Endothelial cells genetically selected from differentiating mouse embryonic stem cells incorporate at sites of neovascularization in vivo

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Summary

Large scale purification of endothelial cells is of great interest as it could improve tissue transplantation, reperfusion of ischemic tissues and treatment of pathologies in which an endothelial cell dysfunction exists. In this study, we describe a novel genetic approach that selects for endothelial cells from differentiating embryonic stem (ES) cells. Our strategy is based on the establishment of ES-cell clones that carry an integrated puromycin resistance gene under the control of a vascular endothelium-specific promoter, *tie-1*. Using EGFP as a reporter gene, we first confirmed the endothelial specificity of the *tie-1* promoter in the embryoid body model and in cells differentiated in 2D cultures. Subsequently, *tie-1*-EGFP ES cells were used as recipients for the *tie-1*-driven puro^r transgene. The resulting stable clones were expanded and differentiated for seven days in the presence of VEGF before puromycin selection. As expected, puromycin-resistant cells were positive for EGFP and also expressed several endothelial markers, including CD31, CD34, VEGFR-1, VEGFR-2, Tie-1, VE-cadherin and ICAM-2. Release from the puromycin selection resulted in the appearance of α-smooth muscle actin-positive cells. Such cells became more numerous when the population was cultured on laminin-1 or in the presence of TGF-β1, two known inducers of smooth muscle cell differentiation. The hypothesis that endothelial cells or their progenitors may differentiate towards a smooth muscle cell phenotype was further supported by the presence of cells expressing both CD31 and α-smooth muscle actin markers. Finally, we show that purified endothelial cells can incorporate into the neovasculature of transplanted tumors in nude mice. Taken together, these results suggest that application of endothelial lineage selection to differentiating ES cells may become a useful approach for future pro-angiogenic and endothelial cell replacement therapies.

Key words: Embryonic stem cells, Endothelial cells, Differentiation, Selection, Angiogenesis

Introduction

The formation of new blood vessels, called angiogenesis, is involved in many physiological and pathological situations, such as embryogenesis, tissue remodeling and regeneration, wound healing and tumor neovascularization. In the adult, this process involves the recruitment and proliferation of either endothelial cells from pre-existing vessels or circulating endothelial progenitor cells (EPCs) originating from bone marrow (Asahara et al., 1997; Folkman, 1995).

In different situations, including advanced age, diabetes or hypercholesterolemia, such neovascularization is impaired, due to the reduction of vascular endothelial growth factor (VEGF) expression and to endothelial cell dysfunction (Couffinhal et al., 1999; Rivard et al., 1999a; Rivard et al., 1999b; Van Belle et al., 1997). Consequently, endothelial cell transplantation as a potential method for treatment of patients with vascular defects has become attractive and challenging in the past few years. This approach could also be used to improve tissue grafting after injury or reperfusion of ischemic tissues.

Several studies have described protocols to isolate and expand the population of circulating EPCs from blood (Asahara et al., 1997; Kalka et al., 2000; Shi et al., 1998). In different animal models of neovascularization, EPCs were shown to participate and enhance the formation of new blood vessels, and consequently increase tissue salvage (Asahara et al., 1999a; Asahara et al., 1999b; Kawamoto et al., 2001; Takahashi et al., 1999). However, EPCs represent a small proportion (0.1-0.5%) of circulating blood cells, and their expansion ex-vivo after harvest takes a considerable amount of time. This aspect is a concern for treating life-threatening conditions in patients. In this context, the development of a strategy for large scale purification of vascular endothelial cells (ECs) is of great interest.

Embryonic stem (ES) cells are derived from the inner cell mass of mouse blastocysts and have the potential of generating all embryonic cell lineages, including endothelial cells (Doetschman et al., 1985; Risau et al., 1988). ES-derived endothelial cells express most known endothelial cell markers, including CD31, VEGFR-2 (Flk-1), VE-cadherin, Tie-1 and Tie-2, and have the potential to form pseudo-vascular

structures when differentiated into embryoid bodies (Vittet et al., 1996).

ES cells have a high proliferation rate and can easily be modified to express transgenes. In this respect, they could be used to genetically select ES-derived endothelial cells for grafting. Approaches employing genetic selection have already been used in ES cells in order to select for cardiomyocytes and neural-precursor cells (Klug et al., 1996; Li et al., 1998). Importantly, ES-derived cardiomyocytes were shown to form stable intracardiac grafts after injection into mice (Klug et al., 1996).

In this report, we used a similar strategy to yield large numbers of ECs from genetically modified ES cells. To this end, undifferentiated ES cells were transfected with a fusion gene consisting of the *tie-1* promoter, specifically expressed in endothelial cells (Iljin et al., 1999; Korhonen et al., 1995), followed by sequences encoding the selection marker puromycin. Expression of the puromycin-resistance gene in ES-derived endothelial cells facilitated their purification during their in vitro differentiation. In addition, we observed that the release of puromycin selection was accompanied by the occurrence of cells positive for α-smooth muscle actin. We also show that purified endothelial cells participate in tumor angiogenesis in vivo.

Materials and Methods

Generation of vectors

Reporter gene constructs containing the *tie-1* promoter were generated by subcloning the *Hin*dIII-*Apa*I fragment of the mouse *tie-1* promoter (Iljin et al., 1999; Korhonen et al., 1995) into the same sites of either the pGL2 basic vector (luciferase reporter plasmid, Promega, Madison, WI) or the pEGFP vector (Clontech, Palo Alto, CA). The pEGFP vector contains the neomycin (G418) resistance gene.

The luciferase gene construct containing the *VE-cadherin* promoter was generated by subcloning the *Sal*I(blunted)-*Xho*I fragment of the mouse *VE-cadherin* promoter [a gift from P. Huber, CEA, Grenoble, France (Gory et al., 1999)] into the *Sma*I-*Xho*I sites of the pGL2 basic vector.

A *Bam*HI-*Kpn*I fragment corresponding to the puromycin-N-acetyl-transferase (puro^r) cDNA was introduced into the same sites of the pGEM-3Zf(+/–) vector containing the *Hin*dIII-*Apa*I fragment of the mouse *tie-1* promoter (Korhonen et al., 1995).

Antibodies

Rat antibodies against mouse CD31 (clone MEC 13.3), mouse CD34 (clone RAM34) and mouse VE-cadherin (clone 75) were from BD Pharmingen (Los Angeles, CA). Rat anti-VEGFR-2 was purchased from Clinisciences (France). Anti-α-smooth muscle actin was purchased from Sigma (Saint-Louis, MO).

Alexa Fluor-conjugated goat anti-mouse antibody, FITC and Alexa Fluor-conjugated streptavidin were from Molecular Probes (Eugene, OR). The biotin-conjugated donkey anti-rat antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). Biotin-conjugated anti-rabbit antibodies were from Amersham Pharmacia Biotech (Sweden). The FITC-conjugated rabbit anti-goat antibody was from Dako (Denmark).

Cell culture

OLA 129 ES cells, a gift from A. Smith (Centre for Genome Research, Edinburgh, UK) were maintained in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-1 and NaPyruvate (Gibco BRL, Germany) containing 10% fetal calf serum (FCS; Dutscher, France), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM β-mercaptoethanol and non essential amino acids (all reagents were from Gibco BRL). This medium will be referred to as complete DMEM. The cells were kept undifferentiated by the addition of recombinant leukemia inhibitory factor (LIF) either purchased from Sigma or produced from COS cells (Smith, 1991). ES cells (5×10^6 cells) were transfected by electroporation with a gene pulser (Eurogentec, Belgium) set at 500 V and 40 μF, in a total volume of 500 μl of PBS.

The established Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection) and PS120 MEK S222D (Brunet et al., 1994) were maintained in DMEM supplemented with 7.5% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. Bovine aortic endothelial cell (BAEC) were a generous gift of H. Drexler (Max-Planck-Institut, Bad Nauheim, Germany). They were maintained in low glucose DMEM supplemented with 5% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. HEK 293 cells were maintained in DMEM supplemented with 8% heat inactivated FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Transfection and luciferase assay

BAEC (1.5×10^5 cells/well), CCL39 (10^5 cells/well) and HEK 293 (2×10^5 cells/well) in 12-well dishes were transiently transfected by $CaPO_4$ precipitation with the indicated plasmids (600 ng/well of the reporter plasmid and 300 ng/well of the CMV β-galactosidase as a control for transfection efficiency). Two days later, cells were washed with cold PBS, and luciferase assays were performed according to the Promega protocol and application guide. Briefly, cells were lysed in lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1, 2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, and 1% Triton X-100) for 15 minutes at room temperature, and the lysate was cleared by centrifugation. Luciferase activity was quantified in a luminometer (EG&G Wallac, Turku, Finland) in a buffer containing 20 mM Tricine, 1.07 mM $(MgCO_3)Mg(OH)_2$, $5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferine, and 530 μM ATP. The protein concentrations were measured using the Biorad protein assay (Hercules, CA).

In vitro differentiation and selection of ES-derived endothelial cells

Two protocols were used, to induce growth and differentiation. In the first one, freshly EDTA/trypsin (Biowhitaker, Walkersville, MD) dissociated ES cells were aggregated into embryoid bodies (EBs) in hanging drops of complete DMEM lacking LIF. 20 μl drops of cell suspension (4×10^4 cells/ml) were placed on the inside of lids of bacteriological Petri dishes. The lids were then placed over PBS-filled Petri dishes and incubated at 37°C; this was designated day 0. After 3-4 days, the resulting EBs were transferred to gelatin-coated 24-well tissue culture plates. In some cases, the medium was supplemented with human rVEGF-165 either purchased from Sigma or produced in our laboratory from *Pichia pastoris* after purification on heparin binding affinity columns, using the pPICZαA vector (Scheidegger et al., 1999) kindly provided by K. Ballmer-Hofer (University of Zürich, Switzerland).

In the second differentiation protocol, freshly dissociated ES cells were plated (3000 cells/cm^2) in a 2D plane, on gelatin-coated dishes in complete DMEM lacking LIF. This method was used for selection of ES-derived endothelial cells. The concentration of puromycin to be applied on *tie-1*-puro^r expressing clones was determined using the non-resistant *tie-1*-EGFP cells as control cells. As *tie-1*-EGFP cells were killed by 0.25 μg/ml antibiotic, 1 μg/ml puromycin was added to the medium of differentiated *tie-1*-puro^r cells during 4 days. When indicated, selected cells were cultured on mouse laminin-1 (Sigma,

20 μg/ml) or in the presence of 5 ng/ml TGF-β1 (TEBU) or 10 ng/ml PDGF-BB (TEBU).

For spheroid formation, purified endothelial cells were aggregated into hanging drops (20 μl drops, 10^5 cells/ml) for 5 days, in complete DMEM lacking LIF and in the presence of 10 ng/ml VEGF.

Genomic PCR for screening ES stable clone

PCR amplification on genomic DNA was carried out with the *Taq* PCR master mix (Qiagen, France) containing *Taq* DNA polymerase, PCR buffer (1.5 mM $MgCl_2$) and dNTPs (200 μM for each dNTPs). After 35 cycles, the products were analyzed on ethidium-bromide-stained 1% agarose gels.

For PCR detection of the *tie-1*-EGFP transgene, we used the following pair of primers: 5′-CCCAACCATCCCCAGATCTG-3′ and 5′-TCCTCGCCCTTGCTCACCAT-3′, that anneal to the 3′ region of the *tie-1* promoter and the 5′ region of the EGFP cDNA, respectively. For PCR detection of the *tie-1*-puro^r transgene, we used the same *tie-1* promoter primer and the primer 5′-GCGACCCACACCTTGCCG-ATG-3′, which anneals to the 5′ region of the puro^r cDNA. The annealing temperature was set at 57°C for both PCR reactions.

RT-PCR

Total RNAs from undifferentiated and from purified B9TP cells were isolated using the RNA-now procedure (Biogentex, France). First-strand cDNAs were generated using reverse transcriptase (Roche, France) and oligo dT (Gibco BRL) using the manufacturer's instructions. For PCR amplification, cDNAs were amplified using the Taq PCR master mix (Qiagen). The amplification parameters were 95°C for 45 seconds, 55°C for 45 seconds and 72°C for 30 seconds, for 35 cycles. To ensure that the RT-PCR assay was semiquantitative, the quantity of equivalent reverse-transcribed RNA chosen was in the linear range of amplification. Hypoxanthine phosphoribosyl-transferase (HPRT) was used as an internal standard. The sequences of the primers used are the following:

VEGFR-2 5′-AGCTCTCCGTGGATCTGAAA-3′ and 5′-CCAAG-AACTCCATGCCCTTA-3′;

VEGFR-1 5′-CGGAAGCTCTGATGATGTGA-3′ and 5′-TATCT-TCATGGAGGCCTTGG-3′;

Tie-1 5′-CTCACTGCCCTCCTGACTGG-3′ and 5′-CGATGTA-CTTGGATATAGGC-3′;

HPRT 5′-GCTGGTGAAAAGGACCTCC-3′ and 5′-CACAGGAC-TAGAACACCTGC-3′.

Tumor formation in nude mice

The incorporation of ES-derived endothelial cells into sites of neovascularization was analyzed by using a tumor transplantation model in athymic nude mice (Harlan, France). To this end, 10^6 ES-derived endothelial cells were co-injected subcutaneously with 10^6 PS120 MEK S222D cells into the left flank of male athymic nude mice (n=6). For controls, 10^6 tumoral cells were injected into the right flank of the same animal, and 10^6 ES-derived endothelial cells were injected into the flank of five other mice.

Mice were euthanized after macroscopic tumor identification and frozen sections (4 μm) were processed for hematoxylin and immunofluorescence staining (see below).

Immunofluorescence staining

Differentiated ES cells, gelatin-bound embryoid bodies, spheroids and tumor sections were fixed in 4% paraformaldehyde for 20 minutes at room temperature, permeabilized with 0.2% Triton X-100 for 5 minutes and blocked in 2% BSA/PBS for 2 hours. Cells were then incubated with the appropriate antibody for 1 hour at room temperature. After three washes in PBS, cells were incubated with the appropriate fluorescent-conjugated secondary antibody or streptavidin conjugates. Preparations were mounted in PBS:glycerol (1:9) and viewed under a Leica microscope.

Results

Activity of *tie1* and *VE-cadherin* promoters in cultured cells

The purpose of this study was to purify endothelial cells from differentiating ES cells through the use of a puromycin-resistance gene driven by an endothelial-specific promoter. We first compared the strength of the tyrosine kinase receptor Tie-1 and the vascular endothelial-cadherin (VE-cadherin) promoters. Both promoters were previously shown to result in vascular endothelial-specific expression in mice (Gory et al., 1999; Iljin et al., 1999; Korhonen et al., 1995).

To this end, luciferase reporter vectors controlled by either the *tie1* or the *VE-cadherin* promoter were transiently transfected in bovine aortic endothelial cells (BAEC) and in non-endothelial cells, namely the fibroblastic CCL39 and the epithelial HEK 293 cells. The constructs were co-transfected with a vector encoding the β-galactosidase gene to monitor transfection efficiency. Relative luciferase activities were normalized to the values obtained with the empty pGL2-basic vector. Fig. 1 shows that the *tie-1* promoter was active in BAEC and, to a lesser extent, in CCL39 cells. The low expression observed in CCL39 cells could reflect the lack of specificity reached in transient expression. In contrast, no activity could be detected in HEK 293 cells. A similar pattern was observed for the *VE-cadherin* promoter, but its relative activity was always lower than that of the *tie-1* promoter. Transient expression of an EGFP reporter gene controlled by the same promoters gave similar results regarding their relative strength (data not shown). Based upon these results, we decided to test the cell-type specificity of the *tie-1* promoter in the embryoid body model.

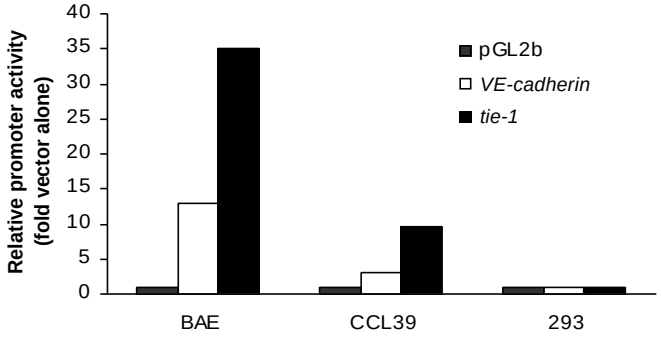

Fig. 1. Activities of *tie-1* and *VE-cadherin* promoters in cultured cells. 600 ng of each luciferase reporter construct were transfected into BAE (bovine aortic endothelial cell), CCL39 and 293 cells (non-endothelial cells). In each assay, 300 ng of the β-galactosidase expression vector were co-transfected in order to normalize for transfection efficiency. 48 hours after transfection, luciferase activity was measured. The relative activity of each construct was expressed as fold induction over the pGL2 basic vector. The results shown in this figure are representative of three independent experiments. Each set of data represents the mean of quadruplicate determinations.

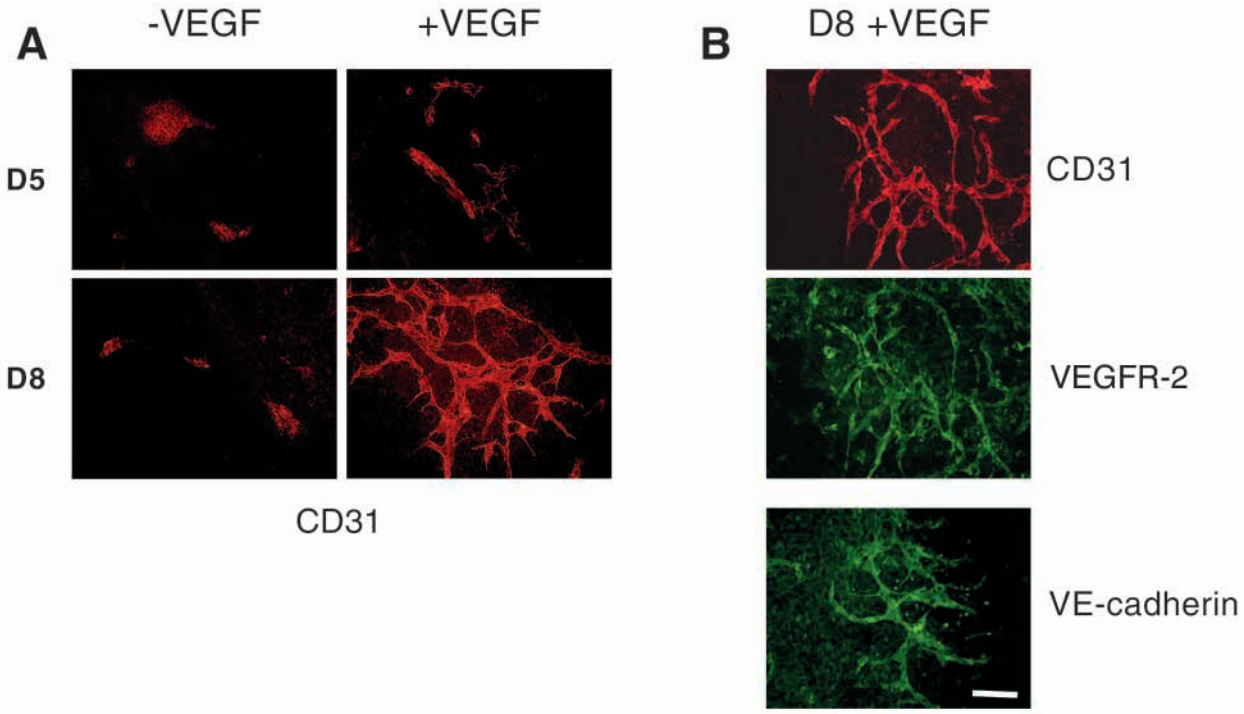

Fig. 2. Expression of vascular markers in embryoid bodies (EBs). ES cells were differentiated into EBs, fixed on day 5 or 8 and stained with antibodies to CD31, VEGFR-2 and VE-cadherin. When indicated, differentiation medium was supplemented with 10 ng/ml human rVEGF-165. (A) While the CD31 antibody stained cell clumps in untreated 5- and 8-day-old EBs, the addition of 10 ng/ml rVEGF promoted the organization of $CD31^+$ cells into pseudo-vascular structures. (B) Vascular endothelial networks in 8-day-old EBs were co-stained for CD31 (red) and VEGFR-2 (green), and similar structures also expressed VE-cadherin (green). Bar, 100 μM.

Endothelial specificity of the *tie-1* promoter in differentiating ES cells

When differentiated into embryoid bodies (EBs), ES cells reproduce the stages of embryonic development, including vasculogenesis and angiogenesis (Risau et al., 1988). We first characterized vascular-like structures in wild-type EBs treated or not with 10 ng/ml VEGF, 5 and 8 days after leukemia inhibitory factor (LIF) removal to induce differentiation. Three different endothelial markers were used: the adhesion molecule CD31, VEGFR-2 and VE-cadherin.

Five days after LIF removal, control EBs contained numerous CD31-positive ($CD31^+$) cell clusters (Fig. 2A), which also stained for VEGFR-2 but not for VE-cadherin (not shown). These cell clusters, in which CD31 staining was concentrated at cell-cell contacts, probably represent colonies of endothelial progenitors. After 8 days of differentiation, stained cells started to reorganize into primitive cord-like structures, and CD31 redistributed from cell-cell contacts to the entire cell membrane. The addition of VEGF accelerated the formation of the vascular network, already apparent at day 5 and fully developed after 8 days of differentiation (Fig. 2A). Cells forming these networks also expressed VEGFR-2 and VE-cadherin (Fig. 2B), indicating that VEGF promoted endothelial cell maturation. The time course of marker expression, in which VEGFR-2 and CD31 are expressed before VE-cadherin, is in agreement with previous studies (Hirashima et al., 1999; Vittet et al., 1996).

To test the cell-type specificity of the *tie-1* promoter in the EB model, undifferentiated ES cells were transfected with the *tie-1*-EGFP construct. G418-resistant clones were screened by PCR to verify transgene integration. Two independent clones, designated A1TG and E3TG, were differentiated into EBs in the presence of VEGF. EGFP protein expression was undetectable until day 6 or 7 of differentiation, whereas CD31 and VEGFR-2 appeared earlier, at day 4 (not shown). Again, this was in agreement with the work of Vittet et al., who showed that expression of CD31 and VEGFR-2 transcripts preceded that of *tie-1* (Vittet et al., 1996). As shown in Fig. 3A, extensive vascular-like structures were visualized by CD31 staining in 10-day-old EBs derived from the A1TG clone (Fig. 3A, top). Cells forming these structures also expressed EGFP. All EGFP-positive ($EGFP^+$) cells were found in the vascular network, thus demonstrating the endothelial specificity of the *tie-1* promoter in the EB model. CD31-positive ($CD31^+$) cell clusters that were negative for EGFP could be detected in some areas. As CD31 is an earlier marker than Tie-1 in the process of endothelial cell maturation, these clusters are likely to represent endothelial progenitors. In contrast, in EBs derived from a selected clone that did not carry the *tie-1*-EGFP transgene, vascular-like structures expressed only CD31 and not EGFP. The punctate green staining apparent in these control EBs represent autofluorescent dead cells (Fig. 3A, bottom).

To further check the tissue specificity of the *tie-1* promoter, cells from embryoid bodies were enzymatically dissociated and replated on coverslips for 1 day. All $EGFP^+$ cells also expressed CD31 as shown in Fig. 3B and all autofluorescent dead cells were lost. Changes in the cell morphology that correlate with EGFP expression are most obvious in this figure. Whereas $CD31^+EGFP^-$ cells were rounded and tightly bound

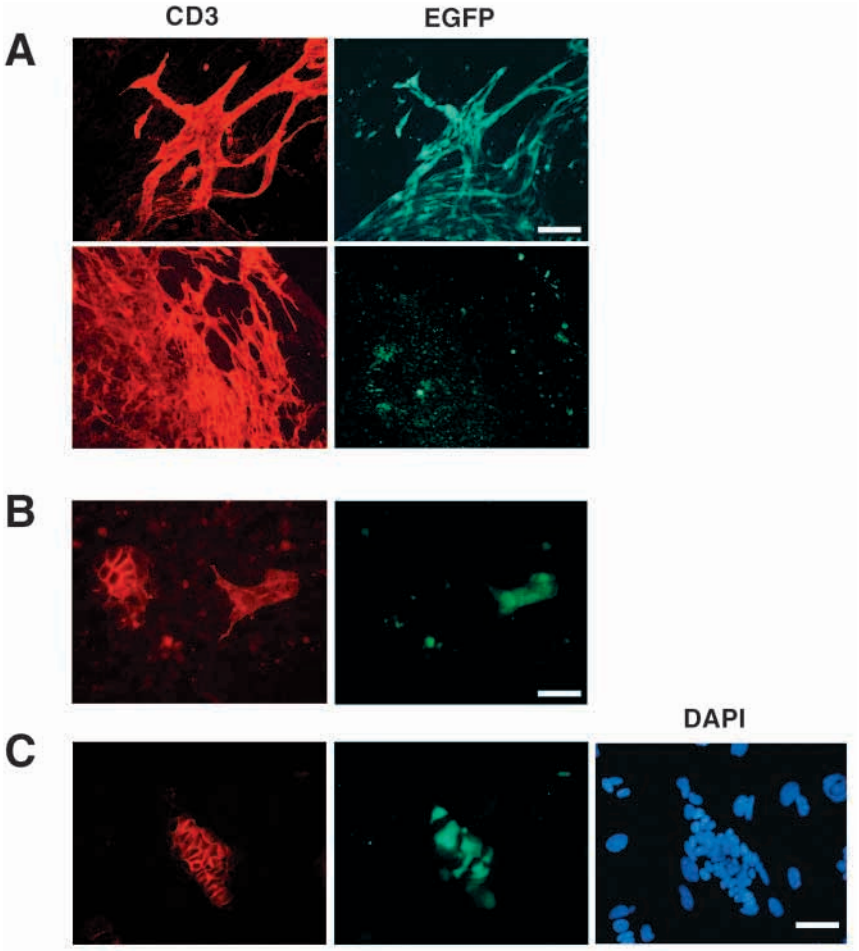

Fig. 3. Analysis of the *tie-1* promoter for endothelial specificity in EBs and 2D culture. (A) *tie-1*-EGFP ES cells were aggregated into EBs and plated on gelatin in the presence of 10 ng/ml of rVEGF. Whole 10-day-old EBs were stained for CD31 (red) to visualize pseudo-vascular structures. Analysis of EGFP fluorescence showed localization of $EGFP^+$ cells within the CD31-stained vascular network in EBs derived from the A1TG clone (top panel). The two photographs on the bottom represent staining of EBs derived from a neomycin-selected control ES-cell clone, which does not carry the *tie-1*-EGFP transgene. Bar, 100 μM. (B) 10-day-old EBs were enzymatically dissociated and single cells were plated on gelatin for 1 day. Immunofluorescence staining for CD31 confirmed that all $EGFP^+$ cells were also $CD31^+$. Note the presence of $CD31^+EGFP^-$ cell clumps, that possibly represent endothelial cell progenitor colonies. Bar, 50 μM. (C) *tie-1*-EGFP ES cells were plated on gelatin-coated slides in the presence of 10 ng/ml rVEGF. After 10 days of differentiation, slides were fixed and stained for CD31. In addition, cell nuclei were stained with DAPI. Bar, 50 μM.

to each other, $CD31^+EGFP^+$ cells were more elongated and CD31 was found on the entire cell membrane, instead of being concentrated at cell-cell contacts.

As an alternative, we also tested another ES differentiation system that was initiated by plating cells in 2D culture without LIF and in the presence of VEGF. This model was previously shown to support endothelial cell differentiation (Hirashima et al., 1999; Nishikawa et al., 1998). Thus, A1TG cells were analyzed for the expression of CD31, CD34 (another marker of the endothelial lineage) and EGFP after 8 days of differentiation in the presence of VEGF. A population of cells expressing CD31, EGFP (Fig. 3C) and CD34 (not shown) was identified. DAPI analysis shows the heterogeneity of the differentiated population, which includes cells that express neither CD31, EGFP (Fig. 3C) nor CD34 (not shown). Since all $EGFP^+$ cells also expressed CD31, this experiment confirmed the endothelial specificity provided by the *tie-1* promoter in the 2D differentiation system.

Puromycin selection of ES-cell-derived endothelial cells

Stable clones carrying both *tie-1*-EGFP and *tie-1*-$puro^r$ transgenes were obtained by co-transfecting the A1TG clone described above with the *tie-1*-$puro^r$ transgene and a hygromycin resistance gene. Hygromycin-resistant clones were screened by PCR for transgene integration (Fig. 4A). Note that positive clones also carry the *tie-1*-EGFP transgene.

Two independent clones, B9TP and C4TP, were induced to differentiate in EBs and in 2D culture in the presence of 10 ng/ml VEGF. We found that puromycin addition to EBs was not appropriate for proper endothelial cell selection. First, the antibiotic could not efficiently penetrate into the whole 3D structures and, second, endothelial cells became surrounded by dying cells, which affected their growth capacity. Prior enzymatic dissociation of EBs was not efficient, since it damaged the cells and gave rise to only small numbers of selected cells. For these reasons, 2D cultures were preferentially used for endothelial cell selection.

Fig. 4B shows the efficiency of selection on differentiated B9TP cells after a 4-day puromycin treatment (1 μg/ml puromycin), started at day 7 of differentiation after LIF removal. Endothelial cells were visualized by both CD31 staining and EGFP expression. Nuclei were stained with DAPI to detect cells present in the field, including $CD31^-EGFP^-$ cells. On the left panel, corresponding to 10-day-old differentiated B9TP cells without puromycin treatment, $EGFP^+CD31^+$ cells are encompassed in a monolayer of cells negative for both markers. After a 4-day puromycin-selection followed by 1 day without the antibiotic to allow cells to recover, 96-98% of cells were positive for CD31 and EGFP. Upon selection, $EGFP^+CD31^+$ cell spreading was less efficient and cells were more tightly associated (Fig. 4B). EGFP protein expression also decreased in some cells when selection was released, but all selected cells retained the CD31 marker, which

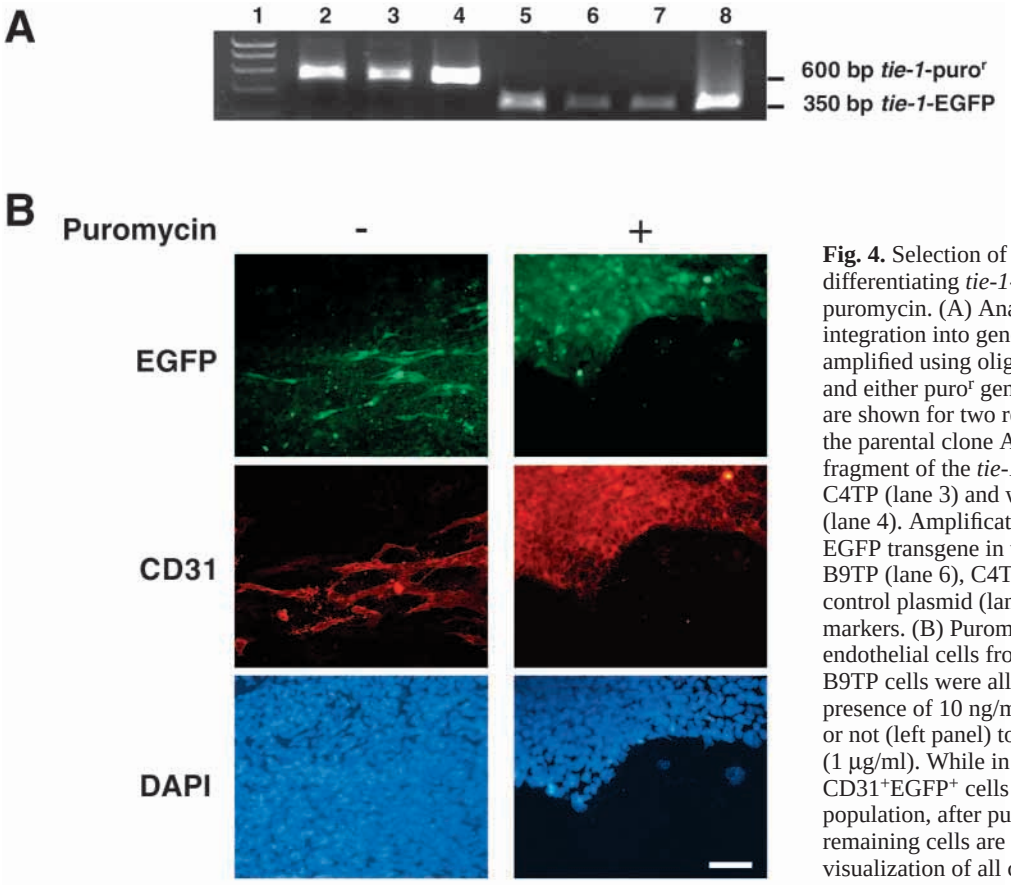

Fig. 4. Selection of endothelial cells from a population of differentiating *tie-1*-EGFP/*tie-1*-puro^r ES cells by puromycin. (A) Analysis of *tie-1*-puro^r transgene integration into genomic DNA. PCR products were amplified using oligonucleotides for the *tie-1* promoter and either puro^r gene or the EGFP cDNA. PCR products are shown for two resistant clones, B9TP and C4TP, and the parental clone A1TG. Amplification of a 500 bp fragment of the *tie-1*-puro^r transgene in B9TP (lane 2), C4TP (lane 3) and with the *tie-1*-puro^r control plasmid (lane 4). Amplification of a 310 bp fragment of the *tie-1*-EGFP transgene in the parental clone A1TG (lane 5), B9TP (lane 6), C4TP (lane 7) and with the *tie-1*-EGFP control plasmid (lane 8). Lane 1, molecular weight markers. (B) Puromycin selection of ES-derived endothelial cells from 2D plane cultures of B9TP cells. B9TP cells were allowed to differentiate for 7 days in the presence of 10 ng/ml rVEGF and subjected (right panel) or not (left panel) to a 4-day puromycin selection (1 μg/ml). While in the absence of puromycin, CD31$^+$EGFP$^+$ cells are present in an heterogenous population, after puromycin selection, almost all remaining cells are CD31$^+$EGFP$^+$. DAPI staining allows visualization of all cells present in the field. Bar, 100 μM.

was then found concentrated at cell-cell contacts. It is possible that environmental changes caused by the removal of all other cell types results in a partial dedifferentiation of endothelial cells towards an endothelial progenitor phenotype.

The C4TP clone gave results similar to B9TP, which clearly demonstrates the efficiency of ES-cell-derived endothelial cell purification through genetic selection using the *tie-1* promoter. Subsequent analyses were performed on the B9TP clone.

Characterization of selected cells

Purified cells were further characterized for the expression of various endothelial markers. We did not visualize VE-cadherin and VEGFR-2 proteins in 2D cultures with conventional immufluorescence techniques. Reasoning that the detection of these antigens could be easier in pseudo-vascular structures than in selected colonies, we aggregated purified CD31$^+$EGFP$^+$ cells into spheroids. After 3 days, spheroids were plated on gelatin, and stained for endothelial markers 2 days later. Fig. 5A shows that although most cells were CD31$^+$, EGFP expression was restricted to certain cells organized into vascular networks. Such structures were also found to express VE-cadherin, VEGFR-2 and CD34. However, VEGFR-2 and CD34 were also expressed in cell clusters negative for EGFP. These clusters probably represent endothelial progenitors.

Expression of three other endothelial markers, VEGFR-1, Tie-1 and ICAM-2, was monitored by a semi-quantitative RT-PCR approach. Fig. 5B shows that VEGFR-2 and VEGFR-1 were expressed at basal levels in undifferentiated cells. However, their expression was strongly increased in purified endothelial cells. In contrast, Tie-1 and ICAM-2 mRNA could be detected only in selected cells.

Interestingly, selected cells contained a small percentage (2-4%) of CD31$^-$/EGFP$^-$ cells when analyzed 24 hours after puromycin removal. These cells were highly spread and expressed α-smooth muscle actin (α-SMA; Fig. 6). Their proportion was strongly enhanced at high cell density and in spheroids. In the light of recent results (Yamashita et al., 2000), we formulated the hypothesis that α-SMA$^+$ cells may be derived from purified endothelial cells themselves or their progenitors. Thus, we tested the effects of TGF-β and laminin-1, two known inducers of SMC differentiation (Arciniegas et al., 1992; Hayashi et al., 1998; Hirschi et al., 1998; Morla and Mogford, 2000) on the purified population. Fig. 6A shows that providing 5 ng/ml of TGF-β or plating cells onto laminin-1 dramatically increased the number of α-SMA$^+$ cells after 2 days. Laminin-1 had the strongest effect, even when cells were cultured in the presence of 10 ng/ml of VEGF. PDGF, a potent growth factor for SMC, was less efficient than TGF-β or laminin-1, suggesting that the occurrence of α-SMA$^+$ cells in the purified endothelial cell population was due to the differentiation of endothelial cells or their progenitors towards an SMC phenotype rather than to their proliferation. Finding cells that expressed both CD31 and α-SMA$^+$ (Fig. 6B) further supported this hypothesis.

Interestingly, and unlike previous results (Yamashita et al., 2000), we did not obtain a pure population of α-SMA$^+$ cells, even after a treatment with either TGF-β1, laminin-1 or PDGF

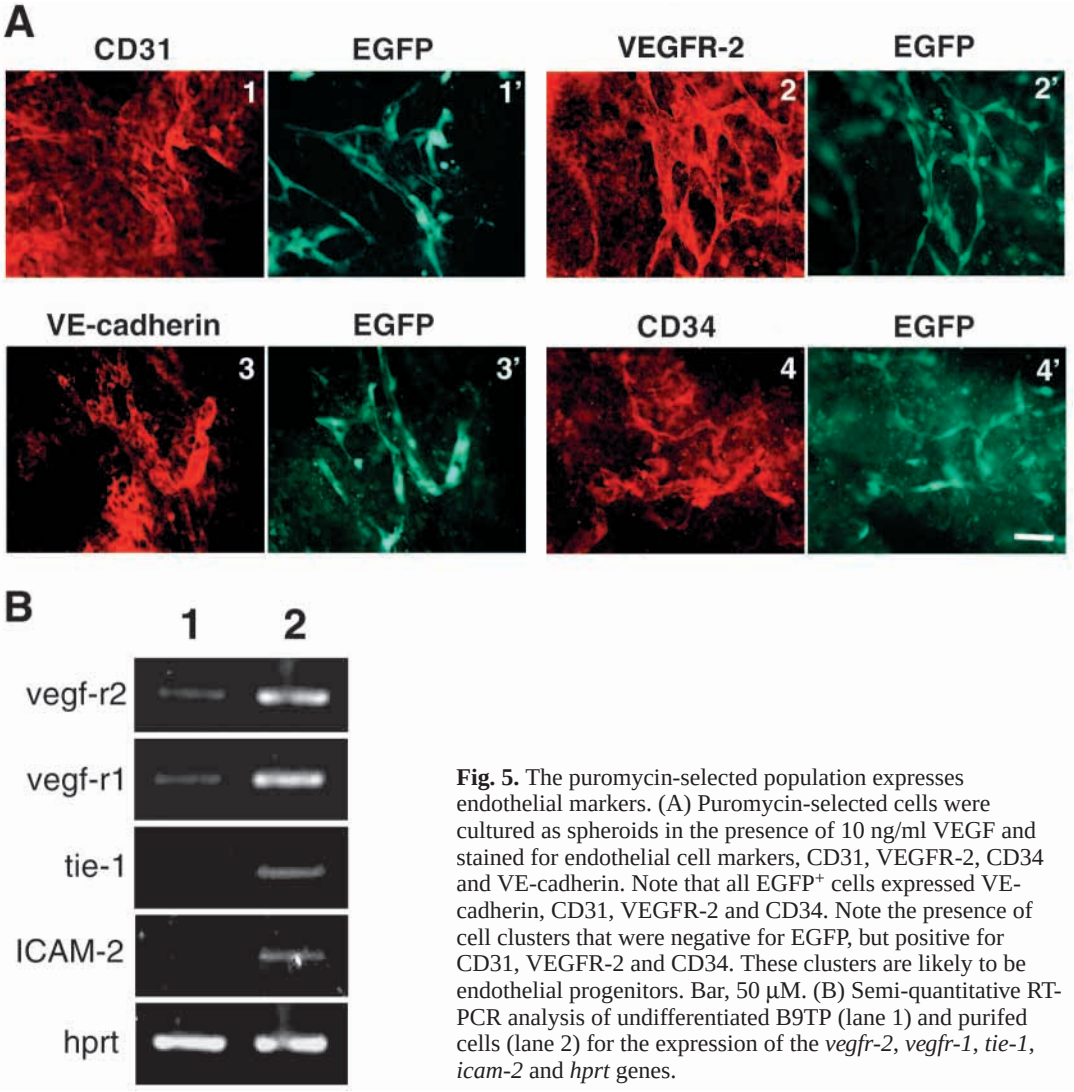

Fig. 5. The puromycin-selected population expresses endothelial markers. (A) Puromycin-selected cells were cultured as spheroids in the presence of 10 ng/ml VEGF and stained for endothelial cell markers, CD31, VEGFR-2, CD34 and VE-cadherin. Note that all EGFP$^+$ cells expressed VE-cadherin, CD31, VEGFR-2 and CD34. Note the presence of cell clusters that were negative for EGFP, but positive for CD31, VEGFR-2 and CD34. These clusters are likely to be endothelial progenitors. Bar, 50 μM. (B) Semi-quantitative RT-PCR analysis of undifferentiated B9TP (lane 1) and purifed cells (lane 2) for the expression of the *vegfr-2*, *vegfr-1*, *tie-1*, *icam-2* and *hprt* genes.

for several days. This might be due to the different stages of cell differentiation at the time of purification. Whereas Yamashita et al. selected VEGFR-2$^+$ progenitors by flow cytometry at day 4 of differentiation, we waited until day 7 before adding puromycin for 4 additional days. It is possible that our selection protocol allows endothelial cells or their progenitors to reach a more advanced stage of differentiation that could prevent some of them from entering the smooth muscle cell lineage.

Incorporation of selected endothelial cells into neovascularization sites in vivo

To investigate the capacity of purified endothelial cells to participate in the process of neovascularization in vivo, we used a tumor transplantation model in athymic nude mice. For that purpose, we chose a clone of PS120 cells expressing an active mutated form of MEK-1 (MEK S222D) conferring a weak tumoral potential in nude mice (Brunet et al., 1994). Six mice were injected subcutaneously with a mixture of 10^6 selected endothelial cells and 10^6 PS120/MEK S222D cells on the left flank, and 10^6 PS120/MEK S222D cells alone on the right flank. Other controls were provided by five other mice injected only with 10^6 endothelial cells. Mice injected with 10^6 endothelial cells alone never developed tumors, even after 5 months. PS120 MEK/S222D cells produced small tumors, if any, visible 3-4 weeks after inoculation; their weight ranged from 0.3 to 70 mg, confirming their previously described low tumorigenic potential. In contrast, co-injection of selected endothelial cells and PS120 MEK S222D resulted in the formation of tumors visible 2 weeks after injection. Three weeks after injection, their weight was almost 10 times higher than control tumors (ranged from 75 to 610 mg). This experiment was performed three times and always gave similar results.

When stained with hematoxylin and eosin, sections of 3-week-old co-injected and control tumors were homogenous and revealed no overt difference (not shown). These tumors were next analyzed for the presence of EGFP$^+$ endothelial cells in the neovasculature (Fig. 7A). Immunofluorescence staining for CD31 showed that the extent of neovasculazisation was similar in both types of tumors. EGFP$^+$ cells were found in microvessels at the periphery of tumors resulting from the co-injection of the two cell types. Surprisingly, there was no EGFP

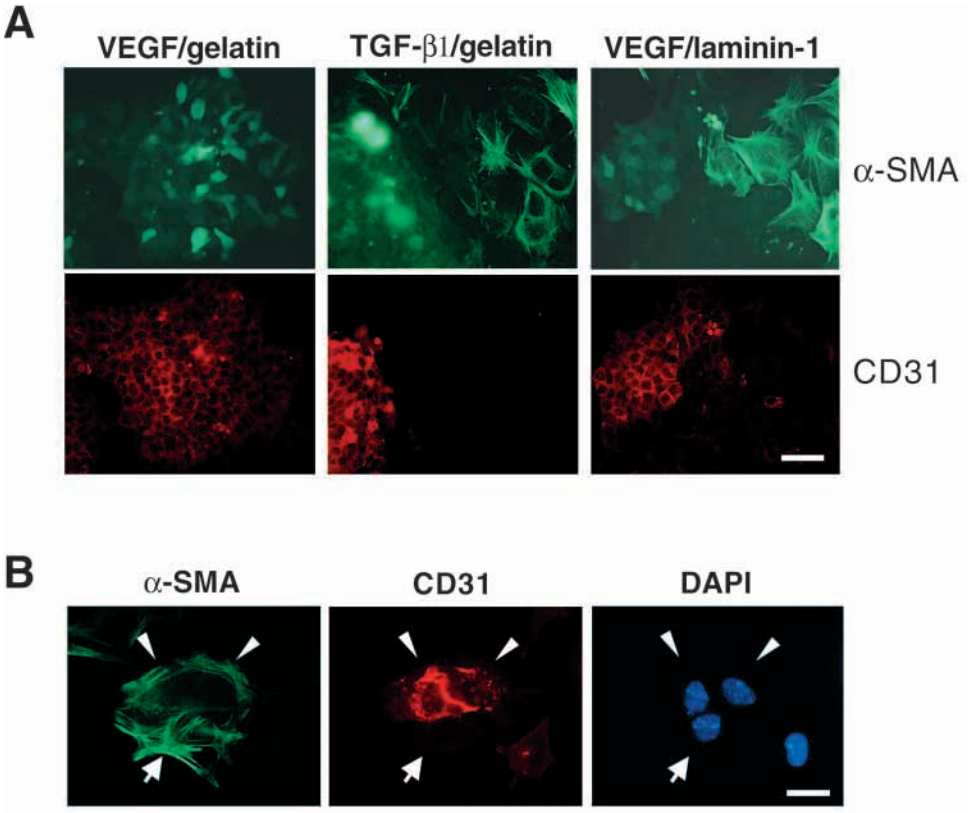

Fig. 6. The puromycin-selected population contains smooth muscle cells. (A) TGF-β1 and laminin-1 increase the number of α-SMA$^+$ cells. Puromycin-selected cells were cultured on either gelatin or laminin-1, in the presence of 10 ng/ml rVEGF or 5 ng/ml TGF-β1, and stained for α-smooth muscle actin (FITC, actin filaments in green) and CD31 (red). The diffuse green staining reveals the expression of EGFP in CD31$^+$ cells that do not contain α-SMA. Bar, 50 μM. (B) CD31/α-SMA double staining. While some cells only expressed α-SMA (arrows), others expressed both α-SMA and CD31 (arrowheads). Bar, 30 μM.

staining in the center of the tumors. Whether this means that injected cells are simply absent in this location or that the *tie-1* promoter is no longer active in these particular cells is not known and will be discussed below.

We also did not detect any EGFP$^+$ cells in tumors resulting from the injection of PS120/MEK S222D alone in the same mice, indicating that injected endothelial cells did not migrate to the other flank of the animals through the circulation.

As puromycin-selected cells could also give rise to α-SMA$^+$ cells in vitro, tumor sections were co-stained for α-SMA and CD31 (Fig. 7B). First, we detected few, if any, α-SMA$^+$ blood vessels in control tumors, suggesting that these vessels were not mature. In contrast, most blood vessels in co-injected tumors were lined by α-SMA$^+$ cells. It is unclear whether these α-SMA$^+$ cells were of host or ES cell origin. In addition, we detected, in the co-injected tumors only, areas rich in α-SMA$^+$ cells, probably derived from the injected endothelial cells. The size difference between co-injected and control tumors might thus be due to the growth of these α-SMA$^+$ cells. This hypothesis and alternatives will be discussed below.

Subcutaneously injected ES cells are known to give rise to teratomas, which are tumors containing various types of differentiated tissues. Even if we found that the expression of oct-4, a marker for undifferentiated ES cells (Yeom et al., 1996), was downregulated in purified cells (data not shown), we thought it was important to check that selected endothelial cells do not give rise to teratomas in vivo. Although we did not detect any tumor formation in mice injected with 10^6 endothelial cells alone, we repeated the experiment using 10^7 endothelial cells instead. This protocol did not result in teratoma formation but hemangiomas did develop 3 weeks after injection (not shown). Hemangiomas consisted of both blood-filled cavities and tumor masses, with cavities surrounded by several layers of endothelial cells positive for EGFP, CD31 and CD34, while the rest of the tumor mass contained mostly CD34$^+$ cells, with occasional CD31 staining.

Discussion

We show here that the *tie-1* promoter drives endothelial-specific EGFP expression in the embryoid body model of vasculogenesis and angiogenesis. We also present a novel approach for genetically selecting endothelial cells from differentiating ES cells, based on the expression of a puromycin resistance gene controlled by the *tie-1* promoter. Selected cells expressed several endothelial markers and were recruited at sites of neovascularisation in vivo in a tumor transplantation model.

A novel experimental model for easily monitoring angiogenesis and the regulation of *tie-1* promoter activity in embryoid bodies

Both *tie-1* and *VE-cadherin* promoters were previously found to drive endothelial-specific expression in vivo (Gory et al., 1999; Korhonen et al., 1995). In the present study, we compared the activity of these two promoters in various cell lines. In agreement with previous reports (Gory et al., 1999; Iljin et al., 1999), both promoters displayed functional activity in endothelial cell lines, but they also exhibited a weaker activity in fibroblast cells in transient transfection experiments. Whereas previous studies have not directly compared their respective activity, our experiments demonstrate that the *tie-1* promoter allows a more potent induction of reporter genes in

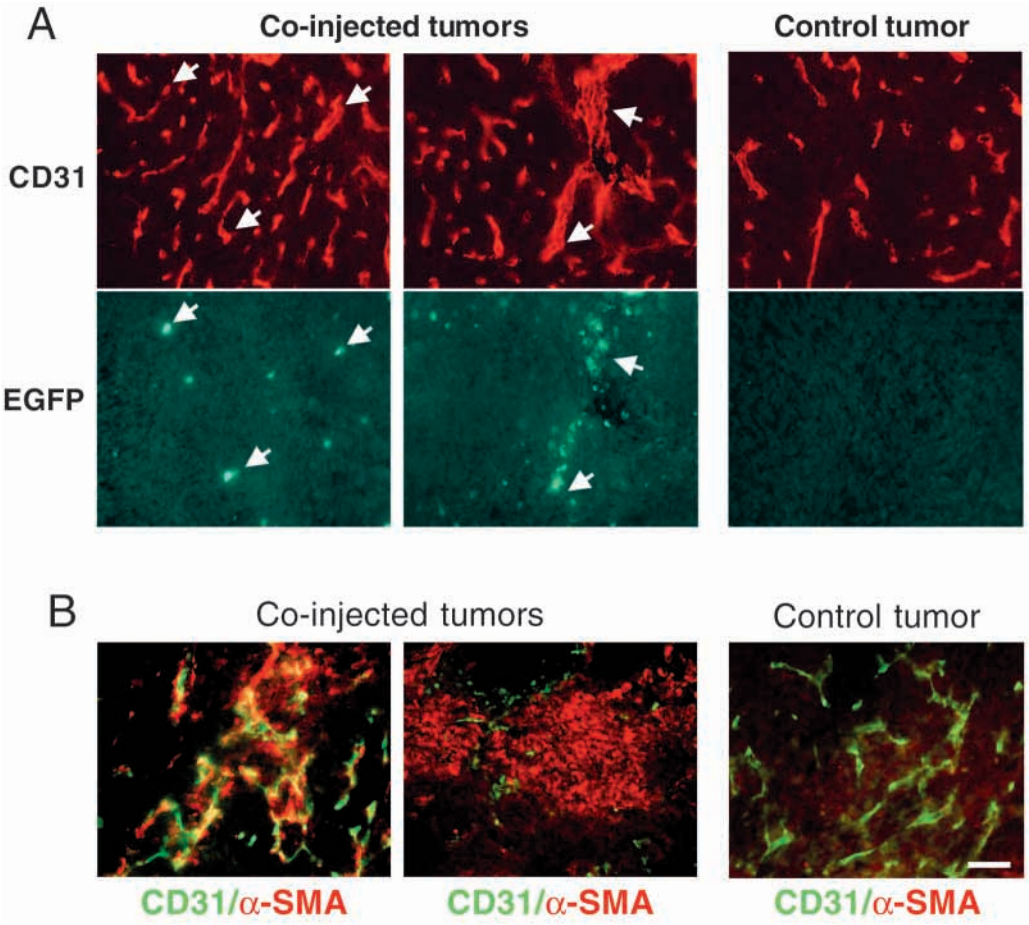

Fig. 7. Puromycin-selected endothelial cells are incorporated into neovessels in vivo. (A) Localization of EGFP$^+$ in cryo-sections of co-injected (left) and control (right) tumors. EGFP$^+$ cells are incorporated into the tumor neovasculature, as visualized by CD31 staining. Arrows indicate EGFP$^+$CD31$^+$ cells present in sections of 'co-injected' tumors. Note that the extent of neovascularization was similar in both types of tumors, but that the EGFP expression was observed only in co-injected tumors. (B) Analysis of α-SMA expression in tumors. Tumor sections were co-stained for α-SMA (red) and CD31 (green). Bar, 100 μM.

endothelial cells. Therefore, we chose the *tie-1* promoter for the rest of the study.

In spite of its weak activity in fibroblast cell lines in transient experiments, the *tie-1* promoter retained its cellular specificity when expressed in differentiating ES cells. Reporter EGFP expression could be detected from day 5 of differentiation in VEGF-treated EBs and from day 6 in 2D cultures. This occurred only 1 day later than the expression of CD31, an early marker for endothelial precursors and endothelial cells (Nishikawa et al., 1998; Vittet et al., 1996). In embryoid bodies, EGFP expression was restricted to cells organized into cord-like structures also expressing CD31. VEGF enhanced the number of both EGFP$^+$ cells and vascular-like structures. However, EGFP was absent from clusters of CD31$^+$VEGFR-2$^+$ cells, which probably represent endothelial progenitors. This confirms previous reports showing that the VEGF receptor R2 is an early marker for endothelial cell precursors (Hirashima et al., 1999; Vittet et al., 1996; Yamaguchi et al., 1993), whereas *VE-cadherin* and *tie-1* transcript expression start at a later stage of differentiation (Hirashima et al., 1999; Vittet et al., 1997; Vittet et al., 1996).

Interestingly, the onset of *tie-1* promoter activity correlated with changes in cell morphology. Whereas CD31$^+$EGFP$^-$ cells were round and formed clumps, CD31$^+$EGFP$^+$ cells displayed elongated processes and reorganized into pseudo-vascular structures. It is not clear whether these morphological changes are induced by Tie-1 expression or whether they are simply a part of a more general maturation program, but it would be interesting to analyze the effects of Tie-1 expression on endothelial cell migration and cytoskeleton organization.

The availability of ES cell clones carrying an integrated *tie-1*-EGFP transgene is of great interest for several reasons. First, we have confirmed the endothelial specificity of the *tie-1* promoter in the EB model. This finding is in agreement with recently published work (Gustafsson et al., 2001). Second, this model can be used to trace the activity of the *tie-1* promoter in single cells within EBs whereas, in most studies published so far, *tie-1* expression was assayed only by RT-PCR in a mix of different cell types. Third, *tie-1*-EGFP clones constitute a good model for easily monitoring the effects of potential pro- or anti-angiogenic factors. Two examples are given by our work, in which VEGF (this study) and bFGF (S.M., unpublished) strongly promoted the formation of EGFP$^+$ vascular-like structures. Finally, these cells can be used to generate mice expressing EGFP in their vasculature. This may help to understand the molecular mechanisms regulating *tie-1* promoter activity, in particular, physiological and pathological conditions.

A novel approach for selecting endothelial cells from differentiating embryonic stem cells

We took advantage of the cellular specificity of the *tie-1* promoter to genetically select endothelial cells from differentiating ES cells, using puromycin as a selection marker. Such an approach has been previously used for selecting neuronal and cardiac cells from a population of differentiating

ES cells (Klug et al., 1996; Li et al., 1998). Starting from a few undifferentiated ES cells, large numbers of cells expressing the endothelial markers CD31, CD34, VEGFR-1, VEGFR-2, Tie-1, VE-cadherin and ICAM-2 can be obtained using this method.

While this work was under way, an alternative method was proposed to isolate vascular progenitors by flow cytometry cell sorting based on VEGFR-2 expression (Yamashita et al., 2000). VEGFR-2$^+$ cells were isolated at day 4 of differentiation and counter-selected for E-cadherin expression, which is a marker for undifferentiated ES cells. VEGFR-2 is an early marker for endothelial progenitors (Hirashima et al., 1999; Vittet et al., 1996; Yamaguchi et al., 1993) and we detected its expression by RT-PCR in undifferentiated ES cells. This was in agreement with the weak *vegfr-2* promoter activity previously observed in undifferentiated ES cells by the same experimental method, using lacZ as a reporter gene (Hidaka et al., 1999). Although LIF removal is theoretically sufficient to induce the differentiation of most ES cells over a few days, we thought it might be advantageous to wait longer than 4 days to avoid contamination by undifferentiated ES cells. For this reason, we chose a promoter regulating the expression of a late marker of endothelial cell maturation, [i.e. *tie-1* (Hirashima et al., 1999; Vittet et al., 1996)]. Because EGFP expression driven by the *tie-1* promoter was not detectable before day 5 or 6 in differentiating ES cells, we waited until day 7 to start selection with puromycin. After 4 days selection, almost all cells (96-98%) were CD31$^+$EGFP$^+$. The other cells expressed α-SMA, a marker for smooth muscle cells. When maintained in culture, α-SMA$^+$ cells later expressed transcripts for two other smooth muscle markers, SM22 and calponin (S.M. and C.G., unpublished). Two known inducers of SMC differentiation, TGF-β1 and laminin-1, strongly increased the proportion of α-SMA$^+$ in the cell population. The fact that some cells express both CD31 and α-SMA suggested the existence of either a common progenitor for endothelial and SMC or a transdifferentiation mechanism from endothelial towards SMC. This finding is reminiscent of previous works by other groups (Arciniegas et al., 1992; DeRuiter et al., 1997; Hirschi et al., 1998; Yamashita et al., 2000). A few days after puromycin removal, CD31$^+$ cells selected with our method adopted a morphology more characteristic of endothelial progenitors than more mature endothelial cells (Bautch et al., 2000). We interpreted this phenomenon as being a possible dedifferentiation of endothelial cells towards a more immature phenotype. Whether a dedifferentiation process is required for further differentiation into smooth muscle cells or whether transdifferentiation may occur between both lineages needs to be explored in more depth. If the common origin for both lineages is confirmed, our differentiation system might serve as a new model for studying the effects of TGF-β1 and laminin-1 on SMC differentiation.

Puromycin-selected endothelial cells are incorporated into neo-vessels in vivo

Yamashita et al. showed that FACS-selected VEGFR-2$^+$ cells participated in the formation of the vascular network in the developing chick embryo, and that they differentiated into both endothelial and vascular smooth muscle cells in vivo (Yamashita et al., 2000). In the present study, we showed that puromycin-resistant cells could be incorporated into microvessels at sites of neovascularisation in a tumor transplantation model in athymic nude mice, as revealed by EGFP fluorescence. Surprisingly, there were no EGFP$^+$ endothelial cells in the vessels found in the core of the tumors, rather they were incorporated into peripheric vessels. The simplest explanation for this phenomenon would be that EGFP$^+$ cells are not integrated at this particular location for unknown reasons. Alternatively, the activity of the *tie-1* promoter might be downregulated in microvessels found in the tumor center. Another member of the Tie family, Tie-2, was shown to be preferentially expressed in vessels at the periphery of certain tumors (Asahara et al., 1999a; Peters et al., 1998). Because Tie-1, like Tie-2, is involved in angiogenesis and blood vessel maturation, it is tempting to speculate that the two promoters might be similarly regulated.

We consistently found that the size of the PS120/MEK S222D tumors was larger when tumor cells were co-injected with purified endothelial cells. Providing exogenous endothelial cells may have promoted the vascularization and subsequent growth of the tumors, as suggested by the presence of EGFP$^+$ cells in newly formed blood vessels. In this respect, it should be noted that, although vascular densities were comparable in control and co-injected tumors, overall angiogenesis was increased as the co-injected tumors were larger. However, there might be other causes for this difference in tumor size. First, we found numerous α-SMA$^+$ cells in co-injected tumors. Although we do not have a marker for these cells (the *tie-1* promoter is not active in α-SMA$^+$ cells), it is possible that they are derived from the injected endothelial population, as they were absent in the control tumors. α-SMA$^+$ cells were found both located around blood vessels and organized in cell aggregates. On the one hand, blood vessel lining by α-SMA$^+$ cells, observed only in the co-injected tumors, may have an additional impact on angiogenesis and subsequent tumor growth. On the other hand, the size of SMC-containing aggregates could be increased by tumor-secreted cytokines, such as members of the TGF family. Alternatively, injected endothelial cells may themselves secrete growth factors, such as PDGF, triggering the survival and proliferation of PS120 MEK S222D cells at the initial stage following injection. Although it is likely that the tumor size difference results from a combination of all these effects, the fact remains that puromycin-selected endothelial cells can be incorporated in tumors at sites of neovascularization. Importantly, experiments performed in mice indicated that endothelial cells selected by our protocol did not produce teratomas over a period of several months when subcutaneously injected in large amounts, suggesting that the purified cells did not contain undifferentiated cells.

Further studies will be necessary to assess the ability of endothelial cells selected by our method to promote revascularization of wounded or ischemic tissues. Their potential ability to give rise to both endothelial and smooth muscle cells in vivo could be particularly advantageous if mature vessels are to be formed. If they prove to be competent, ES-cell-derived endothelial cells could provide an appealing alternative to adult stem cells in the context of pro-angiogenic therapy. As we have shown here, a large number of endothelial cells or progenitors can be obtained from few undifferentiated ES cells and they can also be easily genetically modified. Hence, a combination of stem cell and gene therapy could be of great benefit for treating degenerative diseases in which endothelial cells are involved.

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