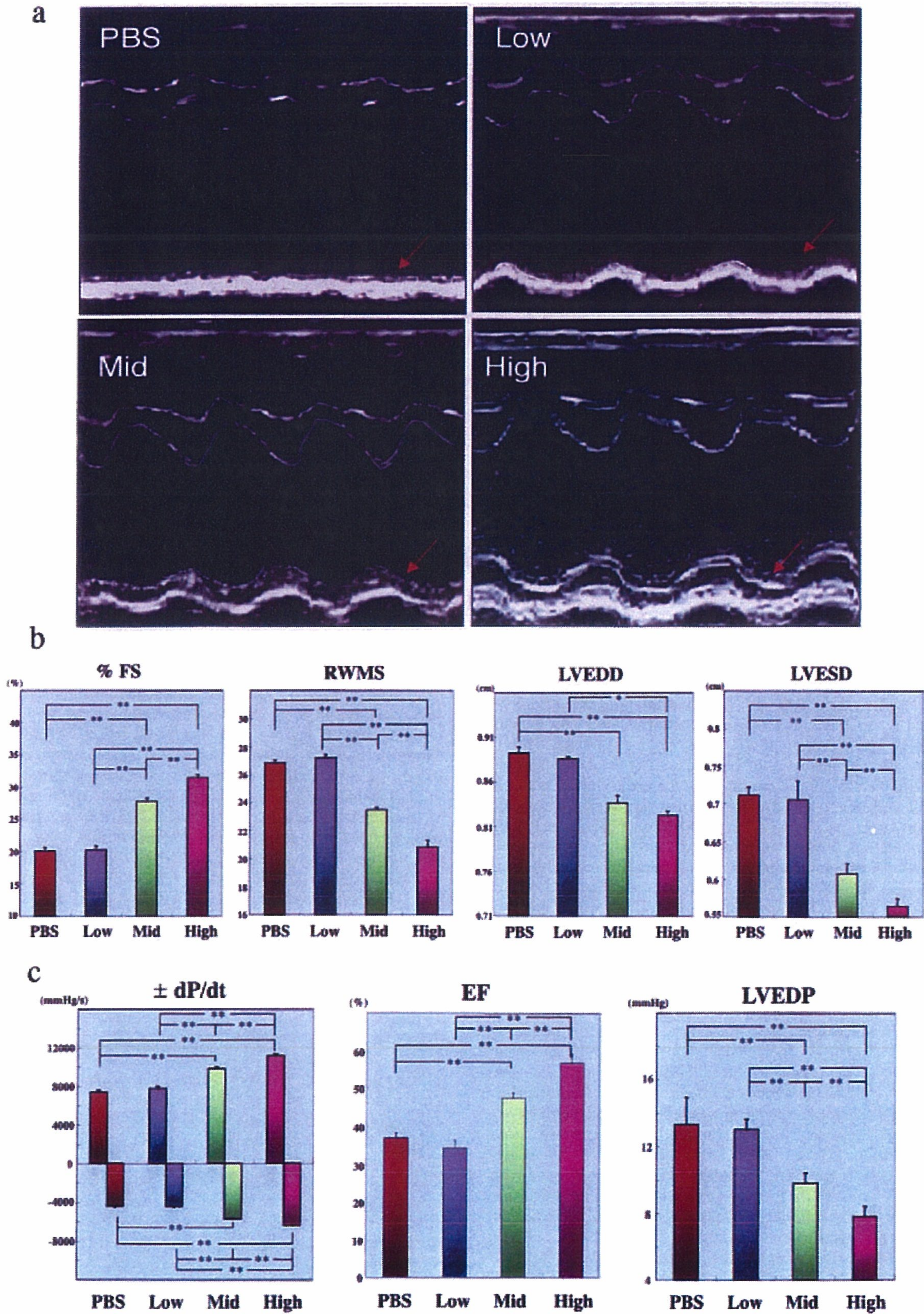


Figure 2. Functional evaluation of treated heart by echocardiography. a, Representative recording of M-mode echocardiography 28 days after cell or PBS administration. Lateral wall motion was dose-dependently preserved (arrow: endocardium in lateral wall). b, Echocardiographic parameters 28 days after cell transplantation. Global and regional LV function was dose-dependently preserved after CD34$^+$ cell transplantation. $^*P<0.05$, $^{**}P<0.01$ (n=12 in all groups). c, Invasive hemodynamic parameters after CD34$^+$ cell or PBS administration at day 28. The functional parameters were dose-dependently preserved after CD34$^+$ cell transplantation. +dP/dt and −dP/dt indicate maximum and minimum derivative of LV pressure (positive values indicate +dP/dt, and negative values show −dP/dt). $^*P<0.05$, $^{**}P<0.01$ (n=12 in each group).

AAC ATC ACA; antisense CTG TCC TTC TTA AAC TCC GCA CAG C; hGAPDH primer sequence (596 bp): sense CTG ATG CCC CCA TGT TCG TC; antisense CAC CCT GTT GCT GTA GCC AAA TTC G; total GAPDH primer sequence (320 bp): sense GTG CCA GCC TCA TGT TCG TC; antisense CGC CAG TGT ACT CCA CGA CAT TTC G; hKDR primer sequence (468 bp): sense CAA ATG TGA AGC GGT CAA CAA AGT T; antisense ATG CTT TCC CCA ATA CTT GTC GTC T.

Statistical Analysis

The results were statistically analyzed with the use of a software package (Statview 5.0, Abacus Concepts Inc, Berkeley, Calif). All values were expressed as mean±SE. Paired t tests were performed for comparison of data before and after treatment. The comparisons among 4 groups were made with 1-way ANOVAs. Post hoc analysis was performed by Fisher protected least significant difference test. Differences of $P<0.05$ were considered statistically significant.

Results

Characterization of Freshly Isolated Peripheral Blood CD34$^+$ Cells

The CD34$^+$ cell fraction had a purity of >99%, as determined by FACS analysis with the use of anti-CD34, anti-CD45, anti-CD31, anti-AC133, anti-KDR, and anti–VE-cadherin monoclonal antibodies (Figure 1a and 1b). The RT-PCR analysis revealed the human-specific gene expression of CD34 and SMA but not of cardiomyocyte markers (hBNP, hcTn-I, hMHC-α, hMHC-β, and hNkx 2.5), hKDR, and hsm22α (Figure 1c).

Transplanted CD34$^+$ Cells Dose-Dependently Preserve LV Function After MI

There were no significant differences in preoperative echocardiographic parameters, LVEDD, LVESD, FS, and RWMS among high, mid, low, and PBS groups. Echocardiography on day 5 revealed that the functional parameters were also similar in all groups (data not shown). Echocardiography performed 4 weeks after cell transplantation demonstrated that LVEDD was significantly smaller in the high group than in the low and PBS groups ($P<0.05$ versus low and $P<0.01$ versus PBS). LVEDD was also significantly smaller in the mid group than in the PBS group ($P<0.01$). However, LVEDD was similar in the high and mid groups and in the low and PBS groups (Figure 2a and 2b). LVESD 4 weeks after MI was significantly smaller in the high group than in the mid group and in the mid group than in the low group (high group, 0.564±0.01; mid group, 0.607±0.013; low group, 0.705±0.025; PBS group, 0.711±0.011 cm; $P<0.01$ for high versus mid and mid versus low) (Figure 2b). FS was significantly greater in the high group than in the mid group and in the mid group than in the low group (high group, 31.4±0.43%; mid group, 27.7±0.45%; low group, 20.2±0.58%; PBS group, 20.0±0.54%; $P<0.01$ for high versus mid and mid versus low) (Figure 2a and 2b). RWMS was significantly better preserved in the high group than in the mid group and in the mid group than in the low group (high group, 20.8±0.46; mid group, 23.4±0.15; low group, 27.2±0.2; PBS group, 26.8±0.24; $P<0.01$ for high versus mid and mid versus low). LVEDD, LVESD, FS, and RWMS 4 weeks after transplantation in the low group were not significantly different from those in the PBS group (Figure 2a and 2b).

Invasive Hemodynamic Parameters After CD34$^+$ Cell or PBS Administration at Day 28

Group	n	Heart Rate, bpm	Systolic Left Ventricular Pressure, mm Hg	Diastolic Left Ventricular Pressure, mm Hg
PBS	12	296±1.9	110±1.9	5.5±1.4
Low	12	302±2.0	112±3.0	3.5±0.5
Mid	12	300±2.4	117±1.8	3.9±0.5
High	12	300±2.1	120±3.1	3.3±0.4

Invasive hemodynamic study performed 4 weeks after transplantation revealed that heart rate and diastolic blood pressure were similar in each group. Systolic blood pressure was significantly greater in the high and mid groups than in the PBS group ($P<0.05$) (Table). The +dP/dt, absolute value of −dP/dt, and EF were significantly greater in the high group than in the mid group and in the mid group than in the low group (+dP/dt: high group, 11 131±106; mid group, 9772±111; low group, 7734±160; PBS group, 7322±233 mm Hg/s; $P<0.01$ for high versus mid and mid versus low) (Figure 2c) (−dP/dt: high group, −6403±209; mid group, −5753±170; low group, −4413±230; PBS group, −4415±212 mm Hg/s; $P<0.01$ for high versus mid and mid versus low) (Figure 2c) (EF: high group, 56.8±2.3%; mid group, 47.5±1.4%; low group, 34.2±2.1%; PBS group, 36.9±1.4%; $P<0.01$ for high versus mid and mid versus low) (Figure 2c). In addition, LVEDP 4 weeks after ischemia was significantly smaller in the high group than in the mid group and in the mid group than in the low group (high group, 7.8±0.6; mid group, 9.8±0.6; low group, 13.0±0.7; PBS group, 13.3±1.6 mm Hg; $P<0.01$ for high versus mid and mid versus low) (Figure 2c). The +dP/dt, −dP/dt, EF, and LVEDP 4 weeks after transplantation in the low group were not significantly different from those in the PBS group.

Thus, transplantation of high and mid doses of CD34$^+$ cells, but not the low dose, significantly preserved global and regional LV function after MI. The functional effect of CD34$^+$ cells was dose-dependently observed.

Morphometric Evaluation of Capillary Density and Infarct Size

Myocardial neovascularization assessed by capillary density on day 28 was dose-dependently enhanced in rats receiving CD34$^+$ cell transplantation (high group, 714.3±25.0; mid group, 535.8±31.0; low group, 320.9±36.0; PBS group, 291.3±19.0/mm^2; $P<0.01$ for high versus mid and mid versus low). Capillary density in the low group was similar to that in the PBS group (Figure 3a and 3b). LV remodeling evaluated by the percent fibrosis area was dose-dependently inhibited in rats receiving CD34$^+$ cell transplantation (high group, 16.0±2.6%; mid group, 22.4±1.9%; low group, 30.7±3.9%; PBS, 31.5±0.7%; $P<0.01$ for high versus mid and mid versus low groups). Percent fibrosis area was similar in the low and PBS groups (Figure 3c and 3d).

Thus, transplantation of high and mid CD34$^+$ cells, but not the low dose, significantly preserved LV structural integrity

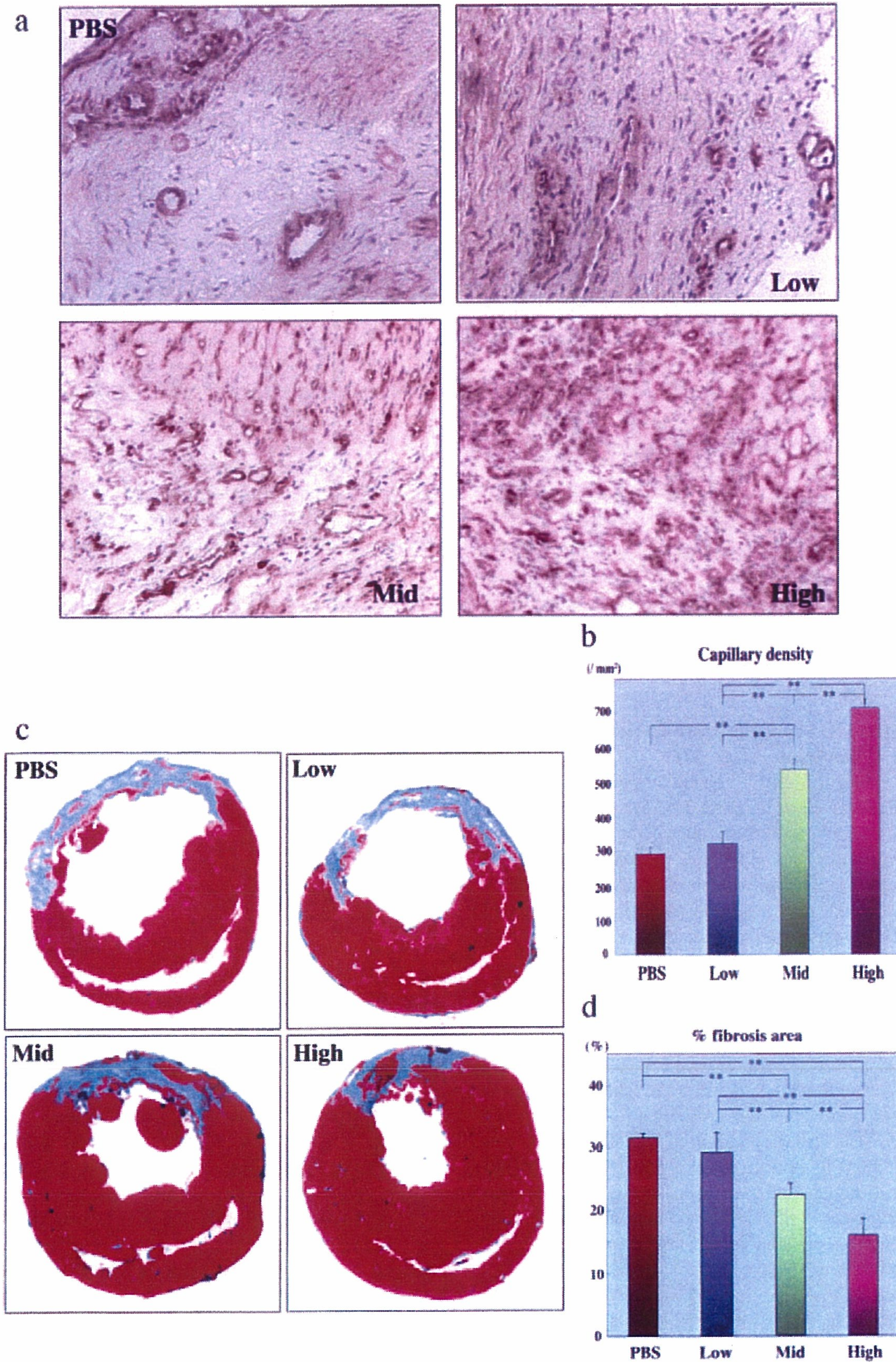


Figure 3. Histological evaluation of myocardial neovascularization and LV remodeling after MI. a, Representative immunostaining for isolectin B4 in each group at day 28 (magnification ×200). b, Capillary density in rats receiving $CD34^+$ cells or PBS at day 28. Ischemic neovascularization was dose-dependently enhanced after $CD34^+$ cell transplantation. $^{**}P<0.01$ (n=8 in all groups). c, Representative Masson trichrome staining at day 28 in each group. d, Ratio of fibrosis area/LV area (percent fibrosis area) at day 28 in each group. LV remodeling after MI was dose-dependently inhibited after $CD34^+$ cell transplantation. $^{**}P<0.01$ (n=8 in each group).

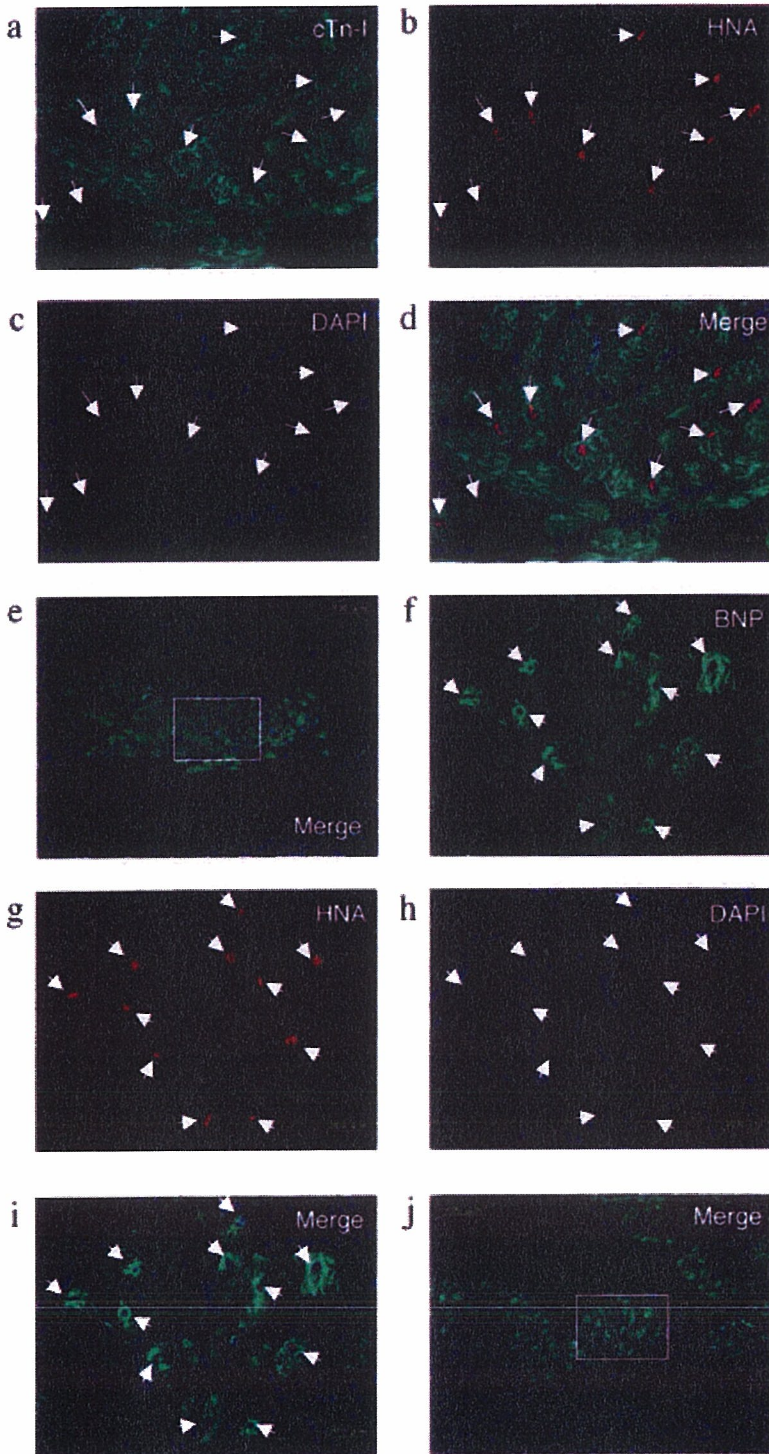
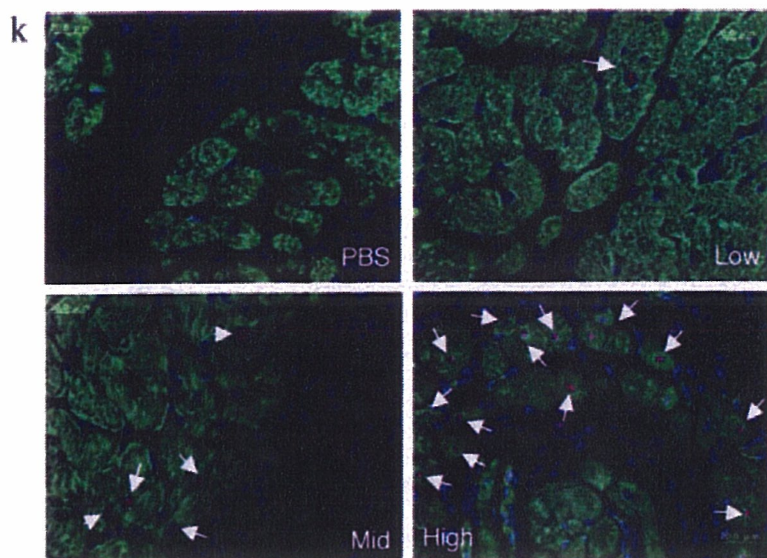


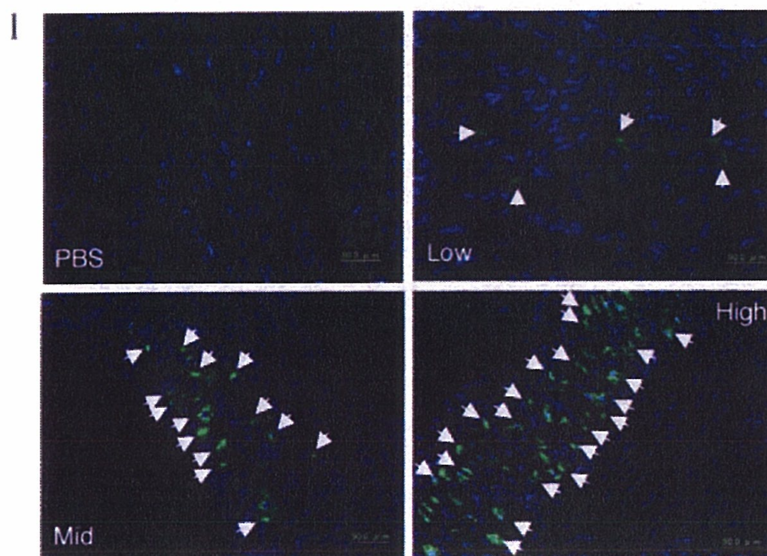
Figure 4. Histological evaluation of human cardiomyocyte development in rat ischemic myocardium. a to e, Representative double immunofluorescence staining for cTn-I and HNA in high-dose group at day 28. Human cardiomyocytes were identified as double-positive cells for cTn-I (green) and HNA (red). a, cTn-I, magnification ×400; b, HNA, ×400; c, DAPI, ×400; d, merge, ×400; e, merge, ×100. White arrows show nuclei of human cardiomyocytes. f to j, Representative double-immunofluorescence staining for hBNP and HNA in high-dose group at day 28. Human cardiomyocytes were identified as double-positive cells for hBNP (green) and HNA (red). f, hBNP, magnification ×400; g, HNA, ×400; h, DAPI, ×400; i, merge, ×400; j, merge, ×100. White arrows show nuclei of human cardiomyocytes. k, Representative double-immunofluorescence staining for cTn-I and HNA at day 28 in each group. Human cardiomyocytes were identified as double-positive cells for cTn-I (green) and HNA (red) (magnification ×400). White arrows show nuclei of human cardiomyocytes. The double-positive cells for cTn-I and HNA derived from transplanted cells were dose-dependently observed in ischemic myocardium (magnification ×400). l, Representative fluorescence immunohistochemical images for hBNP at day 28 in each group. Green fluorescence shows hBNP-positive cells, and blue indicates DAPI for nuclear staining. The hBNP-positive cardiomyocytes derived from transplanted cells were dose-dependently observed in ischemic myocardium (magnification ×200). m, Human cardiomyocytes (CMC) (hBNP-positive cardiomyocytes: black bar) and total (both human and rat) cardiomyocytes (white bar) on day 28 were dose-dependently observed in the ischemic myocardium. *$P<0.05$, **$P<0.01$ (n=8 in all groups).

after MI. The histological efficacy of CD34$^+$ cells was dose-dependently observed.

Transplanted hCD34$^+$ Cells Dose-Dependently Differentiate Into Cardiomyocytes

Differentiated human cardiomyocytes derived from the transplanted CD34$^+$ cells were mainly identified in the rat peri-infarct myocardium by double staining both for cTn-I and HNA (Figure 4a to 4e) and for hBNP and HNA (Figure 4f to 4j). These findings suggest that transplanted CD34$^+$ cells have potency of differentiation into cardiomyocytes. Double immunohistochemistry with hBNP and HNA also revealed specificity of the hBNP antibody for human cells in rat myocardium. Dose-dependent distribution of human cardiomyocytes in rat myocardium was observed both in samples stained with cTn-I and HNA (Figure 4k) and in samples stained with hBNP (Figure 4l). In fact, the numbers of hBNP-positive cardiomyocytes were dose-dependently observed in ischemic myocardium at day 28 (high group, 2480±149; mid group, 1860±141; low group, 423±9; PBS





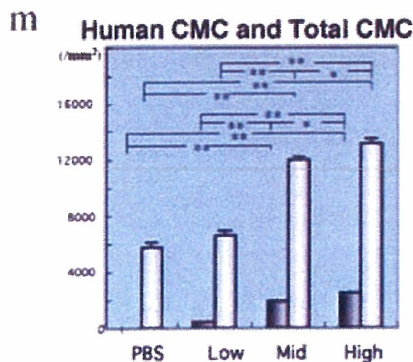


Figure 4. Continued

group, $0\pm0/\mathrm{mm}^2$; $P<0.05$ for high versus mid and mid versus low groups). Total (both human and rat) cardiomyocytes were also dose-dependently observed in ischemic myocardium at day 28 (high group, $13\,102\pm298$; mid group, $11\,936\pm238$; low group, 6564 ± 369; PBS group, $5707\pm300/\mathrm{mm}^2$; $P<0.05$ for high versus mid and mid versus low groups) (Figure 4l and 4m). Similar dose-dependent cardiomyogenesis was observed when CD34$^+$ cells from another patient were used (Figure I in the online-only Data Supplement). These findings strongly suggest that transplanted CD34$^+$ cells may have dose-dependent potency of differentiation into cardiomyocytes.

Transplanted hCD34$^+$ Cells Dose-Dependently Differentiate Into SMCs

Human SMCs derived from the transplanted CD34$^+$ cells were mainly identified in the vasculatures within the peri-infarct area

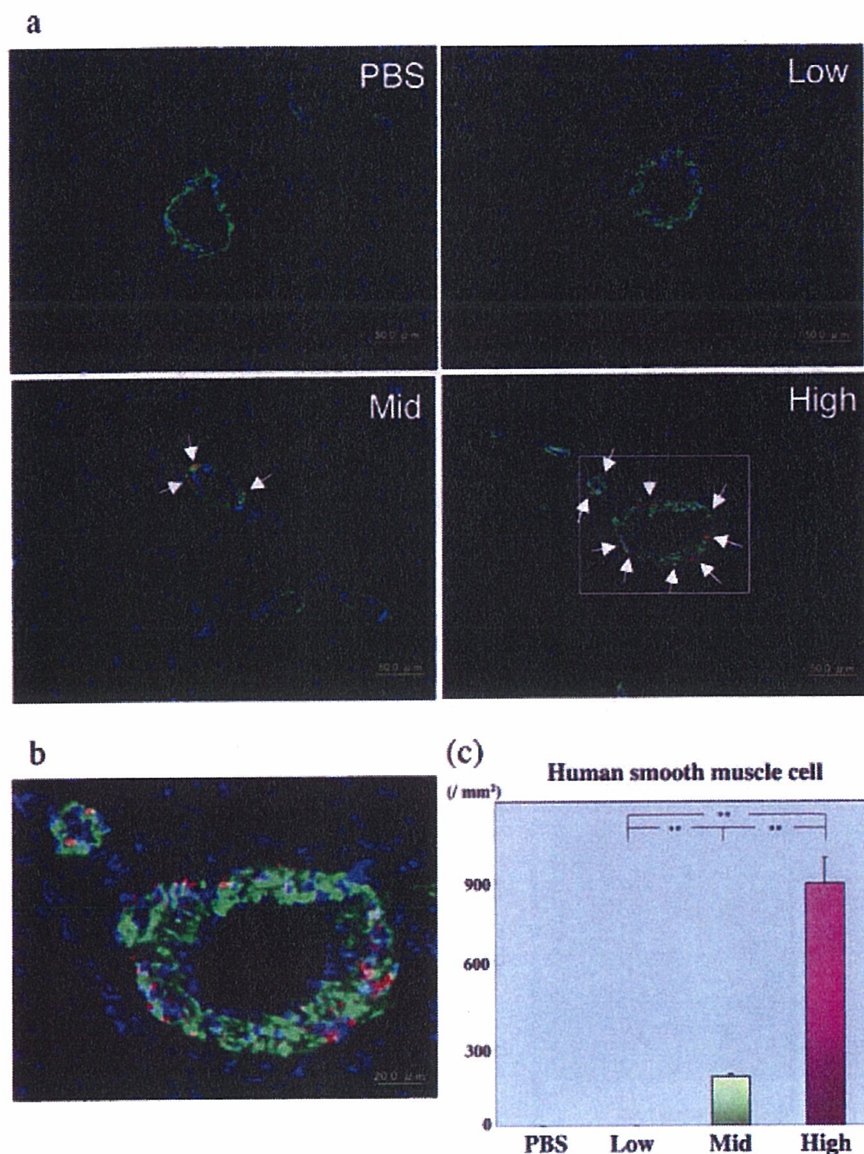


Figure 5. Histological evaluation of human SMC development. a, Representative double-immunofluorescence staining for SMA and HLA-ABC at day 28 in each group (magnification ×200). Human vascular SMCs were identified as double-positive cells for SMA (green) and HLA-ABC (red). b, Representative double-immunofluorescence staining for SMA and HLA-ABC at day 28 in high group (magnification ×400). c, Human SMCs on day 28 were dose-dependently observed in rat ischemic myocardium. **$P<0.01$ (n=8 in each group).

by double staining for SMA and HLA-ABC. Identified human SMCs were dose-dependently observed after CD34$^+$ cell transplantation (high group, 895±95; mid group, 180±11; low group, 0±0; PBS group, 0±0/mm^2; $P<0.01$ for high versus mid and mid versus low groups). In contrast, differentiated human SMCs were not identified in PBS and low groups (Figure 5a to 5c). Similar dose-dependent SMC commitment was observed when CD34$^+$ cells from another patient were used (Figure II in the online-only Data Supplement). These findings suggest that transplanted CD34$^+$ cells may have dose-dependent potency of differentiation into SMCs.

Transplanted hCD34$^+$ Cells Dose-Dependently Differentiate Into ECs

We confirmed the specificity of UEA-1 staining for human ECs using double immunohistochemistry with UEA-1 and HNA (Figure 6a to 6e). Differentiated human ECs derived from the transplanted CD34$^+$ cells were observed in the vasculatures within the peri-infarct area by UEA-1 staining. Identified UEA-1–positive cells were greater in higher-dose groups than lower-dose groups (high group, 3373±363; mid group, 980±211; low group, 226±35; PBS group, 0±0/mm^2; $P<0.05$ for high versus mid and mid versus low groups). In contrast, differentiated human ECs were not identified in the PBS group (Figure 6f, 6g). Similar dose-dependent endothelial commitment was observed when CD34$^+$ cells from another patient were used (Figure III in the online-only Data Supplement).

Thus, locally transplanted CD34$^+$ cells were incorporated not only into ECs but also into mature SMCs, resulting in contribution to vasculogenesis in ischemic myocardium.

FISH Analysis of Transplanted CD34$^+$ Cell–Derived Cardiomyocytes

To determine whether cardiac repair occurred through cell fusion in MI tissue, we performed FISH with human Y chromosomes and rat genome probe. The specificity of the probes was tested in tissues of normal rat heart and rat heart immediately after human cell transplantation. We confirmed that these 2 probes did not cross-react with cells of the other species (data not shown). The FISH analysis revealed the

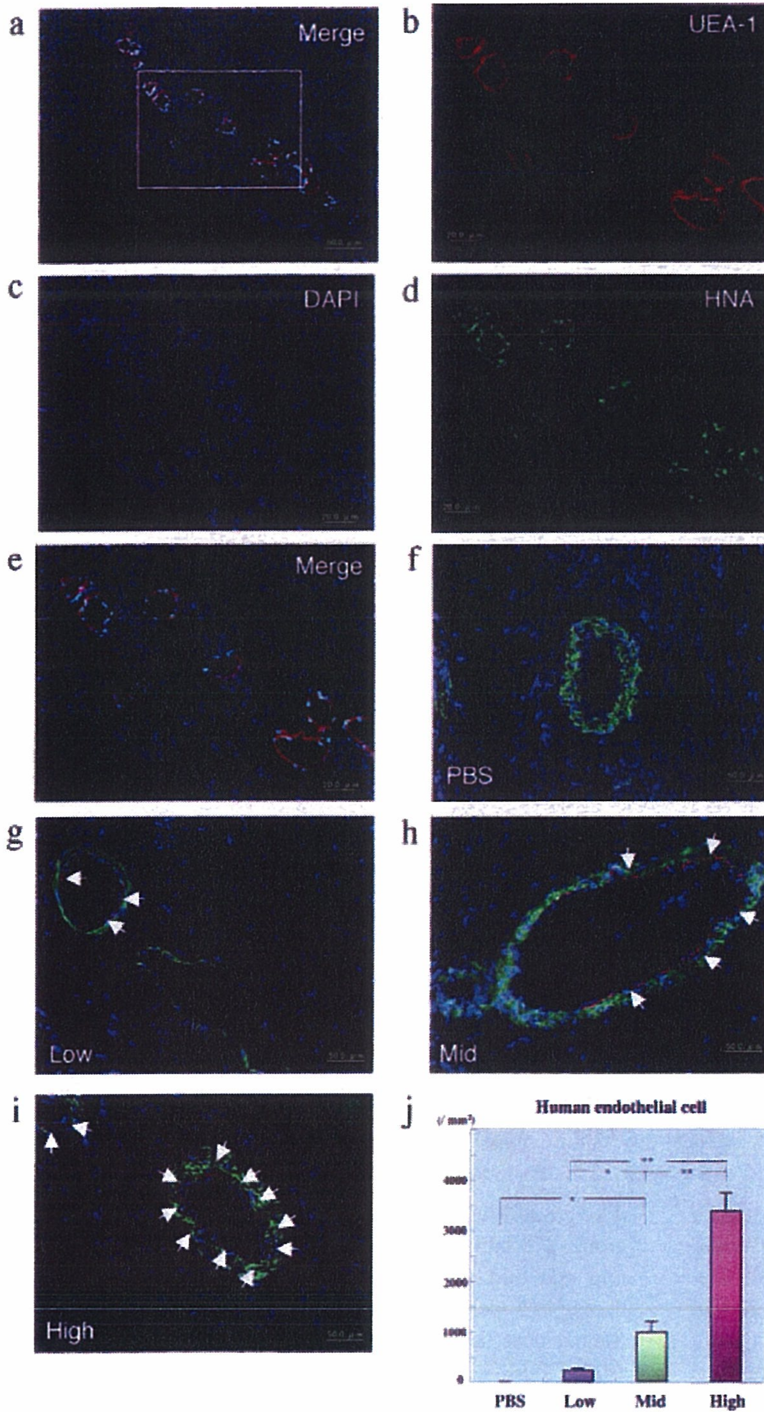


Figure 6. Histological evaluation of human EC development. a to e, Representative double-immunofluorescence staining for UEA-1 (red) and HNA (green) in high-dose group at day 28. Human ECs were identified as double-positive cells for UEA-1 and HNA. a, Merge, magnification ×100; b, UEA-l, ×400; c, DAPI, ×400; d, HNA, ×400; e, merge, ×400. f to j, Representative double-immunofluorescence staining for HNA (green) and UEA-1 (red) at day 28 in each group (magnification ×200). Human ECs were identified as UEA-1–positive cells (arrow). In the PBS group (f), differentiated human ECs were not identified. In the low group (g), differentiated human ECs were rarely identified. In the mid group (h), human ECs were more frequently identified than in the low and PBS groups. In the high group (i), human ECs were further more frequently identified than in other groups. j, Human ECs on day 28 were dose-dependently observed in the ischemic myocardium. *$P<0.05$, **$P<0.01$ (n=8 in all groups).

existence of cardiomyocytes in which human Y chromosome was paired with rat genome (cell fusion) as well as those without genome (no fusion) (Figure 7a to 7d). These findings indicate that both cell fusion and multilineage differentiation without fusion may be involved in transformation of transplanted $CD34^+$ cells into cardiomyocytes.

Dose-Dependent Gene Expression of Human-Specific Cardiac, Smooth Muscle, and Endothelial Lineage Cell Markers in Rat Ischemic Myocardium After $CD34^+$ Cell Transplantation

To further ensure the immunohistochemical results with regard to cardiomyogenesis and vasculogenesis by the molecular approach, we performed RT-PCR with rat ischemic myocardium by using human-specific primer BNP, cTn-I, MHC-α, MHC-β, and Nkx 2.5 as human cardiomyocyte lineage markers, human-specific primer sm22α and SMA as human SMC markers, and human-specific primer CD31 and KDR as human EC markers.

The RT-PCR analysis revealed dose-dependent expression of human-specific cardiomyogenic, arteriogenic, and vasculogenic genes in rat ischemic myocardium after human $CD34^+$ cell transplantation with the use of the NIH Image program (version 1.62) (Figure 8a and 8b). Notably, gene expression of all markers except hSMA was not detected in freshly isolated

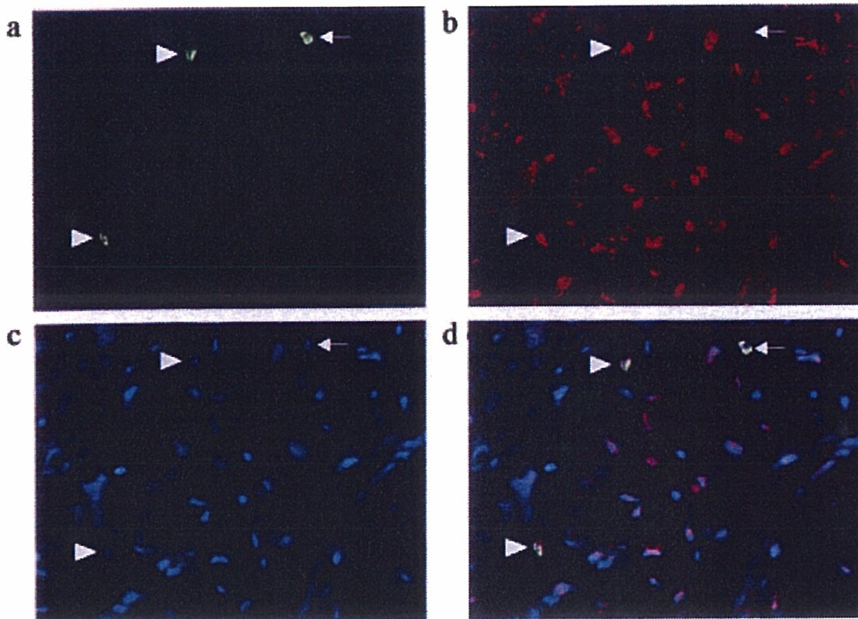


Figure 7. Representative FISH analysis at day 28 with the use of human Y chromosome probe and rat genome probe to assess cardiomyogenesis mechanism. Human cardiomyocytes due to cell fusion (arrow heads) were identified as double-positive cells for human Y chromosomes (yellow) and rat genome (red). Human cardiomyocytes developed without cell fusion (arrow) were identified as only positive cells for human Y chromosome. a, FISH image for human Y chromosome probe; b, FISH image for rat genome probe; c, DAPI staining; d, merged image.

CD34$^+$ cells (Figure 1c) but was dose-dependently augmented in ischemic myocardium after cell transplantation.

Discussion

Abrupt occlusion of coronary arteries causes MI, which leads to massive cardiomyocyte loss and consequently deterioration of cardiac function because cardiomyocytes have severely limited capacity to be divided and thus replace the damaged tissue. Progressive heart failure is a major cause of death or frequent hospitalization in patients after MI. Although MI is classified as vascular (coronary artery) disease, therapeutic strategies should be focused on regenerating not only blood vessels but also cardiac muscle to improve the poor prognosis of the disease.

Compelling evidence suggests that transplantation of bone marrow–derived CD34$^+$ cells or cultured EPC-enriched population contributes to preservation of LV function after MI through enhancing ischemic neovascularization.[10–12] The mechanism of this therapeutic effect was previously considered to be incorporation, differentiation, and proliferation of EPCs for new blood vessel formation.[9,11,26] Recently, Badorff et al[27] reported in vitro transdifferentiation of EPCs into functional cardiomyocytes. Yeh et al[16] also demonstrated in vivo differentiation of CD34$^+$ cells into cardiomyocytes and SMCs in a mouse model of acute MI. Regeneration of SMCs as well as ECs may result in mature vasculogenesis, which is more potent for blood flow recovery in ischemic myocardium compared with capillary formation by EC-only regeneration. These findings lead to a novel concept that CD34$^+$ cell transplantation may contribute to cardiomyogenesis and vasculogenesis, which may be an ideal strategy to treat MI. On the other hand, Balsam et al[18] and Murry et al[19] reported that mouse bone marrow HSCs isolated as Lin$^-$c-Kit$^+$ cells or c-Kit$^+$Thy1.1^{lo}Lin-Sca-1$^+$ cells do not transdifferentiate into cardiomyocytes in infarcted myocardium. Several points should be considered carefully with regard to this discrepancy, such as the difference in species (human versus murine), cell populations (CD34$^+$ cells versus Lin$^-$c-Kit$^+$ cells or c-Kit$^+$Thy1.1^{lo}Lin-Sca-1$^+$), cell doses to transplant, or cell delivery methodologies. Given the controversy, the question is whether improvement of myocardial function after EPC transplantation was due to myocardial preservation through the signal from enhanced vasculogenesis as well as due to regenerative cardiomyogenesis by transplanted cells. To solve this issue, we tried to confirm the lineage potency and the tissue plasticity of CD34$^+$ cells by transplanting the cells into immunodeficient rats with MI in a dose-ranging fashion. From a practical point of view, a significant contribution of cardiomyogenesis and vasculogenesis to LV functional recovery after MI may not be expected if such translineage differentiation is a rare event after CD34$^+$ cell transplantation. To detect the translineage differentiation of human CD34$^+$ cells into rat myocardium, we performed not only immunohistochemistry but also RT-PCR for human-specific markers of cardiomyocytes, SMCs, and ECs. These sensitive assessments revealed dose-dependent augmentation of cardiomyogenesis and vasculogenesis of human CD34$^+$ cells in rat infarcted myocardium. The translineage potential was accompanied with dose-dependent enhancement of capillary density, inhibition of LV fibrosis, and preservation of LV function. These findings suggest that transplantation of a higher dose of CD34$^+$ cells may be more potent for therapeutic application to the damaged myocardium than a lower dose. Another interesting finding in this study is that these favorable effects of CD34$^+$ cells were not significantly observed in the low-dose group (1×10^5 cells/kg). To our knowledge, information about a noneffective dose of CD34$^+$ cells has never been provided. Taken together, the present results strongly suggest the therapeutic importance of the cell dosage in the actual clinical application.

It is unclear what mechanism of CD34$^+$ cells is involved in multilineage commitment and significant incorporation for functional organogenesis. One mechanism for multilineage commitment is the translineage differentiation of

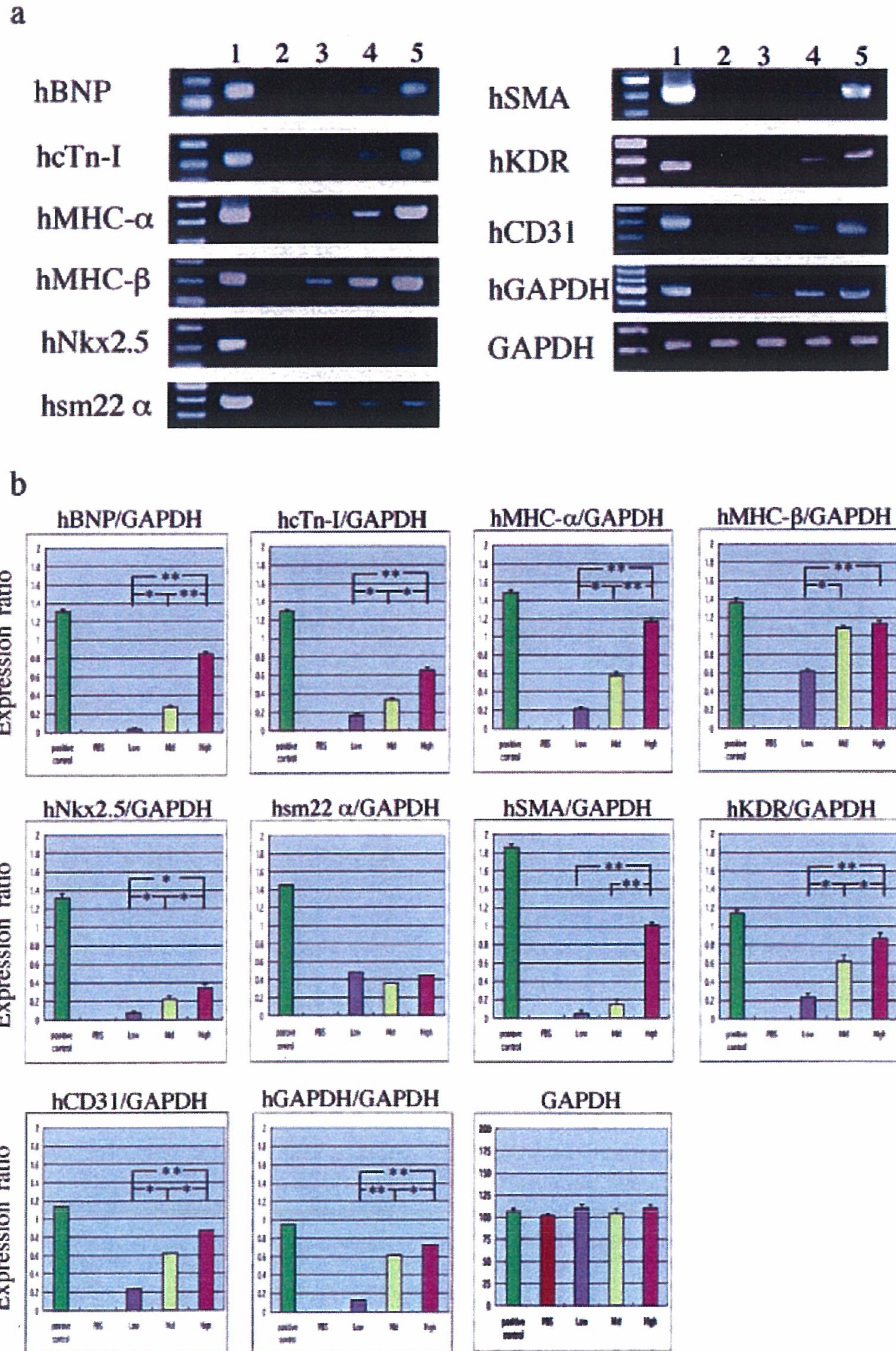


Figure 8. a, RT-PCR analysis to evaluate expression of human-specific genes of cardiomyocyte (hBNP, hcTn-I, hMHC-α, hMHC-β, and hNkx 2.5), SMC (hsm22α and hSMA), and EC (hKDR and hCD31) lineages in rat ischemic myocardium at day 28. Lane 1, human heart (positive control); lane 2, PBS group; lane 3, low group; lane 4, mid group; lane 5, high group. b, The ratio of gene expression of each human-specific marker to total (rat and human) GAPDH in MI tissue was dose-dependently augmented after CD34+ cell transplantation. Gene expression of total GAPDH mRNA levels was similar in all groups. *$P<0.05$, **$P<0.01$ (n=4 in each group).

already committed EPCs or HSCs among CD34+ cells through transdifferentiation or cell fusion. Recently, Zhang et al[17] demonstrated that 70% of newly formed cardiomyocytes derived from CD34+ cells were developed through a cell fusion mechanism between human and mouse cells, whereas CD34+-derived ECs are mainly not developed by cell fusion. The FISH analyses in this study revealed that the mechanism of cardiomyogenic plasticity of CD34+ cells involves both cell fusion and the multilineage differentiation without fusion, although the functional contribution of both mechanisms to myocardiogenesis remains uncertain.

The other possible mechanism of multilineage differentiation is due to the original multipotency of the CD34+ cell population. Recently, peripheral blood CD34+ cells were proved to contain a cell fraction expressing not only hematopoietic and endothelial but also cardiac, muscle, liver, and neural lineage markers after mobilization following G-CSF administration or myocardial ischemia,[28,29] whereas this issue is not determined if multilineage cells are derived from pluripotent stem cell or various lineage progenitor cell mixtures in CD34+ cells. When the magnificent incorporation of CD34+ cell–derived cardiomyocytes and SMCs is taken into account, the mechanism is likely due to programmed lineage commitment in the myocardial ischemia environment, although we have not defined each responsible cell fraction for lineage diversification in CD34+ cells.

The cooperative signal from vasculogenesis to cardiomyogenesis must also be considered further in the regenerative process through multilineage commitments by CD34+ cells. Cardiomyogenesis and vasculogenesis are closely regulated in terms of microenvironmental interaction during the developmental stage. Recently, Shen et al[30] proved the significance of vascular signals for postnatal neural stem cell biology, as formerly indicated in the case of liver and pancreas development in embryo.[31,32] Microenvironmental interaction between myocardial and vascular lineage cells involves not only paracrine regulatory factors but also direct cellular communications in developing CD34+ cells. We speculate that an enhanced vasculogenesis signal may exert cellular commitment and development of CD34+ cells into myocardial cells as a cooperative organogenesis mechanism.

In conclusion, after transplantation of bone marrow–derived CD34+ cells, the collaborative multilineage differentiation potential of CD34+ cells not only into ECs but also into cardiomyocytes and SMCs was enhanced by cell dose escalation and was conducive to heart regeneration in terms of functional and histological recovery through vasculogenesis and cardiomyogenesis.

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Disclosures

None.

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Gene Modified Cell Transplantation for Vascular Regeneration

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Abstract: Cell Transplantation is one of the powerful tools to ameliorate the capillary flow in ischemic condition. EPC (Endothelial Progenitor Cell) was identified in adult peripheral blood and thought to be a suitable candidate for cell transplantation. Also, gene therapy is already promising choice for enhancing angiogenic property. The combination of cell transplantation and gene therapy should be more effective way to regenerate vasculature in ischemic region. Recently, several research reports have come out regarding gene modified cell transplantation. We will mainly focus on the background of EPC, and then gene modified EPC findings in this review.

Keywords: Endothelial Progenitor Cell (EPC), Gene modified cell therapy, VEGF, TERT, Vasculogenesis.

ENDOTHELIAL PROGENITOR CELL (EPC), AS A POTENT CANDIDATE FOR CELL TRANSPLANT

Ischemic leg or heart disease is gradually increasing following several background, such as hypertension, hyperlipidemia, diabetes, and hyperuricemia. Cell transplantation, especially Endothelial Progenitor Cells (EPCs) transplantation, might be the next option for non-curable ischemic diseases compared with conventional therapies. EPCs [Asahara *et al.*, 1997; Asahara *et al.*, 1999; Gunsilius *et al.*, 2000; Shi *et al.*, 1998; Takahashi *et al.*, 1999] have been identified from adult peripheral blood as CD34, Flk-1 or CD133 antigen positive cells, which were considered to share the antigens with hematopoietic stem cell population. EPCs are considered to contribute to be incorporated into foci of neovascularization using animal model experiments. For example, EPCs were observed to be incorporated into foci of neovascularization, especially in ischemic region by myocardial [Kawamoto *et al.*, 2001] and limb [Kalka *et al.*, 2000] ischemia models. This finding, that circulating EPCs may home to sites of neovascularization and differentiate into ECs *in situ*, is consistent with "vasculogenesis", a critical paradigm for embryonic neovascularization, and suggests that vasculogenesis and angiogenesis may act as the complementary mechanisms for postnatal neovascularization. However, enthusiasm for the therapeutic potential of strategies of EPC transplantation is limited because of scarcity of these progenitor cells in adult circulating peripheral blood. For example, adjusting the number of EPCs for injection according to body weight, approximately 6 L of blood would be required for harvesting of EPCs in an average-size patient to administer a dose equivalent to that which yielded therapeutic effects in limb and myocardial ischemia in small animal models. Several strategies may be considered to overcome this issue as follows. The possible strategies would be (1) *in vivo* EPC expansion (2) *ex vivo* EPC expansion (3) local, not systemic, EPC injection (4) modification of EPC by gene transfer. Having demonstrated the potential for endogenous mobilization of BM-derived EPCs, we considered that iatrogenic expansion and mobilization of this putative EC precursor population might represent an effective means to augment the resident population of ECs that is competent to respond to administered angiogenic cytokines. Such a program might thereby address the issue of endothelial dysfunction or depletion that may compromise strategies of therapeutic neovascularization in older, diabetic, and/or hypercholesterolemic animals and patients. Granulocyte macrophage-colony stimulating factor (GM-CSF), which stimulates hematopoietic progenitor cells and myeloid lineage cells, as well as non-hematopoietic cells including BM stromal cells and ECs, has been shown to exert a potent stimulatory effect on EPC kinetics [Takahashi *et al.*, 1999]. Such cytokine-induced EPC mobilization could enhance neovascularization of severely ischemic tissues as well as *de novo* corneal vascularization [Takahashi *et al.*, 1999]. The mechanisms, whereby these EPCs are mobilized to the peripheral circulation, are in their early definitions. Among other growth factors, vascular endothelial growth factor (VEGF) is the most-critical factor for vasculogenesis and angiogenesis [Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Shalaby *et al.*, 1995]. Recent data indicates that VEGF is an important factor for the kinetics of EPC as well. Our studies performed first in mice [Asahara *et al.*, 1999] and subsequently in patients undergoing VEGF gene transfer for critical limb ischemia [Kalka *et al.*, 2000] and myocardial ischemia [Kawamoto *et al.*, 2001] established that a previously unappreciated mechanism by which VEGF contributes to neovascularization is via mobilization of BM-derived EPCs. The similar EPC kinetics modulation has been observed in response to other hematopoietic stimulators, such as granulocyte-colony stimulating factor (G-CSF) [Gehling *et al.*, 2000], angiopoietin-1 [Hattori *et al.*, 2001] and stroma derived factor-1 (SDF-1) [Peichev *et al.*, 2000]. This therapeutic strategy of EPC mobilization has recently been impli-

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cated not only by natural hamatopoietic or angiogenic stimulants but also by recombinant pharmaceuticals. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, inhibit the activity of HMG-CoA reductase, which catalyzes the synthesis of mevalonate, a rate-limiting step in cholesterol biosynthesis. The statins rapidly activate Akt signaling in ECs, stimulate EC bioactivity *in vitro* and enhance angiogenesis *in vivo* [Kureishi *et al.*, 2000]. Recently we and Dimmeler *et al.*, demonstrated novel function for HMG-CoA reductase inhibitors that contributes to postnatal neovascularization by augmented mobilization of bone marrow-derived EPCs through stimulation of the Akt signaling pathway [Dimmeler *et al.*, 2001; Llevadot *et al.*, 2001; Vasa *et al.*, 2000] (Fig. 1). On the other hand, other groups reported that statins effects are regulated by biphasic and dose dependent manners [Urbich *et al.*, 2002; Weis *et al.*, 2002]. Recently, high-dose atorvastatin was reported to result in improvements in hypercholesterolemic induced endothelial dysfunction without improvements of collateral-dependent myocardial perfusion in swine chronic myocardial ischemia. The authors disclosed the increase in Akt phosphorylation, the decrease expression of VEGF, and the increase expression of the antiangiogenic mediator, endostatin [Boodhwani *et al.*, 2006]. These findings suggest that a possible new mechanism for the antiangiogenic effects of statins.

EXPANSION AND MODIFICATION OF EPC

There are several strategies for effective vascular regenerative therapies. Based on the EPC kinetics, a lot of growth factors and chemokines are suitable for EPC expansion. These factors can be added *in vivo* directly, or in culture medium for expanding the target cells, such as EPC. Local injection may suitable to concentrate the abundant number of target cells at ischemic region. However, gene modified cell therapy should be the most effective way from the point of the issue both quantity and quality of transplanted cells (Fig. 2).

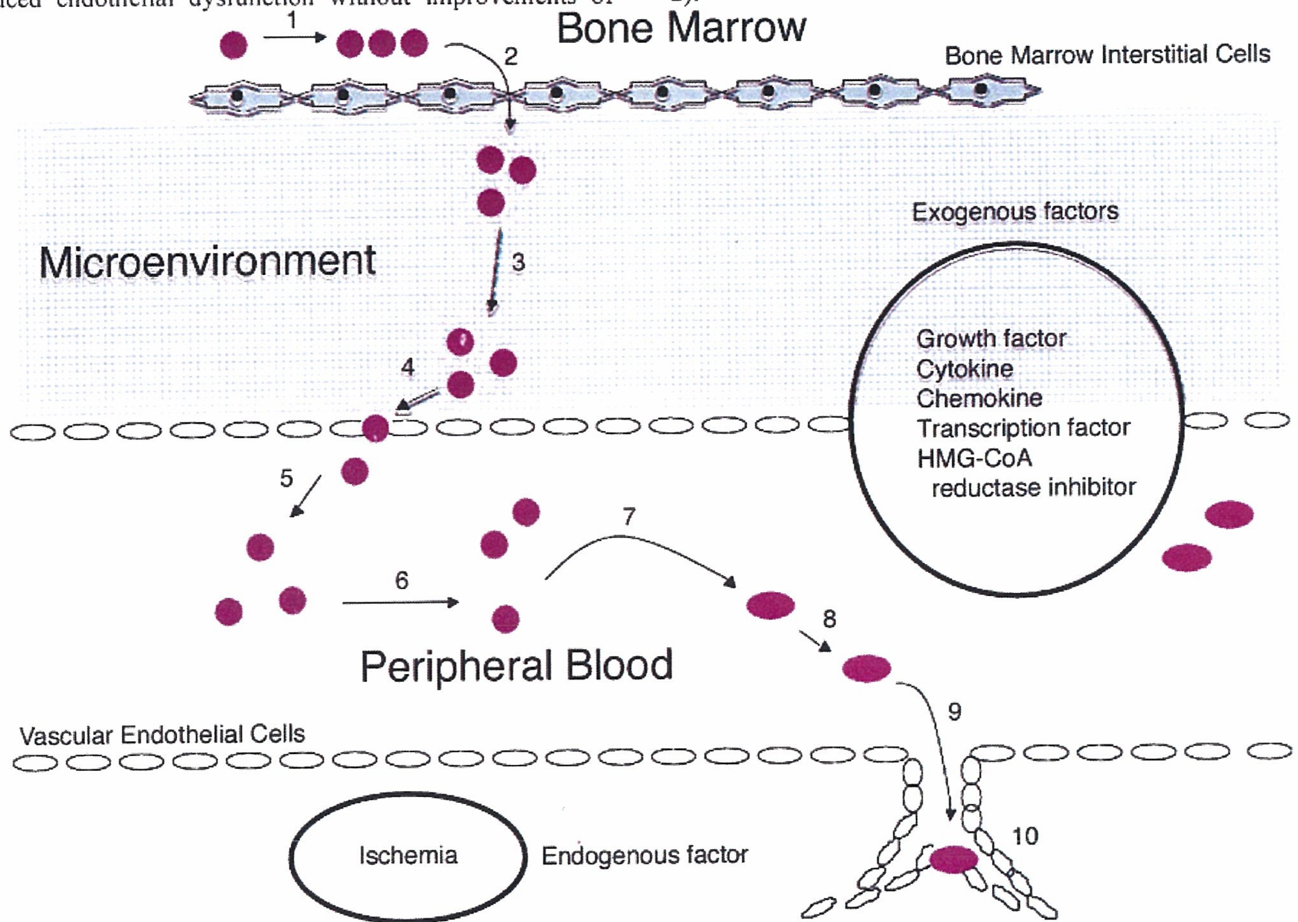

Fig. (1). EPC kinetics.
EPCs are mobilized from bone marrow to peripheral blood in adult following several exogenous and endogenous factors. Mobilized EPCs are recruited and incorporated at the damaged peripheral vasculature as "vasculogenesis" mechanism. The mechanism exists in embryonic vascular development process. Each mobilization step is described as follows.
1. proliferate and differentiate in bone marrow
2. move to bone marrow microenvironment
3.migrate in bone marrow microenvironment
4. move to circulating peripheral blood
5. 6. migrate in peripheral blood
7. differentiate in peripheral blood
8. 9. incorporated into damaged vessels by exogenous and endogenous factors
10. proliferate and differentiate at the site of damaged vessels

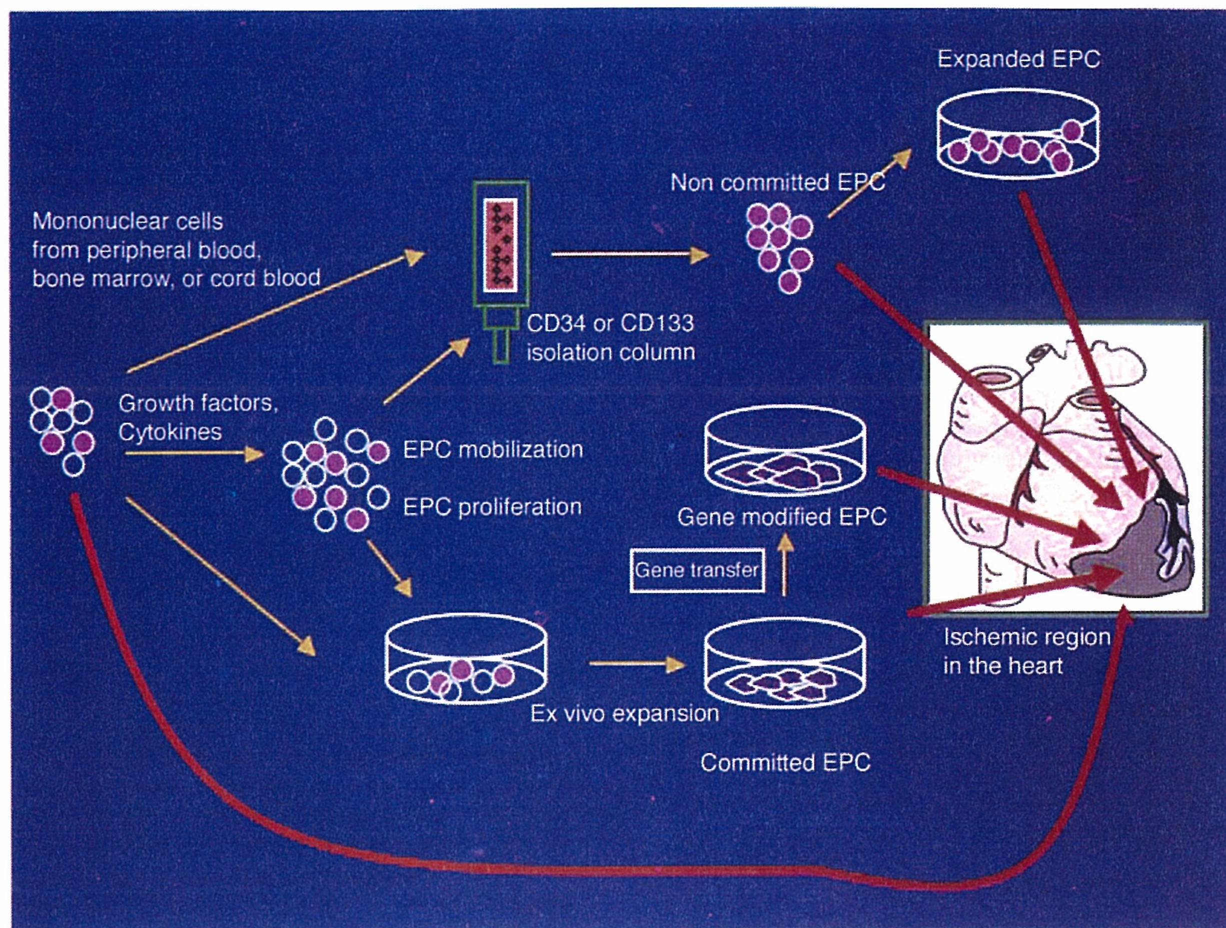

Fig. (2). Clinical application of EPC transplantation.
Several strategies are considered for vascular regeneration therapy based on EPC transplantation. Freshly isolated CD34$^+$ or expanded CD34$^+$ cells from patient's peripheral blood is applicable for autologous cell transplantation. Mononuclear cells are also transplanted in ischemic limb or myocardium. Gene modified cell transplantation could be the more powerful strategy for vascular regeneration because the cell quality is impaired in elder patients. EPC transduced with VEGF or TERT reflects higher potential for vascular regeneration according to enhancement of vessel formation or prolongation of cell life span. Moreover, gene modified cells may act as the "carrier" of each transduced gene. These methods could be the next generation therapy for vascular regeneration.

1. *In Vivo* EPC Expansion

There are several candidates for *in vivo* EPC expansion. Granulocyte macrophage-colony stimulating factor (GM-CSF) has been shown to exert a potent stimulatory effect on EPC kinetics [Takahashi *et al.,* 1999]. Vascular endothelial growth factor (VEGF) is the most-critical factor for vasculogenesis and angiogenesis [Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996; Shalaby *et al.,* 1995]. Indeed, VEGF gene transfer trial shows enhanced vascular flow in ischemic region. Stroma derived factor-1 (SDF-1) [Peichev *et al.,* 2000] could be one of the key factors for EPC kinetics. Previous reports suggested that HMG-CoA reductase inhibitor (statins) mobilized EPCs from BM [Dimmeler *et al.,* 2001; Llevadot *et al.,* 2001]. Granulocyte colony stimulating factor (G-CSF) mobilizes EPCs from bone marrow, and G-CSF mobilized CD34$^+$ cells (EPCs) are applied for clinical trial. We should consider the side effect of each growth factor or chemokine for clinical usage. In the case of cytokine-induced angiogenesis, we should consider both physiological and pathological angiogenesis. Despite improvement of the ischemic condition, neovascularization in atherosclerotic regions could be the trigger for restenosis. Indeed immunohistological assessment suggested that the hemorrhage of neovascularization in atherosclerotic plaques caused acute coronary syndrome [Kolodgie *et al.,* 2003]. Also systemic inflammation derived from cytokine induction may cause unfavorable results in clinical situation. *Ex vivo* expansion might avoid using these stimulators and minimize the side effect for clinical trial.

2. *Ex Vivo* EPC Expansion

Ex vivo expansion of EPCs was performed as follows, total human peripheral blood mononuclear cells were isolated from healthy human volunteers by density-gradient centrifugation with Histopaque-1077 (Sigma) and plated on culture dishes coated with human fibronectin (Sigma). The cells were cultured in endothelial cell basal medium-2 (EBM-2, Clonetics) supplemented with 5% FBS, human vascular endothelial growth factor (VEGF)-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, ascorbic acid, and antibiotics. After

4 days in culture, nonadherent cells were removed by washing with PBS, new medium was applied, and the culture was maintained through day 7.

Ex vivo expansion of EPCs cultured from the peripheral blood of healthy human volunteers typically yields approximately 5.0×10^6 cells per 100ml of blood. Our animal studies [Kalka *et al.*, 2000] suggest that heterologous transplantation required $0.5 \sim 2.0 \times 10^4$ human EPCs /g weight (of the recipient mouse) to achieve satisfactory reperfusion of the ischemic hindlimb. Even with the integration of certain technical improvements, the adjustment of species compatibility by autologous transplantation, and adjunctive strategies (e.g. cytokine supplements) to promote EPC mobilization [Asahara *et al.*, 1999], the primary scarcity of a viable and functional EPC population constitutes a potential limitation of therapeutic vasculogenesis based on the use of *ex vivo* expansion alone.

Recently, CD34+ cells from isolated human peripheral blood mononuclear cells were positively selected using the AutoMACS immunomagnetic separation system (Milteney Biotec). This method allowed us to expand CD34+ cells 10-100 folds as non-adhesive cells. Importantly, this culture method is non-serum culture for clinical application. On the other hand the definition of EPC is updated, for example CD133 is the another candidate surface marker of EPC, and regarding cell source, peripheral blood, bone marrow, and cord blood are useful to isolate EPC.

3. Local, Not Systemically, EPC Injection

Local injection should be effective way to overcome the scarcity of the number of EPCs. We investigated the hypothesis that locally administered SDF-1 could augment the local accumulation of transplanted EPCs, thereby resulting in enhanced neovascularization. EPCs expressed CXCR4 and that the combination of SDF-1 local administration and EPC transplantation had potential as a strategy for therapeutic neovascularization [Yamaguchi *et al.*, 2003].

4. Modification of EPC by Gene Transfer

Given these findings together with the limited quantity of EPCs available even under healthy, physiological conditions, one must consider a strategy that addresses this shortfall and mitigates the possibility of dysfunctional EPCs for therapeutic vasculogenesis in ischemic disorders complicated by ageing, diabetes, hypercholesterolemia, and/or hyperhomocysteinemia. Genetic modification of EPCs to over-express angiogenic growth factors, enhance signaling activity of the angiogenic response, and rejuvenate the bioactivity and/or extend the life span of EPCs constitutes one potential strategy that might address these limitations of EPC transplantation and thereby optimize therapeutic neovascularization.

Our recent findings provide the first evidence that exogenously administered gene-modified EPCs augment naturally impaired neovascularization in an animal model of experimentally induced limb ischemia [Iwaguro *et al.*, 2002]. Transplantation of heterologous EPCs transduced with adenovirus encoding VEGF improved not only neovascularization and blood flow recovery, but also had meaningful biological consequences: limb necrosis and auto-amputation were reduced by 63.7% in comparison with controls. The dose of EPCs used in the current *in vivo* experiments was sub-therapeutic; i.e. this dose of EPCs was 30 times less than that required in previous experiments to improve the rate of limb salvage above that seen in untreated controls. Adenoviral VEGF EPC-gene transfer, however, accomplished a therapeutic effect, as evidenced by a functional outcome, despite a sub-therapeutic dose of EPCs. Thus, VEGF EPC-gene transfer constitutes one option to address the limited number of EPCs that can be isolated from peripheral blood prior to *ex vivo* expansion and subsequent autologous readministration.

Another approach was performed regarding gene modification of EPC. Most somatic cells of humans and other mammals undergo a finite number of cell divisions, ultimately entering a nondividing state termed senescence [Harley *et al.*, 1990; Hastie *et al.*, 1990; Wright *et al.*, 1992]. Loss of telomerase activity has been suggested to constitute the molecular clock that triggers cellular senescence [Greider, 1990; Nakamura *et al.*, 1997]. In contrast to somatic cells, true stem cells and germline cells highly express the catalytic subunit of telomerase (human telomerase reverse transcriptase [hTERT]) [Harrington *et al.*, 1997; Kilian *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997], thus maintaining telomerase activity and full replication of telomeric DNA; these cells (by definition) are thereby able to divide indefinitely [Kolquist *et al.*, 1998]. Although demonstrating regenerative potentials for vascular development, EPCs are not pluripotent, self-renewing stem cells, but rather lineage-committed progenitors, and are thus subject to a Hayflick life span [Hayflick *et al.*, 1961] via replicative senescence. Accordingly, we have deduced that constitutive expression of hTERT might induce delay in senescence and recover/enhance regenerative properties of EPCs. Ectopic expression of the hTERT gene has been investigated as a means to bypass senescence; indeed, this strategy has been used successfully to impart replicative immortality to fibroblasts and retinal pigment epithelial cells [Bodnar *et al.*, 1998] without converting either to a transformed neoplastic phenotype [Jiang *et al.*, 1999; Morales *et al.*, 1999]. The immortalized blood vessel-derived endothelial cells (ECs) similarly exhibited neither evidence of malignant transformation nor loss of functional and morphogenetic characteristics of the parental cells [Yang *et al.*, 1999]. Such hTERT-transduced (Td-hTERT) EC lines appeared more resistant to programmed cell death, exhibited a survival advantage beyond replicative senescence [Yang *et al.*, 1999], and had improved NO production compared with that of control senescent cells [Matsushita *et al.*, 2001]. Also, recent findings suggest that cell therapy in elder patients is not effective despite the abundant mobilization of EPC from bone marrow. The receptiveness might be impaired in aged tissue, or cell, then gene modification is one of the positive tools to overcome the aged impaired condition.

Given the impact on regenerative features, EPC transplantation was performed to assess the corresponding physiological impact *in vivo* after hTERT gene modification. After 1-week *ex vivo* expansion, 1.5×10^4 human EPCs were transduced with Ad/TERT or Ad/GFP and administered intravenously to athymic nude mice with unilateral hindlimb ischemia (n=18 each). Compared with mice transplanted

with Td/GFP, mice transplanted with Td/TERT demonstrated enhanced perfusion measured by LDPI (0.77±0.10 versus 0.47±0.06 in arbitrary units measured by LDPI; P=0.02). The physiological relevance of this finding was underscored by the fact that salvage of the ischemic limb was significantly improved among mice transplanted with Td/TERT versus Td/GFP (P<0.01). Capillary density, evaluated in histologic sections retrieved at day 28 from ischemic hindlimb muscle, was markedly increased in mice receiving Td/TERT versus Td/GFP (224±78 versus 90±40 capillaries/mm^2; P<0.01). Animals treated with Td/TERT or Td/GFP EPCs disclosed no evidence of neoplastic transformation [Murasawa *et al.,* 2002]. These findings have encouraged consideration of potential therapeutic applications of hTERT gene transfer to achieve functional improvement in EPCs through delay in senescence and recovering/enhancing regenerative properties of EPCs.

OTHER GENETIC ENGINEERED EPC

Chen *et al.,* have shown recently that CD34 positive population transduced with Angiopoietin-1 and VEGF represented a favorable effect for myocardial ischemic mice model. They used adenoassociated virus (AAV) [Chen *et al.,* 2005]. Choi *et al.,* have demonstrated that GSK3β transduced EPC augmented angiogenic property both *in vitro* and *in vivo* study. Adenoviral vector was chosen for transduction of GSK3β [Choi *et al.,* 2004]. These findings implicated that genetic modification of EPC could enhance the potential of vascular regeneration and apply for clinical trial in future.

Genetic Engineered Neural Stem Cell (NSC)

Brain ischemia is critical situation for the patients from the point of worsening QOL (Quality of Life). NSC transplantation might improve the brain ischemic situation, such as stroke, Parkinson's disease, or multiple sclerosis and NSC can be genetically engineered for more effective treatment [Alessandri *et al.,* 2004]. One example is that human NSCs transduced with nerve growth factor supplemented the neuronal loss derived from rat brain ischemic model [Andsberg *et al.,* 1998].

CONCLUSION

Cell based therapy is thought to be effective for ischemic diseases, however, we usually need effort to obtain the enough number of high potential autologous cells from the patients. For example, expansion of cells might be useful to overcome quantitative issue. Moreover, cell quality issue should be solved because of the impaired cell function derived from aged patients. Gene engineered cell therapy could be the more powerful strategy in future. Gene modified cells act as carriers of respective genes at the same time. It is necessary to investigate the mechanistic of gene-modified cells for future clinical application.

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Simple screening method for differentially methylated regions of the genome using a small number of cells

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Abstract

Genomic DNA methylation is a major epigenetic mechanism controlling the expression of genetic information. Therefore, identifying regions of the genome that are differentially methylated in different cells is a useful strategy for the study of biological phenomena. To date, several useful screening methods have been established for identifying differentially methylated genomic regions. However, it is impossible to use these methods in fields of study in which it is difficult to obtain a large number of uniform cells, because considerable amounts of genomic DNA are required. Given this situation, we developed a method for preparing large genomic DNA from a small number of cells, and a simple and highly sensitive method for screening for differentially methylated sites. Combined, these two methods comprise a simple screening method, which we named "Differential Methylation Site Scanning" (DMSS), for identifying differentially methylated regions of the genome from a small number of cells. Just 10 cells are sufficient for the method described here.
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Keywords: Differential Methylation Site Scanning; Genomic DNA methylation; Small number of cells

Recently, analyses of whole genomic DNA sequences have been carried out for several species, including human. However, although the DNA sequence is important, it is not the sole determinant of gene expression: genomic DNA can be modified in several ways to control gene expression, and a knowledge of these modifications is important for understanding various biological phenomena. DNA methylation is one such modification mechanism. Genomic DNA methylation changes according to cell type, developmental stage and cell differentiation, and acts to control the expression of genetic information. Therefore, identifying regions in which methylation status changes is a useful strategy for studying biological phenomena. Several useful methods are presently used to screen for differentially methylated regions, for example, restriction landmark genomic scanning [1], methylation-sensitive arbitrarily primed polymerase chain reaction (PCR) [2], and methylation-sensitive representational difference analysis [3]. Microarray technology can also be used to analyze genomic methylation status [4].

Although these methods are useful, it is impossible to use them in fields of study in which it is difficult to obtain a large number of uniform cells, because considerable amounts of genomic DNA are required. Furthermore, for microarray analysis, expensive electronic equipment is needed, at this stage the DNA chips available do not cover the whole genome, and it is difficult to analyze the methylation states of areas that contain repetitive sequences.

Given this situation, we developed a method for preparing a large genomic DNA from a small number of cells, and a simple and highly sensitive screening method for identifying differentially methylated regions. Combining these two methods, we developed a simple screening

Abbreviations: DMSS, Differential Methylation Site Scanning; PCR, polymerase chain reaction.

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method for identifying differentially methylated sites from a small number of cells, which we call "Differential Methylation Site Scanning" (DMSS).

Materials and methods

Cell lines. HeLaS3 and 293T cell lines were provided by Dr. K. Miyazaki (National Cardiovascular Center) and the American Type Culture Collection, respectively.

Preparation of large genomic DNA from a small number of cells. Ten HeLaS3 or 293T cells were collected in 1 μl PBS using sterilized glass capillaries, and were mixed with 3 μl 0.75% NuSieve agarose (TaKaRa)/PBS in mineral oil (to prevent evaporation). After setting the agarose on ice, the blocks of agarose containing the cells were removed from the mineral oil using a sterilized spatula and incubated at 50 °C for 1 h in a mixture of 150 mM EDTA (pH 8.0), 10 mM Tris–HCl (pH 8.0), 1% SDS and 0.1% proteinase K (Invitrogen). The blocks were then washed four times with 5 ml TE for 2 h each and stored at 4 °C.

Digestion of genomic DNA with restriction enzyme in agarose blocks. Agarose blocks containing genomic DNA were incubated twice for 20 min each with 20 μl buffer (33 mM Tris–acetate (pH 7.9), 10 mM Mg–acetate, 500 μM dithiothreitol, 66 mM K–acetate, and 0.01% BSA) in a PCR tube at 4 °C. After completely removing the buffer, to the sample blocks, we added restriction enzyme solution (33 mM Tris–acetate (pH 7.9), 10 mM Mg–acetate, 500 μM dithiothreitol, 66 mM K–acetate, and 0.01% BSA plus 10 U *Hpa*II (Toyobo), *Msp*I (TaKaRa) or dH_2O (control)) to 10 μl and incubated for 2 h at 37 °C.

First PCR. When Alu primers were used for the first PCR, to the solution that had reacted with the restriction enzyme solution, we added 10 μl 10× LA Taq buffer (TaKaRa), 8 μl, 2.5 mM dNTP, 2 μl 25 mM $MgCl_2$, 0.2 μl of each 100-μM Alu primer solution (5′-TAGTA GAGACGGGGTTTCAC-3′/5′-CCTGGGCGACAGAGCGAGAC-3′), 5 U LA Taq (TaKaRa), 5 μl DMSO and dH_2O to 100 μl. PCR was performed using the following conditions: 1 cycle of 94 °C for 3 min, 37 °C for 5 min, and 72 °C for 12 min; followed by 29 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 12 min; with a final elongation stage of 72 °C for 15 min.

When arbitrary primers were used for the first PCR, to the solution that had reacted with the restriction enzyme solution, we added 10 μl 10× Ex Taq buffer (TaKaRa), 8 μl, 2.5 mM dNTP, 2 μl 25 mM $MgCl_2$, 0.2 μl of each of four 100-μM arbitrary primer solutions (5′-GGATCAGCA TNN-3′/5′-CCCGATCAGAANN- 3′/5′-GGGCCGAGTATNN-3′/5′-G CGGCTTAACNN-3′), 5 U Ex *Taq* (TaKaRa), 5 μl DMSO and dH_2O to 100 μl. PCR was performed using the following conditions: 94 °C for 3 min, 37 °C for 5 min, and 72 °C for 2 min; followed by 34 cycles of 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 2 min, with a final elongation stage of 72 °C for 5 min.

Each reaction was duplicated to ensure reproducibility. The amplifications of genomic DNA were checked by agarose gel electrophoresis.

Second PCR. We used the PCR conditions used for fluorescent differential display methods [5] for the second PCR. The reactions were carried out using 1 μl of a 10× dilution of the product obtained from the first PCR as a template, 2 μl 10× PCR Gold buffer (Applied Biosystems), 2 μl 2 mM dNTP, 1.2 μl 25 mM $MgCl_2$, 0.5 μl of each of two 100-μM arbitrary primer solutions (5′-CCCGATCAGAANN-3′/5′-GGGCT AGTTTNN-3′ (in the case of Fig. 2A), 5′-CGGCAGTTACNN/5′-CGCGTATCAGNN-3′ (in the case of Fig. 2B), 5′-TGTCGTAAACNN-3′/5′-CGATCAGGTTNN-3′ (in the case of Fig. 2C)), 0.5 U AmpliTaq Gold (Applied Biosystems), 1 μl DMSO in a total reaction volume of 20 μl. The reactions were performed using the following conditions: 95 °C for 9 min, 40 °C for 5 min and 72 °C for 5 min; then 31 cycles of 94 °C for 15 s, 40 °C for 2 min, and 72 °C for 1 min; with a final elongation stage of 72 °C for 5 min.

Gel electrophoresis. Each PCR product was mixed with the same volume of dye solution (125 mM Tris–HCl (pH 6.8), 192 mM glycine, 10% glycerol, and 0.1% bromophenol blue). Twenty microliters of each sample was added to each well of a 7% polyacrylamide gel in 0.375 M Tris–HCl (pH 8.8). The sample was run at 40 mA for 2.5–3.0 h. After electrophoresis, the gel was stained using SYBR Green I (TaKaRa) solution for 20 min. Bands were visualized by using the LAS-3000 system (Fujifilm) and an image of the gel was printed at actual size. The gel was placed on the print-out, and the piece of gel containing the band of interest was excised using a blade. The gel could then be scanned again to confirm precise excision of the band of interest.

Re-amplification. The piece of gel containing the band of interest was boiled in 100 μl dH_2O, and DNA was recovered by ethanol precipitation in the presence of 0.3 M sodium acetate (pH 5.2) and 1 μl ethachinmate (TaKaRa) as a carrier, and redissolved in 10 μl dH_2O. Re-amplification was carried out using 2 μl of the eluted DNA solution as a template, 2 μl 10× PCR Gold buffer (Applied Biosystems), 2 μl 2 mM dNTP, 1.2 μl 25 mM $MgCl_2$, 0.4 μl of each of two 100-μM arbitrary primer solutions (the same arbitrary primers as used in the second PCR), 0.5 U AmpliTaq Gold (Applied Biosystems), and 1 μl DMSO in a total reaction volume of 20 μl. PCR was performed using the following conditions: 95 °C for 9 min, 30 cycles of 94 °C for 15 s, 40 °C for 2 min and 72 °C for 1 min; with a final elongation stage at 72 °C for 5 min.

Cloning and sequencing of the re-amplified DNA. Re-amplified DNA fragments were cloned into the pT7BlueR plasmid. Each cloned DNA fragment in the plasmids was amplified by colony PCR and purified using the QIAquick PCR Purification Kit (Qiagen). PCR products were then sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The reacted samples were electrophoresed using the ABI 3100 system (ABI).

Methylation status confirmation. Primers were designed in accordance with the sequences of the cloned bands and the surrounding genomic DNA. One microgram of genomic DNA of HeLaS3 or 293T was incubated with 100 μl of a solution of 33 mM Tris–acetate (pH 7.9), 10 mM Mg–acetate, 500 μM dithiothreitol, 66 mM K–acetate, and 0.01% BSA, plus 10 U *Hpa*II (Toyobo), *Msp*I (TaKaRa) or dH_2O (control) for 3 h at 37 °C. PCR was carried out using 1 μl of digested genomic DNA solution as a template, 2 μl 10× PCR Gold buffer (Applied Biosystems), 2 μl 2 mM dNTP, 1.2 μl 25 mM $MgCl_2$, 0.08 μl of each 100-μM primer solution (primer 1: 5′-GGACCCTAGAGAGAACTCT-3′/primer 2: 5′-GATTC CTGACAGCAGCATAA3′ or primer 3: 5′-GAGTCCTTGAGAAG GCAATA-3′ (in the case of Fig. 3A), primer 1: 5′-TGAGCTCTGAC AGTTCATAAT-3′/primer 2: 5′-AACCAGAGCATGACATTCAAA-3′ or primer 3: 5′-TGGGAGGTCCGATGCTCA-3′ (in the case of Fig. 3B), primer 1: 5′-CAACTATCAGGTTTCTTAGCT-3′/primer 2: 5′-GTCC TAGGTGTGGGAAAAAT-3′ or primer 3: 5′-GGCCTGACAGCAGAG ATG-3′ (in the case of Fig. 3C)), 0.5 U AmpliTaq Gold (Applied Biosystems) and 1 μl DMSO, in a total reaction volume of 20 μl. PCR was performed using the following conditions: 95 °C for 9 min; then 30 cycles of 94 °C for 30 s, 55 or 60 °C for 1 min and 72 °C for 30 or 50 s; with a final elongation stage of 72 °C for 5 min.

Results and discussion

Principles of DMSS

The principles underlying DMSS are shown diagrammatically in Fig. 1. After digestion with a methylation-sensitive restriction enzyme, genomic DNA was amplified. At present, there are several useful methods for amplifying small amounts of DNA, for example multiple displacement amplification [6] and degenerate oligonucleotide primed-PCR [7]. In the present study, we used a PCR-based method with repetitive sequence or arbitrary primers. PCR was performed to amplify the long genomic DNA fragments that could be expected to contain at least one restriction enzyme site. In the present study, we refer to this step as the "first PCR". If the product of the first PCR

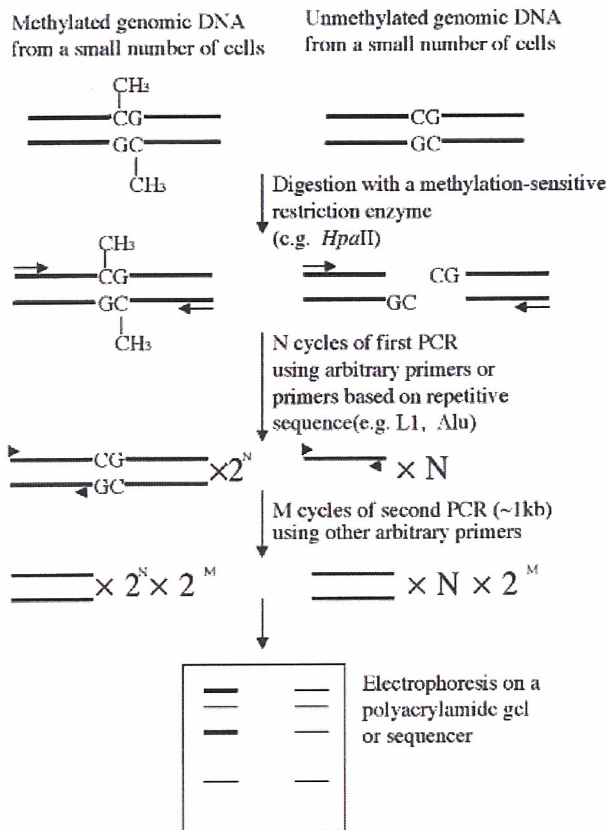

Fig. 1. Schematic diagram of the DMSS method. Genomic DNA is represented by a solid line. Primers for first PCR and second PCR are indicated by the arrows and arrowheads, respectively.

contains methylation-sensitive restriction enzyme sites, the amount of the amplified DNA fragment present will differ for the methylated and unmethylated DNA templates. Next we performed the "second PCR" using the first PCR product diluted tenfold as template DNA and other arbitrary primers, in order to visualize the band pattern on polyacrylamide gels. The amount of second PCR product is a function of the amount of first PCR product. As a result, the band intensity reflects the methylation state of the original template DNA. Using this method, it is possible to efficiently screen for differentially methylated sites over a wide area of the genome using only a small amount of DNA.

Preparation of a large genomic DNA from a small number of cells

For comparing the methylation state of two or more types of cells, it is necessary to reproducibly prepare the intact genomic DNA. To prevent loss and degradation of the whole genomic DNA extracted from a small number of cells, we modified the sample block method used in pulse field gel electrophoresis [8]. This preparation procedure is shown schematically in supplementary Fig. 1. Cells collected in PBS were mixed with low melting-point agarose, and after the agarose was set the cells were digested with proteinase K. In the agarose, large pieces of DNA are protected from physical damage, and cannot be scattered. Using this method, we were easily able to obtain intact genomic DNA from a small number of cells. Furthermore, in low melting-point agarose, DNA can be digested by restriction enzymes.

DMSS using repetitive element primers in the first PCR

To test this newly developed method in a real-life situation, we used the HeLaS3 and 293T cell lines. We digested the genomic DNA of these two cell lines in agarose blocks using *Hpa*II, which is a methylation-sensitive restriction enzyme, or *Msp*I, which is an isochizomer of *Hpa*II but is not methylation sensitive. After digestion with these two restriction enzymes, we amplified the genomic DNA using a modified form of the interspersed repetitive sequence PCR method [9]. We designed the primers with reference to the Alu consensus sequences [10] to amplify the genomic regions that are located between two Alu elements. Alu is a repetitive element that is scattered throughout the human genome in about one million locations [11]. The average distance between two Alu elements is about 3 kb. If PCR is performed using conditions that will amplify more than 10 kb of DNA, a considerable area of the genome can be amplified using the Alu primers.

Fig. 2A shows the DMSS image obtained using Alu primers for the first PCR. Differences in the band patterns may reflect the following differences between HeLaS3 and 293T cell lines: (1) differential methylation states, (2) polymorphism of restriction enzyme sites, or (3) differences in sequences. To distinguish differential methylation states from the other two factors, we compared differential band patterns between HeLaS3 and 293T in not only *Hpa*II-digested DNAs but also undigested and *Msp*I-digested DNAs. Each reaction was duplicated to ensure reproducibility. There are more than 30 bands per lane on the polyacrylamide gel in Fig. 2A. It is thus possible to perform genomic footprinting 1000 times from 10 cells. We detected several bands that differ between HeLaS3 and 293T. We cloned the band indicated by the white arrow in Fig. 2A, which was present in both the undigested DNA and the *Hpa*II-digested 293T DNA, but not in the *Hpa*II-digested HeLaS3 DNA. To check whether this differing band pattern reflects differential methylation of the genomic DNA, we sequenced the band and checked the sequence of the surrounding genome (Accession No. AADD01119569 in GenBank). We found that an *Hpa*II site in the surrounding region but not in the cloned band sequence (Fig. 3A). We designed primers on the basis of the sequences of the cloned band and its surrounding genome and performed PCR using *Hpa*II- or *Msp*I-digested and undigested genomic DNA as templates to check the methylation status of the region in HeLaS3 and 293T