表6　PEI磁気ビーズによるウイルスの濃縮結果

| ウイルス | 宿主 | ウイルスゲノム | 脂質膜 | サイズ（nm） | PEI-磁気ビーズ濃縮 |
|---|---|---|---|---|---|
| モデルウイルス | | | | | |
| サイトメガロウイルス | サル | DNA | + | 180-200 | + |
| ヘルペスウイルスⅠ型 | ヒト | DNA | + | 150-200 | + |
| 水疱性口内炎ウイルス | ウシ | RNA | + | 70-150 | + |
| 同種指向性マウス白血病ウイルス | マウス | RNA | + | 80-110 | + |
| Sindbis ウイルス | ヒト | RNA | + | 60-70 | + |
| アデノウイルス5型（Ad-5） | ヒト | DNA | − | 70-90 | + |
| SV-40ウイルス（SV-40） | サル | DNA | − | 40-50 | + |
| ブタパルボウイルス（PPV） | ブタ | DNA | − | 18-24 | +* |
| ポリオウイルス Sabin 1 型 | ヒト | RNA | − | 25-30 | +** |
| ヒト感染性ウイルス | | | | | |
| ヒト免疫不全ウイルス（HIV） | ヒト | RNA | + | 80-100 | + |
| B型肝炎ウイルス（HBV） | ヒト | DNA | + | 40-45 | + |
| C型肝炎ウイルス（HCV） | ヒト | RNA | + | 40-50 | + |
| A型肝炎ウイルス（HAV） | ヒト | RNA | − | 25-30 | +* |

＊：条件により濃縮されない場合もある
＊＊：PEI磁気ビーズのみでは濃縮されないが，IgM 抗体や抗体と補体の添加により濃縮可能

ズを用いることにより，C型肝炎ウイルスやB型肝炎ウイルスをはじめとして多くのウイルスが濃縮可能であることを報告している（表6）．

## 4. 遺伝子治療薬や細胞治療薬のウイルス安全性確保を目指した将来的な課題

遺伝子治療薬や細胞治療薬などの先端技術医薬品のウイルス等の安全性確保に関しては，多くの検討すべき課題が残されている．また，これらの先端技術医薬品の開発はその周辺技術も含めて急速に進展しており，さらに腫瘍溶解性ウイルスベクターのようにこれまでの概念にない画期的な製品の開発も続いており，このような革新的技術を用いた製品については，その安全性を確保しつつ合理的な規制を行うことが，よりよい医療をできるだけ早く国民に届けることになる．このためにも，高感度・高精度のウイルス安全性検出技術等の基盤技術の開発を進めると共に，適切なリスク評価に基づいた行政施策の立案に資する研究が望まれている．

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# A comparison of the tube forming potentials of early and late endothelial progenitor cells

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ABSTRACT

The identification of circulating endothelial progenitor cells (EPCs) has revolutionized approaches to cell-based therapy for injured and ischemic tissues. However, the mechanisms by which EPCs promote the formation of new vessels remain unclear. In this study, we obtained early EPCs from human peripheral blood and late EPCs from umbilical cord blood. Human umbilical vascular endothelial cells (HUVECs) were also used. Cells were evaluated for their tube-forming potential using our novel *in vitro* assay system. Cells were seeded linearly along a 60 μm wide path generated by photolithographic methods. After cells had established a linear pattern on the substrate, they were transferred onto Matrigel. Late EPCs formed tubular structures similar to those of HUVECs, whereas early EPCs randomly migrated and failed to form tubular structures. Moreover, late EPCs participate in tubule formation with HUVECs. Interestingly, late EPCs in Matrigel migrated toward pre-existing tubular structures constructed by HUVECs, after which they were incorporated into the tubules. In contrast, early EPCs promote sprouting of HUVECs from tubular structures. The phenomena were also observed in the *in vivo* model. These observations suggest that early EPCs cause the disorganization of pre-existing vessels, whereas late EPCs constitute and orchestrate vascular tube formation.



## Introduction

In healthy individuals, endothelial cells are rarely found in the blood. However, mature endothelial cells can be found in the circulation following detachment from injured vessels. For example, circulating endothelial cells substantially increase in a wide variety of pathological conditions associated with profound vascular insult. Therefore, circulating endothelial cells are a useful marker of vascular damage [1,2]. Endothelial progenitor cells (EPCs), derived from bone marrow, are also found in the circulation and are involved in tumor vasculogenesis and wound healing

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[3,4]. The discovery of EPCs in human blood has led to a new paradigm in which vasculogenesis and angiogenesis occur in adult tissues [5]. These results have suggested a potential new approach to the treatment of cardiovascular and ischemic diseases. Preclinical studies have shown that transplantation of human EPCs to nude mice with hind limb ischemia improved blood flow recovery and capillary density resulting in a significant reduction in the rate of limb loss [5–10]. Clinical trials using autologous EPC transplantation have already been performed and significant improvements have been observed in myocardial function and ischemic diseases [11–15]. The strict roles of EPCs in neovascularization are not well understood because various populations of cells with varying differentiation potentials were transplanted.

EPCs consist of two different subpopulations, termed early and late EPCs [16–18]. Although both EPCs are derived from mononuclear cells (MNC) and express endothelial cell markers, they have different morphologies and growth patterns [18,19]. Early EPCs exhibit a spindle-like morphology and the majority of them are derived from CD14(+) subpopulations [16,19–21]. Late EPCs, named after their late outgrowth potential, exhibit a cobblestone morphology and are derived from CD14(−) fractions [17,19]. These two populations have been characterized for production of vascular endothelial growth factor (VEGF), VEGF receptor expression, cytokine secretion, and tube forming activity *in vitro* and *in vivo* [19].

Using photo-catalytic lithography, we have developed a new cell culturing technology for capillary engineering [22]. In this approach, endothelial cells are patterned on a substrate and then transferred to Matrigel. The endothelial cells change their morphology and form tubular vessels as confirmed by electron microscopy and dye microinjection. In the present study, using this novel technology, we focused on the tube-forming activity of early and late EPCs. In addition, we asked whether EPCs were incorporated into preexisting tubular structures.

## Materials and methods

### *Human samples*

Human peripheral blood was provided by 7 healthy human volunteers. Human umbilical cords and cord bloods were obtained from 12 healthy newborns. Informed consent was obtained from all donors, and samples were handled according to the tenets of the Declaration of Helsinki, with the approval of university review boards.

### *Cell isolation*

Blood was diluted 1:1 with PBS containing 2 mM EDTA, and overlayed on Lymphoprep (AXIS-SHIELD, Oslo, Norway). Cells were centrifuged at 1100×*g* for 10 min. The resulting mononuclear cells (MNC) were collected and washed three times in PBS. Human umbilical vein endothelial cells (HUVECs) were isolated enzymatically.

### *Cell culture*

Isolated MNC were resuspended in endothelial basal medium-2 (EBM-2) (Clonetics, San Diego, CA) supplemented with the EGM-2 bullet kit (Clonetics), plated on culture dishes precoated with human fibronectin (Sigma, Saint Louis, MO), and maintained in the medium. To obtain early EPCs from peripheral blood MNC and cord blood MNC, medium was changed every 2 days, and after 5 to 7 days of culture, early EPCs were isolated. Late EPCs were obtained from cord blood as previously described by Gulati et al. [17]. Medium was then changed daily for 7 days and on alternate days thereafter. The characteristic colonies of late EPCs were observed under a phase-contrast microscopy (IMT-2; Olympus Optical, Tokyo, Japan) and these cells were cloned by colony isolation. HUVECs were used at passages 1–3.

### *Cell characterization*

Early and late EPCs were seeded onto a chamber slide glass (Nalge Nunc, Naperville, IL) coated with fibronectin (Sigma) and fixed in 4% paraform aldehyde (PFA) next day. Immunocytochemistry was performed using PE-conjugated anti-human CD31 antibody (555446; BD Pharmingen, San Diego, CA; diluted 1:50) and primary antibodies against human VEGFR2, (sc-6251; Santa Cruz Biotechnology, Santa Cruz, CA; 2 μg/ml) or von Willebrand factor (vWF) (M0616; Dako, Glostrup, Denmark; 1.2 μg/ml), followed with Alexa 488-conjugated anti-mouse IgG (A-11029; Molecular Probe, Leiden, Netherlands; 2 μg/ml). Non-immune mouse $IgG_1$ (sc-3877; Santa Cruz; diluted 1:100) was used as a control. Cell nuclei were stained with 1 μg/ml of To-Pro3 (T3605; Molecular Probe). Samples were observed and photographed under a laser confocal microscope (LSM510META; Carl-Zeiss, Jena, Germany).

### *Flow cytometric analysis of CD14, CD31, CD45 and VEGFR2 expression in early EPCs and late EPCs*

Cells were collected non-enzymatically using Cell Dissociation Buffer (Invitrogen Corp, San Diego, CA) and labeled with FITC-conjugated anti-CD45 monoclonal antibody (Beckman Coulter, Marseilles, France), FITC-conjugated anti-CD31 monoclonal antibody (Pharmingen) and anti-VEGFR2 monoclonal antibody (Santa Cruz Biotechnology) at 4 °C for 30 min. Cells incubated with anti-VEGFR2 monoclonal antibody were subsequently stained with PE-conjugated anti-mouse IgG antibody at 4 °C for 30 min. After washing with PBS containing 1% BSA, flow cytometric analysis was performed with a FACS Calibur (Becton-Dickenson, NJ).

### *Photo-mask preparation*

STK-03 titanium dioxide ($TiO_2$) photo-catalyst aqueous dispersion (Ishihara Sangyo; Osaka, Japan) was diluted to 33 wt.% with isopropyl alcohol, stirred for 1 h and filtered using a 3.0 μm pore size polytetrafluoroethylene filter (Advantec, Tokyo, Japan). Cr-Quartz photo-masks with slit width 60 μm and slit interval 300 μm were cleaned using a VUM-3184 UV-ozone washing machine (Oak Manufacturing, Tokyo, Japan) to

decompose low molecular weight adsorbents on the mask surface. The mask was coated with the $TiO_2$ dispersion by spin-coating at ~700 rpm for 15 s and then baking at 150 °C for 10 min.

### *Substrate preparation*

NA35 polished glass substrates (NH Techno Glass, Yokohama, Japan) were cleaned using the VUM-3184 for 420 s. Next, 1.5 g of heptadecafluorodecyltrimethoxysilane solution (TSL-8233; GE Toshiba Silicone, Tokyo, Japan), 5.0 g of tetramethoxysilane solution (TSL-8114; GE Toshiba Silicone), and 2.4 g of 0.005 N HCl were mixed and stirred for 24 h at room temperature to make fluoro-alkyl-silane (FAS) mixture solution. The mixture was diluted with 1 wt.% isopropyl alcohol, stirred for 15 min, filtered by Chromato-Disc (filter type 0.45 μm; Kurabo) and then coated onto the glass substrate by spin-coating at ~1000 rpm for 15 s. The FAS-coated substrate was then baked at 150 °C for 10 min.

### *Surface modification and patterning*

Both $TiO_2$-coated photo-masks and FAS-coated substrates were immersed and sonicated in deionized water for more than 5 min, and then baked at 120 °C for 5 min. The $TiO_2$-coated side of the photo-mask was irradiated with UV for 15 min at irradiation energy of ~30 J/cm$^2$ to rejuvenate the photo-catalytic activity of $TiO_2$. The FAS-coated side of the glass substrate was placed facing and in contact with the $TiO_2$ layer of the photo-mask and UV-irradiated for several minutes through the mask to form hydrophilic regions on the FAS layer.

### *Cell patterning*

Prior to cell seeding, glass substrates were placed on cell culture dishes. The cells were trypsinized and suspended, and labeled with PKH26 Red (Sigma) according to the manufacturer's instructions. Cells were counted, seeded on the substrates, and incubated for 18 h at 37 °C. A total of $2\times10^5$ cells were seeded per substrate. During incubation, cells in hydrophobic areas moved to hydrophilic areas.

### *Transplant to matrigel*

The patterned cells on the substrate were turned over onto Matrigel (Becton Dickinson, Bedford, TX) and incubated with the culture medium containing 0.2% FBS for 24 h. When the substrate was removed, the cells were transferred from the substrate to the Matrigel.

### *Observation of the tube formation*

Firstly, the pattern of cells on Matrigel was observed by a phase-contrast microscopy (IMT-2; Olympus Optical). Next, live cells which formed tubular structures were labeled with 500 ng/ml Calcein-AM (Molecular Probes) for 30 min in 37 °C, and three-dimensional structures of capillaries were analyzed by a laser confocal microscope. Furthermore, the engineered capillaries on Matrigel were fixed with 4% PFA and stained with 4 μg/ml of anti-human VE-cadherin rabbit polyclonal antibody (210-232-c100; ALEXIS, San Diego, CA), followed by 4 μg/ml of Alexa 488-conjugated anti-rabbit IgG (Molecular Probe). Capillaries were observed by a laser confocal microscope.

### *EPC incorporation into tube-like structure*

Early and late EPCs were pre-labeled with PKH26 Red, mixed with unlabeled HUVECs, and seeded on the substrate. Twenty-four hours after transplant to Matrigel, the cells were labeled with Calcein-AM, the fluorescence of EPCs and HUVECs were analyzed by a laser confocal microscope.

To quantify EPC incorporation into tube-like structure, early and late EPCs were pre-labeled with PKH26 Green. These cells were mixed with HUVECs which were pre-labeled with PKH26 Red, and cultured on the substrate. The fluorescence of EPC and HUVECs was analyzed by a fluorescence microscopy (BZ-8000; Keyence, Osaka, Japan). The incorporation ratio of EPCs into the tube structure was calculated by the cellular area of EPC (μm$^2$)/mm of tube structure.

### *In vivo angiogenesis assay*

We developed an ear vessel occlusion model for an in vitro angiogenesis assay. After occlusion of the murine auricular vessel, cells were introduced into the subcutaneous pocket below the occlusion point, and newly formed vessels were analyzed. Twelve hours before the cell injection, the mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and auricular vessels were occluded. Collateral circulation was observed under a stereomicroscope (LEICA Mz9, Leica Microsystems, Wetzlar, Germany). Early or late EPCs were labeled with PKH26 and suspended in growth media, and $5\times10^4$ of each EPCs were subcutaneously injected into the occluded pinna within 12 h of vessel occlusion. Forty-eight hours after the cell injection, the mice were given 250 μg of *Banderiaea simplicifolia* lectin 1 (BS1-lectin) (Vector Laboratories, Burlingame, CA) intravenously and sacrificed 45 min later. Sections (4-μm thickness) of cell-transplanted pinna were made for histological observation. This experiment was performed using 7-week nude mice and all procedures were carried out with the full approval of the ethical committee of Tokyo Medical and Dental University.

### *Calculations and statistical analysis*

The statistical significance of differences in the data was evaluated by use of analysis of Welch's t-test or Student t-test. A value of $P<0.05$ was accepted as statistically significant.

## Results

### *Characterization of two types of EPCs*

MNC were harvested from human peripheral blood of healthy individuals or from cord blood and seeded on fibronectin-coated dishes. Following 5 to 7 days of cultivation, adherent cells were observed in clusters. The cells exhibited a spindle-

like shape (Fig. 1A, left) and displayed several endothelial cell markers including CD31, vWF and VEGFR2 (Fig. 1B, left). When MNC were isolated from human cord blood, early EPCs were also observed after 3 days of cultivation. Their morphology and expression of endothelial markers used here were almost the same as those of early EPCs derived from peripheral blood (data not shown). They proliferated and thereafter gradually disappeared over the next 1 week.

After early EPCs had disappeared, colonies with different morphology emerged over 12–21 days of cultivation. They exhibited a cobblestone morphology, spindle-like shape (Fig. 1A, right) and displayed several endothelial cell markers such as CD31, vWF and VEGFR2. Also, eNOS expression was observed in both early and late EPCs (data not shown). Thus, these cells were late EPCs as reported by Hur et al. [18]. To characterize two types of EPCs, we carried out a flow

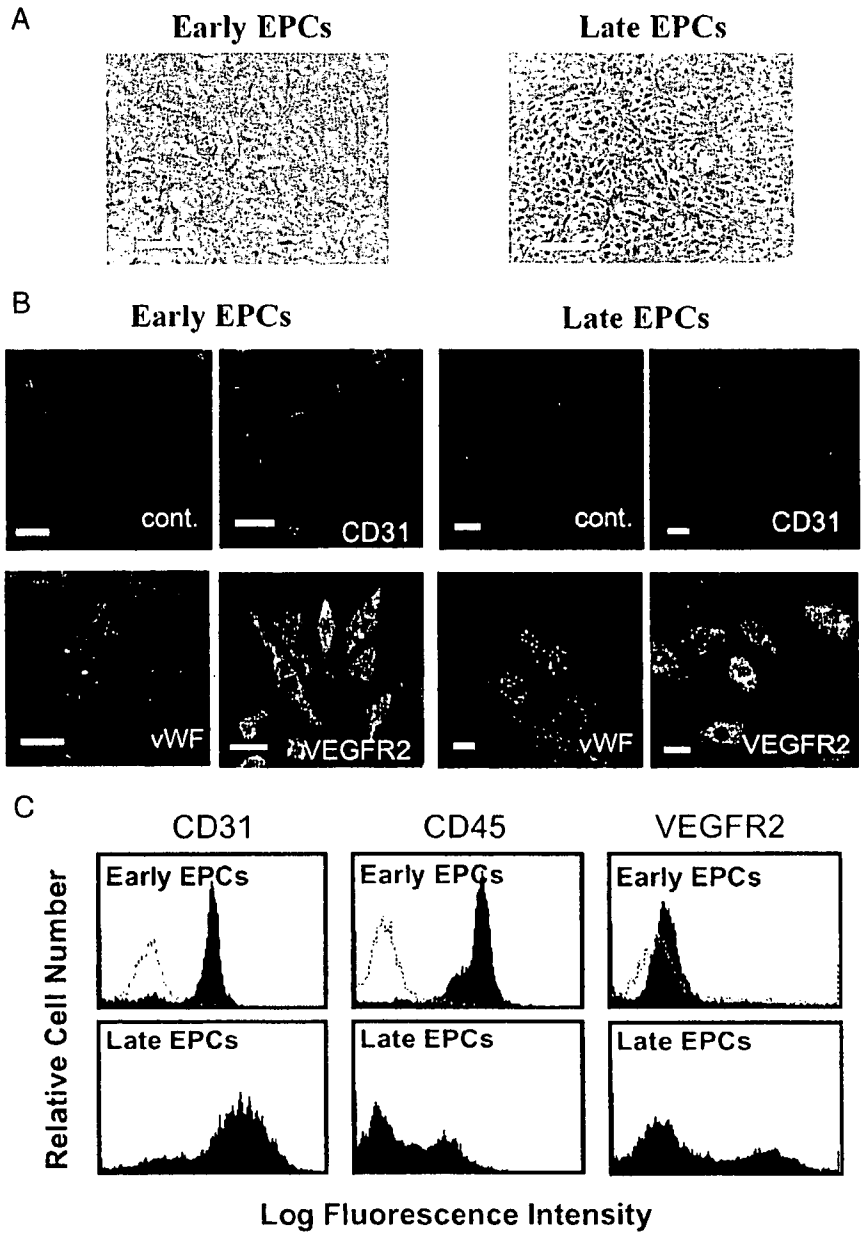


**Fig. 1 – Morphological and immunophenotypical characterization of early and late EPCs. (A) Early EPCs cultured for 7 days and late EPCs cultured for 14 days. Scale bar = 100 μm. (B) Immunocytochemistry of CD31, vWF and VEGFR-2 was demonstrated. Scale bar = 20 μm. Shown are representative data from 7 independent experiments using early EPCs isolated from different peripheral blood and 7 independent experiments using late EPCs isolated from different cord blood with similar results. (C) Flow cytometric analysis of CD31, CD45 and VEGFR2 expression in early EPCs and late EPCs. Shown are representative data from 5 independent experiments using early EPCs and late EPCs isolated from different cord blood with similar results. Isotype controls are overlaid in a dot line on each histogram for each surface antigen tested.**

cytometric analysis of CD31, CD45 and VEGFR2 expression in early EPCs and late EPCs derived from cord blood (Fig. 1C). Similar to the immunocytochemical staining, the expression of CD45 in late EPC was weaker than that in early EPCs, while VEGFR2 expression in late EPCs was stronger than that in early EPCs. CD14 expression was observed only in early EPCs (data not shown).

### *Tubular structures could be constructed by late EPC, but not by early EPC*

To assess the tube-forming activities of early and late EPCs, both cell types were analyzed by a novel method which promoted capillary formation in vitro. Early EPCs, late EPCs and HUVECs were distributed linearly on a specific substrate

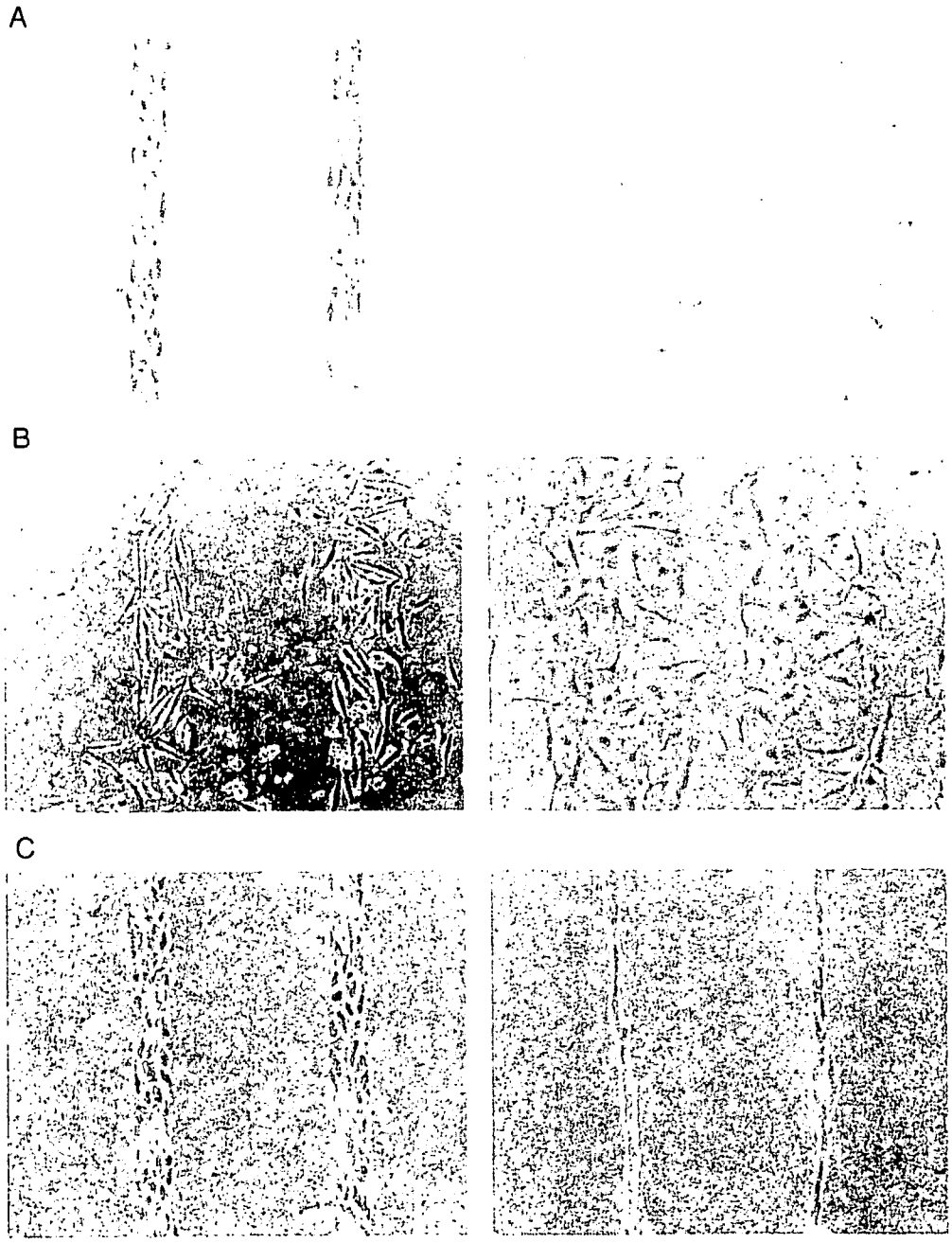


**Fig. 2 – Tube forming activities in early and late EPCs compared with HUVECs. Cells ($2 \times 10^5$) were seeded on the glass substrate and cultured for 18 h (A–C, left), then transplanted to Matrigel. Twenty-four hours after transplantation (right), HUVECs formed tubular structures (A). Early EPCs spread on Matrigel and did not form tubular structures (B). Late EPCs formed tubular structures (C).**

(Fig. 2, left) and were subsequently transferred to Matrigel (Fig. 2, right). During the transfer of patterned cells to Matrigel, HUVECs changed cellular morphology to form capillary-like structures. Twenty-four hours after cell transfer, the substrate was removed and tubular structures were readily observed by a light microscopy (Fig. 2A). Electron microscopic observation revealed that the luminal structure consisted of four to five HUVECs [22]. In contrast, linearly arrayed early EPCs did not form tubular structures (Fig. 2B). The time course of tube formation in late EPCs was quite similar to that in HUVECs (Fig. 2C).

After HUVECs or late EPC were loaded with calcein-AM, the tubular structures were assessed with a laser confocal microscope. The luminal structure formed by late EPCs was observed as well as that by HUVECs (Figs. 3A, B). When tubes formed by HUVECs and late EPCs were stained for VE-

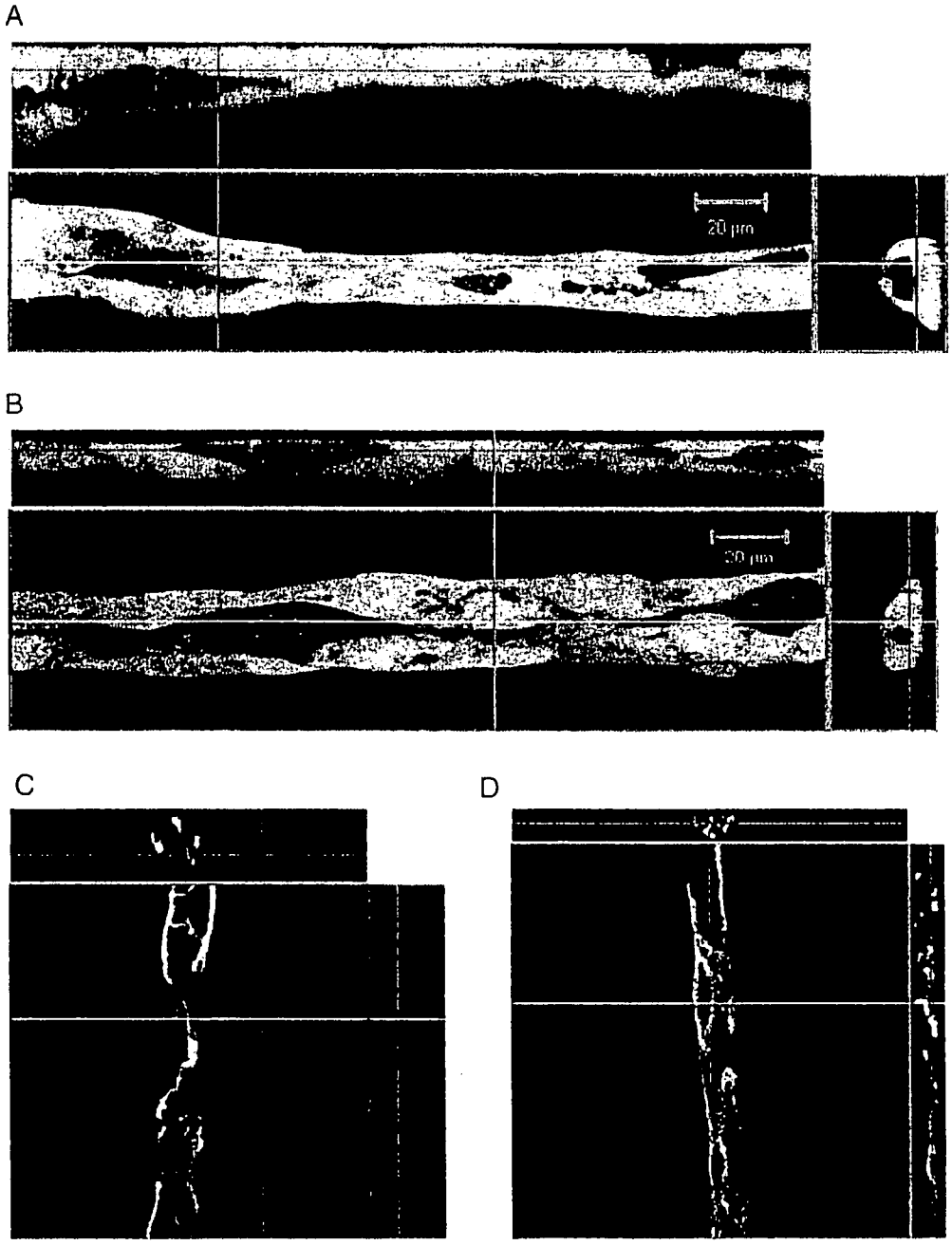


**Fig. 3 – Three-dimensional images of tubular structures formed by HUVECs and late EPCs. Tubular structures formed by HUVECs (A) and late EPCs (B) were stained with calcein-AM. The luminal structure was observed with a laser confocal microscope. Scale bar=20 μm. Immunostaining of HUVEC (C) and late EPCs (D) tubular structures with anti-VE-cadherin. The localization of VE-cadherin was observed at cell–cell junctions.**

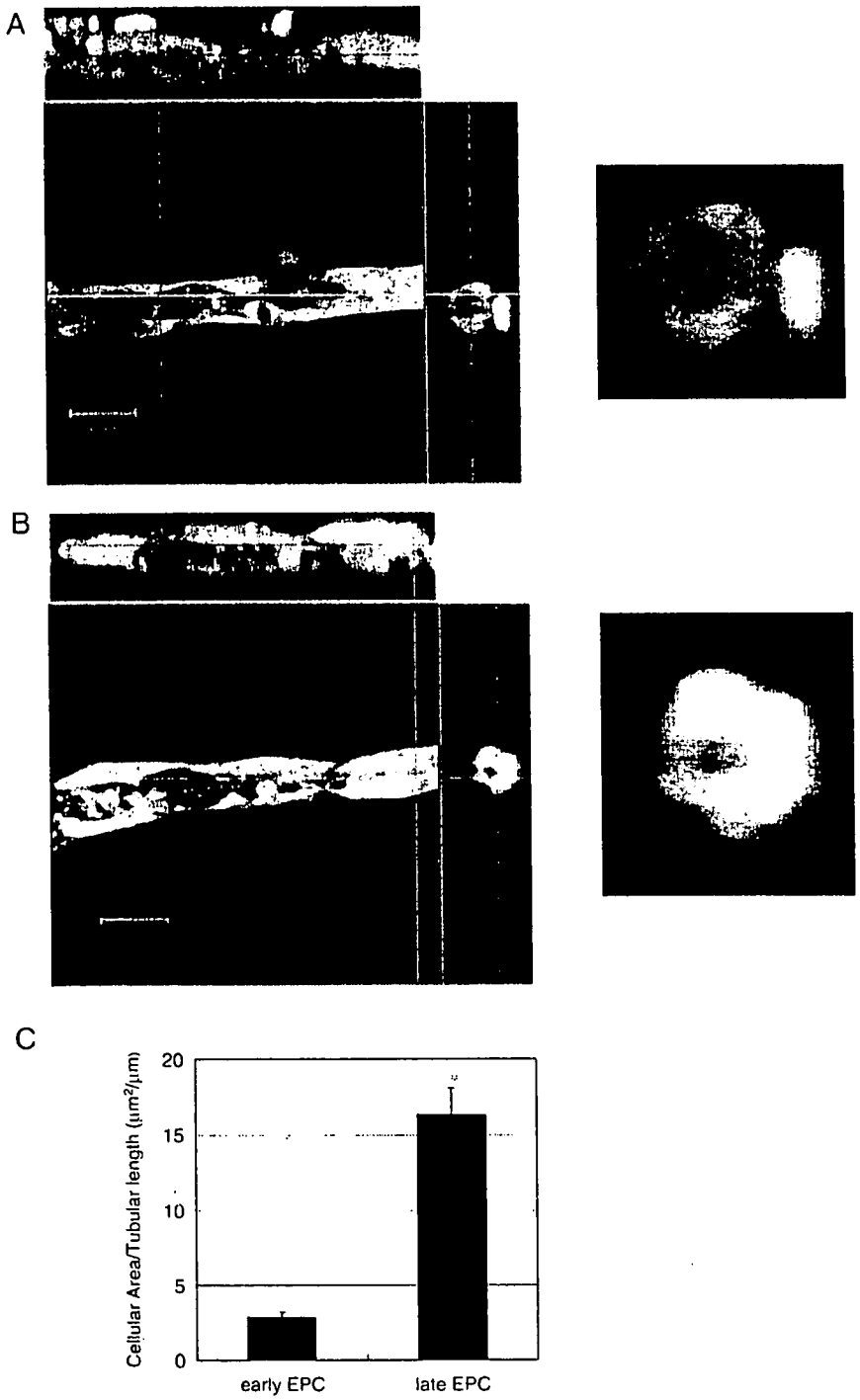


Fig. 4 – Cooperative tube-forming activity of EPCs and HUVECs. After early or late EPCs were stained with PKH26 Red, EPCs and HUVECs were mixed and co-cultured on the substrate, and then transplanted to Matrigel. The ratio of EPCs to HUVECs was 1:10. (A) Tubular structures formed by these EPCs and HUVECs were then labeled with Calcein-AM. Tubular structure constructed with both early EPCs (yellow) and HUVECs (green). Tubular structures were observed three dimensionally with a laser confocal microscopy. The early EPCs attached to the tube surface and were not incorporated into the tubular structure formed by HUVECs. Scale bar=20 μm. (B) Tubular structure constructed with both late EPCs (yellow) and HUVECs (green). The late EPCs participated in tube formation with HUVECs. Scale bar=20 μm. (C) The incorporation of EPCs into tube structure was calculated by the area of EPC in the indicated length of tubular structure. Value is the mean and SE (standard error) of 30. $^{**}P<0.01$ vs. early EPCs.

cadherin, a specific endothelial cell–cell adhesion molecule, we observed VE-cadherin at cell–cell junctions (Figs. 3C, D).

*Late EPCs, but not early EPCs, participated in tube formation with HUVECs*

When EPCs are mixed with HUVECs and incubated on Matrigel, EPCs are incorporated into the tubular structure formed by HUVECs [5]. To confirm this, early EPCs or late EPCs were mixed with HUVECs and they were applied to the same tube formation systems. To distinguish the EPCs from HUVECs, the EPCs were labeled with PKH26 Red. Tubular structures formed by these EPCs and HUVECs were then labeled with Calcein-AM. The fluorescence of EPC and HUVECs was observed with a laser confocal microscopy. The early EPC as well as the late EPCs were observed in the tubular structure. However, by a three-dimensional imaging, we demonstrated that the early EPCs were only attached to the tube surface and were not incorporated into the tube structure formed by HUVECs (Fig. 4A). In contrast, the late EPCs participated in tube formation with HUVECs (Fig. 4B). To quantify the incorporation of EPCs into tube structure was calculated by the area of EPC per the indicated length of tubular structure. The incorporation of late EPCs into tubular structures was over 5-fold larger than that of early EPCs (Fig. 4C).

*Late EPCs integrated into pre-existing tubular structures formed by HUVECs while early EPCs caused tubular sprouting*

In order to investigate whether EPCs could substitute for HUVECs in preformed tubular structures, EPCs and HUVECs were independently cultured. HUVECs labeled with PKH26 Red were seeded onto the substrate and EPCs labeled with PKH26 Green were suspended in Matrigel. The patterned HUVECs on the substrate were transferred to the EPC-containing Matrigel. The late EPCs migrated toward the tubular structures and were observed adjacent after 6 h (Fig. 5A) and attached to the tubes 10 h later (Fig. 5B). Thereafter, they were incorporated into the tubular structure 24 h later (Fig. 5C). In contrast, early EPCs did not migrate toward the tubes and were not

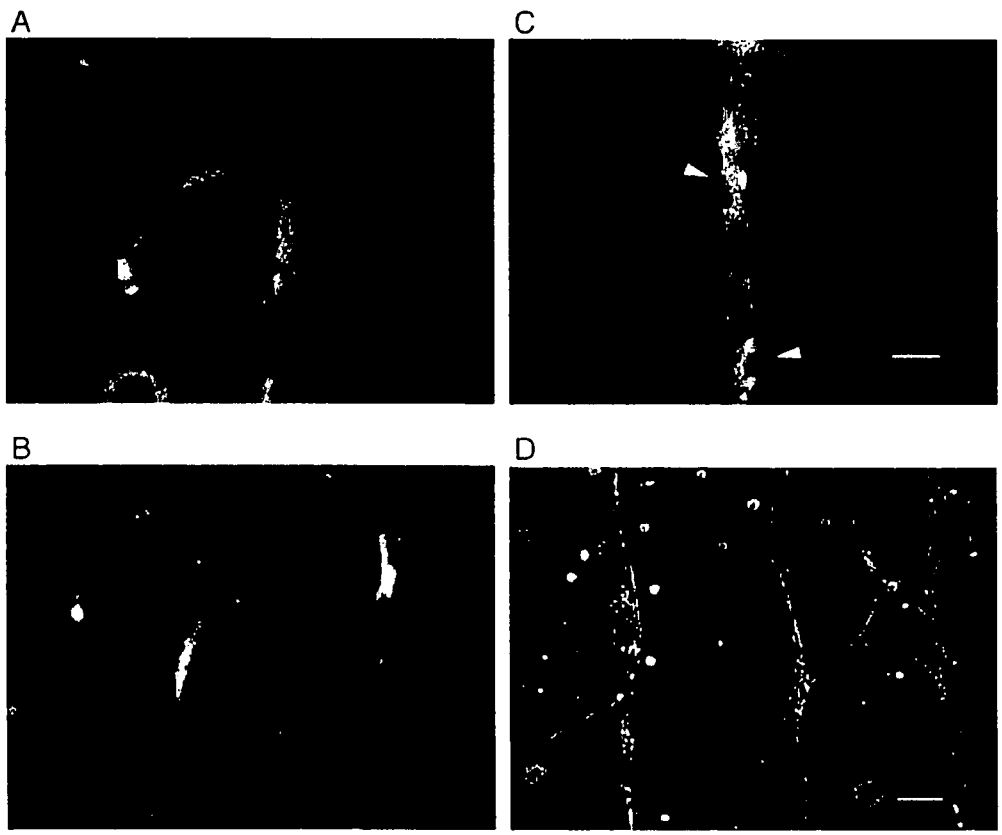


**Fig. 5 – Incorporation of EPCs into preformed tubular structures. HUVECs labeled with PKH26 Red were seeded on the substrate and EPCs labeled with PKH26 Green were suspended in Matrigel. The patterned HUVECs on the substrate were transferred to the EPC-containing Matrigel. (A) Six hours after transplantation, late EPCs were migrating toward the tubular structure preformed by HUVECs. (B) Ten hours after transplantation, late EPCs were attached to the tube. (C) Twenty-four hours after transplantation, late EPCs were incorporated into the tubular structure preformed by HUVECs (yellow arrow heads). Scale bar = 30 μm. (D) Twenty-four hours after transplantation, early EPCs had not been incorporated into the tubular structure preformed by HUVECs. The HUVECs in the tubular structure were sprouting and migrating. Scale bar = 100 μm.**

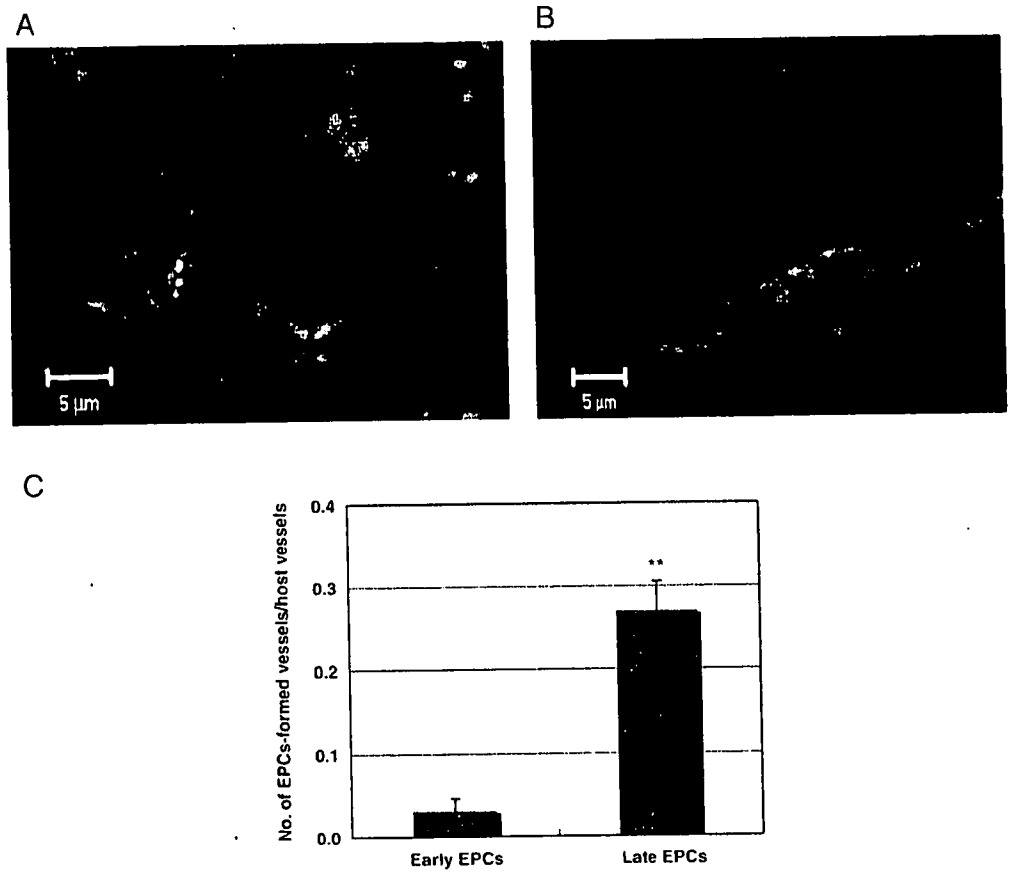


**Fig. 6 – *In vivo* angiogenesis assay. After occlusion of murine auricular vessel, early (A) or late (B) EPCs labeled with PKH26 Red were subcutaneously injected into the occluded pinna. Forty-eight hours after the cell injection, the mice were given BS1-lectin (green) intravenously and sacrificed. Scale bar=5 μm. (C) The numbers of blood vessels derived from each EPCs observed in 5 sections were normalized to the number of recipient blood vessels. The data are shown as the mean and SE of three mice and data are mean of 5 fields/mouse.**P<0.01 vs. early EPCs.**

incorporated during the observation period. However, after HUVECs were transferred to Matrigel containing early EPCs, the HUVECs in the tubular structures initiated sprouting and migrated toward early EPCs as evidenced by branching patterns between the tubular structures (Fig. 5D).

*In vivo angiogenesis assay*

To confirm the tube forming activity of each EPCs *in vivo*, the vessels derived from implanted EPCs were observed in the ear vessel occlusion model, early or late EPCs labeled with PKH26 Red were injected into the occluded pinna. Blood vessels derived from the late EPCs were observed in the pinna, but only a few blood vessels derived from early EPCs were observed (Fig. 6).

## Discussion

We investigated the tube-forming capacity of early EPCs and late EPCs isolated from human peripheral blood and umbilical cord blood, respectively. In the previous report, it was demonstrated that late EPCs were enriched in umbilical cord blood compared with adult peripheral blood [23]. Therefore, we isolated late EPCs from human umbilical cord blood. We used our novel method for capillary engineering which makes use of photo-catalytic lithography. Traditional assays for assessing characteristics of tube formation *in vitro* have been carried out by cultivation of endothelial cells in type I collagen and Matrigel. However, these methods could not distinguish between tube formation and morphological changes in the cells. The luminal structure of the vascular tube made in our present method was confirmed by an electron microscopy, a confocal laser microscopy and dye microinjection [22]. This technique is unique because the method allows one to focus on the process of tube formation. In contrast, tube formation in collagen and Matrigel evaluates the total activity of endothelial cells including migration, invasion and tube formation [24]. Using this novel method, we demonstrated that late EPCs participated in the formation of tubular structures with mature endothelial cells, i.e., HUVECs. It is interesting that late EPCs migrated toward and adjacent to pre-existing tubular structures and finally were incorporated

into the structure itself. In contrast, early EPCs could not form tubular structures, and they induced migration and sprouting of HUVECs present in the tubular structure. In a previous study using Matrigel, Hur et al. [18] demonstrated that early EPCs were incorporated into tubules when co-cultured with HUVECs, although the formation was weaker than that of late EPCs. However, that paper demonstrated the incorporation of EPCs into network structures of HUVECs on Matrigel, but could not show the incorporation of EPCs in tubular structures.

Our results showed that early EPCs were not capable of constructing tubular luminal structures even when they were co-cultured with mature endothelial cells. Instead, they stimulated the migration and sprouting of HUVECs from the tubular structure. These phenomena may be interpreted by the release of some growth factors from early EPCs. In our experiment, the amount of secreted interleukin-8 from early EPCs was significantly higher than that from late EPCs ($0.81 \pm 0.07$ vs. $0.44 \pm 0.02$, ng/ml, $P < 0.01$). It has been also reported that early EPCs secrete angiogenic factors such as VEGF [18,21], hepatocyte growth factor (HGF), and granulocyte-colony stimulating factor (G-CSF) [21]. These factors are known to stimulate endothelial cell migration and proliferation. In vivo experiments, implanted late EPCs in the occlusion tissues cause to construct new blood vessels by themselves, but early EPC could not. This result may be discussed with a hierarchy of EPCs [23]. It has been also reported that the different types of cells derived from peripheral blood have distinct actions in healing activity [25]. In this study, we demonstrated specific and distinct behaviors of early and late EPCs in tube formation and how they affected mature ECs in tube formation.

Although numerous reports have demonstrate that early EPCs have the potential to construct new blood vessels by themselves [5,6], some studies argued that early EPCs were not actually incorporated into newly formed vessels [26,27]. This discrepancy may be due to marked heterogeneity of EPCs used in their experiments. Numerous studies have fractioned EPC by phenotypic markers such as CD34, CD133, and VEGFR2 and various methods of EPCs isolation have been reported [5,6,28,29]. Among them CD34 has been often used for the marker of EPCs. However, Romagnani et al. showed that CD14 positive MNC-derived EPCs, which had been fractioned as CD34 negative, express very low level of CD34 using an antibody-conjugated magnetofluorescent liposomes (ACMFL) technique [30]. From these results, they suggested that $CD14^{+}$ $CD34^{low}$ cells are the major source of early EPCs obtained from human peripheral blood MNC. This subset exhibited clonogenicity and multipotency to differentiate not only into endothelial cells, but also into osteocytes, or neural cells [30]. This report suggests that it is difficult to sort EPCs into highly defined fractions by ordinary FACS technique. In fact, each EPC population in previous studies is supposed to have a different phenotype even from the same source. To avoid these complexities, we collected and isolated early and late EPCs by focusing on their characteristics such as morphologies and proliferation pattern, and compared their tube-forming activities in short duration in vitro assays.

While the regenerative potential of EPCs has been demonstrated in animal models of myocardial and limb ischemia, the number of EPC available for transplantation is very important for cell-based therapy. Because EPCs are derived from a limited endogenous pool, it is necessary to expand the number of EPCs in vitro or modulate phenotypes of EPCs. Iwaguro et al. reported that VEGF gene transfer in EPCs stimulated neovascularization in an in vivo model [31]. Murasawa et al. demonstrated that gene transfer of human telomerase reverse transcriptase into EPCs enhanced their angiogenic properties, mitogenic and migratory activities, and cell survival [32]. From the standpoint of cellular proliferation and phenotypic stability, late EPCs are superior to early EPCs. Therefore, the use of late EPCs for tissue engineering has been challenged [33–35]. With our method of generating transplantable capillary networks, the formation of tubular structures in vitro is a necessary precondition. The data presented here are thus important since the results demonstrate that late EPCs are a candidate for tissue engineering.

In conclusion, our data provide the first definitive evidence that early EPCs promote angiogenesis through migration and proliferation of mature endothelial cells, whereas late EPCs can form blood vessels. These results suggest that early EPCs and late EPCs have different roles in neovascularization in vivo. Finally, we expect that the novel culture system using a patterned substrate might be useful for future in vitro analyses of neovascularization.

## Acknowledgments

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ARTICLES

# 薬の名前
## ステムを知れば薬がわかる
Stems used in drug names ; For the better understanding of pharmacological actions of drugs

第18回

国立医薬品食品衛生研究所
川崎ナナ，内田恵理子
NANA KAWASAKI, ERIKO UCHIDA
*National Institute of Health Sciences*

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## はじめに

本連載第17回（本誌2007年12月号）では，抗悪性腫瘍薬のステムとして，

「-sulfan」：メタンスルホネート系抗悪性腫瘍薬
「-mustine」：β-クロロエチルアミノ構造を持つ抗悪性腫瘍薬
「-tepa」：チオテパ系誘導体
「-ribine」：ピラゾフリン系リボフラニル誘導体
「-trexate」：葉酸類縁化合物
「-trexed」：チミジル酸合成酵素阻害薬
「mito-」：細胞核に対して毒性を有する抗悪性腫瘍薬

を紹介した。

今回は，生物薬品の第６回目として，酵素性医薬品のステムを紹介する。

## 「-ase」：酵素

「-ase」は，酵素（Enzyme）を示すステムである。「-ase」は，さらに「-uplase」（ウロキナーゼ型プラスミノーゲンアクチベーター），「-teplase」（組織プラスミノーゲンアクチベーター），「-diplase」（プラスミノーゲンアクチベーターと他の酵素との融合タンパク質），「-lipase」（リパーゼ活性を持つ酵素），「-dismase」（スーパーオキシドジスムターゼ活性を持つ酵素）などのサブステムに分けられる。

### (1)「-ase」：タンパク質分解酵素

「-ase」は，タンパク質分解酵素（proteinase）の（サブ）ステムとしても使用される。タンパク質分解酵素を示すサブステム「-ase」を持ち，わが国で承認されている医薬品にKallidinogenase（カリジノゲナーゼ），Serrapeptase（セラペプターゼ），L-Asparaginase（L-アスパラギナーゼ），Pronase（プロナーゼ），Urokinase（ウロキナーゼ）およびTisokinase（チソキナーゼ）がある。このうち，カリジノゲナーゼ，セラペプターゼおよびウロキナーゼは日局収載品目である。

カリジノゲナーゼは，血液中のキニノーゲンに特異的に作用してキニンを遊離するキニノゲナーゼ（カリクレイン）の１種で，ブタ膵臓由来のものが医薬品として使用されている。遊離したキニンは，末梢血管の拡張なら

びに微小循環速度の亢進を介して血流増加作用を示す。わが国でカリジノゲナーゼは，高血圧症，メニエール症候群，閉塞性血栓血管炎(ビュルガー病)における末梢循環障害の改善および更年期障害，網脈絡膜の循環障害改善薬として適用されている。

セラペプターゼは，セラチア(*Serratia*)属細菌から精製したタンパク質分解酵素である。わが国では，手術後および外傷後，慢性副鼻腔炎，乳汁うっ滞における腫脹の緩解，ならびに気管支炎，肺結核，気管支喘息の喀痰喀出困難および麻酔後の喀痰喀出困難に適用される。

L-アスパラギナーゼは，321個のアミノ酸残基からなるサブユニット4つで構成されるタンパク質である。血中のL-アスパラギンを分解し，アスパラギン要求性腫瘍細胞を栄養欠乏状態にすることによって抗腫瘍作用を発揮する。日本および米国では急性白血病(慢性白血病の急性転化を含む)および悪性リンパ腫の治療に用いられている。

プロナーゼは，放線菌*Streptomyces griseus*が産生するタンパク質分解酵素である。わが国では，胃内視鏡検査における胃内粘液の溶解除去や消化異常症状の改善を目的に使用されている。また，イソプロテレノールとの混合剤が持続性気管支拡張・粘液溶解剤に適用されている。

その他「-ase」を持つ品目で，海外で承認されている医薬品にRasburicase(ラスブリカーゼ)，Streptokinase(ストレプトキナーゼ)，Pegaspargaseがある。

ラスブリカーゼは，*Saccharomyces cerevisiae*から産生される遺伝子組換え尿酸オキシダーゼである。悪性腫瘍あるいは化学療法に起因して発現する高尿酸血症治療薬として欧米で使用されている。わが国では2007年にJANに収載された。

Pegaspargaseは，大腸菌で産生されたL-アスパラギナーゼに，分子量約5,000のポリエチレングリコールを共有結合させたPEG化タンパク質で，米国で承認されている。なお，「Peg-」はポリエチレングリコール(PEG)が結合していることを意味する接頭語である(本連載第5回(本誌2006年12月号)参照)。

その他タンパク質分解酵素を示すサブステム「-ase」を持つ医薬品としてINNには，Brinase，Ocrase，Promelase，Sfericaseが収載されている。

INNには「-ase」を持たないタンパク質分解酵素もいくつか収載されている。わが国で承認されている医薬品ではBromelain(ブロメライン)がある。ブロメラインは，パイナップルの果汁，または葉茎の搾汁より調製したタンパク質分解酵素で，手術後および外傷の腫脹の緩解，慢性気管支炎や気管支喘息の喀痰困難の改善，熱傷や化膿創などの創傷面の壊死組織の分解除去・洗浄およびそれに伴う治癒促進に用いられている。

「-ase」を持たないタンパク質分解酵素性医薬品としては，欧州で承認され，JAN収載品目でもあるChymotrypsin(キモトリプシン)や，米国で承認されているChymopapainもある。その他，INNにはBatroxobin，Defibrotide，Fibrinolysin(human)，Sutilainsが収載されている。

タンパク質分解酵素には血栓溶解系に関与するものもある。循環血液中には血栓溶解作用を持つプラスミンの前駆体であるプラスミノーゲンが存在する。循環血液中のプラスミノーゲンは，プラスミノーゲンアクチベーターによって560番目のアルギニンと561番目のバリンの間が切断されて，活性型のプラスミンとなる(図1)。プラスミンは，血栓の主成分であるフィブリンを分解することにより血栓を溶解する。哺乳類のプラスミノーゲンアクチベーターは，ウロキナーゼ型のプラスミノーゲンアクチベーターと組織型のプラスミノーゲンアクチベーターに大別される。前者は古くは尿中に見出されたことから，現在でも単にウロキナーゼと呼ばれる。

ウロキナーゼは，セリンプロテアーゼの1つで，411個のアミノ酸残基からなる糖タンパク質である。ウロキナーゼは，プロウロキナーゼ(411個のアミノ酸残基からなる1本鎖糖タンパク質，分子量約55,000)として産生された後に，プラスミンやカリクレインによって158番目のリジンと159番目のイソロイシンの間が限定分解されることによって生じるA鎖(N末端側ペプチド，分子量約22,000)およびB鎖(C末端側ペプチド，分子量33,000)からなる2量体タンパク質である。ウロキナーゼは，活

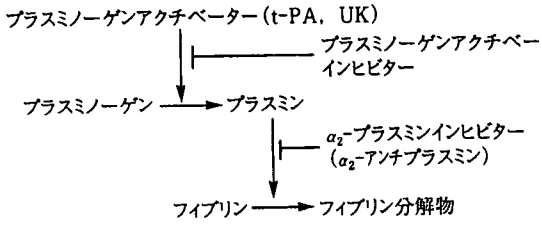



**図1　血栓溶解機構**
t-PA：組織プラスミノーゲンアクチベーター
UK：ウロキナーゼ

性型高分子量ウロキナーゼ(分子量約55,000)または，さらに分解された低分子量ウロキナーゼ(分子量約31,500)として存在する。

INNに収載されているウロキナーゼは，「A plasminogen activator isolated from human sources」とあるようにヒト由来である。わが国で承認され，日局に収載されているウロキナーゼは，ヒト尿から精製した高分子量型ウロキナーゼである。わが国では，急性心筋梗塞における冠動脈血栓の溶解剤として適用されている。ヒト尿由来ウロキナーゼは欧州でも承認されており，EPに収載されている。EP収載ウロキナーゼは高分子量および低分子量ウロキナーゼを含むものである。

米国で承認されているウロキナーゼは，尿由来ではなく，組織培養(新生児腎細胞)由来のもので，分子量約32,000の低分子量ウロキナーゼである。また，INNにはUrokinase Alfaが収載されており，これは遺伝子組換え型糖タンパク質で，非ヒト哺乳動物由来細胞で産生された高分子量ウロキナーゼである。

欧米で血栓溶解剤として使用されているストレプトキナーゼも，プラスミノーゲンアクチベーターである。ストレプトキナーゼは，黄色ブドウ球菌由来のタンパク質分解酵素で，プラスミノーゲンをプラスミンに活性化する。わが国では販売されていない。

### (2)「-uplase」：ウロキナーゼ型プラスミノーゲンアクチベーター

「-uplase」はウロキナーゼ型プラスミノーゲンアクチベーター(urokinase-type plasminogen activator)に共通のサブステムで，JANにはNasaruplase(ナサルプラーゼ(細胞培養))が収載されている。

ナサルプラーゼは，ヒトプロウロキナーゼと同一のアミノ酸配列を持つ糖タンパク質である。ナサルプラーゼは，ヒト腎臓に由来する2倍体細胞の培養により繊維芽細胞をクローン化し，株化した細胞で産生される。急性心筋梗塞における冠動脈血栓の溶解を効能とした血栓溶解剤として承認されている。

ナサルプラーゼと同一のアミノ酸配列を持ち，糖鎖が異なる品目として，INNにNasaruplase Betaが収載されている。これは，マウス細胞で産生される遺伝子組換え糖タンパク質である。Nasaruplaseは，Alfaが収載されず，Betaが収載されている唯一の例である。その他，「-uplase」を持つ品目としてINNにはSaruplaseが収載されている。

### (3)「-teplase」：組織プラスミノーゲンアクチベーター類

「-teplase」は，組織プラスミノーゲンアクチベーター(t-PA)類(tissue-type-plasminogen activator)に共通のサブステムである。t-PAは，ウロキナーゼと同様にプラスミノーゲンの560番目のアルギニンと561番目のバリンの間を切断することによって，プラスミノーゲンをプラスミンに活性化する(図1)。t-PAは，主に血管内皮細胞で産生される527個のアミノ酸残基からなる分子量約70,000の1本鎖糖タンパク質で，分子内の3カ所(Asn117，184，448)に糖鎖が結合している。N末端側から，フィンガードメイン，EGFドメイン，クリングル1ドメイン，クリングル2ドメイン，Catalyticドメインから構成されている(**図2**)。t-PAは，プラスミンによりCatalyticドメイン上の275番目のアルギニンと276番目のイソロイシンの間が切断されると，重鎖(N末端側)と軽鎖(C末端側)からなる2本鎖t-PAになる。2本鎖t-PAになると十分な活性を発揮するが，1本鎖でも2本鎖t-PAの約1/10の酵素活性を有する。t-PAがウロキナーゼと大きく異なる点は，フィブリン親和性を有する点である。t-PAは血栓に特異的に結合してプラスミノーゲンを活性化するので，血栓溶解効率が高い。これに対してウロキナーゼは，循環血液中のプラスミノーゲンを活性化するために，生じたプラスミンが特異的インヒビター($\alpha_2$-プラスミンインヒビター等)で失活したり，出血傾向を引き起こしたりする。t-PAの高いフィブリン親和性には，フィンガードメインとクリングル2ドメインが関与しているといわれている。

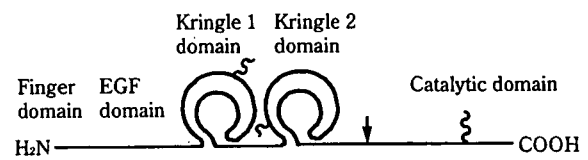
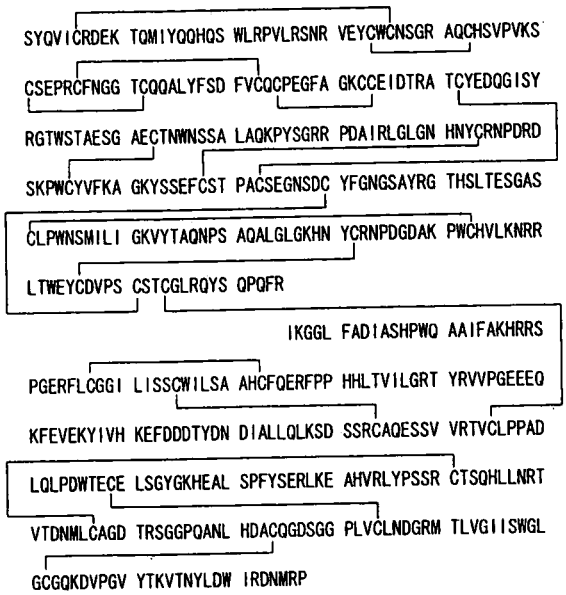



**図2　t-PAの構造**
フィンガードメイン：1～43番目
EGFドメイン：44～91番目
クリングル1ドメイン：92～173番目
クリングル2ドメイン：180～261番目
Catalyticドメイン：276～527番目
∿∿∿：糖鎖
→：プラスミン限定分解部位

サブステム「-teplase」を持ちわが国で承認されている医薬品として，Alteplase(アルテプラーゼ)，Monteplase(モンテプラーゼ)およびPamiteplase(パミテプラーゼ)がある。




N117，N184，N448：糖鎖結合

**図3　アルテプラーゼの一次構造，ジスルフィド結合と糖鎖結合部位**

アルテプラーゼは，遺伝子組換えヒト組織プラスミノーゲンアクチベーターで，CHO細胞によって産生される(図3)。虚血性脳血管障害急性期に伴う機能障害の改善(発症後3時間以内)および急性心筋梗塞における冠動脈血栓の溶解(発症6時間以内)に適用される。アルテプラーゼは，海外でも承認されており，EPおよびUSPにも収載されている。わが国では日局収載候補品目になっている。

t-PAは，血中からの消失が速く，静脈投与する場合は点滴投与が必要とされている。t-PAの肝臓での代謝には，クリングル1ドメイン上のAsn117に結合している高マンノース型糖鎖やEGFドメインが関与していると考えられている。そこで，血中での滞留時間を延長させるために，遺伝子工学的にt-PAを改変する研究が進められた。現在ではさまざまな改変型t-PAが血栓溶解薬として使用されている。

モンテプラーゼは，t-PAの84番目のシステインをセリンに変換した改変型t-PAで，ベビーハムスター腎細胞により産生される。わが国では急性心筋梗塞における冠動脈血栓の溶解(発症後6時間以内)および不安定な血行動態を伴う急性肺塞栓症における肺動脈血栓の溶解に適用されている。

パミテプラーゼは，t-PAのクリングル1ドメインを欠失させることによって血中半減期を延長し，かつt-PAが2本鎖に解離しないように天然型t-PAのN末端から275番目のアルギニンをグルタミン酸に変換してフィブリン親和性を回復させた改変型t-PAで，CHO細胞によって産生される。パミテプラーゼは，急性心筋梗塞における冠動脈血栓の溶解(発症後6時間以内)に適用されている。

サブステム「-teplase」を持つその他の品目としてJANには，Duteplase(デュテプラーゼ)，Lanoteplase(ラノテプラーゼ)，Silteplase(シルテプラーゼ)，Nateplase(ナテプラーゼ)，およびEcolteplase(エコルテプラーゼ)が収載されている。欧米では，TenecteplaseとReteplaseが承認されているが，いずれもJAN未収載である。

Tenecteplaseは，CHO細胞で産生されるt-PA改変体で，103番目および117番目のアミノ酸残基がそれぞれアスパラギンおよびグルタミンに変換され，さらに296～299番目のアミノ酸残基がアラニンに変換されている。Reteplaseは，クリングル2ドメインとCatalyticドメインからなる改変体で，大腸菌で産生される糖鎖非結合タンパク質である。

その他サブステム「-teplase」を持つ医薬品として，INNにはAnistreplaseおよびDesmoteplaseが収載されている。

「-ase」の項で述べたTisokinase(チソキナーゼ)は，t-PAを示すサブステム「-teplase」を持たないが，天然型t-PAである。チソキナーゼは，527個のアミノ酸残基からなる糖タンパク質で，ヒト肺に由来する2倍体繊維芽細胞で産生される。血栓溶解剤として承認されている。

### (4)「-diplase」：プラスミノーゲンアクチベーターと他の酵素との融合タンパク質

「-diplase」はプラスミノーゲンアクチベーターと他の酵素との融合タンパク質に与えられたサブステムである。サブステム「-diplase」を持つ品目として，AmediplaseがINNに収載されている。Amediplaseは，t-PAのクリングル2ドメインとプロウロキナーゼのC末端側ドメインから構成される遺伝子組換えキメラ型プラスミノーゲンアクチベーターである。

### (5)「-lipase」：リパーゼ活性を持つ酵素

リパーゼ(lipase)活性を持つ酵素にはサブステム

「-lipase」が与えられている。リパーゼはグリセロールエステルを加水分解し，脂肪酸を遊離する酵素である。サブステム「-lipase」を持ちINNに収載されている品目として，遺伝子組換えヒト胆汁酸塩活性化リパーゼであるBucelipase Alfaや，*Rhizopus arrhizus*が産生するリパーゼRizolipaseがある。

### (6)「-dismase」：スーパーオキシドジスムターゼ活性を持つ酵素

「-dismase」は，スーパーオキシドジスムターゼ(Superoxide dismutase)活性を持つ酵素に共通のサブステムである。スーパーオキシドジスムターゼは，異性化酵素(isomerase)の1種で，スーパーオキシドアニオンラジカルの不均化反応(下式)を触媒する。

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

スーパーオキシドジスムターゼは，細胞内に発生したスーパーオキシドアニオンラジカル濃度を低下させることにより，DNA，膜脂質，タンパク質，炭水化物の酸化的損傷を抑制し，酸素障害を防御している。サブステム「-dismase」を持ちINNに収載されている品目として，LedismaseとSudismaseがある。また，「-dismase」を持たないが，INNに収載されているOrgoteinは赤血球由来スーパーオキシドジスムターゼである。そのPEG化体PegorgoteinもINNに収載されている。

ステム「-ase」を持つその他の酵素性医薬品として，INNやJANには多くの糖分解酵素も収載されている。それらは本連載の第21回で紹介する予定である。

以上，今回は，酵素を示すステム「-ase」を持つ医薬品の中から，タンパク質分解酵素，ウロキナーゼ型プラスミノーゲンアクチベーター類，組織プラスミノーゲンアクチベーター類，プラスミノーゲンアクチベーターと他の酵素との融合タンパク質，リパーゼおよびスーパーオキシドジスムターゼ活性を持つ酵素を紹介した。


■参考文献

本稿作成に使用した参考文献は，本連載第5回(本誌2006年12月号)に記載している。また，以下の文献を参考にした。

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HUMAN MUTATION 0, 1–8, 2008

RESEARCH ARTICLE

# A Human Phospholamban Promoter Polymorphism in Dilated Cardiomyopathy Alters Transcriptional Regulation by Glucocorticoids

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*Communicated by Nancy B. Spinner*

**Depressed calcium handling by the sarcoplasmic reticulum (SR) Ca-ATPase and its regulator phospholamban (PLN) is a key characteristic of human and experimental heart failure. Accumulating evidence indicates that increases in the relative levels of PLN to Ca-ATPase in failing hearts and resulting inhibition of Ca sequestration during diastole, impairs contractility. Here, we identified a genetic variant in the PLN promoter region, which increases its expression and may serve as a genetic modifier in dilated cardiomyopathy (DCM). The variant AF177763.1:g.203A > C (at position –36bp relative to the PLN transcriptional start site) was found only in the heterozygous form in 1 out of 296 normal subjects and in 22 out of 381 cardiomyopathy patients (heart failure at age of 18–44 years, ejection fraction = 22 ± 9%). In vitro analysis, using luciferase as a reporter gene in rat neonatal cardiomyocytes, indicated that the PLN-variant increased activity by 24% compared to the wild type. Furthermore, the g.203A > C substitution altered the specific sequence of the steroid receptor for the glucocorticoid nuclear receptor (GR)/transcription factor in the PLN promoter, resulting in enhanced binding to the mutated DNA site. These findings suggest that the g.203A > C genetic variant in the human PLN promoter may contribute to depressed contractility and accelerate functional deterioration in heart failure. Hum Mutat 0,1–8, 2008.** © 2008 Wiley-Liss, Inc.

KEY WORDS: promoter; polymorphism; transcriptional factor; GR; GRE; cardiomyopathy; PLN; SR Ca-ATPase

## INTRODUCTION

Heart failure is a multifactorial syndrome in which intrinsic myocardial dysfunction contributes to cardiac dilation and diminished ejection performance, leading to progressive cardiac deterioration or sudden death [Richardson et al., 1996; Seidman and Seidman, 2001]. Genes causally associated with cardiomyopathy have been identified through nonbiased genetic analysis or by candidate gene studies in experimental system [Geisterfer-Lowrance et al., 1996; Franz et al., 2001]. Thus, molecular modifiers of heart failure include mutations of genes that encode cytoskeletal, sarcomeric, nuclear membrane, and calcium handling sarcoplasmic reticulum (SR) proteins. These findings have implicated pathogenic mechanisms whereby perturbation of structural integrity, contractile force dynamics, and calcium regulation within the cardiac myocyte intrinsically contribute to myocardial disease.

Abnormal calcium homeostasis is a prototypical mechanism for contractile dysfunction in failing cardiomyocytes. Depressed calcium cycling in experimental and human heart failure reflects, at least in part, impaired calcium sequestration by the SR [Chien, 2000; MacLennan and Kranias, 2003]. Calcium sequestration is mediated by a Ca-transport ATPase (SERCA2a), whose activity is modulated by alteration in the expression and phosphorylation of phospholamban (PLN; MIM# 172405) [Luo et al., 1996; Simmerman and Jones, 1998]. In experimental models, expression levels of PLN closely correlate with basal contractile parameters and their responses to β-agonists [Luo et al., 1994; Kadambi et al., 1996; Brittsan et al., 2000; Dash et al., 2001]. In human heart failure, the levels of PLN are increased relative to SERCA2a, resulting in higher inhibition of the Ca-pump's Ca-affinity, which impairs relaxation [Beuckelmann et al., 1992; Meyer et al., 1995; Hasenfuss, 1998]. As a double insult, the phosphorylation status of PLN is decreased, leading to increased inhibitory function and further depression of SR Ca-cycling. Thus, PLN is a major

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