Sox2 Transduction Enhances Cardiovascular Repair Capacity of Blood-Derived Mesoangioblasts


Rationale: Complementation of pluripotency genes may improve adult stem cell functions.

Objectives: Here we show that clonally expandable, telomerase expressing progenitor cells can be isolated from peripheral blood of children. The surface marker profile of the clonally expanded cells is distinct from hematopoietic or mesenchymal stromal cells, and resembles that of embryonic multipotent mesoangioblasts. Cell numbers and proliferative capacity correlated with donor age. Isolated circulating mesoangioblasts (cMABs) express the pluripotency markers Klf4, c-Myc, as well as low levels of Oct3/4, but lack Sox2. Therefore, we tested whether overexpression of Sox2 enhances pluripotency and facilitates differentiation of cMABs in cardiovascular lineages.

Methods and Results: Lentiviral transduction of Sox2 (Sox-MABs) enhanced the capacity of cMABs to differentiate into endothelial and cardiomyocytes in vitro. Furthermore, the number of smooth muscle actin positive cells was higher in Sox-MABs. In addition, pluripotency of Sox-MABs was shown by demonstrating the generation of endodermal and ectodermal progenies. To test whether Sox-MABs may exhibit improved therapeutic potential, we injected Sox-MABs into nude mice after acute myocardial infarction. Four weeks after cell therapy with Sox-MABs, cardiac function was significantly improved compared to mice treated with control cMABs. Furthermore, cell therapy with Sox-MABs resulted in increased number of differentiated cardiomyocytes, endothelial cells, and smooth muscle cells in vivo.

Conclusions: The complementation of Sox2 in Oct3/4-, Klf4-, and c-Myc-expressing cMABs enhanced the differentiation into all 3 cardiovascular lineages and improved the functional recovery after acute myocardial infarction. (Circ Res. 2010;106:1290-1302.)

Key Words: circulating progenitors ■ reprogramming ■ differentiation ■ Sox2

stem cell therapy is a potential therapeutic option for treating ischemic cardiovascular diseases. Several types of adult stem or progenitor cells have been shown to improve recovery after ischemic damage and contribute to vasculogenesis and possibly cardiomyogenesis, bone marrow-derived cells being the most extensively studied to date. Overall, the clinical trials using bone marrow-derived cells for patients with acute or chronic ischemic heart disease demonstrated the safety of the procedure and generally documented improvements of heart function and clinical outcome.1-5 However, the increase in contractile recovery was modest in most trials (for review see the recent metaanalysis of Lipinski et al6). The modest effects of the transplanted cells have been attributed to rather low engraftment and survival as well as their limited cardiomyogenic capacity.7,8 Additionally, increasing age and risk factors for coronary artery disease significantly reduce the functional activity of bone marrow-derived and circulating cells in patients.9-11 Aging is known to affect stem/progenitor cell function in animal models and may lead to exhaustion of the endogenous stem cell pools.12 Therefore, we investigated whether subsets of circulating progenitor cells might exist during early human postnatal development and during childhood. In the circulating blood of adults, different subsets of hematopoietic progenitor cells, endothelial progenitor cells and -after mobilization- mesen-
chymal stromal cells (MSCs) have already been described.\textsuperscript{13–15} Here, we identified multipotent, clonally expandable, telomerase-positive circulating cells in the blood of children, which are distinct from previously identified circulating hematopoietic and endothelial progenitor cells. The isolated cells can be induced to differentiate to the principle cell types of 3 cardiovascular lineages and form skeletal muscle. Phenotypically, the isolated cells resemble previously described vessel-associated mesoangioblasts.\textsuperscript{16} The isolated cells express 3 of the 4 pluripotency genes\textsuperscript{17} that have been shown to reprogram somatic cells to pluripotency (reviewed by Nishikawa et al\textsuperscript{18}), namely Klf-4, c-Myc, and low levels of Oct3/4. The cells however lack Sox2 expression. We therefore reasoned that complementation with Sox2 may be sufficient to enhance the differentiation capacity of these bone-derived mesoangioblast-like cells. Thus, we investigated the phenotypic and functional consequences of ectopic Sox2 expression.

Methods

Cell Isolation and Culture From Human Peripheral Blood

Blood samples were collected from patients who underwent open heart surgery with cardiopulmonary bypass. The ethics review boards of the universities Giessen and Marburg, and Frankfurt approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient or patient’s parent. Mononuclear cells were isolated by Ficoll density gradient centrifugation. Detailed protocols are available in the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

Myocardial Infarction, Cell Injection, and Functional Evaluation

Myocardial infarction was induced by permanent ligation of the left coronary artery in 10- to 12-week-old athymic NMRI nude mice (Harlan). Soon after ligation, animals were randomized to receive 1\times10^6 cells or PBS (both 50 \muL), which were injected intramuscularly into the border zone at 3 different sites. After 2 weeks (for unmodified cell injection; Figure 3) and 4 weeks (green fluorescent protein [GFP] and Sox2 transduction; Figure 7), cardiac catheterization was performed for functional analysis by using 1.4F micromanometer-tipped conductance catheter (Millar Instruments Inc.). Left ventricular pressure and its derivative were continuously monitored with a multiple recording system. Then the data were confirmed in a second group of mice using noninvasive monitoring of cardiac ventricular pressure and its derivative were continuously monitored with a multiple recording system. Then the data were confirmed in a second group of mice using noninvasive monitoring of cardiac function by high quality echocardiography at day 0, day 14, and day 28. All data were acquired under stable hemodynamic conditions.

Preparation of Lentiviral Stocks

Preparation of lentiviral stocks were performed as described.\textsuperscript{19} In brief, self-inactivating lentiviral vectors containing the enhanced GFP gene, \alpha myosin heavy chain (MHC) promoter (accession number: U71441) GFP, endothelial NO synthase (eNOS) promoter (accession number: AF387340) GFP, or the human SOX2 gene under the control of a spleen focus-forming virus (VSV-G) pseudotyping. After 8 hours, the medium was replaced by EBM supplemented with EGM SingleQuots and 20% FBS. The supernatant was collected every 24 hours for 2 days, pooled (200 to 250 mL), and filtered through 0.22-\mum filters.

Lentiviral Transduction

For lentiviral transduction, isolated cells were transduced at the second to third passage. Transduction was carried out by adding viral supernatant to the EBM supplemented with EGM SingleQuots and 20% FBS. After 6 hours, medium was changed and the cells were transduced a second time.

All other methods are outlined in detail in the Online Data Supplement.

Results

Characterization of Blood-Derived Progenitor Cells in Children

Blood-derived circulating mononuclear cells were obtained from individuals undergoing cardiopulmonary bypass for cardiac surgery. Cells were cultivated on fibronectin-coated dishes and first clones were detected after 1 to 2 weeks (Figure 1A). Overall, 1.1 to 2.1 colonies were detected per ml blood after 2 weeks of culture. Cells isolated from the first sequential 12 patients (8 children and 4 adults) were continuously cultured to define the long term proliferation capacity (Figure 1 and Online Table I). Cells showed a spindle-shaped morphology (Figure 1A), exhibited a high proliferative capacity, and were cultured for 24.6\pm1.7 passages (Figure 1B). The proliferative capacity was highly correlated with the donor age (Figure 1C) and was associated with high telomerase activity, which was detected at least until the 15th passage (Figure 1D; Online Figure I, A). Onset of senescence, as determined by acidic \beta-galactosidase staining, started after the 20th passage (0.25%) and approximately 10% acidic \beta-galactosidase–positive cells were detected after 29 passages (Figure 1A). Characterization of the surface marker expression by flow cytometry and RT-PCR revealed that the cultivated cells express the mesenchymal markers CD13, CD73, and CD44, the endothelial markers CD105, Tie2, and KDR (type II vascular endothelial growth factor receptor), but were negative for the endothelial/hematopoietic marker CD31 and the hematopoietic markers CD34, CD133, and CD45 (Figure 1E and 1F). Online Table II provides a summary of the expression of various markers by RT-PCR and flow cytometry of the cultivated cells compared to other cells. Although MSCs comprise heterogenous cells, which show variable marker gene expression, the high expression of KDR and Tie2 distinguishes the isolated cells from MSCs (Figure 1F and 1G; and Ball et al\textsuperscript{20}), multipotent adult progenitor cells,\textsuperscript{21} and umbilical cord-derived unrestricted stem cells (for summary, see Online Table III).\textsuperscript{22}}
although the cells were $CD105^+KDR^-$, the absence of $CD31$ and lack of the hematopoietic markers $CD34$ and $CD45$ distinguish the cultivated juvenile cells from circulating endothelial and hematopoietic progenitor cells (Figure 1F and elsewhere$^{23-25}$). When comparing the marker expression profile to previously described cells, these children-derived cells resemble mesoangioblasts, which are multipotent progenitors of mesodermal tissue originally isolated from the embryonic dorsal aorta and characterized by the expression of mesenchymal and endothelial markers.$^{26}$ Consistent with the phenotype of mesoangioblasts isolated from adult tissue,$^{27}$ children-derived cells expressed the proteoglycan NG2, a marker for pericyte-derived cells (Online Figure I, B).

**Blood-Derived Mesoangioblasts Can Be Clonally Expanded**

To elucidate whether the cells can be grown clonally from single cells, we performed limiting dilution assays using cells from the second to third passage. The clonal efficiency estimated by single cell deposition was $9.5 \pm 2.0\%$. Eighteen expanded single cell-derived clones were further characterized, and all clones showed the characteristic expression of $CD73^+$ and $KDR$ but lacked expression of $CD45$ (Figure 1H and 1I). To determine whether the cultured cells indeed originate from $CD73^+$ circulating cells, we sorted $CD73^+$ and $CD73^-$ cells from peripheral blood mononuclear cells before culturing the cells. The number of colonies was 10.3 times higher when $CD73^+$ cells were used as starting population for the culture compared to nonsorted cells, whereas only very few colonies were occasionally observed in the $CD73^-$ fraction (Online Figure I, C). These data suggest that the expression of $CD73$ indeed defines circulating mesoangioblast (cMAB).

**Differentiation of Blood-Derived Mesoangioblasts**

Next, we assessed the lineage-directed differentiation potential by culturing the isolated cells (third to fifth passage) under conditions favoring endothelial, smooth or skeletal
muscle and cardiomyocyte differentiation. Endothelial differentiation was determined by using Matrigel assays. Tube-like structures were detected in vitro (Figure 2A) and perfused vascular structures were formed in implanted Matrigel plugs in vivo (Figure 2C and Online Figure II) similar to the structures detected after implantation of human umbilical venous endothelial cells (HUVEC) (Figure 2B; Online Figure II, A). Implanted cells connected to the mouse vasculature leading to increased perfusion of the plugs (Online Figure II, B) and expressed the endothelial marker CD31 (Online Figure II, C). Consistent with the capacity of the cells to differentiate to the endothelial lineage, the transcription
factors Hex and Fli-1, known to play a key role in vascular development,28,29 were highly expressed (Figure 2D). Differentiation into smooth muscle cells was induced by addition of transforming growth factor (TGF)β1, whereas cultivation of the cells on dishes coated with Jagged-1 protein in combination with fibroblast growth factor 8 induced the expression of smooth muscle-specific proteins in >60% of all cells (Figure 2E; Online Figure III, A). The mRNA expression of the muscle marker smooth muscle actin was additionally documented (Online Figure III, B). Skeletal muscle differentiation was tested by coculturing human mesoangioblasts with C2C12 mouse myogenic cells and scoring the % of human nuclei fused into myotubes. As reported in Online Figure IV, more than 10% of human nuclei fused with mouse myoblasts indicating a significant myogenic potency, even though, as in embryonic mouse mesoangioblasts, spontaneous myogenesis did not occur.16

To induce differentiation into the cardiac lineage, we incubated mesoangioblasts with Wnt3a, which was previously shown to promote cardiac differentiation of embryonic stem cells.30,31 Wnt3a increased the expression of α-MHC in vitro (Online Figure V, A). Furthermore, when blood-derived mesoangioblasts were cocultured with neonatal rat cardiomyocytes for 6 days32,33 they acquired features of cardiomyocytes as demonstrated by the expression of α-sarcomeric actinin by immunostaining (Online Figure V, B) and human troponin T mRNA using human specific primers designed to span the region from exon 6 to exon 10 (Online Figure V, C). The identity of the human troponin T was confirmed by sequencing (Online Figure V, C). The increase in the extent of cardiac differentiation in terms of troponin T expression levels after 6 days of coculture with rat cardiomyocytes, as assessed by RT-PCR, was 8-fold higher compared to endothelial progenitor cells used in similar previous studies (data not shown). Furthermore, we cultured embryonic body–like structure from 10000 GFP-transduced cells by using the hanging drop technique and then cocultured the embryonic bodies with neonatal rat cardiomyocytes. GFP-positive cells contracted after 10 days of culture (see Online Figure VI and Movie I). In addition, using a modified coculture assay with type I atelocollagen membrane to separate the mesoangioblasts and neonatal cardiomyocytes and exclude fusion,34 mesoangioblasts differentiated to α-sarcomeric actinin positive cardiomyocytes after 10 days of culture (Figure 2F).

In line with these results, mesoangioblasts also expressed various transcription factors important for myogenic differentiation such as GATA-4, Mef-2C, and Tbx5 (Figure 2G and 2H; Online Figure VII, B). Interestingly, before inducing cardiac differentiation, the children-derived cells strongly expressed Islet-1 (Figure 2G and 2H), a transcription factor expressed by multiple cell lineages but also shown previously to define a multipotent primordial cardiovascular progenitor cells during development.35 The expression of Nkx2.5 was more heterogeneous (Figure 2G and 2H). Taken together, the blood-derived mesoangioblast-like cells are multipotent and can be directed to differentiate into the 3 distinct cardiovascular cell lineages in vitro.

Transplanted Blood-Derived Mesoangioblasts Differentiate to Endothelial, Smooth Muscle, and Cardiac Muscle in Ischemic Models In Vivo

To determine the potential functional benefit using blood-derived cells for therapeutic applications, human cells were injected in a nude mouse model of hind limb ischemia. After 14 days, the recovery of blood flow was significantly greater in mice treated with cMABs compared to PBS-treated control mice (Figure 3A and 3B). In addition, cells were injected intramyocardially in mice after induction of myocardial infarction. After 2 weeks, cell-treated mice exhibited a significantly, but modestly improved cardiac function with lower left ventricular end-diastolic pressures (46.2±11.0% reduction) and improved diastolic function ( Tau: 18.2±5.4% reduction, Figure 3C). The in vivo differentiation of the injected cells was assessed by immunostaining and human-specific RT-PCR. Injected cells differentiated to endothelial cells, smooth muscle cells, and cardiomyocytes in vivo (Figure 3D through 3F; Online Figure VIII, A through D; and Online Figure IX, A through C; for clonally derived cells). Moreover, cardiac and endothelial differentiation was confirmed by human specific RT-PCR of troponin T and Tie2, respectively (Figure 3G). Survival and retention of human cells was confirmed by human specific GAPDH expression, which was measured by quantitative PCR (Figure 3H). In addition to the capacity of the injected cells to contribute to tissue regeneration by differentiation to the cardiovascular cell lineages, cells expressed and secreted a variety of proangiogenic cytokines and cardioprotective factors known to contribute to improved infarct healing (Online Figure X).

Expression of Stem Cell Markers and Pluripotency Associated Genes in cMABs

Because our results demonstrate that blood-derived cMABs can be clonally expanded from single cells and are capable of differentiating into all 3 cardiovascular lineages, we characterized the expression of markers associated with stemness and pluripotency. Among the 4 factors (Oct3/4, Klf4, c-myc, and Sox2, which are sufficient to induce pluripotency in human fibroblasts,36) cMABs, after the second to third passage or after single cell expansion, expressed Oct3/4, Klf4, and c-myc, whereas Sox2 expression was below the detection limit (Figure 4A through 4D). The expression of the active Oct4A isoform was confirmed by immunostaining with an Oct4A antibody and by subcloning and sequencing of the Oct4A specific PCR product (see the Online Data Supplement). However, whereas c-myc and Klf4 were expressed at levels similar to embryonic stem cells, expression of Oct3/4 was lower (<10%) in cMABs compared to embryonic stem cells (Figure 4A and 4B). Moreover, another important stem cell marker, Nanog,35,36 was not detected (Figure 4A), whereas the endodermal transcription factor Sox17, which plays an important role in cardiac specification,37 was abundantly expressed (Figure 4A).

In accordance with the expression levels observed by RT-PCR and Western blot, chromatin immunoprecipitation revealed histone modifications associated with active transcription (histone H3 acetylation and H3 lysine 4 trimethylation) and low repressive heterochromatin markers (trim-
Figure 3. Cell therapy after induction of hind limb ischemia (A and B) or myocardial infarction (MI) (C through G). A and B, Blood-derived cMABs (1×10^6) were injected intramuscularly after ischemia and function was analyzed 2 weeks after operation. Representative images of Doppler flow in peripheral blood-derived cMABs or PBS control group (A) and summary of data (B). *P<0.05. C, Pressure-volume loop analysis using Millar catheter was performed 2 weeks after induction of myocardial infarction (MI). Quantification (LVEDP and Tau) are shown. *P<0.01 vs sham; #P<0.05, ##P<0.01 vs MI PBS group. n=3 (sham), n=7 (PBS), and n=6 (cMAB). D through F, Immunofluorescent images of smooth muscle actin, von Willebrand factor (vWF), and α-sarcomeric actinin. Injected cells were identified by human Alu probes (E) or human nuclear antigen (hNA) (D and F). Blue arrows indicate human smooth muscle cells. The white arrow indicates hNA-positive cardiomyocytes (F). G, Human-specific RT-PCR of Tie2, troponin T, and GAPDH in hearts injected with cMAB or PBS. H, Human GAPDH was measured by quantitative real-time PCR. RNA was isolated from the total hearts of mice. Cell number was calculated by standard curves. n=3 (MI PBS) and n=5 (MI cMAB).
ethylated H3 lysine 9 and trimethylated H3 lysine 27) at the Klf4 promoter region (Figure 4E). In contrast, the repressive histone modification trimethyl-H3 lysine 27 strongly prevails at the Sox2 promoter (Figure 4E). Oct3/4, which was expressed at rather low levels in cMABs compared to embryonic stem cells, demonstrated an intermediate histone modification pattern with predominantly active marks in its promoter region (Figure 4E). Thus, excessive transcription repressive histone modifications of Sox2 may limit the pluripotency and stemness characteristics of blood-derived cMABs.

Complementation of Sox2 Enhances Differentiation Capacity and Therapeutic Effects of cMAB

Because, of the 4 pluripotency genes, only Sox2 was completely silenced, we hypothesized that Sox2 transduction may enhance the multipotency of the cells. Therefore, we overexpressed Sox2 by lentiviral vectors and confirmed Sox2 expression by RT-PCR (Figure 5A), quantitative PCR (data not shown) and on protein level (Online Figure XI, A). Sox2-transduced cMAB demonstrated a re-expression of the pluripotency associated gene Nanog (Figure 5B and 5D) and an increase in Oct3/4 (Figure 5C), although the expression was still lower compared to embryonic stem cells. To evaluate the potential of Sox2-transduced cMABs to generate progeny of the 3 germ layers, we induced differentiation in hepatocytes and neuronal cells in vitro according to published protocols.16,38,39 Sox2-transduced cMABs efficiently differentiate to CK18 and α-H9251-fetoprotein expressing hepatocytes (Online Figure XII), and nestin and Tuj1-positive neuronal cells (Online Figure XIII). Endothelial and cardiac differentiation was further quantified by using reporter gene assays, in which GFP is expressed under the control of the eNOS and α-H9251-MHC promoter, respectively. Sox2-transduced cMABs expressed eNOS-promoter-driven GFP and formed vascular networks in vitro and vivo (Figure 6A through 6C and data not shown). Furthermore, Sox2-transduced cMABs showed a rapid and more efficient induction of α-MHC-promoter driven GFP expression after coculture with rat neonatal cardiomyocytes (Figure 6D through 6F). Importantly, Sox2-transduced cMABs but not unmodified cMABs express MHC-promoter driven GFP when exposed to conditioned medium to induce cardiac differentiation (Figure 6G and 6H). Differentiation to smooth muscle cells, which was very efficient in control cells
(41±7.4%) was further augmented when Sox2-transduced cMABs were exposed to TGFβ (61±9.4%, Figure 6I). Likewise, skeletal myogenic differentiation was also increased, though not dramatically by Sox2 expression (Online Figure IV). These data document that Sox2-transduced cells have the potency to differentiate into all 3 germ layers in vitro, at variance with untransduced cells whose potency is more restricted to solid mesoderm.

To test whether Sox2-transduced cells exhibited improved therapeutic potential, we compared the effects of injecting Sox2-transduced cMABs in comparison to GFP-transduced control cMABs in nude mice after induction of acute myocardial infarction. In mice that had been randomized to the treatments, cardiac function was significantly improved in mice receiving Sox2-overexpressing cells compared to mice treated with GFP-transduced control cells after 4 weeks (Figure 7A; Online Figure XIV, A and B). In addition, measurement of cardiac function by echocardiography in a second group of mice confirmed that mice receiving Sox2-transduced cMABs showed an improved WMSI compared to PBS (84.4±11%) and GFP-transduced cMAB-treated mice (92.2±5.7%). Likewise, fractional shortening in Sox2-transduced cMAB-treated mice was 154.6±22.7% compared to PBS controls and 138.2±13.8% compared to GFP-transduced cMABs. Furthermore, administration of Sox2-overexpressing cells resulted in increased numbers of α-sarcomeric actinin, smooth muscle actin, and von Willebrand factor-positive human cells compared to the injection of GFP-transduced control cMAB as shown by immunostaining and quantitative PCR (Figure 7B through 7E). To confirm cardiac differentiation, we transplanted Sox2-transduced cMABs expressing GFP under the control of the αMHC promoter. GFP-expressing α-sarcomeric actinin positive cells were detected in the border zone of the infarcts (Figure 7F and Online Figure XV). These data suggest that Sox2 transduction enhances the cardiovascular repair capacity as well as the ability to differentiate to endothelial, smooth muscle and cardiac cells. However, although Sox2-transduced cMAB formed embryoid body-like structures in vitro (Online Figure XI, B and C) and differentiate to all 3 germ layers in vitro, we never observed teratoma formation when subcutaneously injecting Sox2-transduced cMABs into nude mice (6 months observation; n=9). Likewise, spontaneously contracting embryoid bodies did not develop in culture. These data indicate that, although Sox2 transduction led to re-expression of Nanog and increased the expression of Oct3/4, the expression levels of pluripotency genes appear to be insufficient to reprogram cMAB to fully induced pluripotent cells resembling embryonic stem cells.

**Discussion**

Our studies identify a novel subset of circulating human progenitor cells, that can be expanded in vitro to large numbers, are capable to differentiate into all 3 distinct cardiovascular cell lineages in vitro and in vivo, secrete proangiogenic and cardioprotective factors, and mediate significant functional improvements after therapeutic administration in models of ischemia and infarction, specifically when transduced with Sox2.

Marker expression by the isolated cells is clearly distinct from all subsets of hematopoietic or endothelial progenitor cells described so far, as shown by the absence of CD45, CD34, CD133, CXCR4 and myeloid markers, such as CD14 in bulk cultures and in single cell-derived colonies. Whereas the expression of mesenchymal markers is shared by bone marrow–derived and blood-derived MSCs and multipotent adult progenitor cells, the very high expression of the endothelial marker KDR and Tie2 is unique for the circulating cells isolated in the present study. Moreover, multipotent adult progenitor cells do not express CD73 and CD44, which were highly expressed on the children-derived circulating progenitor cells isolated in the present study. Phenotypically, these blood-derived cells may therefore represent a correlate of embryonic dorsal aorta–derived mesoangioblasts present after birth and in the adult. Indeed, the majority of the markers being expressed on blood-derived cMABs are similar to the marker profile of previously described mouse mesoangioblasts isolated from aorta or the heart itself (see Online Table II) and both cell populations are positive for Nkx2.5, GATA-4, Isl-1, and Tbx-5, however both mouse and human mesoangioblasts isolated from either heart or skeletal muscle are strongly positive for alkaline phosphatase, whereas human cMABs, like embryonic mesoangioblasts, are not (data not shown). Interpretation of the phenotype by
Figure 6. Sox2 increased the differentiation into cardiovascular cell lineages in vitro. A through C, cMABs were transduced with lentiviral vectors expressing GFP under control of the eNOS promoter (eNOSp-GFP, illustrated in A) with or without Sox2 and endothelial differentiation was induced by vascular endothelial growth factor, erythropoietin, basic fibroblast growth factor, and interleukin-6 for 7 days. eNOSp-GFP was detected by fluorescence (B) and FACS (C). D through H, cMABs were transduced with lentiviral vectors expressing GFP under control of the αMHC promoter (αMHCp-GFP, illustrated in D) with or without Sox2. Cardiac differentiation of Sox2-transduced cMAB was induced by coculture with rat neonatal cardiomyocytes (E and F) or by conditioned medium derived from cardiomyocytes without coculture for 7 days (G and H). αMHCp-GFP was detected by fluorescence microscopy (E and G) and FACS (F and H). I, Smooth muscle differentiation of Sox2-transduced cMABs compared to GFP-transduced cMABs (control) was induced by TGFβ (7 days) and detected by smooth muscle actin (SMA) immunostaining. n=3.
Figure 7. Sox2 increased the differentiation and therapeutic capacity of cMABs. A, Mice hearts were injected with PBS, GFP-transduced cells, and Sox2-transduced cells (each $1 \times 10^6$), and cardiac function were measured by Millar catheters 4 weeks after induction of myocardial infarction. Left ventricular end diastolic pressure (LVEDP), maximum dp/dt (dp/dt max), minimum dp/dt (dp/dt min), and tau (Weiss) were measured in n=7 (PBS), n=7 (GFP), and n=8 (Sox2) treated mice. B, Quantification of the differentiation into cardiomyocytes, smooth muscle, and endothelial cells in hearts treated with GFP and Sox2-transfected cells by immunochemistry (B) and quantitative PCR (C). B, \(\alpha\)-Sarcomeric actinin, SMA, and vWF-positive human hNA-positive cells were counted (6 to 12 sections per group). C, Quantitative PCR of human-specific troponin T (TnT), SM22, and eNOS, n=5. D through E, Representative immunofluorescent image of smooth muscle actin (SMA) (red) (D) and vWF (red) (E). Human nuclear antigen (hNA) (green) was used to identify human cells. Yellow arrows indicate SMA\(^+\) (red) or vWF\(^+\) (red) hNA\(^+\) (green) cells. Pink arrow indicates hNA\(^+\)/SMC\(^+\) cell. F, GFP expression in section of mice after acute myocardial infarction injected with Sox2-transduced cMABs expressing \(\alpha\)MHC promoter-driven GFP. Yellow arrows indicate \(\alpha\)-sarcomeric actinin\(^+\) (red) and GFP\(^+\) (green) cells.
using marker gene expression obviously has its limitations, because cells may change marker gene expression during culture. However, FACS sorting revealed that the expression of CD73 defines the cell population, which gives rise to the colonies. Lineage tracing will be helpful to determine the population of the circulating versus tissue-resident mesangioblasts in mouse models. However, such studies will not give insights into the relation of these cells in humans, and human studies are obviously complicated by ethical constraints which limit the availability of blood and tissue for research particularly in children.

The novel finding of the present study that the cultured mesangioblast-like cells and single cell-derived colonies express 3 of 4 pluripotency genes, Oct3/4, c-Myc, and Klf4, respectively, is both exciting and intriguing. However, our findings also demonstrate that the levels of Oct3/4 expression are low compared to those observed in embryonic stem cells, whereas Klf4 and c-Myc are expressed at comparable levels. Detailed analysis of epigenetic control of the promoter regions of the pluripotency genes revealed histone modifications associated with active transcription for Klf4, but excessive repressive histone modifications at the promoter of Sox2, which was not expressed in cMABs, and active histone H3 acetylation but not H3K4 trimethylation at the Oct3/4 promoter. Thus, the active transcription of the pluripotency genes Klf4 and c-Myc in combination with KDR, which defines cardiovascular progenitor cells in human embryonic stem cells, might provide a rational explanation for the capacity of mesangioblast-like cells to differentiate into all 3 cardiovascular lineages, whereas full plasticity is limited by the rather low expression of Oct3/4 and the completely repressed transcription of Sox2. Indeed, transduction of cMABs with Sox2 induced a re-expression of Nanog and increased expression of Oct3/4, a finding which is consistent with the transcriptional network induced by Sox2. Interestingly, transduction with Sox2 enhanced the differentiation capacity of cMABs to the cardiovascular lineages and significantly improved the therapeutic potential compared to control cMABs in the myocardial infarction model. Moreover, Sox2-transduced cMAB can be efficiently differentiated to cell lineages of all 3 germ layers such as hepatocytes and neuronal cells, indicating that Sox2 complements the set of pluripotency genes and repgrams cMABs. However, despite the observation that Sox2-transduced cells formed some embryoid body-like structures and differentiated to cell lineages of the 3 germ layers in vitro, we did not detect teratoma formation in vivo, indicating that the cells may not be pluripotent. This might be explained by the fact that even after Sox2 transduction the expression of Oct3/4 and Nanog was significantly lower compared to embryonic stem cells. The cells may be similar to the partially reprogrammed cells described previously. In contrast to the lack of teratoma formation and full reprogramming when cMABs were transduced with Sox2, others recently have reported that adult murine neuronal stem cells can be fully reprogrammed and generated teratoma after overexpression of one single factor Oct4. Interestingly, in contrast to neuronal stem cells, cMAB expressed high levels of p53 and p21, factors that have been recently shown to prevent induced pluripotent cell formation (Online Figure XVI). Nevertheless, for therapeutic purposes in the future, the lack of full pluripotency in Sox2-transduced cMABs might be an advantage by reducing the risk for adverse effects such as teratoma formation. However, the use of lentiviral vectors for transducing the cells with Sox2, as in the present study, would not be clinically applicable. First alternative strategies using plasmids and/or small molecules for reprogramming were recently described and might be helpful for clinically oriented studies. Moreover, pharmacological or genetic interventions to reduce the excessive transcriptional repressive histone modifications of the Sox2 promoter may be conceived to further increase the plasticity and therapeutic potential of this unique blood-derived cell population.

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Disclosures

S.D. and A.M.Z. are founders and advisors of t²cure GmbH.

References


Novelty and Significance

What Is Known?

- Cell therapy is a promising option to improve cardiac function after acute myocardial infarction or heart failure.
- Several types of cells have been used, including the recently discovered reprogrammed induced pluripotent cells.

What New Information Does This Article Contribute?

- We identified a novel progenitor cell type in the circulating blood in humans.
- Complementation of a pluripotency gene (in this case Sox2) increased the differentiation capacity of the cells in vitro and in vivo and improved functional recovery after myocardial infarction model compared with control cells.

We identified a novel progenitor cell type in the circulating blood in humans that is distinct from previously identified hematopoietic or endothelial progenitor cells and that resembles embryonic aorta resident mesoangioblasts in mice. The isolated cells, which we termed circulating mesoangioblasts, expressed 3 of 4 pluripotency genes (namely Oct4, Klf4, c-myc) and differentiated into all 3 cardiovascular lineages in vitro and in vivo. Therapeutic application improved cardiovascular repair in 2 different animal models. In addition, we addressed the question of whether complementation of the fourth pluripotency gene Sox2 increased the therapeutic benefit. Indeed, cells that were transfected to express Sox2 further improved the functional recovery after acute myocardial infarction compared with control cells. Furthermore, Sox2-expressing cells formed more cardiac and vascular cells in the injured heart. In summary, the present study identifies a novel circulating progenitor cell type in humans that might be suitable for therapeutic application.
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Supplementary Methods

Cell isolation & culture from human peripheral blood

Blood samples were collected from patients who underwent open heart surgery with cardiopulmonary bypass. Patients with known genetic disorder such as troponin mutation, Down syndrome, or CATCH22 were excluded. Mononuclear cells (MNCs) were isolated by Ficoll density gradient centrifugation. MNCs were plated at $8 \times 10^6$ cells/ml on a fibronectin-coated dish in endothelial basal medium supplemented with 1 μg/mL hydrocortisone, 12 μg/mL bovine brain extract, 50 μg/mL gentamicin, 50 ng/mL amphotericin-B, 10 ng/mL epidermal growth factor and 20% fetal bovine serum. After 7 days in culture, non-adherent cells were discarded and cells were cultured for additional 7 days in the same medium. On day 15, cells were detached by 0.25% Trypsin-EDTA (GIBCO) and were seeded at $5 \times 10^4$ cells/ml on fibronectin-coated dishes. When cells reached 80% confluency, cells were subsequently passaged at $5 \times 10^4$ cells/ml and were used for following analyses. Patient characteristics of the first 12 patients (including 4 adults) who donated blood for cell isolations and long term culture assays shown in figure 1 are provided in table S1. For the entire manuscript, cells were isolated of a total of 56 young patients. All patients suffered congenital heart disease requiring surgery (13 with single ventricle, 6 ventricular septal defect, 6 transposition of great artery, 5 tetralogy of Fallot). The donor age ranges from 0 to 9 years (average 1.9±0.3 years old) and 59% were male. All outgrowing cells of each patient showed rigorous proliferation capacity with a population doubling time of 1.38 ± 0.02 days. We routinely used passages 3-5 except for Figure 1.

Single cell expansion

For single cell clonogenic expansion, cells of 2nd-3rd passage were labelled with CM-Dil (Invitrogen) and seeded at a single cell density on fibronectin-coated 96well plates. Single-cell deposition was confirmed microscopically and wells containing more than one cell were excluded.
**Other cells**

Human umbilical venous endothelial cells (HUVEC) were purchased from Cambrex and cultured in endothelial basal medium supplemented with 1 μg/mL hydrocortisone, 12 μg/mL bovine brain extract, 50 μg/mL gentamicin, 50 ng/mL amphotericin-B, 10 ng/mL epidermal growth factor and 10% fetal bovine serum. Mesenchymal stromal cells (MSCs) were isolated by density-gradient centrifugation with Ficoll from bone marrow of healthy human volunteers. The CD34⁺ hematopoietic progenitor cells were isolated from human peripheral blood by immunomagnetic purification. Human mesoangioblasts were obtained from the tissue of ascending aorta from explanted hearts in collaboration with Dr. G. Cossu. Aortic tissue was explanted on gelatin-coated dishes and outgrowing cells were used for the experiments.

**Flow cytometry**

Following antibodies were used for fluorescence activated cell sorting (FACS): Phycoerythrin (PE)-conjugated anti-CD13, CD14, CD31, CD34, CD44, CD45, CD73, CD146 (BD Biosciences), CD105, CD144, KDR (R&D), CD133 (Miltenyi Biotec), Fluorescein (FITC)-conjugated anti-CD117 (Santa Cruz), and isotype-matched PE, or FITC-conjugated mouse immunoglobulins. Samples were analyzed by a flow cytometer, BD FACS Calibur cell sorter (BD Biosciences, San Jose, CA).

**RT-PCR**

Total RNAs were isolated by using TRIzol (Invitrogen) or RNeasy Mini Kit (Qiagen). RNA was subjected to RT-PCR by using SuperScript First Strand Synthesis System (Invitrogen). Primer sequences are available upon request.

**Quantitative RT-PCR**

Quantitative RT-PCR was performed on LightCycler 1.2 (Roche Diagnostics) or StepOnePlusTM Real Time PCR System (Applied Biosystems). Primer sequences are available upon request.

**Quantification of engrafted cells**
Human specific GAPDH was measured by StepOnePlusTM Real Time PCR System (Applied Biosystems). CT values were compared to a standard curve to quantify the number of viable cells per sample.

**Subcloning and sequencing**

RT-PCR products of human troponin T and Oct4A were purified by Gel Extraction kit (Qiagen) and were subcloned by using pGEM-T Easy vector (Promega) or TOPO TA cloning kit (Invitrogen). 3 clones (troponin T) and 4 clones (Oct4A) were collected and each clone was amplified and sequences were analyzed (SeqLab, Germany or AGOWA). Troponin T (between exon 6 and 10): forward primer; GAAGAAGAAGAGGAAGCAAAAGGAG, reverse primer; TCCTTCTCCGCTCATTC. Oct4A (between exon 1 and 2): forward primer; GCAAGCCCTCATTTCAACCAG, reverse primer; AATAGAACCCCAAGGGTGAG.

**Chromatin immunoprecipitation (ChIP) Assay**

For each immunoprecipitation, approximately 5x10^6 cells were crosslinked with 1% formaldehyde for 5 min. at room temperature in the presence of glycine (1.25 M). The cell lysates were sonicated by using a Branson 450 Sonifier with 4 pulses for 5 sec. with 30% Output to sheer chromatin-DNA complex. For histone ChlPs, the EZ-Magna ChIP kit (17-409 Millipore) was used according to the manufacturer's protocols. In this assay, 5 µg of Acetyl-H3 (06-599 Millipore), H3K9me3 (07-442 Millipore), H3K27me3 (07-449 Millipore), H3K4me3 (07-473 Millipore), pan Histone H3 (Abcam), and 5µg of IgG (12-370 Millipore), were used. The total input or immunoprecipitated DNA was determined by quantitative RT-PCR with the equation: ΔCt (threshold cycle) of each sample = mean of Ct_{(IP)} - mean of Ct_{(input)}. Relative expressions are presented as 2^{-ΔCt} and normalized to the pan histone H3 (Abcam). Primer sequences are available upon request.

**Telomerase activity**

Telomerase activity was measured with a Telomerase ELISA Assay Kit (Millipore) according to the manufacturer’s instructions. 1x 10^6 cells were used for each experiment.
**In vitro endothelial, smooth muscle, and cardiac differentiation assay in vitro**

Several differentiation assays were performed as summarized in Table S3. Endothelial differentiation using eNOSp-GFP transduced cells with or without Sox2 transduction was induced by culturing in endothelial differentiation medium as summarized in Table S3 for 7 days. Immunofluorescence was performed to confirm smooth muscle cells (SMC) differentiation using anti-smooth muscle actin antibody after 14 days of culture. To induce cardiac differentiation, cells were cultured with neonatal rat cardiomyocytes (CM) using atelocollagen membrane (CM-6, KOKEN, Japan) that is permeable for only small molecules (less than 5000 MW). Two days after culturing rat cardiomyocytes on the top of the atelocollagen membrane, the membrane was plated upside down on the culture dish. Then, cMAB were cultured for 10 days. Alternatively, cells were co-cultured with CM for 6 days as previously described with a minor change. In brief, cells were added onto CM at a ratio of 1:6 two days after CM isolation. After 6 days, cells were used for detection of cardiac marker gene expression. Moreover, cells were cultured on non-coated dishes and Wnt3a (100 ng/ml) and dexamethasone (10 nM) were added without co-culturing rat neonatal CM. In addition, αMHCp-GFP cells were co-transduced with or without Sox2 and cells were cultured on gelatine coated dishes in cardiomyocytes conditioned medium. After 7 days, GFP expressions were analyzed by flow cytometry.

**Hind limb ischemia, cell injection and functional evaluation**

The *in vivo* angiogenic capacity was examined in an unilateral hind limb ischemia model using 8- to 10-week-old athymic NMRI nude mice (Harlan, Borden, Germany). The proximal portion of the right femoral artery including the superficial and the deep branch and the distal portion of the saphenous artery were occluded with an electrical coagulator. Then $1\times10^6$ cells in 50 µl PBS were injected intramuscularly at 4 different sites. The overlying skin was closed by using surgical staples. After 2 weeks, we determined the ischemic /nonischemic limb blood flow ratio by using a laser Doppler blood flow imager (Laser Doppler Perfusion Imager System, moorLDI-Mark 2; Moor Instruments, Wilmington, DE). Data are expressed as the ratio of ischemic to nonischemic hind limb.
**Skeletal muscle differentiation assay**

In order to have a five-fold excess of myoblasts versus cMABs, 22,857 C2C12 myoblasts/cm² were seeded together with 5,714 GFP or Sox2-cMABs/cm² in 3.5 cm² dishes (Nunc) on day 0 in a medium containing 50% of C2C12 proliferating medium (DMEM (Sigma)) containing 10% FBS and 50% of cMABs medium.

Two days after (day 2), the medium was switched to DMEM containing 2% horse serum to promote myogenic differentiation. Half of the media were changed every other day. The co-culture was checked daily for GFP positive myotubes. On day 7, the dishes were fixed with 4% PFA for 5 minutes and stained for LaminA/C (mouse monoclonal, 1/300, Novocastra) and Myosin Heavy Chain (MyHC, rabbit polyclonal, 1/500, details in Minasi et al., Development 2002) with the proper secondary antibodies (Molecular Probes). The assay was done twice in triplicate. The quantification was done counting the number of LaminA/C positive nuclei inside MyHC positive myotubes versus the total number of LaminA/C positive nuclei per field. Twenty-five random 20X fields (containing high and low density areas) per co-culture type were counted and the assay was quantified using microsoft excel (two tailed, paired T test).

**Tube Formation Assay (In Vitro Matrigel Assay)**

Cells (2×10⁵) were cultured in a 12-well plate (Greiner) coated with 200 µL of Matrigel Basement Membrane Matrix (BD Biosciences). Tube length was quantified after 48 hours by measuring the cumulative tube length in four random microscopic fields with a computer-assisted microscope using the program KS300 3.0 (Zeiss).

**In Vivo Matrigel Plug Assay**

This assay was performed as described previously ⁵. Briefly, CM-Dil (Invitrogen) labeled or GFP expressing cells (1×10⁶) were resuspended in 30 µl PBS and mixed with 500 µl of Matrigel Basement Membrane Matrix (BD Biosciences) containing 15 U of heparin (Sigma-Aldrich). The cell–matrigel mixture was injected subcutaneously into 6- to 8-week-old female athymic nude mice (Harlan) along
the abdominal midline. After 7 days, blood vessel growth in Matrigel plugs was quantified by analysis of CD31-stained sections using microscopy. In some experiments, fluorescence labeled lectin was perfused 30 minutes before sacrificing the mice. For hemoglobin analysis, the Matrigel plug was removed after 7 days and homogenized in 130 µL of deionized water. After centrifugation, the supernatant was used in the Drabkin assay (Sigma-Aldrich) to measure hemoglobin concentration. Stock solutions of hemoglobin are used to generate a standard curve. Results are expressed relative to total protein.

ELISA
Quantification of secreted pro-angiogenic cytokines in supernatant of HUVEC, adult endothelial progenitor cells (EPC), and children-derived circulating mesoangioblasts (cMAB) culture. After collecting supernatants (10 ml from 75cm² dish), the cell number was counted (3.3-6.6 x 10⁶ cells per flask) and the results were normalized per 10000 cells. ELISA of insulin-like growth factor (IGF), stromal-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) was performed according to the manufacturer’s instructions. All ELISA kits were purchased from R&D.

Histological analysis
For morphological examination, the hearts of the mice and Matrigel plugs were fixed with 10% buffered formalin and embedded in paraffin, processed for light microscopy, and stained with hematoxylin and eosin. Alternatively, the hearts of mice and Matrigel plugs were embedded in optimal cutting temperature compound (O.C.T. compound), and were quickly frozen in liquid nitrogen and cut in 6 µm sections.

Immunostaining
For immunochemistry of cultured cells, cells were fixed with 4% paraformaldehyde. After permeabilization with 0.2% saponin (Sigma), cells were incubated with the respective antibodies (smooth muscle actin; Sigma, alpha sarcomeric actinin; Sigma, GATA4; Santa Cruz, Nkx2.5; Santa
Cruz, Oct3/4; Cell Signaling, Isl-1; R&D, nanog; Abcam, Sox2; Abcam, nestin; Abcam, Cytokeratin 18 (CK18); Chemicon). The specimens from frozen tissue sections were fixed with 4% paraformaldehyde, followed by staining with the respective antibodies (CD31; Chemicon, human nuclear antigen; Chemicon, smooth muscle actin; Sigma, alpha sarcomeric actinin; Sigma, von Willebrand factor; Sigma). Human nuclear antigen, smooth muscle actin, alpha sarcomeric actinin were conjugated with Alexa Fluor 488, Alexa Fluor 546 (or 555), and Alexa Fluor 647 Monoclonal Antibody Labeling Kit (Molecular Probes) according to the manufacturer’s instructions. Nuclei were counterstained with To-pro-3 iodide, Sytox Blue (both Molecular Probes) or DAPI according to the manufacturer’s instructions. The images were recorded by confocal microscope (LSM510-META, Carl Zeiss, Oberkochen, Germany).

**Immunoblot Analysis**

cMABs and MSC were lysed with lysis buffer (Cell Signaling) containing 1 mM phenylmethanesulfonyl fluoride. After centrifugation, the supernatants were collected and subjected to electrophoresis in 10% SDS-polyacrylamide gels. Proteins were transferred to poly vinylidene difluorid membrane, and incubated with anti-Tie2 (BD), anti-nanog (Abcam), anti-Oct3/4 (Oct4A, Cell Signaling), anti-Klf4 (Abcam), anti-c-myc (Santa Cruz), anti-Sox2 (Abcam), or anti-beta actin (Sigma) overnight at 4 °C. Bound antibodies were visualized by using horseradish peroxidase (HRP)-conjugated sheep anti-mouse or donkey anti-rabbit antibody (both Amersham).

**Fluorescence in situ hybridization**

6 µm sections were prepared from formalin-fixed, paraffin-embedded tissue blocks according to standard procedures. After deparaffinization, sections were subjected to heat-induced epitope retrieval by boiling for 22 minutes in 1 mmol/L sodium citrate buffer (pH 8.0). Sections were fixed with 1% paraformaldehyd/PBS on ice for 10 minutes.

The human Alu probes were used for fluorescence in situ hybridization (FISH) 6. Probes were
dehybridized for 4 min. at 71°C before incubation of probes with samples (4 min. at 80°C). Overnight hybridization at 37°C was followed by a stringent wash (2× SSC with 50% formamide 2 times for 10 minutes, 2 x SSC for 5 minutes, NP-40 /2 x SSC for 5 minutes, 2 x SSC for 5 minutes at 42°C).

Sections were blocked for 30 minutes with bovine serum albumine and incubated with antibodies against smooth muscle actin (mouse IgG, 1:400, DAKO) for 1 hour. After washing, cells were incubated with Alex Fluor 647-conjugated secondary antibodies (1:200) (donkey anti-mouse IgG, Molecular Probe). Nuclei were stained with DAPI (DAPI Mounting medium, Vector Laboratories) or Cytox blue (1:2000, Molecular Probes).

All images were aquired using confocal microscopy (Zeiss LSM510 system, Germany).

**Echocardiography**

In order to control initial infarct size and check the left ventricular function, we performed echocardiographic analysis (Visualsonics; Vevo770). Initial infarct size was evaluated by left ventricular diastolic dimension (LVDd) and wall motion score index 7 soon after coronary ligation. Echocardiography was performed by a blinded single observer.

**Teratoma formation assay**

In order to check whether Sox2-transduced cells form teratoma in vivo, we injected cells into 15x CD1/Nude mice (Charles River). Therefore, 1.5x10^6 cells in 100 µl NaCl were injected subcutaneosly in the dorsal flank (9 mice received Sox2-transduced cMABs, 2 mice received GFP-transduced cMABs, 2 mice received Hela cells) and 2 mice were used as vehicle control. Hela cells that have been used as positive control formed teratomas.

**Statistics**

Data are expressed as mean±SEM. Unpaired two-tailed students t-test, Mann-Whitney U-test (to compare 2 groups) or ANOVA (≥ 3 groups) were used for the comparison between groups based on the original data. Single regression analysis was performed to investigate the relation of donor age and population doubling.
References


Online Figure I, Koyanagi et al

(A) Representative gel detecting telomerase activity. Heat inactivation was used as negative control. (B) RT-PCR of proteoglycan NG2 in peripheral blood derived circulating mesoangioblasts (cMAB) isolated from 2 children, mesoangioblasts (MAB) obtained from human aorta, and human heart are shown. GAPDH is identical to Fig. 2D. (C) Colony number in peripheral blood derived sorted CD73+ versus CD73- cells after 2 weeks. Non-sorted cells are shown as control.
Online Figure II: Matrigel plug assay of implanted children-derived cells (2×10^5) in vitro (A) and (1×10^6) in vivo (B-E). Summary of tube length after 48 hours (A; in vitro) and hemoglobin measurement after 7 days (B; in vivo) are shown. Human umbilical vein endothelial cells (HUVEC) are used as positive control for in vitro tube formation.

(C) CD31 staining of CM-Dil-labeled human cells in in vivo matrigel plug assays (14 days).

(D) Morphology of in vivo matrigel plug assays. PBS control and cMAB are shown.
Online Figure III: Confocal images of differentiated smooth muscle cells (SMA; green, Nuclei; blue) after culture on Jagged-1 coated dish + FGF8b + heparin for 1 or 7 days as indicated.

(B) RT-PCR of smooth muscle actin (SMA) after 3 days of stimulation with cMABs with Jagged 1, FGF8b and heparin. Right panel shows the quantification (n=3). For details regarding concentrations see supplementary table 4.
Online Figure IV: Koyanagi et al

Online Figure IV:
cMABs contribution to myogenesis in vitro and in vivo. (A) Live imaging of GFP-cMABs, cultured with C2C12 myoblasts showing GFP positive myotubes. (B) Quantification of myogenic fusion in the co-culture assay. Error bars indicate s.e.m. and asterisk is P= 0.018 (two tailed, paired T test). (C)[c1, c3] Representative pictures showing GFP [c1] and Sox2 [c3] cMABs in co-culture with C2C12 murine myoblasts. Lamin A/C stains human nuclei and the residual cytoplasmic fluorescence is due to GFP (both in green); arrowheads show human nuclei inside myotubes; arrows point to mouse nuclei adjacent to cMABs with weak LaminA/C signal; asterisks indicate two myotubes, the left one with GFP positive cytoplasm. Z stacks showing lamin A/C positive cells inside myosin positive myotubes are showed in the merged pictures. [c2, c4] In vivo differentiation of GFP [c2] and Sox2 [c4] cMABs after intramuscular injection of 5 x 10^4 cells into the tibialis anterior muscle of mdx/SCID mice. Human nuclei and dystrophin are both labeled in red by anti-LaminA/C and Dys2 mouse monoclonal antibodies.
Online Figure V: (A) Cardiac gene expression without co-culture. Cells were treated with Wnt3a (100 ng / ml) and dexamethasone (10 nM) for 3 days in DMEM / F-12 (Ham) (1:1) with 10% FBS. α-myosin heavy chain (α–MHC) gene was detected.

(B) Representative immunofluorescence of alpha sarcomeric actinin positive human cells after co-culture with rat cardiomyocytes. Arrow indicates differentiated cells. Nuclei (blue), α-sarcomeric actinin (red), and human nuclear antigen (green). Bar indicate s50 µm.

(C) Troponin T mRNA was detected by using human specific RT-PCR in mesoangioblasts which have been co-cultured with CM for 7 days. The products of RT-PCR of troponin T (TnT) was subcloned and sequenced. Sequences are 100% identical with human TnT.
Online Figure VI  Koyanagi et al

(A) Phase contrast of EB and rat CM feeder. Yellow arrow indicate rat CM cluster.

(B) GFP positive cMAB EB, which are contracting (see Online Movie1).

Online Figure VI : 10000 GFP transduced cells were cultured in DMEM / F-12 (Ham) (1:1) with 10% serum replacement with L-glutamin, non-essential amino acid, alpha mercaptoethanol, and basic FGF (50µM) using hanging drop technique. After 3 days, the embryoid body like structure (EB) was transferred to a floating condition in same medium and replaced onto rat CM after 2 days. After 10 days with co-culture images and movies were taken.

(A) Phase contrast of EB and rat CM feeder. Yellow arrow indicate rat CM cluster.

(B) GFP positive cMAB EB, which are contracting (see Online Movie1).
Online Figure VII:  (A) Immunostaining of GATA4, Nkx2.5, and Isl1 in human MSC. The set-up for detecting the immunofluorescence is identical as in Fig. 2H.  
(B) Expression of cardiac transcription factors in human heart (positive control), MSC and cMABs. qPCR of n=3-4 experiments are shown.
Online Figure VIII: Stainings of hearts after acute myocardial infarction treated with cMAB

(A-B) Immunofluorescent images of smooth muscle actin (red) in the infarcted region. Human Alu probes (green) was used to detect human cells. Nuclei were stained by DAPI. Blue arrows indicate human Alu-positive smooth muscle cells. Yellow arrow suggests differentiated Alu-positive endothelial cells.

(C-D) Differentiated cardiomyocytes are indicated by human nuclear antigen (hNA)-positive (green) α-sarcomeric actinin-positive cells (red). Nuclei are stained with DAPI (blue). White arrows indicate hNA positive actinin positive cells. Pink arrow indicate non-differentiated cells. Bar indicates 20µm (C) and 50µm (D).
Online Figure IX: Stainings of hearts after acute myocardial infarction treated with single cell-derived cMAB. Immunofluorescent image of alpha-sarcomeric actinin (A), smooth muscle actin (B), and von Willebrand factor (vWF)(C) in the infarcted region. Human nuclear antigen antibodies (hNA) (green) were used to detect human cells. Nuclei were stained with DAPI. White arrows indicate hNA-positive cells which are expressing the respective differentiation markers. Bar indicates 50 µm or 20 µm.
Online Figure X: Quantification of secreted pro-angiogenic cytokines in supernatants of HUVEC, adult endothelial progenitor cells (EPC), and children-derived circulating mesoangioblasts (cMAB) culture. Data obtained by ELISA of insulin-like growth factor (IGF), stromal-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) are shown. Data are results per 10000 cells.
Online Figure XI: Sox2 transduction using lentiviral vectors in cMAB. (A) Sox2 expression after Sox2 transduction. Bar indicate 100 µm. Untransduced cells served as control. SOX2-transduced cells formed embryoid body-like structure after transfer to gelatin-coated dishes (B and C).
Online Figure XII: Differentiation of Sox2-transduced cMABs. Immunostaining (A) and qPCR (B) after induction of hepatocyte differentiation. n=3 each.
Online Figure XIII: Differentiation of Sox2-transduced cMABs. Immunostaining (A, C, and D) and qPCR (B) after induction of neural differentiation. n=3 each
Online Figure XIV  Koyanagi et al

Echocardiographic data after injection of PBS or Sox2-transduced cMABs. Wall motion score index (A; Zhang Y, Yeghiazarians Y et al, AJP 2007) was measured at day 0, day 14, and day 28 (A). Time course of percent fractional shortening (%FS) (B), was measured by a blinded observer. No difference in all parameters at day 0 indicate that initial infarct size was similar in both groups. n = 8 (PBS) and n=4 (SOX2).
Online Figure XV: Stainings of hearts after acute myocardial infarction treated with Sox2-transduced cMAB which express αMHCp-driven GFP. Immunofluorescent images of GFP under control of alpha-sarcomeric actinin promoter (A) and untransduced control (B).
Online Figure XVI: Koyanagi et al

TP53 (A) and p21 (B) expressions (qPCR) in neuronal stem cells (NSC), and cMAB.
Online Table I  Koyanagi et al

**Patient characteristics of first 12 patients (in Figure 1)**

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summary

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PD: population doubling
DORV: double outlet right ventricle
LVOTO: left ventricular outflow tract obstruction
CAD: coronary artery disease
TGA: transposition of great artery
AVSD: atrial and ventricular septal defect
VSD: ventricular septal defect
Marker profiles of human umbilical vein endothelial cells (HUVEC), mesoangioblasts (MAB) from human aorta, CD34 positive cells, mesenchymal stem cells (MSC), children-derived MAB-like cells, and umbilical cord blood-derived cells are shown. Confirmed data by both FACS and RT-PCR are summarized. -, +, ++, +++ indicate no expression (0%), low expression (1-40%), high expression (40-80%), and highly expression (80-100%) by FACS measurement, respectively. Umbilical cord blood-derived cells were obtained from umbilical cord blood with the same protocol as used for culturing children-derived MAB-like cells.
Comparison of the marker expressions of CD45-negative human stem / progenitor cells described in the literature

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<tr>
<td>Mesenchymal marker</td>
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<tr>
<td>CD13</td>
<td>high</td>
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<td>-</td>
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</tr>
<tr>
<td>CD73</td>
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<td>-</td>
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<tr>
<td>Hematopoietic marker</td>
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</tr>
<tr>
<td>CD45</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>CD133</td>
<td>-</td>
<td>- or low</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Adhesion / Integrin marker</td>
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<tr>
<td>CD18</td>
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<td>CD29</td>
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</tr>
<tr>
<td>CD44</td>
<td>high</td>
<td>- or low</td>
<td>high</td>
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</table>

Marker profiles of CD45-negative human stem / progenitor cells as described in the cited references are summarized. MAB: mesoangioblasts, BM: bone marrow, UCB: umbilical cord blood, MAPC: multipotent adult progenitor cells, USSCs: Unrestricted Somatic Stem Cells

References:
Characteristics of nude mice, which were used for the detection of functional improvement after cell implantation in the myocardial infarction (MI) experiments. HR; heart rate, bpm; beat per minute, LVESP; left ventricle end-systolic pressure, LVEDP; left ventricle end-diastolic pressure
* indicates p< 0.01 vs sham. # and ## indicates p< 0.05 and p<0.01 vs MI PBS group, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=3)</th>
<th>MI+PBS (n=7)</th>
<th>MI+MAB (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (weeks)</strong></td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>684±10</td>
<td>525±84</td>
<td>520±117</td>
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<tr>
<td><strong>LVESP (mmHg)</strong></td>
<td>99±20</td>
<td>93±12</td>
<td>86±5</td>
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<tr>
<td><strong>LVEDP (mmHg)</strong></td>
<td>4.8±0.3</td>
<td>11.8±2.3 *</td>
<td>6.4±3.2 ##</td>
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<tr>
<td><strong>Tau (msec)</strong></td>
<td>4.5±0.9</td>
<td>6.7±1.0 *</td>
<td>5.5±0.9 #</td>
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</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>MI+PBS (n=7)</th>
<th>MI+MAB (GFP) (n=7)</th>
<th>MI+MAB (SOX2) (n=8)</th>
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</thead>
<tbody>
<tr>
<td><strong>Age (weeks)</strong></td>
<td>12</td>
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<td>12</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>462±11</td>
<td>423±30</td>
<td>462±20</td>
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<tr>
<td><strong>Pmax (mmHg)</strong></td>
<td>92±2</td>
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<td>93±2</td>
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<tr>
<td><strong>LVESP (mmHg)</strong></td>
<td>91±2</td>
<td>90±2</td>
<td>91±2</td>
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<tr>
<td><strong>LVEDP (mmHg)</strong></td>
<td>27.1±1.9</td>
<td>18.0±1.5 **</td>
<td>13.3±0.7 **; #</td>
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<tr>
<td><strong>dP/dt max</strong></td>
<td>6526±450</td>
<td>6350±122</td>
<td>7902±650 #</td>
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<tr>
<td><strong>dP/dt min</strong></td>
<td>4414±394</td>
<td>4927±297</td>
<td>6251±501 **; #</td>
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<tr>
<td><strong>Tau (msec)</strong></td>
<td>13.3±1.2</td>
<td>11.0±1.0</td>
<td>9.3±0.6 *</td>
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</tbody>
</table>

* and ** indicates p<0.05 and p< 0.01 vs MI+PBS. # indicates p< 0.05 vs MI MAB (GFP) group, respectively.
<table>
<thead>
<tr>
<th>Endothelial differentiation</th>
<th>VEGF</th>
<th>bFGF</th>
<th>IL-6</th>
<th>erythropoietin</th>
<th>none</th>
<th>IMDM with 15% FBS +insulin</th>
<th>7 days</th>
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</thead>
<tbody>
<tr>
<td>smooth muscle differentiation</td>
<td>TGFβ1 (5 ng/ml)</td>
<td>fibronectin</td>
<td>DMEM with 10% FBS</td>
<td>14 days</td>
<td></td>
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<tr>
<td>condition 1</td>
<td>FGF8b (30 ng/ml)</td>
<td>Jagged-1 (10mg/ml)</td>
<td>DMEM + medium 199 (4:1) with 8% FBS</td>
<td>3 days, 7 days and 14 days</td>
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<tr>
<td>smooth muscle differentiation</td>
<td>Heparin (10 μg/ml)</td>
<td>collagen (non-permeable membrane)</td>
<td>DMEM + medium 199 (4:1) with 8% FBS</td>
<td>10 days</td>
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<tr>
<td>condition 2</td>
<td>Co-culture with rat CM (non-fusion assay)</td>
<td>gelatine</td>
<td>DMEM + medium 199 (4:1) with 8% FBS</td>
<td>6 days</td>
<td></td>
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<tr>
<td>cardiac myocytes differentiation</td>
<td>Co-culture with rat CM gelatein DMEM / medium 199 (4:1) with 8% FBS</td>
<td>Wnt3a (100 ng / ml)</td>
<td>none</td>
<td>DMEM / F-12 (Ham) (1:1) with 10% FBS</td>
<td>3 days</td>
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<tr>
<td>condition 3</td>
<td>Dexamethasone (10 nM)</td>
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</tbody>
</table>

In vitro differentiation assay of children derived circulating mesoangioblasts-like cells for endothelial cells, smooth muscle cells (SMC), and cardiomyocytes (CM). Stimuli, matrixes, medium, and time of culture are indicated.
<table>
<thead>
<tr>
<th></th>
<th>stimuli</th>
<th>matrix</th>
<th>medium</th>
<th>duration</th>
</tr>
</thead>
</table>
| Neuronal differentiation | bFGF (20 ng/ml)  
EGF (10 ng/ml) | none   | DMEM:F12 with N2 and B27 supplement + insulin + heparin | 7 days and 28 days (no medium change after 7 days) |
| Hepatocyte differentiation | bFGF (10 ng/ml)  
HGF (20 ng/ml)  
Nicotinamide (0.6 g/l) | none   | IMDM                                             | 7 days                 |

Conditions used for the in vitro differentiation assays to differentiate cMABs to neuronal cells (Modified protocol according to Journal of Neuroscience Research 2002; 69:894) and to hepatocytes (protocol according to Hepatology, 2004; 40: 1275). Stimuli, matrixes, medium, and time of culture are indicated.