

Isolation of Putative Progenitor Endothelial Cells for Angiogenesis

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Putative endothelial cell (EC) progenitors or angioblasts were isolated from human peripheral blood by magnetic bead selection on the basis of cell surface antigen expression. In vitro, these cells differentiated into ECs. In animal models of ischemia, heterologous, homologous, and autologous EC progenitors incorporated into sites of active angiogenesis. These findings suggest that EC progenitors may be useful for augmenting collateral vessel growth to ischemic tissues (therapeutic angiogenesis) and for delivering anti- or pro-angiogenic agents, respectively, to sites of pathologic or utilitarian angiogenesis.

Postnatal neovascularization is thought to result exclusively from the proliferation, migration, and remodeling of fully differentiated ECs derived from preexisting blood vessels (1). This adult paradigm, referred to as angiogenesis, contrasts with vasculogenesis, the term applied to the formation of embryonic blood vessels from EC progenitors, or angioblasts (2).

Vasculogenesis begins as a cluster formation, or blood island, comprising angioblasts at the periphery and hematopoietic stem cells (HSCs) at the center (3). In addition to this spatial association, angioblasts and HSCs share certain antigenic determinants, including Flk-1, Tie-2, and CD34. Conceivably, then, these progenitor cells may derive from a common precursor (3, 4).

The demonstration that HSCs from peripheral blood can provide sustained hematopoietic recovery is inferential evidence for circulating stem cells (5). Here, we have investigated the hypothesis that peripheral blood contains cells that can differentiate into ECs (6). We exploited two antigens that are shared by angioblasts and HSCs to isolate putative angioblasts from the leukocyte fraction of peripheral blood. CD34 is expressed by all HSCs but is lost by hematopoietic cells as they differentiate (7). It is also expressed by many including most activated ECs in the adult (8). Flk-1, a receptor for vascular endothelial growth factor (VEGF) (9), is also expressed by both early HSCs and ECs but ceases to be expressed during hematopoietic differentiation (10, 11).

CD34-positive mononuclear blood cells (MB^{CD34+}) were isolated from human peripheral blood by means of magnetic beads

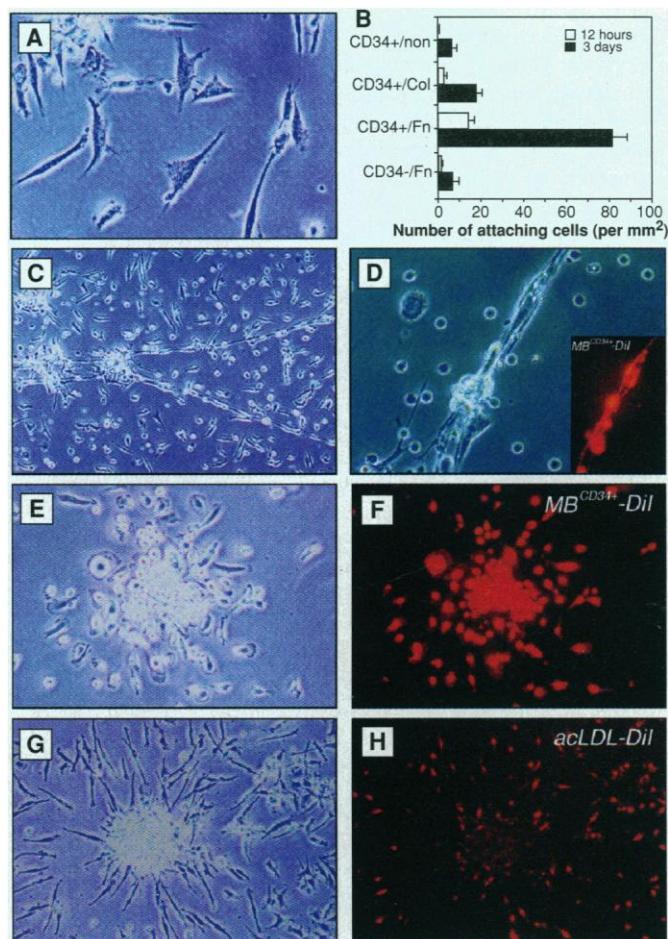
coated with antibody to CD34 (Dyna, Lake Success) (12). Fluorescence-activated cell sorting (FACS) analysis (13) indicated that $15.7 \pm 3.3\%$ of selected cells compared with $<0.1\%$ of the remaining cells expressed CD34. CD34-depleted cells (MB^{CD34-}) were used as controls. An antibody to Flk-1 was used for magnetic bead selection of Flk-1-positive mononuclear

blood cells (MB^{Flk1+}); among MB^{Flk1+} cells, $20.0 \pm 3.3\%$ were Flk-1 positive.

The MB^{CD34+} and MB^{CD34-} cells were plated separately (14) on tissue culture plastic, collagen type I, or fibronectin. When plated on tissue culture plastic or collagen at a density of 1×10^3 cells/mm², a limited number of MB^{CD34+} attached, became spindle shaped, and proliferated for 4 weeks. A subset of MB^{CD34+} plated on fibronectin promptly attached and became spindle shaped within 3 days (Fig. 1A); the number of attaching cells (AT^{CD34+}) in culture increased with time (probability $P < 0.05$, by analysis of variance) (Fig. 1B). Attached cells were observed only sporadically among MB^{CD34-} cultures, including cells followed for up to 4 weeks on fibronectin-coated plates.

To confirm that the spindle-shaped cells were derived from CD34-positive cells, we labeled MB^{CD34+} cells with the fluorescent dye DiI and coplating them with unlabeled MB^{CD34-} cells on fibronectin at an overall density of 5×10^3 cells/mm²; the ratio of the two cell types was identical to that of the original mononuclear cell population (1% MB^{CD34+} , 99% MB^{CD34-}). After 7 days, DiI-labeled cells derived from the MB^{CD34+} culture, which initially account-

Fig. 1. Attachment, cluster formation, and capillary network development by progenitor ECs in vitro. (A) Spindle-shaped attaching cells (AT^{CD34+}) 7 days after plating MB^{CD34+} (50 cells/mm²) on fibronectin in standard medium (14). (B) Number of AT^{CD34+} cells 12 hours and 3 days after culture of MB^{CD34+} on plastic alone (CD34+/non), collagen coating (CD34+/Col), or fibronectin (CD34+/Fn), and MB^{CD34-} on fibronectin (CD34-/Fn). Network formation (C) and cord-like structures (D) were observed 48 hours after plating coculture of MB^{CD34+} , labeled with DiI, with unlabeled MB^{CD34-} cells (ratio of 1:100) on fibronectin. At 12 hours after coculture, MB^{CD34+} -derived cells had formed multiple clusters (E and F). After 5 days, uptake of acLDL-DiI was detected in AT^{CD34+} cells at the periphery but not the center of the cluster (G and H).



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ed for only 1% of the blood cells, accounted for $60.3 \pm 4.7\%$ of total attaching cells as analyzed by FACS. Coincubation with MB^{CD34-} cells increased the proliferation rate to more than 10 times that of MB^{CD34+} plated alone. Cocultures of MB^{CD34+} and MB^{CD34-} cells also showed enhanced MB^{CD34+} differentiation, including the formation of cellular networks and tube-like structures on fibronectin-coated plates (Fig. 1, C and D). These structures consisted principally of DiI-labeled MB^{CD34+} -derived cells (Fig. 1D). Furthermore, within 12 hours of coculture, multiple clusters had formed (Fig. 1E) that contained mostly MB^{CD34+} -derived cells (Fig. 1F). These clusters comprised round cells centrally and sprouts of spindle-shaped cells at the periphery. The appearance and organization of these clusters resembled that of blood island-like cell clusters observed in dissociated quail epiblast culture, which gave rise to ECs and vascular structures in vitro (3). AT^{CD34+} cells at the cluster periphery took up DiI-labeled acetylated low density lipoprotein (acLDL), whereas the round cells did not (Fig. 1, G and H); the latter detached from the cluster several days later. The MB^{Flk1+} cells behaved similarly.

To evaluate whether MB^{CD34+} cells progressed to an EC-like phenotype, we assayed them for the expression of leukocyte and EC markers. Freshly isolated MB^{CD34+} cells, AT^{CD34+} cells cultured on fibronectin for 7 days, and human umbilical vein endothelial cells (HUVECs) were incubated with fluorescent-labeled antibodies and analyzed by FACS (Fig. 2). Leukocyte common antigen CD45 was identified on 94.1% of freshly

isolated cells but disappeared after 7 days of culture (Fig. 2). In freshly isolated MB^{CD34+} cells, $15.7 \pm 3.3\%$ were $CD34+$, $27.6 \pm 4.3\%$ were $Flk-1+$, and $10.8 \pm 0.9\%$ were $CD34+, Flk-1+$. Expression of CD34, CD31, Flk-1, Tie-2, and E selectin—all markers of the EC lineage (11, 15)—was greater in AT^{CD34+} cells after 7 days of culture than in freshly isolated MB^{CD34+} cells.

Additional analyses (16) of AT^{CD34+} cells after 7 days of culture showed limited ($6.0 \pm 2.4\%$ cells) expression of CD68, a marker of the monocyte-macrophage lineage; positive immunostaining for factor VIII, ulex europaeus agglutinin-1 (UEA-1), CD31, endothelial constitutive nitric oxide synthase (ecNOS), and E selectin; and more than 80% uptake of DiI-labeled acLDL.

To confirm an EC-like phenotype of AT^{CD34+} cells, we documented expression of ecNOS, Flk-1/KDR (Flk-1 is also known as VEGFR-2 in mouse, and KDR is the human homolog of VEGFR-2), and CD31 mRNA at 7, 14, and 21 days by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 3A). Evidence for ecNOS and Flk-1/KDR in AT^{CD34+} cells was also demonstrated in a functional assay. Nitric oxide was produced in the cells in response to the EC-dependent agonist acetylcholine (Ach) and the EC-specific mitogen VEGF (Fig. 3B); the latter response also confirms that the cells express a functional Flk-1 receptor (17).

To determine if MB^{CD34+} cells contribute to angiogenesis in vivo, we used mouse and rabbit models of hindlimb ischemia. For administration of human MB^{CD34+} cells, $C57BL/6J \times 129/SV$ background athymic

nude mice were used to avoid potential graft-versus-host complications. Two days after creating unilateral hindlimb ischemia by excising one femoral artery, we injected mice with 5×10^5 DiI-labeled human MB^{CD34+} or MB^{CD34-} cells into the tail vein. Histologic examination 1 to 6 weeks later revealed numerous (Fig. 4A) including proliferative (Fig. 4, C and D) DiI-labeled cells in the neovascularized ischemic hindlimb. Nearly all labeled cells appeared integrated into capillary vessel walls. In MB^{CD34+} -injected mice, $13.4 \pm 5.7\%$ of all CD31-positive capillaries contained DiI-labeled cells, compared with $1.6 \pm 0.8\%$ in MB^{CD34-} -injected mice (18). By 6 weeks, DiI-labeled cells were clearly arranged into capillaries among preserved muscle structures (Fig. 4, I and J).

No labeled cells were observed in the uninjured limbs of either MB^{CD34+} - or MB^{CD34-} -injected mice. DiI-labeled cells consistently colocalized with cells immunostained for CD31 (Fig. 4, B, F, and J), Tie-2 (Fig. 4G), and UEA-1 lectin (16). In contrast, in hindlimb sections from mice injected with MB^{CD34-} , DiI-labeled cells were typically found in stroma near capillaries, but they did not form part of the vessel wall nor did they colocalize with cells that stained with antibodies to either UEA-1 or CD31 (Fig. 4, K and L).

In a second set of mouse experiments, 1×10^4 MB^{Flk1+} cells were isolated from

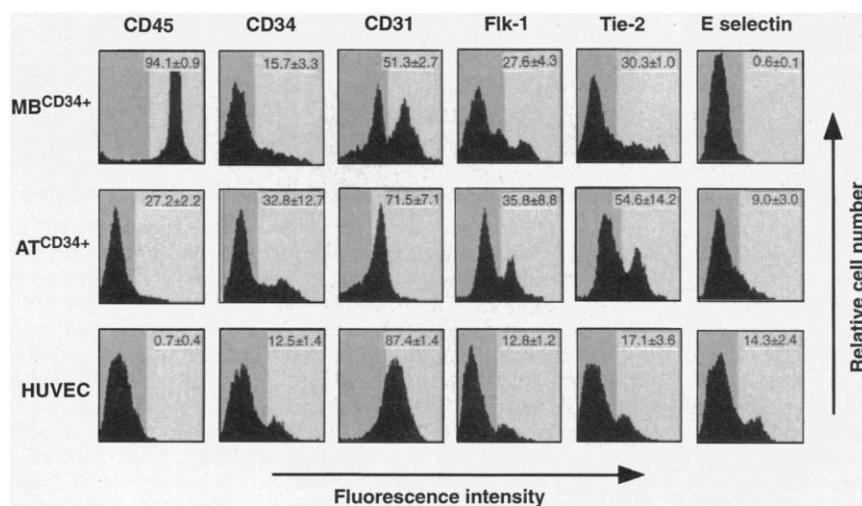


Fig. 2. FACS analysis of freshly isolated MB^{CD34+} and AT^{CD34+} cells after 7 days in culture, and HUVECs. Cells were labeled with fluorescent antibodies to CD45 (DAKO, Carpinteria); CD34, CD31 (Bioss); Flk-1, Tie-2 (Santa Cruz); and E selectin (DAKO). Similar results were obtained in three or more experiments. The shaded area of each box denotes negative antigen gate, and the white area denotes positive gate. Numbers are the mean \pm SEM percentage of cells for all experiments determined by comparison with corresponding negative control labeling.

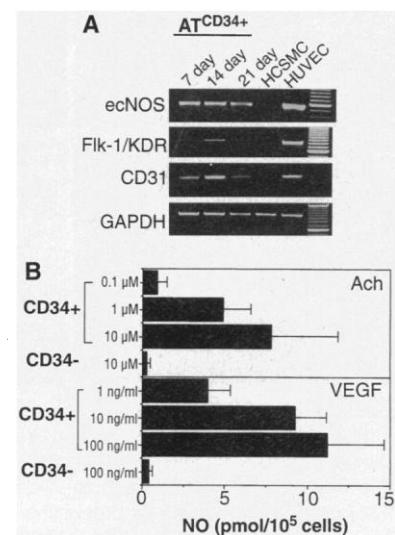


Fig. 3. Progenitor ECs express ecNOS, Flk-1/KDR, and CD31 mRNA and release NO. (A) Complementary DNA (from 10^6 cells) was amplified by PCR (40 cycles) with paired primers (23) (B) NO release from AT^{CD34+} and AT^{CD34-} cells cultured in six-well plates was measured as described (24). NO production was measured in a well with incremental doses of VEGF and Ach. HUVECs and bovine aortic ECs were used as positive controls, and human coronary smooth muscle cells (HCSMCs) as negative control. The values are means \pm SEM of 10 measurements for each group.

whole blood of 10 transgenic mice constitutively overexpressing β -galactosidase (β -Gal) (all mice were Flk-1^{+/+}). MB^{Flk1+} or MB^{Flk1-} cells were injected into nontransgenic mice of the same genetic background that had hindlimb ischemia of 2 days duration. Immunostaining of ischemic tissue, harvested 4 weeks after injection, for β -Gal demonstrated incorporation of cells expressing β -Gal in capillaries and small ar-

teries (Fig. 4M); these cells were identified as ECs by staining with antibody to CD31 (anti-CD31) and BS-1 lectin.

In vivo incorporation of autologous MB^{CD34+} cells into foci of neovascularization was also tested in a rabbit model of unilateral hindlimb ischemia. MB^{CD34+} cells were isolated from 20 ml of blood obtained by direct venipuncture of normal New Zealand White rabbits immediately

before surgical induction of unilateral hindlimb ischemia (19). Immediately after surgery, freshly isolated autologous DiI-labeled MB^{CD34+} were reinjected into the ear vein of the same rabbit. Histologic examination of the ischemic limbs 4 weeks later revealed that DiI-labeled cells were localized exclusively to neovascular zones of the ischemic limb (Fig. 4, N and O) and were incorporated into $9.7 \pm 4.5\%$ of the capillaries that

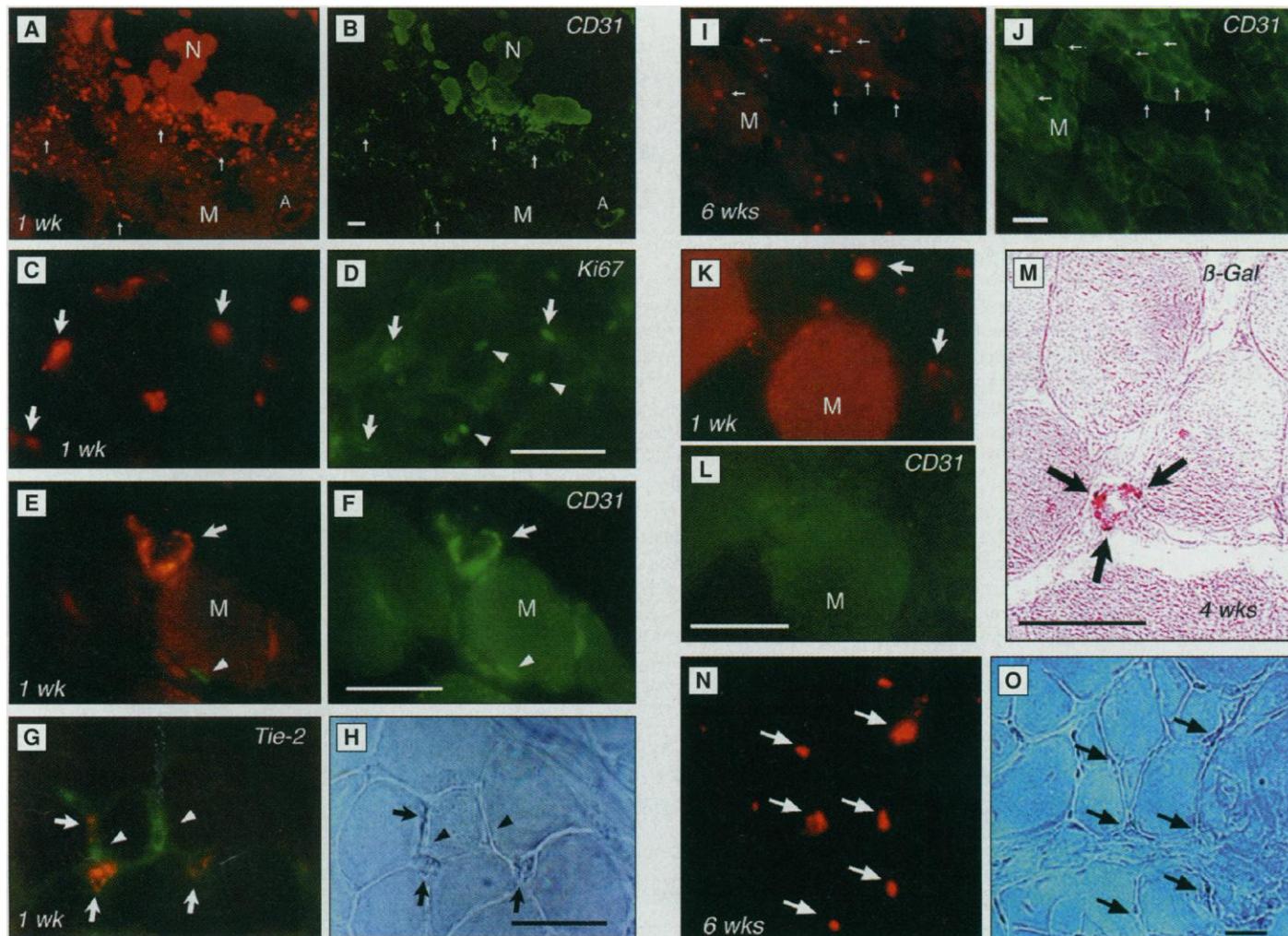


Fig. 4. Heterologous (panels A to L), homologous (M), or autologous (panels N and O) EC progenitors incorporate into sites of angiogenesis in vivo. (A and B) DiI-labeled MB^{CD34+} (red, arrows) between skeletal myocytes (M), including necrotic (N) myocytes 1 week after injection; most are colabeled with CD31 (green, arrows). Note a preexisting artery (A), identified as CD31-positive, but DiI-negative. (C and D) Evidence of proliferative activity among several DiI-labeled MB^{CD34+}-derived cells (red, arrows), indicated by coimmunostaining for antibody to Ki67 (Vector Lab, Burlingame, California) (green). Proliferative activity is also seen among DiI-negative, Ki67-positive capillary ECs (arrowheads); both cell types contribute to neovascularization. (E) DiI (red) and CD31 (green) in capillary ECs (arrows in E and F) between skeletal myocytes, photographed through a double filter 1 week after DiI-labeled MB^{CD34+} injection. (F) A single green filter shows CD31 (green) expression in DiI-negative capillary ECs integrated into the capillary with native (DiI-negative, CD31-positive) ECs (arrowheads in E and F). (G) Immunostaining 1 week after MB^{CD34+} injection showing capillaries comprising DiI-labeled MB^{CD34+}-derived cells expressing Tie-2 receptor (green). Several MB^{CD34+}-derived cells (arrows) Tie-2 positive and integrated with some Tie-2-positive

host capillary cells (arrowheads) identified by the absence of red fluorescence. (H) Phase-contrast photomicrograph of the same section shown in (G) indicates the corresponding DiI-labeled (arrows) and -unlabeled (arrowheads) capillary ECs. (I and J) Six weeks after administration, MB^{CD34+}-derived cells (red, arrows) colabel for CD31 in capillaries between preserved skeletal myocytes (M). (K and L) One week after injection of MB^{CD34-}, isolated MB^{CD34-}-derived cells (red, arrows) are observed between myocytes but do not express CD31. (M) Immunostaining of β -Gal in a tissue section harvested from ischemic muscle of C57BL/6J, 129/SV mice 4 weeks after the administration of MB^{Flk1+} isolated from transgenic mice constitutively expressing β -Gal. (Flk-1 cell isolation was used for selection of EC progenitors because of the lack of a suitable antibody to mouse CD34.) Cells overexpressing β -Gal (arrows) were incorporated into capillaries and small arteries; these cells were identified as ECs by anti-CD31 and BS-1 lectin (16). (N and O) Section of muscle harvested from rabbit ischemic hindlimb 4 weeks after administration of autologous MB^{CD34+} cells. Red fluorescence in (N) indicates localization of MB^{CD34+}-derived cells in capillaries seen (arrows) in the phase-contrast photomicrograph in (O). Each scale bar is 50 μ m.

consistently expressed CD31 and reacted with BS-1 lectin.

In summary, our findings suggest that cells isolated with anti-CD34 or anti-Flk-1 can differentiate into ECs in vitro. The in vivo results suggest that circulating MB^{CD34+} or MB^{Flk1+} cells may contribute to neovascularization in adult species, consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis (2, 3). A potentially limiting factor in strategies designed to promote neovascularization of ischemic tissues (20) is the resident population of ECs that is competent to respond to administered angiogenic cytokines (21). This issue may be successfully addressed with autologous EC transplants. The fact that progenitor ECs home to foci of angiogenesis suggests potential utility as autologous vectors for gene therapy. For anti-neoplastic therapies, MB^{CD34+} cells could be transfected with or coupled to antitumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by transfection of MB^{CD34+} cells to achieve constitutive expression of angiogenic cytokines or provisional matrix proteins or both (22).

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12. Single-donor human peripheral blood was obtained with a 20-gauge intravenous catheter. The first 3 ml was discarded, and the leukocyte fraction was obtained by Ficoll density gradient centrifugation. The cells were plated on plastic tissue culture for 1 hour to avoid contamination by differentiated adhesive cells.
13. MB^{CD34+}, MB^{CD34-}, and MB^{Flk1+} cells (>1 × 10⁶ of each) were analyzed with anti-CD34 (Biodesign, Kennebunkport, ME) and anti-Flk-1 (Santa Cruz Biotechnologies, Santa Cruz, CA).
14. The medium for all cell culture experiments was M-199 with 20% fetal bovine serum and bovine brain

extract (Clonetics, San Diego).

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18. The mean percent of Dil-labeled capillaries among total CD31-positive capillaries was determined by averaging counts made in 10 randomly selected fields (×400).
19. New Zealand White rabbits (3.8 to 4.2 kg, n = 4, Pine Acre Rabbitry, Norton, MA) underwent ligation of the popliteal and saphenous arteries distally, the external iliac artery proximally, and all femoral arterial branches, after which the femoral artery was excised [S. Takeshita et al., *J. Clin. Invest.* **93**, 662 (1994); L. Q. Pu et al., *Circulation* **88**, 208 (1993); R. Baffour et al., *J. Vasc. Surg.* **16**, 181 (1992)].
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23. Glycerinaldehyde phosphate dehydrogenase (GAPDH) was used as a positive control. The paired primers used (sense/antisense) were as follows: for ecNOS,

AAG ACA TTT TCG GGC TCA CGC TGC GCA CCC/ TGG GGT AGG CAC TTT AGT AGT TCT CCT AAC [548–base pairs (bp) PCR product]; for Flk-1 (KDR), CAA CAA AGT CCG GAG AGG AG/ATG ACG ATG GAC AAG TAG CC (819-bp PCR product); for CD31, GCT GTT GGT GGA AGG AGT GC/GAA GTT GGC TGG AGG TGC TC (645-bp PCR product); for GAPDH, TGA AGG TCG GAG TCA ACG GAT TTG/CAT GTG GGC CAT GAG GTC CAC CAC (983-bp PCR product).

24. NO release was measured with a NO-specific polarographic electrode connected to a NO meter (Iso-NO, World Precision Instruments, Sarasota, FL). AT^{CD34+} or AT^{CD34-} cells cultured in six-well plates were washed and then bathed in 5 ml of filtered Krebs-Henseleit solution. Cell plates were kept on a slide warmer (Lab Line Instruments, Melrose Park, IL) to maintain temperature between 35° and 37°C. The sensor probe was inserted vertically into the wells, and the tip of the electrode was positioned 2 mm under the surface of the solution.
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Somatic Frameshift Mutations in the BAX Gene in Colon Cancers of the Microsatellite Mutator Phenotype

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Cancers of the microsatellite mutator phenotype (MMP) show exaggerated genomic instability at simple repeat sequences. More than 50 percent (21 out of 41) of human MMP⁺ colon adenocarcinomas examined were found to have frameshift mutations in a tract of eight deoxyguanosines [(G)₈] within BAX, a gene that promotes apoptosis. These mutations were absent in MMP⁻ tumors and were significantly less frequent in (G)₈ repeats from other genes. Frameshift mutations were present in both BAX alleles in some MMP⁺ colon tumor cell lines and in primary tumors. These results suggest that inactivating BAX mutations are selected for during the progression of colorectal MMP⁺ tumors and that the wild-type BAX gene plays a suppressor role in a p53-independent pathway for colorectal carcinogenesis.

The MMP pathway for colon cancer is characterized by genomic instability that leads to the accumulation of deletion and insertion mutations at simple repeat sequences (1–3). The fixation of these slippage-induced replication errors as mutations (4) is associated with defects in DNA mismatch repair (5). Colorectal MMP⁺ tumors frequently contain frameshift mutations in the type II transforming growth factor-β (TGF-β) receptor gene (6) but are usually wild type for the p53 tumor suppressor gene (1, 7). In addition to its central role in cell growth arrest (8), p53 also plays a role in apoptosis in response to DNA

damage (9). The p53 protein transactivates BAX (10), a member of the BCL2 gene family (11) that promotes apoptosis (12).

The human BAX gene contains a tract of eight consecutive deoxyguanosines in the third coding exon, spanning codons 38 to 41 (ATG GGG GGG GAG) (12). To determine whether this sequence is a mutational target in MMP⁺ tumor cells, we amplified by the polymerase chain reaction (PCR) the region containing the (G)₈ tract from various MMP⁺ tumor cell lines. This analysis revealed band shifts suggestive of insertions and deletions of one nucleotide in some of these tumor cells (Fig. 1A). Prostate (DU145) and colon (LS180) tumor cells exhibited PCR patterns indistinguishable from those amplified from plasmids containing a BAX fragment with mutant (G)₉ and (G)₇ tracts (Fig. 1A, P9 and P7),

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