile of undifferentiated stem cells (Figure 2). miR-302a, belonging to the cluster 302, whose expression is regulated by OCT4 and potentially SOX2 and NANOG, was also more expressed in SSEA-1+ cells versus SSEA-1- cells or HUESCs. This phenomenon could be related to the dual role of this miRNA in both pluripotency and then in specification of HUESCs toward the mesendoderm (33). Another miRNA expressed in HUESCs was also upregulated in SSEA-1+ cells in agreement with its high expression when HUESCs differentiate within embryoid bodies (30). Expression of miR-615 and miR-135 connected to the pluripotency network in ESCs was unchanged (34). Of note, the miR-302bcd, also involved in the pluripotency transcriptional network (34, 35), was downregulated in SSEA-1+ cells when compared with SSEA-1- cells, while it appeared as upregulated when compared with expression level in HUESCs (Figure 2).

Figure 2
Heat map representative of the miRNA profile between SSEA-1+ and HUESCs and SSEA-1- and SSEA-1+ cells. Numbers beside the figure represent miRs. Total RNA was extracted from HUES, SSEA-1-, and SSEA-1+ cells and hybridized to a miRNA microarray. Experiments were performed 6 times and log2 ratio values are shown in the heat map.

We next looked at expression of mesodermal and cardiac lineage marker genes by quantitative PCR (Q-PCR). Freshly sorted SSEA-1+ cells expressed T brachyury-like (7) and FLK1, 2 characteristic genes of the early mesoderm. Furthermore, these cells expressed mesoderm posterior 1 (MESP1), the earliest known marker of cardiogenesis (23); TBX6, a mesodermal muscle gene upstream of MESP1 (24); TBX5, a ventricular marker; and TBX18, a marker of both epicardium (25) and the left ventricle (26). SSEA-1+ cells also expressed myocardin (MYOCD), GATA4, MEF2C, and NKX2.5, key smooth muscle and cardiac transcription factors of the primary heart field, as well as ISL1 and TBX20, the latter more reminiscent of the secondary heart field. While expressing TBX18, SSEA-1+ whole population expressed, to a small extent, Nestin (NES) or PAX3, 2 markers found in neural crest cells (Figure 1C). TBX3, a marker of the cardiac conduction system (27) among other lineages, was not detected (data not shown). We could not detect any other marker of the hematopoietic or skeletal lineage (CD45, CD34, N-cadherin, MyoD) or any endodermal (SOX17, HEX, FOXA1) (data not shown) or ectodermal (SOX1) marker. Flow cytometry analysis confirmed that a minority (5%–7% in 2 separate experiments) of SSEA-1+ cells were Flk1+ at the time of sorting (data not shown). TBX6 and MESP1/2 were readily detected in most cells at the level of the protein, right after sorting (Figure 1C, right). A similar gene profile could be found in 5 HUES cell lines tested (HUES-24, HUESC-26, HUES-9, H9, I6).
The cardiovascular population of SSEA-1+ progenitors is clonogenic and multipotent. To characterize the differentiation potential of SSEA-1+ progenitors toward several cell lineages, we plated isolated SSEA-1+ progenitors as suspensions of single cells on mitotically arrested E14 mouse embryonic fibroblasts (MEFs) in 96-well plates for 5 days, stained them with DAPI, and scanned them using an Arrayscan. Despite being plated by limiting dilution at high or low density (5,000 down to 1 single cell/well), cells generated clones within 5 days. The number of clones per well tightly correlated with the starting number of plated cells (Figure 3A).

One single cell (monitored by phase contrast microscopy, using the wild-type HUES-24 cell line, or by GFP expression, using the HUES-9 Oct4GFP cell line) was able to generate a clone within 7 days (Figure 3, A–C). In another set of experiments, a single SSEA-1+ cell was observed while carrying out cytokinesis as early as 2 days after plating (Figure 3A, above the graph). The limiting dilution experiments were confirmed by adding a single cell into a well of a 96-well plate using a micropipette and monitoring the formation of a colony (data not shown). Altogether, this demonstrates that SSEA-1+ cell–derived colonies originate from single cells and not from cell clusters, eliminated by thorough trypsinization and filtering (40 μm) before the passage through the sorting magnetic column.

After 1 week in culture on MEFs in the absence of SU5402, BMP2-induced SSEA-1–sorted cell–derived colonies still expressed SSEA-1, together with 4 cardiac-restricted transcription factors, ISL1, MEF2C, NKX2.5, and TBX5. A majority of cells from most colonies expressed these markers (Figure 3D), pointing to a rather homogeneous population of cardiac progenitors. ISL1, MEF2C, NKX2.5, and TBX5 were all located into the nucleus. FLK1, poorly expressed in the freshly sorted SSEA-1+ cell population (5%–7%) (data not shown), was found later at the membrane of a proportion, but not in all cells, after 1 week of culture on feeder cells in the absence of SU5402. In contrast, HUES-derived SSEA-1+ cells did not express NKX2.5, ISL1, MEF2C, and TBX5 but still expressed OCT4, SOX2, and NANOG as assessed by immunofluorescence (data not shown).

Addition of PDGF (10 ng/ml) or VEGF (50 ng/ml) to BMP2-induced SSEA-1+ cells cultured on MEFs strongly triggered expression of smooth muscle actin and myosin as well as of CD31, respectively, while only 12% of freshly sorted BMP2-induced SSEA-1+ cells (Supplemental Figure 2A) were CD31+ (Supplemental Figure 2B). Plated on collagen 1–coated dishes and treated with VEGF (50 ng/ml for 10 days), 80%–90% of SSEA-1+ cells acquired the morphology of endothelial cells and expressed high levels of CD31, CD34, and FLK1 mRNAs as well as the protein CD31 for 30% of them (Supplemental Figure 2C) but not cardiac troponin T (data not shown), as investigated by immunofluorescence.

When plated on human fibroblasts in the presence of 5% fetal calf serum or in a mix of human cardiac fibroblasts and cardiomyocytes, 10% and 60%–80% of cells, respectively, expressed actinin arranged in sarcomeric units within a week (Figure 3D and Figure 4A). A similar effect was observed in 70% of cells when cells were treated with the conditioned medium of a coculture of human cardiac fibroblasts and cardiomyocytes.

Interestingly, SSEA-1+ cells in culture on MEFs for 4 days expressed some genes specific for the secondary heart lineage. This was further enhanced by FGF8 (5 × 10^{-8} M) (36) treatment of cells for an additional 4 days. FGF8, indeed, definitively turned on expression of TBX1, retinaldehyde dehydrogenase 2 (RALDH2) (37, 38), HES1 (39), and FOXH1 (40) (Supplemental Figure 3A). The FGF8 effect was also observed when genes were monitored at the level of single colonies (data not shown).

To better quantify the cardiogenic fate of BMP2-induced SSEA-1+ cardiovascular progenitors cultured for 1 week on MEFs, immunostained cells cultured in 96-well plates were scored by high-content cell imaging (HCCI), using an Arrayscan, in 2 experiments. This technological approach allowed us both to quantify and to visualize cells positive for each cardiac marker. The BMP2-induced SSEA-1+ population expanded as cardiovascular progenitors within colonies (Figure 3E, right) expressed NKX2.5 and MEF2C (63% and 91%, respectively) as well as ISL1 (89%). The cells lost expression of OCT4 and SOX2 (data not shown). This quantitative analysis by HCCI was confirmed by flow cytometry (data not shown).

To assess the transcriptomic homogeneity of the clones, individual colonies grown for 7 days on MEFs were randomly picked up through a micropipette and screened for gene expression by RT-Q-PCR. These experiments revealed that each colony expressed, to a similar and high extent, NKX2.5, MEF2C, ACTC1, and MYOCD and, to a lesser degree, FLK1. ISL1 and TBX20 were downregulated in 50% of colonies; NES and PAX3 were expressed in 50% of clones, actually those expressing ISL1 and TBX20. None of the colonies expressed SOX17, an endodermal marker (Supplemental Figure 3B). The multipotentiality of a single clone of SSEA-1+ cells was further demonstrated by cutting single colonies grown on MEFs for 5 days into several cell clusters. Cells were then separately replated on collagen 1–coated dishes and treated with PDGF or VEGF or plated on human fibroblasts. In the later case, cardiomyocytes (Supplemental Figure 4A) were observed 2–3 weeks later, while CD31-positive cells (Supplemental Figure 4B) and smooth muscle actin-positive cells (Supplemental Figure 4C) were present in growth factor treated cells after 1 week (Supplemental Figure 4).

In the perspective of a potential use of SSEA-1+ progenitors in customized regenerative medicine, we used iPES cells. BMP2-induced SSEA-1+ cells could also be derived from iPES cells (Supplemental Figure 5A). A similar pattern of expression of cardiac proteins (i.e., ISL1, MEF2C, and NKX2.5) was found in BMP2-triggered SSEA-1+ cell–derived and sorted cells cultured for 1 week on MEFs, as assessed by immunofluorescence. Furthermore, a minor population of BMP2-induced SSEA-1+ iPES cells (4%) stained positive for CD31 (Supplemental Figure 5B).

Cardiovascular BMP2-induced SSEA-1+ progenitors differentiate into mature cardiomyocytes ex vivo. To test the cardiogenicity and the functionality of BMP2-induced SSEA-1+ progenitors prior to animal graft experiments, we used an ex vivo experimental set up. In order to track the fate of cells in culture, Rhesus ESCs (ORMES-2 cell line) were genetically engineered to express GFP under the transcriptional control of the human cardiac α-actin promoter. BMP2-induced SSEA-1+ Rhesus cardiac progenitors were then cocultured for 4 weeks with human atrial myocytes, cardiac fibroblasts, or a mix of cardiomyocytes and fibroblasts (Figure 4). After 4 weeks, microscopic examination revealed the presence of GFP-positive cells (Figure 4) in the 3 cultures, thereby confirming that human cardiac/fibroblastic cells provide the paracrine environment that triggers the cardiomyogenic differentiation of BMP2-induced SSEA-1+ progenitors. The extent of differentiation of BMP2-induced SSEA-1+ cardiac progenitors was prominent and reached 70% ± 10% (in 3 experiments) for cells cocultured on the mix of cardiac fibroblasts and cardiomyocytes or conditioned medium from
cardiac progenitors and stained with an sarcomeric actin network and became hypertrophied, indicating on fibroblasts, they started to form more mature sarcomeres. When cultured on myocytes alone, cells remains as fetal (sarcomeric situation, a high proportion of cells (60%–80%), as monitored in mature cardiomyocytes expressing organized and sarcomeric muscles [SM] myosin in absence of growth factors or challenged by PDGF or VEGF, or cocultured of BMP2-induced SSEA-1

Figure 3

BMP2-induced SSEA-1+ cardiac progenitors give rise to cardiac, endothelial, and smooth muscle cells. (A–C) Cell clonogenicity. BMP2-induced SSEA-1+–sorted cells were plated at different densities (5,000, 500, 50, 5, and 1 cell/well) in 96-well plates containing MEFs. Five days later, cells were stained with DAPI to be scanned using an Arrayscan. (A) The orange arrow indicates that the colony was generated in a single cell-containing well. Cell colonies were numbered using the Cellomics viewer software. (A and B) A colony generated from a single SSEA-1+ cell (arrows) and visualized 2 (top image) or 5 (A) days later (above the graph). (C) A single SSEA-1+ cell derived from the HUES-9 pOct-4-GFP cell line that was plated in a microwell of 96-well plates and visualized by fluorescence right after plating (top image) or 5 days later (bottom GFP and transmitted light images). (D) Immunofluorescence of BMP2-induced SSEA-1+ cells cultured for 5 days on MEFS in the absence of growth factors or challenged by PDGF or VEGF, or cocultured with human cardiac fibroblasts. Top left corner insets show high-magnification images of localization of markers. The inset in the actin panel shows a high-magnification image of sarcomeres. Experiments were done in triplicate with similar results. (E) BMP2-induced SSEA-1+ cells cultured for 5 days on MEFs immunostained with anti-SMA antibody. No cells were labelled with an anti-SMA antibody. The remaining areas within the scar (Figure 5C). Thus, BMP2 nonresponsive SSEA-1+ and still pluripotent cells proliferated and differentiated within the tumor and should thus be removed before transplantation.

While untreated ORMES cells featured less than 10% SSEA-1+ cells, 50% ± 5% of BMP2-challenged cells expressed the antigen (Figure 5D). To confirm the phenotype of SSEA-1+ cells, the gene expression profile of both SSEA-1+ and BMP2-induced SSEA-1+ cell populations was then compared by real-time RT-PCR. As shown in Figure 5E and as found in HUESC derivatives, the BMP2-induced SSEA-1+ ORMES cells featured a high expression of T, TBX6, TBX20, ISL1, TBX5, MEF2C, and ACTC1 (Figure 5A), thereby demonstrating a cardiac print of these cells and confirming the strong cardiac inductive action of BMP2 in nonhuman primate cells. To assess the fate of these cells in vivo, we developed a nonhuman primate model of myocardial infarction by subjecting Rhesus monkeys to a percutaneous 90-minute coronary occlusion/reperfusion protocol, followed, 2 weeks later, by open-chest cell transplantation in the infarcted area.

In a first series of pilot experiments, 107 BMP2 cardiac-committed cells expressing cardiac genes (Figure 5A) were transplanted in the scar area of 2 immunosuppressed primates. The primates were euthanized after 2 months. Confocal microscopy of the explanted hearts showed clusters of GFP-positive cells throughout the injected areas within the scar (Figure 5B). Unfortunately, a careful pathological examination of the myocardium and other organs (e.g., lung, liver) revealed that 1 out of the 2 primates injected with nonsorted cells had developed a microtromatoma in the scar area (Figure 5C).

While untreated ORMES cells featured less than 10% SSEA-1+ cells, 50% ± 5% of BMP2-challenged cells expressed the antigen (Figure 5D). To confirm the phenotype of SSEA-1+ cells, the gene expression profile of both SSEA-1+ and BMP2-induced SSEA-1+ cell populations was then compared by real-time RT-PCR. As shown in Figure 5E and as found in HUESC derivatives, the BMP2-induced SSEA-1+ ORMES cells featured a high expression of T, TBX6, TBX20, ISL1, TBX5, MEF2C, and NKX2.5, while these genes were barely detectable in the SSEA-1+ cell population. In contrast, BMP2-induced SSEA-1+ cells dramatically lost expression of most pluripotency genes (i.e., CRIPTO, NANOG, and LEFTY), in comparison with SSEA-1+ cells (Figure 4E, inset). Of note, OCT4 was still expressed at a higher level in BMP2-induced SSEA-1+ cells than in the SSEA-1+ cell population, as also observed in human BMP2-induced SSEA-1+–sorted cells. When cultured on MEFs, the SSEA-1+ ORMES–sorted cells expressed to a great extent after 1 week TBX20, ISL1, MEF2C, NKX2.5, and ACTC1. T, whose expression was high in freshly sorted cells, was downregulated. The pluripotency genes NANOG, LEFTY, CRIPTO (also known as TDFG1), and OCT4 were all downregulated (Figure 5F).

Infarcted myocardia of 8 immunosuppressed primates were thus transplanted with 2 × 107 SSEA-1+ cardiac progenitors and 3 were transplanted with cell medium. The primates were euthanized after 2 months. Confocal microscopy of the explanted hearts showed clusters of GFP-positive cells throughout the injected areas within the scar (Figure 5H), and this strong cardiac differentiation potential of BMP2-induced SSEA-1+ cardiac progenitors was further confirmed by PCR using GFP-specific prim
ers (data not shown). These cells were surrounded by CD31- and SMA-positive cells, suggesting either a recruitment of these cells toward the grafted cells or a differentiation of SSEA-1+ cells into these lineages as found in vitro. We quantified, by imaging GFP and morphometry, the extent of the graft within the scar in 60 myocardial sections. The scar area was 284,620 ± 82,816 μm², and the extent of GFP+ cell recolonization was 65,251 ± 46,056 μm², which represented 20% ± 10% of the scar. The ORMES-derived cardiomyocytes expressed α-actinin, set in sarcomeric structures, featuring an adult size (Figure 5H, inset) and expressing CX43 (Figure 5H). The ventricular phenotype of these cells was demonstrated by expression of the ventricular isoform of myosin light chain 2, associated with the presence of myosin light chain kinase, featuring both diffuse distribution and a sarcomeric organization (41) (Figure 5H, inset). Thus, as shown in a rat model of myocardial infarction (42), the scar environment turned out to be potent enough to trigger differentiation of cardiac-committed primate ESCs. No teratoma was detectable in any of the 8 primates that had received sorted SSEA-1+ cells in any of the investigated organs (myocardium, brain, liver, spleen, pancreas, duodenum, lung, gonads, or bones). This finding strongly suggested that some uncommitted SSEA-1+ cells (50% of the injected population), which were not responsive to BMP2 and thus still undifferentiated pluripotent cells, were at the origin of the tumor, thereby confirming that purification of the progenitor cell population is mandatory for the safety of the procedure. The safety of delivering this SSEA-1+ cell population was further supported by another set of experiments performed in 10 humanized immunodeficient Rag2−/−, γ chain−/−, CS−/−, MHC−/− mice (43). In this series, none of the intracardiac injections of the SSEA-1+, ISL1+, NKX2.5+, MEF2C+, and TBX5+ cardiac progenitors resulted in teratoma at a 4-month follow-up (180 cardiac and other organs sections examined; data not shown).

Discussion
Herein, we have identified a very early population of OCT4+ cardiac progenitors expressing the membrane antigen SSEA-1. SSEA-1 expression was employed as one of the earliest markers of HUESCs or iPSC cell loss in pluripotency, to sort out the cells both responsive and unresponsive to BMP2.

Our findings first revealed that BMP2, in the absence of activin (i.e., in serum-free defined medium), acts in a combinatorial manner with Wnt3a, whose expression was triggered by the morphogen, to drive pluripotent stem cells toward an early mesodermal and cardiogenic fate in vitro, as expected from both their in vivo role in defining the posterior primitive streak and mesoderm (44, 45). Several data suggest that the BMP2-induced SSEA-1+–sorted cell population is likely to be more reminiscent of the early cardiac progenitors allocated to the epiblast. When sorted out, these cells still feature a high level of OCT4 (i.e., above the level required to maintain cell pluripotency), as confirmed by the epigenetic status (high H3K4 methylation) of its promoter. Despite their early stage of specification, SSEA-1+ OCT4+ cells acquired a genetic and epigenetic signature, including a profile of miRNA expression, characteristic of pancreatic progenitors. They lost pluripotency, as indicated by NANOG, CRIPTO, LEFTY, and SOX2 downregulation concomitantly with the expression of SSEA-1. While the remaining presence of OCT4 in the absence of other pluripotency genes might
be questionable, its level of expression above the one of undifferentiated stem cells also reflects the first step of cell specification toward a cardiogenic fate (19, 22, 46). Such a gene profile, together with expression of the proteins TBX6 and MESP1/2, indicates that this progenitor somehow reflects the very early cardiac-determined OCT4+ cell population found in the embryonic epiblast (2, 22). In agreement with the last statement, SSEA-1+ cells gained expression of several miRNAs, including 3 (miR-20, miR-17-92, and miR-106) reported to be turned on when ESCs acquire a cardiac fate (31) or are likely important for mesendodermal differentiation (33) and proliferation of cardiac progenitors (32). Interestingly, miRNAs known to be the major targets of the OCT4/SOX2/NANOG transcriptional pluripotency network, miR-302s (34, 47, 48), were dramatically downregulated in SSEA-1+ versus SSEA-1- cells, supporting the idea that SSEA-1+ cells lost their pluripotency to move along a cardiac differentiation pathway. On the other hand, it could be surprising that SSEA-1+ expressed more mRNA-302bed than HUESCs. However, at the time of sorting (when RNAs were extracted for profiling miRNAs), the progenitors still expressed a level of OCT4 above that of HUESCs. As mRNA-302 as well as miRNA-135 and miRNA-615 are primarily induced by OCT4 (or lost when OCT4 is downregulated in the presence of SOX2 and NANOG) (34), this explains that expression of these miRNAs is maintained or even increased. It should be further stressed that their exact role in pluripotency remains to be established and that like miR-106 and miRNA-363, they might be involved at later stages in cell lineages differentiation (30, 33).

Finally, when sorted they express a series of genes (T, TBX6, MESP, ISL1, TBX20, NKK2.5, MEF2C, MYOCD, and TBX18, etc.) and proteins (TBX6 and MESP1/2) that altogether confer them the status of mesodermal cardiogenic cells. Expression of these genes is expected to be sustained as the epigenetic status of their promoter as well as their occupancy by RNA PolII and phosphorylated serine 5 PolII revealed an active chromatin (H3K4 methylation) and suggest an active transcription, respectively. However, the methylated H3K27 marks still on indicate a differential regulation of both activating (K4) and repressive marks and that the gain in K4 methylation is sufficient to turn on the promoter as shown by gene transcription. They further acquire in culture the expression of the proteins (ISL1, NKK2.5, MEF2C, and TBX5 but not SMA), which altogether indicates that they are cardiac progenitors. NKK2.5, ISL1, MEF2C, and TBX5 are intranuclear. TBX5 cellular localization depends upon the stage of cardiac development (49). Thus, its nuclear localization indicates that despite the early stage of SSEA-1+ cardiac progenitors when sorted, they quickly acquire maturity when in culture.

HCCI, together with cell cytometry analysis, further revealed that the majority of cells expressed these markers, 70%-90% expressing ISL1, MEF2C, and NKK2.5 proteins. Thus, these findings show that the BMP2-induced SSEA-1+ population shares some similarities with the recently reported KDR-low cell population (15). However, only 5%-7% of the BMP2-induced SSEA-1+ population is FLK1+ (or KDR+) at the level of the protein, as assessed by flow cytometry after 4 days of BMP2 treatment, thus suggesting that KDR+ cells represent a minor fraction of the sorted SSEA-1+ progenitors. As in the KDR-low population (15), c-KIT is also absent from SSEA-1+ progenitors as well as from the cardiac field in the embryo (50). However in contrast to KDR-low-sorted cells (15), our progenitors did not express SMA if not challenged by PDGF. In fact, our cardiac cell population was sorted out 2 days earlier than the KDR-low cells, which accounts for the finding that it expressed both OCT4+ and MESP1 proteins in addition to cardiac-specific markers like NKK2.5+ and TBX5+, as assessed by HCCI. SSEA-1+ MESP1/2+ cardiovascular progenitors also stand at a much more early developmental stage than the previously isolated ISL1+ cells (51). In contrast to the latter, they feature a great potential to become ventricular myocytes ex vivo and when engrafted in vivo. The in vivo differentiation of ISL1+ cells (51) remains to be shown. Furthermore, our progenitor population was generated using a short protocol (BMP2-inducing WNT3A), which tends to recapitulate the in vivo induction of the primitive streak and subsequently of cardiogenic mesoderm (17, 44). The most interesting feature of the BMP2-induced SSEA-1+ cells is that they retain the capability to differentiate toward the 3 cardiovascular lineages (i.e., cardiac, endothelial, and smooth muscle). When cultured on fibroblasts or a mix of cocultured human fibroblasts and cardiomyocytes, or in the presence of a conditioned medium of both fibroblasts and cardiomyocytes, they mostly developed a myofibrillogenesis typical of a cardiac cell and expressed CX43, MLC2v, and β-MHC. In contrast, when treated with PDGF or VEGF, they gave rise to smooth muscle and endothelial cells, respectively. Thus, these findings point to a multipotentiality of BMP2-induced SSEA-1+ cardiovascular progenitors. To further characterize the identity of these cells, we carried out single-clone real-time PCR on SSEA-1+ cells in culture on MEFs. Interestingly, while most of the clones expressed pancreatic markers (MESP1, NKK2.5, MEF2C, and ACTC1), 50% expressed ISL1 and more specifically TBX20, a marker of the secondary heart lineage, suggesting that some ISL1+ clones at the level of the protein (Figure 3, D and E) started to downregulate expression of this transcription factor, while some other kept it. In line with these findings, SSEA-1+ cells expressed some of the genes specific for the secondary heart lineage (TBX1, RALDH2, HES1, and FOXH1; Supplemental Figure 3A), and this was dramatically enhanced by FGF8. ISL1+ and TBX20+ clones also expressed NES and PAX3, 2 neural crest and/or stem cell markers, and featured the highest levels of TBX18, which marks both the epicardium (25) and left ventricle (26). These data are in agreement with the interplay between the cardiac neural crest and the secondary heart field (52, 53), a process dependent upon BMP receptors and mediated through neural crest derivatives in the epicardium (54). Alternatively, the NKK2.5+, MEF2C+, TBX5+, TBX20+, ISL1+, PAX3+, and NES+ population might represent the most multipotent and early precuror (i.e., cardiovascular stem cell) population among the SSEA-1+ cells. Interestingly, a similar population was recently described as a cardiac neural crest-derived dormant multipotent stem cell in adult heart (55). Thus, these findings suggest that SSEA-1+ early cardiovascular progenitors, each derived from a single cell (Figure 3, A–C), can segregate into multiple cardiac lineages (primary, secondary heart lineages and potentially cardiac neural crest and epicardium), when challenged by FGFs and other signals emerging from fibroblasts.

The last and very peculiar property of BMP2-induced SSEA-1+-sorted cells is their potential to engrat in a diseased primate myocardium. When replaced in vivo in a myocardial environment, they differentiate into mature ventricular cardiomyocytes expressing MLC2v and MLCK required for the assembly of the sarcomeres (56) as expected from their early expression of TBX5, while retaining a bi- or tri-potentiality to also potentially generate endothelial and smooth muscle cells, although this remains to be confirmed in a xenogenic graft model. They further express CX43 at their membrane and respond to electrical stimulation, suggesting a potential...
electrical coupling with the neighboring cells, which remains to be further investigated. Their engraftment turned out to be safe, as their status of definite cardiovascular progenitors prevented them from hyperproliferating, as confirmed by the absence of formation of teratoma in primate treated with sorted SSEA-1- cells or in immunodeficient Rag-/-, B6.Cg-Tg(γ chain-/-), C57/6, Mbc-/- humanized mice, while SSEA-1-scored pluripotent cells gave rise to microteratomas in primate myocardium and huge teratomas in mice.

Altogether, our findings demonstrate that pluripotent stem cells give rise to a single pool of early OCT4+ and SSEA-1+/brachury+/MESP+ cardiogenic mesodermal progenitors. Under specific conditions, the cells further differentiate into cardiac progenitors of the distinct primary and secondary heart lineages or potentially differentiate into cardiac neural crest cells, expressing NKKX2.5, MEF2C, and TBX5 with or without ISL1, TBX20, PAX3, and NES. Finally, challenged with signals derived from cardiac myofibroblasts and adult myocytes, they acquire a fate of differentiated cardiomyocytes. Challenged with other growth factors (PDGF, VEGF), they also acquire the fate of smooth muscle cells expressing smooth muscle actin and myosin or of early endothelial cells expressing CD31, CD34, and Flk1, respectively.

Thus, we have identified from pluripotent stem cells and characterized a very early cardiovascular progenitor, segregating later into the first and second heart lineages (57). Furthermore, we have provided the means to easily sort out this cell population to a great extent. How this population is generated in the cell plates before sorting is likely to be a 2-step process. Under BMP2 challenge and in the absence of both FGF (in the presence of SU5402) and activin (in the absence of serum), the OCT4 upregulated SSEA-1 in the absence of both FGF (in the presence of SU5402) and activin (in the absence of serum), the OCT4 upregulated SSEA-1-cells sorted after 4 days BMP2 treatment. Data were normalized to GAPDH and to the SSEA-1-population right after sorting. (F) Kinetic of gene expression in SSEA-1-cells expressed as fold changes versus SSEA-1-cells normalized to undifferentiated ORMES-2, following 4 days BMP2 treatment after sorting and following 7 days culture on MEFs. (G) H&E staining of the myocardial section from a postinfarction primate having received the medium (left image, sham) or the cells together (right image) with an image illustrating the low background of the green GFP fluorescence channel in the non-cell-grafted myocardium (middle panel). (H) Upper panels show, from left to right, GFP-expressing cells 2 months after engraftment of actin promoter EGFP and SSEA-1-ORMES cells, x40 original magnification of a myocardial section grafted with SSEA-1-ORMES.

Figure 5
SSEA-1-cells safely engrafted in infarcted primate myocardium. (A) BMP2-induced gene expression in ORMES cells. (B) GFP-expressing cells following engraftment of actin promoter EGFP ORMES cells in the scar area of the myocardium. (C) Teratoma formed after cell engraftment (the left and right images show low and high magnification, respectively). (D) Flow cytometry analysis of FITC-conjugated SSEA-1-ORMES cells. (E) Q-PCR of mesodermal, cardiac genes (or pluripotency genes, inset) expressed in SSEA-1-cells sorted after 4 days BMP2 treatment. Data were normalized to GAPDH and to the SSEA-1-population right after sorting. (F) Kinetic of gene expression in SSEA-1-cells expressed as fold changes versus SSEA-1-cells normalized to undifferentiated ORMES-2, following 4 days BMP2 treatment after sorting and following 7 days culture on MEFs. (G) H&E staining of the myocardial section from a postinfarction primate having received the medium (left image, sham) or the cells together (right image) with an image illustrating the low background of the green GFP fluorescence channel in the non-cell-grafted myocardium (middle panel). (H) Upper panels show, from left to right, GFP-expressing cells 2 months after engraftment of actin promoter EGFP and SSEA-1-ORMES cells, x40 original magnification of a myocardial section grafted with SSEA-1-ORMES.

Cardiac development is a dynamic process that is tightly orchestrated by the sequential expression of multiple transcription factors working in a combinatorial manner. As such, the different cardiac progenitor cell populations described heretofore are not “competitive” but likely reflect differences in the developmental stage which impacts on the nature and extent of the differentiat-
Thirty cells, were used to set low- and high-intensity thresholds set as a fixed threshold value to scan the wells of SSEA-1 (19). SSEA-1+ cells were plated by limiting dilution into 96-well plates (from 1,000 down to a single cell per well), stained 7 days later with DAPI, and scanned at a magnification of x10, using the Health Cell Profiling application from Cellomics. The size and fragmentation of objects were set to identify only clusters of cells (cell colonies). The number of colonies was then scored using the software VHCS (Cellomics). To ensure that wells contained a single cell, we also used the HUES-9 pOct-4-GFP cell line and monitored the presence of the cell by epifluorescence.

**Culture of human fibroblasts and human atrial myocytes.** Cells were isolated from the right atrial appendage, collected from 3-month-old patients during surgery required to relieve congenital diseases, and cultured as previously described (60). Approval of human tissue collection, care, and use was given by the ethics committee of Hospital Kremlin Bicêtre (AP-HP), Kremlin Bicêtre, France (Committee, protocol 04-26). Parents of patients provided informed consent prior to their participation in the present study.

**Generation of α-actin promoter GFP ORMES-2 cell line.** The 10^6 ORMES-2 cells, cultured as described above, were nucleofected with 3 μg of vector pActin-GFP, purified using a Pharmingen kit and linearized. Cells were then cultured on neomycin-resistant MEFs for 2 days and G418 (100 μg/ml) was added for the next 10 days. Colonies were cut with a needle and picked out of the dish to be further cultured and amplified. Clones were genotyped using GFP primers and further expanded.

**Monitoring of gene expression by real-time Q-PCR.** To monitor gene expression in separate single clones, single cell-derived colonies grown for 7 days on MEFs were picked up randomly using a needle and used for RNA extraction (Zymo research kit). RT-Q-PCR was performed using the LightCycler 1.5 as described previously (46) and analyzed according to Pfaffl et al. (59).

**ChIP.** The ChIP assay was performed according to the Fast ChIP protocol (61). Briefly, cells were crosslinked for 15 minutes with 1% formaldehyde, and the reaction was stopped for 5 minutes by 125 mM glycine. The cell pellet was then washed with cold PBS, and the cells were lysed with the ChIP buffer (61). One hundred μg chromatin and 3 μg antibody were used in each assay. The antibodies used were the anti-PolII (Santa Cruz Biotechnology Inc.), anti-phospho serine 5 PolII (Covance), the anti-H3trMeK4, and anti-H3trMeK27 (UBI or Abcam), and they were added overnight at 4°C. The Dynabead (protein A–conjugated or anti-IgM–conjugated phosphoserine 5 PolII; Invitrogen) was used to bind the immunocomplex H3-DNA or PolII-DNA. No antibody and nonrelevant rabbit IgG were used as negative controls.

**miRNA profiling.** Total RNA was extracted from cells using the mirVANA kit (Ambion). Five μg of RNA for each condition were subjected to dye/swap labelling and were hybridized on printed chips of 2,000 miRs, originating from 44 species; each spot was done in quadruplet. Raw data were analyzed to obtain a log2 intensity value for each spot. Log2 intensity was taken for each spot for both sorted SSEA-1+ cells and control conditions.
(sorted SSEA-1–cells or HUESCs) and a log2 ratio was obtained. Data were clustered using Cluster view as previously described (63). The heat map was generated using Tree View software. Data were validated by real-time PCR. Ten ng of total RNA extracted with mirVana kit were used in a RT reaction, using stem-loop microRNA specific primers (Applied Biosystems). Subsequently, the diluted RT product was used in a Q-PCR reaction with TaqMan miR-specific probes (Applied Biosystems). Normalization was done using small RNA RNU48 as a reference.

### Myocardial infarction model
Induction anesthetia of mice was performed with propofol (3 mg/kg) and sufentanil (0.5 mg/kg), and maintenance anesthetia was ensured with the same drugs, albeit, at a lower dose (1 mg/kg and 0.25 mg/kg, respectively). Intravenous atracurium besilate (1 mg/kg) was added whenever required, and all animals received heparin (50 U/kg) during the procedure. A myocardial infarction was created in Macaca Mulatta Rhesus monkeys (average weight, 8–10 kg) by a 90-minute balloon inflation in the left circumflex coronary artery, followed by reperfusion and angiographic confirmation of the patency of the revascularized infarct vessel. The occurrence of myocardial necrosis was consistently documented by a postprocedural rise in creatine phosphokinase values. Two weeks later, primates underwent a left thoracotomy and received unsorted or freshly sorted SSEA-1–ESC-derived progenitors or just cell medium over the scar and at its borders. The monkeys were treated with either FK-506, started 5 days before cell transplantation at the dose of 1 mg/kg/d, or cyclosporine, given intramuscularly for 3 days and then by oral gavage at doses adjusted to target serum drug concentrations in the range of 100 ng/ml. Treatments were continued until sacrifice. Primates were humanely euthanized at 2 and 3 months after transplantation. The area of infarction and cell grafting was identified by sutures that had been placed circumferentially at the time of cell injections. This area was cut into 2 halves, of which 1 was used for assessment for teratoma. The second half was divided into 4 blocks, which were snap frozen in isopentane cooled in liquid nitrogen and subsequently processed as cryosections for identification and characterization of GFP–grafted cells. In addition, a whole-body autopsy was performed in each animal and used for the search for extracardiac tumors. The experiments with primates were authorized by the Institutional Review Board on Ethics for primate experiments, IMASSA.

### Cell transplantation in immunosuppressed mice
The 10^5 BMP2-induced SSEA-1–cells were injected in the myocardium (n = 8), after opening the chest, or subcutaneously in the neck (n = 10) of immunodeficient Rag2−/−, γ chain−/−, C5−/−, MHC−/− mice humanized mice. Undifferentiated cells and SSEA-1–cells were also injected subcutaneously in the neck (n = 5 in each group). Four months later, the animals were euthanized and each organ was collected, sliced, and examined for the presence of tumor.

#### Statistics
Results are presented as mean ± SEM of 3–8 experiments in each experimental condition. Significance of data was evaluated by a 2-tailed Student’s t test. A P value of less than 0.05 was considered significant.
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