Induced pluripotent stem cell (iPSC)-derived Flk-1 progenitor cells engraft, differentiate, and improve heart function in a mouse model of acute myocardial infarction

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Aims
Induced pluripotent stem cell (iPSC)-derived cardiovascular progenitor cells represent a suitable autologous cell source for myocardial regeneration as they have the capability to form myocardial cells and to contribute to revascularization. As a first proof of concept we evaluated the potential of a murine iPSC-derived cardiovascular progenitor population, which expresses the surface marker foetal liver kinase-1 (Flk-1), to restore myocardial tissue and improve cardiac function after acute myocardial infarction (MI) in mice.

Methods and results
iPSC-derived Flk-1pos vs. Flk-1neg cells were selected by fluorescence activated cell sorting (FACS) and injected into the ischaemic myocardium of left anterior descending coronary artery (LAD)-ligated mice. Addressing safety aspects we used an octamer binding factor 4 (Oct4)-enhanced green fluorescent protein (eGFP) expressing iPSC clone from the transgenic Oct4-eGFP reporter mouse strain OG2 to enable FACS-based depletion of undifferentiated cells prior to transplantation. Infarcted animals were treated with placebo (phosphate-buffered saline, n = 13), Flk-1neg cells (n = 14), or Flk-1pos cells (n = 11; 5 × 10⁵ cells each). Heart function was evaluated by magnetic resonance imaging and conductance catheter analysis 2 weeks postoperatively. Cardiovascular in vitro and in vivo differentiations were investigated by immunofluorescence staining. Treatment with Flk-1pos and Flk-1neg cells resulted in a favourable myocardial remodelling and improved left ventricular function. Engraftment and functional benefits were superior after transplantation of Flk-1pos compared with Flk-1neg cells. Furthermore, Flk-1pos grafts contained considerably more vascular structures in relation to Flk-1neg grafts.

Conclusion
iPSC-derived Flk-1pos progenitor cells differentiate into cardiovascular lineages in vitro and in vivo and improve cardiac function after acute MI. This proof of concept study paves the way for an autologous iPSC-based therapy of MI.

Keywords
Cardiovascular progenitor cells • Induced pluripotent stem cells • Myocardial infarction • Myocardial regeneration

Introduction
Pluripotent stem cells have the ability to differentiate into cell types of all three germ layers, including cardiac and vascular derivatives. It is still a matter of debate whether more or less mature stem cell-derived progenies represent the most suitable cell source for cardiac repair. Premature embryonic stem cell (ESC)-derived progenitors with the potential to differentiate into both cardiac and...
vascular cell types have been identified as Flk-1pos, Isl1pos, Mesp1pos, or Nkx2.5pos populations.3–11

Recently, reprogramming of somatic cells into a pluripotent state was demonstrated.12–14 These induced pluripotent stem cells (iPSCs) are essentially identical to ESCs, but offer the possibility to generate autologous patient-specific pluripotent stem cells. It has been shown that murine and human iPSCs are able to form multipotent cardiovascular progenitors and mature cardiomyocytes similar to those derived from ESCs.15–20

In contrast to cardiomyocytes, cardiovascular progenitors might not only restore myocardial tissue, but would also contribute to revascularization. In addition, progenitors may be more robust to survive in the hostile graft environment.2 However, complete removal of contaminating pluripotent cells with teratogenic potential from cell suspensions containing cardiovascular progenitor cells is challenging.

For the first time we evaluated the intramyocardial transplantation of murine iPSC-derived foetal liver kinase-1-positive (Flk-1pos) cardiovascular progenitors. As Flk-1 is expressed on the cell surface, selection can be achieved without genetic manipulations. The iPSC-derived Flk-1pos and Flk-1neg cell fractions were both analysed for their potential to give rise to cardiomyocytes, smooth muscle, and endothelial cells in vitro and in vivo. Safety concerns regarding teratoma induction were addressed by taking advantage of the Oct4-promoter-dependent expression of eGFP to deplete contaminating undifferentiated cells prior to cell injection. The ability of iPSC-derived Flk-1pos progenitors to form vascularized myocardial tissue and to improve heart function was assessed in a mouse model of acute myocardial infarction (MI).

**Methods**

**Generation of a transgenic murine iPSC clone**

Embryonic fibroblasts from octamer binding factor 4 (Oct4)-enhanced green fluorescent protein (eGFP) expressing OG2 mice11 were reprogrammed by the overexpression of Oct4, Sox2, c-Myc, and Klf4.12 To trace transplanted cells, iPSC clones were further transduced by a lentiviral vector mediating the ubiquitous nuclear expression of the fluorescent protein Venus—a more stable variant of yellow fluorescent protein (YFP).22 Studies were performed on a selected subclone termed B4. Differentiation into Flk-1pos cells is described in detail in the online Supplementary material.

**Animal care**

Surgery and animal care were provided following the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, volume 25, no 28, revised 1996) and in accordance with institutional and federal regulations.

**Myocardial infarction model**

A total of 49 immunodeficient SCID beige mice (15–19 g, 8–10 weeks, Charles River, Germany) were used. Myocardial infarction (MI) was induced as described in the online Supplementary material. Aliquots of 15 μL cell suspension (5 × 10^5 Flk-1pos or Flk-1neg cells) in phosphate-buffered saline (PBS) or PBS alone were injected into the anterior left ventricular (LV) wall after left anterior descending coronary artery (LAD)-ligation. Animals were divided into a sham-operated group (sham, n = 7), a placebo-treated infarct group (PBS, n = 13), and infarct groups treated with iPSC-derived progenitor cells (Flk-1pos, n = 14; Flk-1neg, n = 11). Follow-up was 2 weeks. Four animals were used for teratoma experiments.

**Magnetic resonance imaging**

A 7 Tesla (300 MHz) scanning system (PharmaScan, Bruker, Ettlingen, Germany) with a 160 mm horizontal bore was used for magnetic resonance imaging (MRI) as detailed in the online Supplementary material. On postoperative day 2, infarct size was determined by contrast-enhanced MRI. Cardiac function was evaluated on Day 13.

**Conductance catheter analysis**

On Day 14, conductance catheter (CC) analysis was performed according to standard protocols (see online Supplementary material). Pressure–volume loops were recorded in steady state to acquire volumetric and functional parameters. Afterwards, animals were sacrificed for histological evaluation.

**Histology and immunostaining**

Hearts were processed in standard fashion and histological morphology, and immunostaining was performed as described in the online Supplementary material. iPSC-derived grafts were detected by their intrinsic nuclear Venus (nucVenus) expression. Graft size was measured using a pixel-based approach. Data were obtained by computer-assisted morphometry (Image J 1.40 g, NIH, USA).

**Statistics**

GraphPad Prism 5.03 was used for statistical analysis. If not stated otherwise, data are given as mean ± SEM. Continuous variables were analysed with either paired or unpaired Student’s t-test or with one-way ANOVA followed by Tukey’s multiple comparison test, as appropriate. Linear regression analysis was performed to correlate continuous data. Differences were considered significant at P < 0.05. All reported P-values are two-sided.

**Results**

The pluripotent iPSC clone shows cardiovascular differentiation potential in vitro

Pluripotency of the transgenic iPSC clone B4 was demonstrated by the expression of pluripotency markers and its ability to form teratomas (see Supplementary material online, Figures S1 and S2).

For cardiovascular differentiation in vitro, iPSCs were differentiated using an embryoid body (EB)-based protocol (see Supplementary material online). On Day 17 of differentiation, 77.7 ± 12.4% of iPSC-derived EBs (76 ± 17 EBs per experiment, n = 3) had spontaneously contracting areas (see Supplementary material online, Figure S2), indicating efficient cardiac differentiation. In addition, EB-derived differentiation cultures contained cells which were immunopositive for the cardiomyocyte markers cardiac troponin T (cTnT) and connexin 43 (Cx43). Cells expressing the endothelial marker Cd31 and the smooth muscle marker α-smooth muscle actin (α-SMA) were also present (see Supplementary material online, Figure S2).
FACS-sorting of iPSC-derived Flk-1 progenitor cells

The highest percentage of Flk-1-Allophycocyanin (APC)\textsuperscript{pos} cells was found on Day 5 (Figure 1A). This Day 5 population was used for the isolation of nucVenus\textsuperscript{pos}/Oct4-eGFP\textsuperscript{neg}/Flk-1-APC\textsuperscript{neg} and nucVenus\textsuperscript{pos}/Oct4-eGFP\textsuperscript{neg}/Flk-1-APC\textsuperscript{pos} populations (termed Flk-1\textsuperscript{neg} and Flk-1\textsuperscript{pos} cells) by fluorescence activated cell sorting (FACS) (Figure 1B–G). Whereas the original unsorted cell population contained 24.3 ± 11.8% (n = 13) Flk-1\textsuperscript{pos} cells, these cells were successfully enriched in the Flk-1\textsuperscript{neg} fraction (91.1 ± 4.6%, n = 12) and depleted in the Flk-1\textsuperscript{pos} population (2.7 ± 2.0%, n = 24; Figure 1H). Additional depletion of Oct4-eGFP\textsuperscript{pos} cells markedly reduced contaminating pluripotent Oct4-eGFP\textsuperscript{pos} cells from 15.5 ± 13.0% (unsorted, n = 10) to 3.8 ± 3.3% in the Flk-1\textsuperscript{neg} population (n = 23) and to 0.6 ± 0.6% in the Flk-1\textsuperscript{pos} population, (n = 12; Figure 1I). These results correlated with the percentage of colonies formed by Oct4 immunopositive cells following cultivation of the selected populations in pluripotency-supporting medium (see Supplementary material online, Figure S3).

Figure S4 (see Supplementary material online) shows representative pictures of re-aggregates 2 days after re-aggregation of Flk-1\textsuperscript{neg}/Flk-1\textsuperscript{pos} cells in vitro, demonstrating the distinct proliferation potential of Flk-1\textsuperscript{neg} and Flk-1\textsuperscript{pos} populations resulting in larger re-aggregates formed by Flk-1\textsuperscript{pos} compared with Flk-1\textsuperscript{neg} cells.
Flk-1 progenitor cells form cardiovascular cell types in vitro

Quantitative real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) of Day 5 sorted Flk-1<sup>−</sup> and Flk-1<sup>+</sup> cells (Figure 2A) confirmed the successful enrichment of Flk-1 expression in the Flk-1<sup>+</sup> fraction. Oct4 mRNA expression was reduced in the Flk-1<sup>−</sup> fraction vs. unsorted cells and even more reduced in the Flk-1<sup>+</sup> population. Flk-1<sup>+</sup> cells showed an accumulation of cardiovascular markers (Mesp1, Nlk2,5, cTnT and Cd31) in comparison with unsorted and Flk-1<sup>−</sup> cells. α-SMA transcripts were significantly depleted in the Flk-1<sup>+</sup> population in contrast to unsorted and Flk-1<sup>−</sup> cells.

Re-aggregates of Flk-1<sup>+</sup> cells were cultivated up to Day 21 of differentiation and developed spontaneous beating areas (see Supplementary material online, Movie S1). Immunostaining revealed the presence of cTnT, α-SMA and Cd31 expressing cells (Figure 2B–D).

Intramyocardial transplantation of Flk-1 progenitors prevents adverse remodelling and improves cardiac function

Compared with infarct sizes determined by MRI on Day 2 post MI, Masson’s Trichrome staining on Day 14 showed enlarged infarcts in the PBS group as a sign for adverse remodelling. In contrast, infarct size decreased in cell-treated animals (Figure 3B). On an average, Flk-1<sup>+</sup> cell grafts were considerably larger than Flk-1<sup>−</sup> cell grafts and led to improved LV wall thickening (Figures 3C and 4). Transplantation of both Flk-1<sup>−</sup> and Flk-1<sup>+</sup>
cells resulted in an improved area of viable myocardium within the infarct zone (Figure 3D). However, beneficial effects on myocardial remodelling were more pronounced in Flk-1 pos cell-treated animals as opposed to the Flk-1 neg group (Figures 3C and 4).

LAD-ligation resulted in a significant reduction in LV function and in a volume overload in ischaemic controls (PBS) compared with sham-operated animals (see Supplementary material online, Table S2 and Figure S6). On Day 13, MRI revealed an improved LV ejection fraction (LV-EF) in Flk-1 pos cell-treated animals in comparison with the PBS group (Figure 5A). Conductance catheter measurements correlated well with MRI findings (Figure 5C), although values were typically underestimated by CC assessment. Based on the CC data, transplantation of both Flk-1 neg and Flk-1 pos cells resulted in an improved LV function and decreased LV volume load (Figure 5). Relative increase in LV-EF compared with placebo-treated (PBS) controls was 43.5% for animals injected with Flk-1 neg cells and 67.9% for animals with Flk-1 pos cell treatment. In summary, cardiac function was superior in animals injected with Flk-1 pos cells (Figure 5, see Supplementary material online, Table S2 and Figures S6 and S7).

Flk-1 progenitor cells form cardiovascular cell types in vivo

The YFP variant Venus tagged with a nuclear location signal enabled clear-cut identification of donor cells after transplantation into the highly autofluorescent host myocardium (Figure 6).

Immunostaining revealed the presence of cTnT immunopositive cardiomyocytes in grafts of Flk-1 neg controls. Graft size (in mm³) as determined from Masson’s Trichrome staining and fluorescence microscopy (Flk-1 neg = 0.13 ± 0.05; Flk-1 pos = 0.74 ± 0.20; *P < 0.05).

Figure 3 Intramyocardial transplantation of Flk-1 pos cells prevents adverse myocardial remodelling and improves left ventricular (LV) wall thickness after myocardial infarction. (A) Representative morphological appearance of Masson’s Trichrome stained myocardial sections 2 weeks after operation; magnification depicts Flk-1 pos cell graft. (B) Infarct size (%): after 2 days (magnetic resonance imaging, MRI): PBS = 32 ± 2; Flk-1 neg = 36 ± 3; Flk-1 pos = 35 ± 3; after 14 days (Masson’s Trichrome staining, Masson’s): PBS = 43 ± 2; Flk-1 neg = 21 ± 11; Flk-1 pos = 19 ± 7. (C) LV wall thickness after 14 days (µm): PBS = 322 ± 15; Flk-1 neg = 449 ± 38; Flk-1 pos = 773 ± 75. (D) Viable myocardium (% of infarct area): PBS = 15 ± 1; Flk-1 neg = 26 ± 2; Flk-1 pos = 46 ± 4. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4 Flk-1 pos cell-derivatives form larger grafts compared with Flk-1 neg controls. Graft size (in mm³) as determined from Masson’s Trichrome staining and fluorescence microscopy (Flk-1 neg = 0.13 ± 0.05; Flk-1 pos = 0.74 ± 0.20; *P < 0.05).
Flk-1 progenitor cells from murine iPSCs

Although, FACS-sorting of Flk-1neg/pos cells was combined with sorting against Oct4-eGFP, a minor proportion of pluripotent Oct4-eGFPpos cells remained, especially in the Flk-1neg cell fraction (Figure 1). Most likely, those contaminating cells gave rise to clusters of Oct4-eGFPpos cells within donor grafts, which were predominantly detected in Flk-1neg cell grafts (Figure 6, white arrows). However, especially Flk-1pos progenitor cells formed remarkable grafts 14 days after cell transplantation in vivo (Figures 4 and 6) and larger re-aggregates in vitro (see Supplementary material online, Figure S4), suggesting a considerably high proliferation potential that was not related to contaminating pluripotent cells. The proliferative potential of Flk-1pos progenitor cells in combination with their cardiovascular differentiation capacity may be particularly useful for in vivo formation of large vascularized myocardial tissue, even though the question arises, whether such proliferation could be controlled in vivo.

Both Flk-1pos and Flk-1neg cells differentiated into a cardiomyocyte phenotype, as shown by the expression of cardiac differentiation markers in vitro and in vivo and the development of contracting areas after cultivation of re-aggregates (Figures 2A and B, 6A–F, see Supplementary material online, Movie S1). However, we were not able to identify a structural organization and alignment as well as a typical cross-striation pattern of donor cardiomyocytes within iPSC-derived grafts. In our opinion, these cells represent an early and immature cardiomyocyte phenotype. However, despite the lack of well-aligned mature donor cardiomyocytes, we observed a beneficial remodelling and improved ventricular function.

Discussion

The generation of iPSCs is considered as a breakthrough in the development of novel cellular therapies. A series of studies demonstrated that iPSCs form functional cardiovascular cell lineages with similar efficiency as ESCs. Various ESC- and iPSC-derived cardiovascular progenitor populations characterized by the expression of different molecular markers have been described. Flk-1, as a surface molecule, allows for selection of those progenitor cells without genetic manipulation and the general cardiovascular potential of iPSC-derived Flk-1pos cells in vitro has been reported recently.

In the present study, we evaluated the capacity of murine iPSC-derived Flk-1pos cells to form vascularized heart tissue following acute MI in mice and to restore heart function.

online, Figure S8). Those Flk-1 and Cd31 immunopositive cells formed vessels that were connected to the host's vasculature as indicated by the presence of erythrocytes within the donor-derived vascular structures (Figure 6I and O, red arrows). Small numbers of α-SMA immunopositive cells in Flk-1pos and Flk-1neg grafts could also be detected (data not shown).

In correlation with the in vitro data (see Supplementary material online, Figure S3), restricted areas of Oct4-eGFPpos undifferentiated iPSCs were present especially in grafts of transplanted Flk-1neg cells (Figure 6, white arrows).

Figure 5 Intramyocardial transplantation of Flk-1pos cells improves cardiac function and reduces ventricular dilatation after myocardial infarction. Haemodynamic evaluation by magnetic resonance imaging (MRI, Day 13; A + C) and conductance catheter analysis (CC; Day 14; B–F). (A) Left ventricular ejection fraction (LV-EF, %) as measured by magnetic resonance imaging on Day 13: PBS = 23 ± 1; Flk-1neg = 28 ± 2; Flk-1pos = 32 ± 3. (B) Left ventricular ejection fraction (%) as measured with conductance catheter on Day 14: PBS = 16 ± 1; Flk-1neg = 23 ± 2; Flk-1pos = 27 ± 2. (C) Volumetric parameters and left ventricular ejection fraction are underestimated by conductance catheter analysis. Values derived from both methods correlate with each other (R² = 0.87; P < 0.001). (D) End-diastolic volume (EDV [µL]): PBS = 27 ± 3; Flk-1neg = 23 ± 1; Flk-1pos = 19 ± 2. (E) End-systolic volume (ESV [µL]): PBS = 24 ± 2; Flk-1neg = 21 ± 1; Flk-1pos = 15 ± 1. (F) Preload adjusted maximal power (mW/µL²): PBS = 28 ± 3; Flk-1neg = 73 ± 6; Flk-1pos = 87 ± 19. *P < 0.05, **P < 0.01.
Figure 6  Flk-1<sup>pos</sup> and Flk-1<sup>neg</sup> cells form intramyocardial grafts 14 days after cell injection. Flk-1<sup>pos</sup> grafts show more vascular structures. (A–F) Representative cryosections with high (A/B + D/E) and moderate numbers (C + F) of cTnT immunopositive cells (red). (B) Magnification from (A). (E) Magnification from (D). (G–L) Flk-1 immunopositive cells (red). (H) Magnification from (G), corresponding phase contrast (I). (K) Magnification from (J), corresponding phase contrast (L). (M–R) Cd31 immunopositive cells (red). (N) Magnification from (M), corresponding phase contrast (O). (Q) Magnification from (P), corresponding phase contrast (R). Oct4-eGFP<sup>pos</sup> cells (white arrows). Donor-derived vessels with erythrocytes (red arrows). Scale bars: 100 μm.
compared with placebo treatment. This finding is most likely the result of a multimodal effect based on geometric LV wall stabilization and paracrine mechanisms rather than attributed to a contractile ‘de novo myocardium’. Positive effects were more pronounced after treatment with Flk-1<sup>pos</sup> cells, which can be explained by the formation of larger grafts and enhanced neovascularization with donor-derived vessels that appeared to be connected to the recipient’s circulation (Figures 4 and 6l and O, see Supplementary material online, Figure S8).

As a limitation of this study, we did not extend our observation period to >2 weeks. Additional long-term studies have to be done to investigate whether the proliferative potential of Flk-1<sup>pos</sup> cardiovascular progenitors is advantageous to achieve larger graft sizes or rather induces tumour-like growth, which hinders structured organization of mature myocardium. The expansive growth of Flk-1<sup>pos</sup> cells within 2 weeks following transplantation might argue against a direct intramyocardial injection. We believe that these cells are most suitable for tissue engineering approaches that will allow for directed differentiation as well as control of proliferation and maturation in vitro before transplantation.

In conclusion, the FACs-sorting of iPSCs using the cell surface marker Flk-1 is a feasible technique to provide sufficient amounts of cardiovascular progenitor cells. We identified iPSC-derived Flk-1<sup>pos</sup> cardiovascular progenitors as useful candidates for cardiac cell therapy. Flk-1<sup>pos</sup> cell-derivatives were superior to Flk-1<sup>neg</sup> cells with respect to engraftment, infarct size reduction, and functional outcome.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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**References**


