Endothelial Progenitor Cells Derived From CD34⁺ Cells Form Cooperative Vascular Networks

Sufen Guo^{1,2}, Yongxia Cheng^{1,2}, Ying Ma¹ and Xianghong Yang¹

¹Department of Pathology, China Medical University Affiliated Shengjing Hospital, Shenyang, ²Department of Pathology, MuDanJiang Medical University, Mudanjiang

Key Words

Endothelial progenitor cell • Endothelial-like cell • Smooth muscle-like cell • Vasculogenesis • Cooperative vascular networks

Abstract

Studies were conducted to investigate whether endothelial progenitor cells (EPCs) derived from CD34⁺ cells could differentiate to endothelial- and smooth muscle-like cells, respectively. Differentiation was induced by either basic fibroblast growth factor (bFGF) or platelet-derived growth factor-BB (PDGF-BB). Further, the contribution of these cells to cooperative blood vessel formation was examined. Immunofluorescence microscopy and Western blot analysis were used to assess vascular smooth muscle (SMC) or endothelial (EC) cell differentiation. SMC (α -SM actin and calponin) or EC (CD31 and von Willebrand factor, vWF) markers showed significant expression. In contrast, expression of the stem/progenitor cell marker CD133 gradually decreased. To quantify the percentage of positive cells of smooth muscle cell marker and endothelial cell marker, flow

cytometric analysis was performed, and the results demonstrated that up to 83.76% of PDGF-BB treated-cells were positive for α -SMA marker and up to 89.27% of bFGF-treated cells were positive for vWF marker. To assess functional properties of the endothelial- and smooth muscle-like cells and to demonstrate an association of the two cell types in vascular structures, we evaluated the capacity of these cells to form angiogenesis-like networks on Matrigel. Both endothelial-like cells plated in isolation and co-cultured with smooth muscle-like cells showed formation of vascular networks on Matrigel while smooth muscle-like cells alone did not. Co-culture of fluorescently labeled endothelial- (PKH26, red fluorochrome) and smooth muscle-like cells (PKH67, green fluorochrome) revealed that these cells formed cooperative networks of tubular structures on Matrigel. Collectively, our findings suggest that EPCs can differentiate into both smooth muscle- and endothelial-like cells and contribute to the development of cooperative vascular networks. Notably, this differentiation could also provide a stable foundation for maintenance of the newly formed vascular networks.

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Introduction

Stem/progenitor cells have been used for cell-based therapy to promote postnatal vasculogenesis in several clinical situations including ischemic heart disease and peripheral artery disease. Although transplanting bone marrow-derived mononuclear cells or CD34⁺ cells has already been applied to the treatment of critical limb ischemia via endothelial cell differentiation and formation of vascular networks [1-4], little information is available regarding whether the newly formed vessels are prone to regression. As these new vessels are simply composed of endothelial cells (ECs) and lack associated pericytes and/or smooth muscle cells (SMCs), questions arise as to the stability of the new vessels.

ECs can initiate but not complete angiogenesis, periendothelial cells being essential for vascular maturation [5, 6]. This maturation requires communication between ECs and supporting cells such as SMCs and pericytes [7-9]. Importantly, pericytes and SMCs have been shown to be key-determining factors in maintaining the stability of blood vessels. Specifically, contact between pericytes/SMCs and ECs has been suggested to stabilize vessels, promote endothelial survival [10, 11], and inhibit endothelial cell proliferation [12-14]. Collectively, the available literature indicates that the formation of a mature and functional vascular network requires both ECs and pericytes/SMCs [15-17].

However, the establishment of stable and functional blood vessel networks is a complex process that requires a cocktail of angiogenic factors to stimulate vessel sprouting and remodeling of the primitive vascular network [8]. At present, most preclinical and clinical studies have shown that the provision of some factors as single agents results in the formation of stabilized blood vessels of only limited duration [5, 8, 18]. Indeed, it has been recently shown that delivery of a combination of FGF-2 and PDGF-BB can induce the formation of long-lasting blood vessels [19, 20]. With respect to the origin of the necessary cell types, studies have demonstrated that ECs and SMCs can be derived from a common progenitor cell [21, 22]. Recent data, however, also suggest that SMCs may originate from bone marrow-derived cells in the adult [23-25].

Based on the above information, we have hypothesized that EPCs may differentiate into both endothelial-like cells and smooth muscle-like cells following treatment with either bFGF or PDGF-BB and participate in cooperative blood vessel formation. As a

consequence, a stable foundation of the newly formed vascular networks may be efficiently established.

Materials and Methods

Isolation, Culture and Characterization of EPCs Derived From CD34⁺ Cells

All animal studies were reviewed and approved by the Animal Care and Use Committee of China Medical University (Shenyang, China). Mononuclear cells were separated from rat bone marrow by Ficoll density gradient centrifugation by differences in time taken for adherence. Briefly, bone marrow cells were flushed from the femurs and tibias of Wistar rats (40–50g) into phosphate buffered solution (PBS). The cell suspension was then loaded on a Ficoll-Paque density gradient (specific gravity=1.083, Tianjin Haoyang Biological Manufacture Co. Ltd, China) and centrifuged at 2000 rpm for 25 minutes. The cells were isolated from the white layer between the Ficoll-Paque reagent and the blood plasma and then washed twice in PBS. An aliquot of the cell suspension (1×10^6 cells) was then plated onto (1%) gelatin-coated 25cm² tissue culture flasks and cultured in a humidified (5% CO₂) incubator at 37°C for 24 hours. The cell culture medium used in these studies was Dulbecco's modified essential medium (DMEM, pH 7.4, Gibco, USA) supplemented with 20% fetal bovine serum (Gibco, USA), 100 U/ml penicillin, 100µg/ml streptomycin, 10Mm HEPES (Sigma, USA), and 10 U/ml heparin (Sigma, USA). After 24 hours of culture, CD34⁺ cells were isolated from non-adherent cells under sterile conditions, using flow cytometry (BD FACSCALIBUR) with an FITC-conjugated anti-CD34 antibody (Santa Cruz Biotechnology, USA). CD34 negative cells (vascular endothelial cells) were used as a control. Isolated CD34⁺ cells (1×10^6 cells) were then plated onto 25cm² tissue culture flasks and cultured in the above medium supplemented with 20 ng/ml vascular endothelial growth factor (VEGF, Peprotech, England), 1 ng/ml fibroblast growth factor (FGF, Peprotech), and 2 ng/ml insulin-like growth factor (IGF, Peprotech). After five days of culture, cells were tested for uptake of 1, 1'-dioctadecyl-3,3',3'-tetramethylindo-carbocyanine percholorate-labeled acetylated low-density lipoprotein (DiI-ac-LDL, Molecular Probes, USA) and binding of fluorescein isothiocyanate-conjugated Ulex europaeus lectin (FITC-UEA-I, Vector, USA). In brief, adherent cells were incubated for four hours with DiI-ac-LDL at 37°C. Cultures were then washed, fixed with 1% formaldehyde (10 minutes), and washed again with PBS. FITC-UEA-I was added at a concentration of 10 µg/ml and incubated for another hour at 25°C. Following washing twice, fluorescence images were recorded using a confocal microscope (Olympus FV 1000, Japan).

Induction culture conditions

After five days of EPC culture, the medium was replaced by either the control medium (DMEM, supplemented with 20% fetal bovine serum, 10Mm HEPES, 10 U/ml heparin, and antibiotics) or a control medium containing rat bFGF (30ng/ml)

(Peprotech, USA) or PDGF-BB(40ng/ml) (Sigma, USA [19, 22] The cultures were incubated for up to 14 days, changing the respective mediums at two- to three-day intervals.

Immunofluorescence staining

Cells grown on chamber slides were washed three times in PBS, fixed in 4% paraformaldehyde for 15 minutes, permeabilized with Triton X-100 0.5% (w/v in distilled water) for 15 minutes, and incubated at 37°C for one hour with PBS containing 3% BSA (Sigma) to block non-specific binding sites. Then, bFGF-induced cells (FICs) and control cells were incubated with primary antibodies for the rabbit polyclonal platelet-endothelial cell adhesion molecule-1 (PECAM-1) (CD31) (1:200, Santa Cruz Biotechnology), von Willebrand factor (vWF), mouse monoclonal anti- α -SM actin (α -SMA) and calponin (1:100, Santa Cruz Biotechnology, USA). PDGF-BB-induced cells (PICs) and control cells were incubated with primary antibodies for mouse monoclonal α -SMA, calponin, rabbit polyclonal vWF and CD31. After washing, cells were incubated with appropriate secondary antibodies (bovine anti-mouse IgG-FITC or mouse anti-rabbit IgG-PE; Santa Cruz Biotechnology, USA). Images were obtained by confocal microscopy, and fluorescence intensities were measured to evaluate SMC or EC markers.

Western blot analysis

Cultured cells were washed twice with ice-cold PBS and then lysed in 1 ml of ice-cold lysis buffer containing 50mM Tris pH8, 100mM NaCl, 1% Triton X-100, 0.5%NP40, 50mM EDTA, 1 mM PMSF, 10 AM leupeptin, 0.2TIU/ml aprotinin, 2mM sodium orthovanadate, 40mM h-glycerophosphate, 50mM NaF, and 100 AM phenylarsine oxide. Homogenates were then centrifuged at 12,000xg for 15 minutes. The supernatants were collected and mixed with concentrated (5x) Laemmli sample buffer, boiled for 5 minutes, and centrifuged 12,000xg for 15 minutes. Protein samples were separated by electrophoresis using 6.5-10% acrylamide/bisacrylamide (29:1) gels containing SDS and then transferred to PVDF membranes. Membranes were blocked by incubation in TBS containing 5% non-fat milk powder for 2 hours at room temperature. The membranes were incubated overnight at 4°C with an antibody against CD133, α -SM actin, calponin, CD31 or vWF(1:100, Santa Cruz, USA) and GAPDH or β -actin (1:2000, Santa Cruz, USA). After washing, the membrane was incubated with horseradish peroxidase-conjugated second antibodies (1:2000) and protein bands detected by enhanced chemiluminescence (Pierce, USA). The intensities of bands were quantified using Quantity One software (Bio-Rad, USA).

Fluorescence Activated Cell Sorting (FACS)

Cells cultured for 2 weeks were harvested with 2 mmol/L EDTA/PBS, and then suspended in PBS with 0.1% bovine serum albumin at 1×10^6 cells per tube. After blocking, cells were incubated for 30 minutes on ice with rabbit polyclonal vWF antibody or mouse monoclonal α -SMA antibody, followed by incubation with mouse anti-rabbit IgG-FITC antibody or bovine

anti-mouse IgG-FITC antibody (Santa Cruz Biotechnology, USA), respectively. As negative controls, different FITC or PE conjugated isotype antibodies (Santa Cruz Biotechnology, USA) were used. After staining, cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry (BD FACSCALIBUR). Positive cells were counted and compared with cells not exposed to the growth factors.

In vitro tube formation

Growth factor-treated ECs and SMCs were labeled with PKH26 (red fluorochrome) and PKH67 (green fluorochrome) (Sigma, USA), respectively. Matrigel (60 μ L per well; BD Biosciences, USA) was added to 24-well plates and incubated at 37°C for one hour. Endothelial-like cells and smooth muscle-like cells (labeled and non-labeled, 5 to 6x10⁵ cells/ well) were plated separately or together at a ratio of 3:1 (endothelial-like to smooth muscle-like cells) in 300 μ L of DMEM/20% FBS, then cultured at 37°C with 5% CO₂, and monitored frequently by phase contrast microscopy. After tube formation was observed, images were obtained with a fluorescence phase contrast microscope (Nikon, Japan).

Statistical analysis

Data are presented as mean $\pm$ SEM. All experiments were repeated in triplicate on three independent occasions. Mean values were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni test. *P* value <0.05 was considered to be statistically significant.

Results

Isolation of CD34⁺ cells

Using an FITC-conjugated anti-CD34 antibody analysis by flow cytometry showed that 76.3% of the purified cells were positive for CD34 (Fig.1).

EPC characterization

Consistent with an EPC phenotype, immunostaining demonstrated that most adherent cells were positive for both uptake of DiI-ac-LDL and binding of FITC-UEA-I (Fig.2) [26-29].

FGF and PDGF - treated cell cultures

After four days' treatment of EPCs with growth factors or vehicle, control cells (non-induced cells, NICs) showed an elongated sprout appearance (Fig. 3A) while FICs displayed a cobblestone appearance (Fig. 3B) and PICs appeared spindle-shaped (Fig. 3C). After eight days, NICs appeared polygonal in shape (Fig. 3D), while FICs showed a flat elongated appearance (Fig. 3E) and PICs displayed a "peak and valley" morphology (Fig. 3F).

Fig. 1. Characterization of purified cells using flow cytometry with an FITC-conjugated anti-CD34 antibody. CD34 negative cells (smooth muscle cells) were used as a control. The result showed 76.3% of the cells to be positive for CD34.

Fig. 2. Characterization of EPCs. On day 5 of culture, adherent cells showed uptake of DiI-ac-LDL and binding of FITC-UEA-I. Red fluorescence represents cells positive for DiI-ac-LDL uptake, and green fluorescence shows cells positive for FITC-UEA-I binding. Endothelial progenitor cells are demonstrated in the merged confocal image (yellow).

Confocal microscopy

To evaluate the phenotype of differentiated cells, we examined the expression of the smooth muscle markers, α -SMA and calponin, and the endothelial cell markers, CD31 and vWF. As expected, under growth factor treated conditions, FICs and PICs displayed, respectively, a prominent fluorescent staining of endothelial cell markers and smooth muscle markers at days 4 and 8 (Fig. 4A-F; G-L). Up to day 8, FICs did not stain positive for SMC markers (α -SMA and calponin), and PICs did not stain positively for EC markers (vWF

and CD31) (Data not shown). Compared with NICs, fluorescence intensity of FICs and PICs was increased at day 4 while being further increased at day 8 ($P < 0.05$ or $P < 0.01$, Fig. 5A, B). NICs remained negative for SMCs markers, while also negative for ECs markers except CD31.

Protein expression in cultured cells

To further confirm the presence of endothelial- and smooth muscle-specific proteins in cells treated with growth factors but not in control cultures, cell lysates from

Fig. 3. Morphology of differentiated cells. On day 4, non-induced cells (NICs) appeared as elongated sprouts (A, arrow); bFGF-induced cells (FICs) displayed a cobblestone appearance (B, arrow); and PDGF-BB-treated cells (PICs) demonstrated a spindle shape (C, arrow). On day 8, NICs appeared polygonal in shape (D, arrow), while FICs had a flat elongated appearance (E, arrow), and PICs displayed a “peaks and valley” morphology (F, arrow). Objectivex20, scale bar 50 μ m.

Fig. 4. Characterization of vascular cell phenotype. Endothelial-like cells were characterized by the expression of the specific markers, CD31 (A–C) and von Willebrand Factor (D–F), while smooth muscle-like cells showed expression of the contractile markers, α -Smooth Muscle Actin (α -SMA: G–I) and calponin (J–L). Images were obtained using confocal microscopy. FICs showed expression of specific EC markers (B, C and E, F). PICs showed the positive expression of specific SMC markers (H, I and K, L), while NICs had weak expression of CD31 (A). Up to day 8, FICs did not stain positive for SMC markers (α -SMA and calponin), and PICs did not stain positively for EC markers (vWF and CD31) (Data not shown). Objectivex20, scale bar 50 μ m.

each cell type were subjected to SDS-PAGE and immunoblotting. The treated cells were confirmed to have vWF and CD31 or α -SMA and calponin protein, while control cells were negative for α -SMA and calponin expression and positive for CD133 and CD31 protein. Until day 4, FICs expressed both CD133 and CD31 protein, while PICs also showed protein α -SMA and calponin protein (Fig. 6A and B). At eight days of culture, a similar pattern in protein expression was observed with the exception of CD133, which decreased with time.

Compared with control cells, α -SMA and calponin expression of PICs and vWF and CD31 expression of FICs was markedly increased at day 4 and further increased at day 8. CD133 gradually decreased over this time period. ($P < 0.05$ or $P < 0.01$, Fig. 7A1, A2 and A3 or B1, B2 and B3).

Fluorescence Activated Cell Sorting(FACS)

FACS analyses were performed to quantify the percentage of cells positive for smooth muscle and

Fig. 5. Time-dependent increases of cell marker expression in growth factor-treated cells. Quantification of fluorescence intensity showed greater expression of both EC (A) and SMC (B) markers to be observed at day 8 compared with either NIC or 4-day treated cells; (#) 4-day versus control, (*) 8-day versus control, (&) 8-day versus 4-day. (*, # and &: $P < 0.05$ and **, ##, and &&: $P < 0.01$). Results are shown as mean $\pm$ SEM.

Fig. 6. Protein expression in bFGF or PDGF-BB-treated cells. FICs showed increased expression of the EC marker, vWF and CD31 at days 4 and 8 (A) while PICs displayed the SMC markers, α -SMA and calponin at both time points (B). CD133 showed a time-dependent decrease in expression in treated cells.

Fig. 7. Expression of protein markers in growth factor-differentiated cells. At days 4 and 8, expression of CD133 protein was markedly decreased (A1 and B1), while CD31, vWF, α -SMA, and calponin were all increased (A2 and A3 or B1 and B2) (#, 4-day group compared with 0-day group; *, 8-day group versus 0-day group and &, 8-day group versus 4-day group, *, # and &: $P < 0.05$ and **, ##, and &&: $P < 0.01$). The data are expressed as mean $\pm$ SEM.

endothelial cell markers. Trypsinized differentiated cells were labeled as described in above. Within the differentiated cell area, as determined by forward and

sideward scattering, 10,000 events were collected and the percentage of positive cells was determined. CD34 negative cells (vascular endothelial cells) were used as a

Fig. 8. FACS analyses performed to quantify the percentage of marker positive cells. During culture of smooth muscle-like cells and endothelial-like cells only nearly 2% of NICs were positive for α -SMA and vWF marker(B), but up to 89.27% of FICs were positive for vWF marker (C) and up to 83.76% of PICs were positive for the α -SMA marker(D). IgG was used as a negative control (A). Compared with NICs, the number of positively staining FICs and PICs was markedly greater (FICs and PICs versus NICs, **: $P < 0.01$; panels E and F).

control (Fig. 8A). The specific markers (α -SMA and/or vWF) were detected in nearly 2% of NICs (Fig. 8B). Up to 89.27% of the induced cells were positive for the vWF marker (Fig. 8C), whereas up to 83.76% of cells induced were positive for α -SMA marker (Fig. 8D). Group data showed that the number of positive FICs and PICs was markedly greater than that determined in NICs (Fig. 8 E and F).

Endothelial- and smooth muscle-like cells co-assemble into vascular networks

To test for functionality of endothelial- and smooth muscle-like cells, we evaluated the capacity of these cells to undergo formation of angiogenesis-like networks on Matrigel. As shown in Figure 8, endothelial-like cells plated alone (Fig. 9A) and co-cultures of endothelial- and smooth muscle-like cells (Fig. 9C) formed vascular networks on

Fig. 9. Images of tube formation in cultures of endothelial- and smooth muscle-like cells. Cells (5 to 6×10^5 cells/well) were plated separately or together on Matrigel at a ratio of 3:1 (endothelial-like cells: smooth muscle-like cells). Endothelial-like cells cultured alone (A, arrow) and co-cultured with smooth muscle-like cells (C, arrow) formed vascular networks, while smooth muscle-like cells alone did not (B, arrow). Objective $\times 10$, scale bar $50 \mu\text{m}$.

Fig. 10. Labeled endothelial- and smooth muscle-like cells co-cultured on Matrigel (5 to 6×10^5 cells/well). Co-culture of PKH26-labeled endothelial-like cells (red) and PKH67-labeled smooth muscle-like cells (green) formed a cooperative network of tubular structures (A, B, and C). Objective $\times 10$, scale bar $50 \mu\text{m}$.

Matrigel, while smooth muscle-like cells alone did not (Fig. 9B). Co-culture of fluorescently labeled endothelial (red) - and smooth muscle (green) - like cells on Matrigel showed that these cells formed a cooperative network of tubular structures (Fig. 10A, B, and C).

Discussion

This study set out to test the hypothesis that bone marrow CD34-derived EPCs may participate in cooperative blood vessel formation by differentiating into endothelial- and smooth muscle-like cells. Collectively, the results provide strong evidence to support this hypothesis. In the initial series of studies, we examined the effects of bFGF and PDGF-BB on EPC morphology using phase-contrast microscopy. After four days of exposure of EPCs to control conditions or growth factors, NICs appeared as elongated sprouts while, in contrast, FICs displayed a cobblestone appearance and PICs formed a spindle shape. After eight days, NICs demonstrated polygonal shape, while PICs displayed “peaks and valley” arrangement, consistent with the appearance of SMCs, and FICs showed a flat elongated morphology. To further evaluate the cell phenotype of differentiated cells, we examined the expression of specific markers, including α -SMA and calponin, known to assess vascular SMC differentiation [30–32], and vWF and CD31 to identify an EC phenotype [33, 34]. As expected, EPCs cultured in DMEM with 20% FCS and PDGF-BB or bFGF, respectively, showed classic SMC or EC specific markers at days 4 and 8. In contrast, there no evidence of SMC-specific markers or adult EC-specific markers (vWF) in NICs. Western blot analysis confirmed the immunohistochemistry studies showing the presence of specific markers in the growth factor-differentiated cells while NICs, similarly derived from bone marrow EPCs, were negative for α -SMA and calponin expression. In contrast, EPCs treated with bFGF or PDGF-BB for eight days showed an absence of CD133 protein, a stem/progenitor cell marker. Thus, the overall pattern is consistent with FICs and PICs losing their stem/progenitor cell characteristics and showing the potential for differentiating into endothelial- or smooth muscle-like cells.

Tissue engineering or neovascularity in ischemic diseases needs a quantity of seed cells. In order to analysis the positive rate of growth factor-treated cells, we examined how much EPCs can differentiate into endothelial- or smooth muscle-like cells by FCS. The results showed that the positive rate of NICs was about 0.2%, the positive rate of the PICs was up to 83.76%, and the positive rate of the FICs was up to 89.27% at days 8.

The intent of manipulating stem/progenitor cells is for them to be induced to exhibit the functional properties

of corresponding adult cells. To further investigate the biological function of endothelial-like cells or smooth muscle-like cells in modulating arteriogenesis and angiogenesis, we cultured endothelial-like cells and smooth-like cells on Matrigel. The results showed endothelial-like cells forming vascular networks, while smooth muscle-like cells alone did not demonstrate this capacity. When co-cultured on Matrigel, endothelial- and smooth muscle-like cells formed cooperative networks of tubular structures. Importantly, this cooperative network may offer a basic structure for longer-term vascular stability, a primary aim of our studies. Although these co-cultured networks likely involved an interplay between endothelial- and smooth muscle-like cells, the EC only networks would lack this interaction with SMCs, perhaps rendering them more susceptible to regression.

The findings of our studies suggest the potential for EPC differentiation into endothelial-like cells or smooth muscle-like cells under the culture conditions in which the medium is supplemented with bFGF or PDGF-BB. Previous studies have shown that PDGF-BB is implicated in embryonic smooth muscle cell differentiation [18], and that PDGF-CC, a heterodimer of PDGF-BB, promotes differentiation of bone marrow progenitors into vascular cells [35]. Consistent with these observations, our studies support a potential role for PDGF-BB (40 ng/ml) in differentiation of smooth muscle cells from bone marrow-derived EPCs. A number of studies have previously shown that EPCs can differentiate into EC [36–38] with a cytokine mix containing epidermal growth factor (EGF), bFGF, and vascular endothelial growth factor (VEGF), while the present experiments showed EPCs could differentiate into endothelial-like cells following treatment with bFGF 30ng/ml alone.

Thus, expansion of these cells may provide sufficient quantities of seed cells for tissue engineering approaches for the treatment of vascular disease [17, 21, 39]. This may also circumvent problems associated with using in vitro mature SMCs (harvested from blood vessels) which can switch their phenotype from a contractile (healthy) to a proliferative (pathological) phenotype [40, 41], a current strong limitation for blood vessel tissue engineering [42]. Clearly, the mechanisms that underlie the differentiation of EPCs require further study. Thus, we are currently testing whether co-implantation of growth factor-treated ECs and SMCs will establish new stable blood vessels *in vivo* and whether the new blood vessels have the ability to anastomose with the host vasculature.

Abbreviations

bFGF (basic fibroblast growth factor); PDGF-BB (platelet-derived growth factor-BB); NICs (non-induced cells); FICs (bFGF-induced cells); PICs (PDGF-BB-induced cells).

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