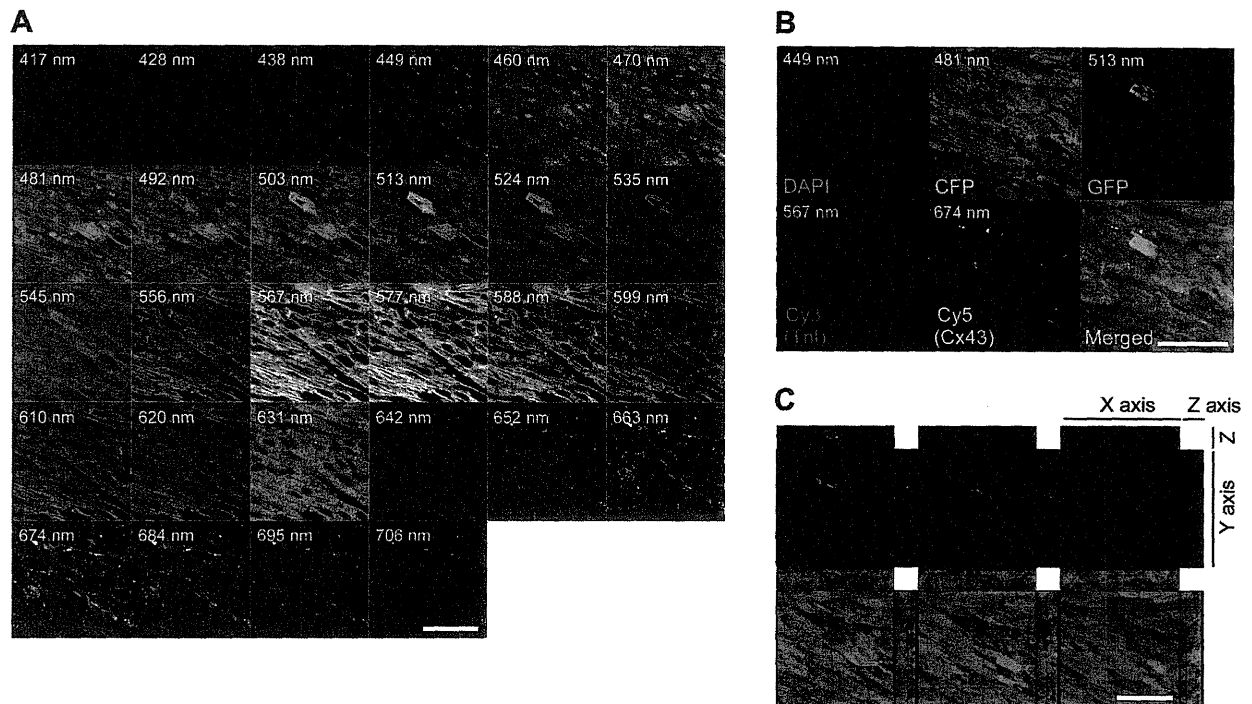

Figure 3. CFP expression in the GFP^{+} BM-derived cardiomyocyte of a CFP-transgenic recipient mouse. (A) Representative image of fluorescence detection from cardiac tissue of the CFP-transgenic recipient transplanted with GFP^{+} BM cells. Detected fluorescence images between 417 and 706 nm wavelength at 10–11 nm interval from a cardiac section stained with anti-TnI (Cy3), anti-Cx43 (Cy5), and DAPI are shown sequentially. (B) The expressions of DAPI, CFP, GFP, Cy3, and Cy5 in the cardiomyocyte shown in (A). Composition of GFP and CFP was examined simultaneously from each detected image between 449 and 663 nm wavelength (linear unmixing analysis). Detection wavebands used to visualize each fluorescence are described in each fluorescence image. The cardiomyocyte shown in (A) expressed donor-derived GFP, recipient-derived CFP, cardiac TnI, and Cx43. (C) Three dimensional analysis of the cardiomyocyte shown in (A) and (B) at the position of each nucleus. In every cross-section of three planes including nuclei, this cardiomyocyte expressed both GFP and CFP. Merged images (B and C) were obtained from the same confocal plane. Scale bars = 20 μm.
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heart and the negative reaction to mouse heart and human cord blood. By contrast, ryanodine receptor 2 was not detected in any cardiac specimens derived from human HSC-engrafted recipients. Although connexin 43 was expressed in all of the recipients, the expression of connexin 43 was not specific to human cardiomyocytes since it was expressed in human cord blood consistent with previous reports showing connexin expression on several hematopoietic cells [28] (see Table S3). Although the frequency of cell fusion between donor hematopoietic cells and host cardiomyocytes is extremely low, we detected the cardiomyocyte-specific expression of the human cardiomyocyte structural gene, cardiac troponin C, but not that of other human cardiomyocyte structural genes such as ryanodine receptor 2 or human cardiac transcription factors in the cardiac tissue of recipient mice.

Discussion

We have obtained three findings by tracing the fate of genetically marked BM cells *in vivo*. First, HSCs generate cardiomyocytes along with hematopoietic reconstitution. Second, myeloid lineage cells are primary intermediates for cardiomyocyte generation. Third, cell fusion with recipient-derived cardiomyocytes is required for the generation of BM-derived cardiomyocytes. In this study, transplantation of cells derived from GFP-transgenic mouse into CFP-transgenic recipients enabled us to determine the mechanism for the generation of donor marker-positive cardiomyocytes. Moreover, confocal imaging and analyses for the separation of these two fluorescences led us to address the long-term question as to the issue of cell fusion or transdifferentiation.

Transplantation of purified HSCs to newborn syngeneic mice with cardiac injury resulted in the most frequent appearance of donor-marker^{+} cardiomyocytes in cardiac tissues. The results suggest the two possibilities, direct contribution of HSCs to cell fusion with cardiomyocytes and cell fusion between myeloid progeny and cardiomyocytes. Previous reports suggest that purified HSCs do not generate cardiomyocytes when directly transplanted into myocardium [10,20,21], but can generate cardiomyocytes following BM transplantation at a low frequency [4,10,12]. We have shown that cell fusion between BM-derived myeloid cells and host-derived cardiomyocytes results in the generation of donor-marker^{+} cardiomyocytes. The frequency of BM-derived cardiomyocytes in our study (~0.04%, see Materials and Methods S1) and other rodent studies (0.001–0.02%) [4,10,20] are similar to those of host marker-expressing cardiomyocytes in human cardiac transplantation (0.04–0.16%) [29,30], which may be the frequency of fusion between circulating myeloid cells from BM and cardiomyocytes in mammals in general.

Cells of myeloid lineage have been shown to contribute to skeletal myocytes [31,32], hepatocytes [33,34], or vascular endothelium [35] *in vivo*. We report here contribution of BM-derived myeloid progenitors to the generation of cardiomyocytes. Actually, several plasma membrane molecules related with cell

fusion were up-regulated in myeloid lineage cells than lymphoid lineage cells confirmed by qRT-PCR (see Figure S3). Expression levels of integrin beta 1 [Itgb1] or signal-regulatory protein alpha [Sirpa] in CMP were 1.73 or 1.82 fold higher than those in CLP, respectively (n = 3). Of the cells derived from GMPs, monocytic cells are likely candidates as fusion partners since they have a physiological capacity for cell fusion [36]. Based on the finding that $CD11b^{low}$ cells, rather than $CD11b^{high}$ cells, within $Gr\text{-}1^{-/low}c\text{-}Kit^{-}$ BM fraction generated cardiomyocytes (unpublished observations), $CD11b^{low}$ immature monocytes may be the population contributing to the appearance of $donor\text{-}marker^{+}$ cardiomyocytes similar to the previous report describing the regeneration of skeletal muscle [32].

The number of donor-derived cells in recipient BM was very low following the transplantation of $Lin^{-/low}CD45^{-}$ cells in our study (Table 1), consistent with previous studies [37,38]. Mesenchymal stem cells in the BM are considered to be relatively resistant to irradiation compared with HSCs [37,38]. Therefore, the lack of donor-derived cardiomyocytes following systemic infusion of $Lin^{-/low}CD45^{-}$ cells into sublethally irradiated mice may be due to inefficient engraftment of injected MSCs in the recipient BM. Intra-BM injection of 10^5 GFP-labeled clonally purified MSCs (CMG cells) resulted in the generation of donor-$marker^{+}$ cardiomyocytes [11]. Our results, together with the report showing little ability of MSCs to give rise to cardiomyocytes following intravenous or intramyocardial transplantation [8], imply that administration route and cell preparation need to be extensively assessed for cell therapy using MSCs. We examined whether BM-derived mesenchymal cells gain a cardiomyocyte phenotype through differentiation or cell fusion with a coculture experiment using GFP-expressing BM mesenchymal cells and adult cardiomyocytes. In the experiment, one GFP^{+} cell expressing cardiomyocyte-specific antigens was detected (unpublished observations). However, the function of the cardiac-maker positive cell as a cardiomyocyte is unknown. A previous study demonstrated that mesenchymal stromal cell-derived cells acquiring cardiomyocyte phenotypic characteristics do not exhibit sufficient electrical property [39]. Additionally, improvement of cardiac function via mesenchymal stem/stromal cell transplantation is thought to be mediated by paracrine mechanism in addition to the direct contribution of cardiac marker-positive mesenchymal stem/stromal cells in themselves as contractile cardiomyocytes [40,41]. Recent report suggests that mesenchymal stromal cells recruit monocytes/macrophages secreting IL-10 or TGF-β [41]. Macrophages contributing to anti-inflammatory status could be one of the determinants for tissue regeneration in a mouse model for myocardial infarction [41]. Therefore, whether HSCs and MSCs act synergistically to regenerate cardiac tissue or to inhibit apoptosis in damaged cardiac tissue should be evaluated by using a large animal model in the future.

To establish regenerative medicine for heart failure patients, long-term improvement in cardiac function is important. Self-renewing cardiac-resident stem/progenitor cells [42,43] could maintain a pool for cardiomyocyte turnover [44,45]. BM could be one of the reservoirs that can give rise to cardiac-resident stem cells [46] and cells of multiple lineages [47]. Indeed, cardiomyocyte renewal in adult mammals is demonstrated under the physiological condition [48] or after injury [1]. Therefore, it should be demonstrated in the future to what degree BM-derived stem/progenitor cells and cardiac stem/progenitor cells contribute to turnover and regeneration of cardiomyocytes in the physiological or pathological conditions and whether BM-derived stem/progenitor cells can generate cardiac stem/progenitor cells.

Finally, we have demonstrated the contribution of myeloid lineage cells to cardiomyocytes through cell fusion. Recent reports suggest that stem cell fusion with the cardiomyocyte could add contractile function to the injured heart [49], and BM cell transplantation can restore impaired cardiac function [7]. Identification of the specific cell type and factors involved in cell fusion may lead to the enhancement of therapeutic effects of ongoing clinical cell therapy. In our qPCR analysis, two out of 6 donor cardiomyocyte-specific genes were detected in recipient cardiac tissues. Considering the low frequency of cell fusion, future *in vitro* and *in vivo* experiments might be required to determine as to how far reprogramming process occurs following cell fusion. Altogether, our findings suggest that myeloid progenitors may potentially serve as a readily available source of effecter cells for targeted cellular therapy of cardiac disorders through cell fusion.

Materials and Methods

Mice

C57BL/6 mice transgenically expressing enhanced GFP driven by the cytomegalovirus (CMV) enhancer/chicken β-actin promoter were kindly provided by Dr. M. Okabe (Osaka University). C57BL/6 mice transgenically expressing enhanced CFP driven by the CMV enhancer/chicken β-actin promoter, NOD/SCID/$IL2r\gamma^{null}$ mice, and $NOD.Rag1^{null}IL2r\gamma^{null}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). $C57BL/6.Rag2^{null}IL2r\gamma^{null}$NOD-Sirpa mice were generated in our laboratory. All mice were bred and maintained under defined flora. Experiments were performed according to the guidelines approved by the Institutional Animal Committee of Kyushu University (Approval ID: 18–68). PB sampling from recipient mice was performed under deep inhalation anesthesia. BM and heart were dissected out from donor/recipient mice sacrificed by cervical dislocation under deep inhalation anesthesia.

Purification of Donor Cells

BM cells were harvested from GFP-transgenic mice at 8–12 weeks of age by flushing femurs and tibiae. MNCs were isolated by gradient centrifugation. Rat anti-mouse lineage antigen (BD Pharmingen, Caltag) and sheep anti-rat IgG antibody-conjugated immunomagnetic beads (Dynal) were used to deplete mature hematopoietic cells from MNCs. For separation of $Lin^{-/low}CD45^{+}$ cells and $Lin^{-/low}CD45^{-}$ cells and enrichment of $Lin^{-/low}Sca\text{-}1^{+}$ cells or $Lin^{-/low}c\text{-}Kit^{+}$ cells by magnetic cell sorting, immunomagnetic microbeads (Miltenyi Biotec) were used. For purification of each BM stem/progenitor fraction by FACS, enriched BM cells were labeled by fluorescence-conjugated antibodies (see Materials and Methods S1). Umbilical cord blood cells were collected during normal full-term delivers after obtained written informed consent (provided by Kyushu Block Red Cross Blood Center, Japan Red Cross Society). Experiments using cord blood were performed according to the guidelines approved by the Institutional Committee of Kyushu University (Approval ID: 17–114). MNCs were separated by Ficoll-Hypaque density-gradient centrifugation. Lineage-depleted cord blood cells were obtained magnetically (Miltenyi Biotec). Sorting of $CD34^{+}CD38^{-}$ subfractions was accomplished by staining lineage-depleted cord blood cells with fluorescein isothiocyanate (FITC)-conjugated anti-CD34, and phycoerythrin (PE)-conjugated anti-CD38 (BD Biosciences). Cell sorting was performed on a FACSAria (BD).

Transplantation

Unfractionated or purified BM cells from GFP mice were injected intravenously into C57BL/6 mice or CFP-transgenic mice

within 48 hours of birth after 560 cGy irradiation. Left ventricular wall injury was induced by puncturing newborn recipient heart with a 29 G needle following transplantation of donor BM cells. Five to ten thousand $Lin^{-}CD34^{+}CD38^{-}$ cells from human cord blood were transplanted into immune-compromised mice after irradiation.

Flow Cytometric Analysis of Recipient PB and BM

PB was collected from retro-orbital plexus of recipients, and BM cells were harvested from femurs and tibiae. The percentage of GFP^{+} cells in PB and BM of recipients was examined by flow cytometric analysis using FACSCalibur (BD). PB and BM of recipients were labeled with each biotin-conjugated anti-mouse lineage antigen (CD3e, CD11b, B220, Gr-1, and TER-119) antibody, subsequently labeled with Cy3-conjugated streptavidin (Jackson ImmunoResearch) to recognize myeloid cells (CD11b, Gr-1), T lymphocytes (CD3e), and B lymphocytes (B220) by flow cytometric analysis. FACS lysing solution (BD) was used to lyse red blood cells following antibody labeling.

Histological Analysis

Recipient hearts were removed and perfused with PBS to eliminate blood cells immediately after they were sacrificed by cervical dislocation. For immunofluorescence staining, recipient hearts were sectioned into 100-μm slices from apex to base with a vibratome (DTK-1000, D.S.K.) following fixation in 4% paraformaldehyde and dehydration in 70% ethanol. Vibratome-sectioned cardiac slices were stained with primary antibodies to CD45 (DakoCytomation), CD11b (BD Pharmingen), vimentin (Sigma), cardiac troponin I (TnI; Santa Cruz Biotechnology), and connexin43 (Cx43; Chemicon). Secondary antibodies conjugated with Cy3 or Cy5 (Jackson ImmunoResearch) were used to visualize primary antibodies. Forty contiguous cardiac sections per a recipient mouse were carefully examined for the presence of GFP^{+} cardiomyocytes by laser-scanning confocal microscopy (LSM-GB200, Olympus). In the transplantation of GFP^{+} BM cells into CFP-transgenic mice, linear unmixing analysis was employed to distinguish GFP^{+} donor-derived cells, $GFP^{+}CFP^{+}$ fused cells, and CFP^{+} recipient-derived cells as described previously [12] (LSM510 META, Carl Zeiss, see Materials and Methods S1).

Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted from recipient heart, non-recipient mouse heart, human cord blood, and 10^4 cells each of sorted mouse hematopoietic cells. Reverse transcription was performed using SuperScript II or SuperScript III (Invitrogen) according to the manufacturer's instructions. For human-specific cardiac transcription factors and cardiomyocyte structural genes, qRT-PCR was performed on the synthesized complementary deoxyribonucleic acid (cDNA) with Platinum Quantitative PCR SuperMix (Invitrogen) on LightCycler 480 (Roche) to determine cycle thresholds (C_T) of amplification. Human heart QUICK-Clone cDNA (Clontech) was used as a positive control. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. For mouse adhesion molecules, qRT-PCR was performed on Applied Biosystems 7500 real-time PCR system with TaqMan$^{(R)}$ primer-probe sets provided from Applied Biosystems. 18 s rRNA was used as the internal control. Sequences of dual-labeled fluorogenic probes and gene-specific primers (Sigma-Aldrich), and product ID of TaqMan$^{(R)}$ Gene Expression Assay (Applied Biosystems) are listed on Table S4. For initial normalization to the housekeeping gene, the difference in C_T value (ΔC_T = [C_T gene of interest] − [C_T human GAPDH or 18 s rRNA]) was determined on each sample. Relative expression levels of each mRNA were calculated as the ratio to levels of human heart or mouse CLP cDNA using the comparative C_T ($2^{-\Delta\Delta Ct}$) method [50].

Statistical Analysis

Results are presented as mean +/− standard deviation (S.D.). Probability values were calculated by using the Mann-Whitney U test (non-parametric independent two-group comparison) or the Pearson's correlation coefficient test. A probability value of less than 0.05 was considered to be statistically significant.

Supporting Information

Figure S1 Differentiation capacities of $Lin^{-/low}CD45^{-}$ cells. (A) CD45 expression of $Lin^{-/low}$MNCs, separated $Lin^{-/low}CD45^{+}$ cells, and separated $Lin^{-/low}CD45^{-}$ cells. (B) Bright-field and fluorescence image of $GFP^{+}Lin^{-/low}CD45^{-}$ cells following culture. Polygonal or spindle-shaped adherent GFP^{+} cells were recognized as mesenchymal cells. (C) *In vitro* differentiation of $Lin^{-/low}CD45^{-}$ cells into osteoblasts (left) or adipocytes (right) by induction using differentiation media for each. Differentiation into osteoblasts or adipocytes was confirmed by alkaline phosphatase staining (left; red) or Oil red O staining (right; lipid vacuoles are stained in red), respectively. (D) Bone section of recipients transplanted with $GFP^{+}Lin^{-/low}CD45^{-}$ cells stained with anti-GFP (red, Cy3), anti-CD45 (yellow, Cy5), and DAPI (blue). Bright-field image is shown at leftmost. $GFP^{+}CD45^{-}$ cells were present within bone cortex (white arrows). These results suggest that separated $Lin^{-/low}CD45^{-}$ cells contain MSCs that can differentiate into multiple mesenchymal lineages. Merged images were obtained from the same confocal plane. Scale bars = 20 μm.
(TIF)

Figure S2 Flow cytometric analysis of recipient hematopoietic tissues. Representative image of flow cytometric analysis of recipient PB and BM transplanted with FACS-purified LSKs, and recipient PB transplanted with total myeloid progenitors (My-P) or CLPs. In LSK recipients, donor-derived GFP^{+} myeloid cell lineage (Gr-1^{+} or $CD11b^{+}$), B cell lineage ($B220^{+}$), and T cell lineage ($CD3e^{+}$) were confirmed. Donor-derived myeloid cell lineage (Gr-1^{+} or $CD11b^{+}$) was predominantly present in recipients transplanted with total myeloid progenitors, and donor-derived B/T cell lineage ($B220^{+}$ or $CD3e^{+}$) was predominantly present in recipients transplanted with CLPs.
(TIF)

Figure S3 Relative cDNA expression of adhesion molecules in myeloid and lymphoid lineages. The averages of relative cDNA expressions from three qRT-PCR experiments are indicated by histogram with positive standard deviation. Expression levels of Itgb1 and Sirpa in CMP were higher than those in CLP. Expression levels of Itgb1, Sirpa, and Adam9 in myeloid derivatives (neutrophil, monocyte) were higher than those in lymphoid derivatives (T lymphocyte, B lymphocyte). In the four adhesion molecules examined (Itgb1, Sirpa, Adam9, and Adam12), Adam12 was not detected in any of the samples. Gene names corresponding to each gene symbol, probes and primers information are described in Table S4.
(TIF)

Table S1 The number of donor-derived cardiomyocytes after serial transplantation.
(XLS)

Table S2 CFP expression of donor-derived GFP^{+} cardiomyocytes in CFP recipient mice.
(XLS)

Table S3 Relative cDNA expression to human heart.
(XLS)

Table S4 Probes and primers for quantitative real-time polymerase chain reaction (qRT-PCR).
(XLS)

Materials and Methods S1 Detailed information on purification of donor BM cells, cell culture and differentiation assay of $Lin^{-/low}CD45^{-}$ cells, and histological analysis.
(DOC)

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Author Contributions

Discussion: MH KA. Conceived and designed the experiments: MF FI MH KA. Performed the experiments: MF FI YN TY KM HS TK K. Nakamura. Analyzed the data: MF FI YN YS KT KS KO K. Nagafuji. Contributed reagents/materials/analysis tools: TK K. Nakamura. Wrote the paper: MF FI YN YS.

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