Suppression of angiogenesis by lentiviral delivery of PEX, a noncatalytic fragment of matrix metalloproteinase 2

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Modulation of the balance between pro- and antiangiogenic factors holds great promise for the treatment of a broad spectrum of human disease ranging from ischemic heart disease to cancer. This requires both the identification of angiogenic regulators and their efficient delivery to target organs. Here, we demonstrate the use of a noncatalytic fragment of matrix metalloproteinase 2 (termed PEX) delivered by lentiviral vectors in different angiogenesis models. Transduction of human endothelial cells with PEX virus suppressed endothelial invasion and formation of capillary-like structures without affecting chemotaxis *in vitro*. Lentiviral delivery of PEX blocked basic fibroblast growth factor-induced matrix metalloproteinase 2 activation and angiogenesis on chicken chorioallantoic membranes. PEX expression also inhibited tumor-induced angiogenesis and tumor growth in a nude mouse model. Thus, our study shows that lentiviral vectors can deliver sufficient quantities of antiangiogenic substances to achieve therapeutic effects *in vivo*.

Angiogenesis is defined as the sprouting of new blood vessels from existing vessels (1, 2). During angiogenesis, invasive endothelial cells recruit matrix metalloproteinases (MMPs) to remodel the extracellular matrix and the basal lamina—an important physical barrier between the endothelial and connective tissue (3, 4). Two members of the MMP family of pericellular proteinases, MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa), display the highest enzymatic activities against type IV collagen, the main constituent of the basal lamina (5). MMP-2 is the most widely distributed MMP (6), and its expression correlates with an invasive phenotype: MMP-2 is highly expressed in stromal cells close to the invasive front of metastasizing tumors (7) and it is localized on the surface of angiogenic endothelial cells (8). Most MMPs are secreted as inactive zymogens (proMMP) that require proteolytic activation by serine proteases such as plasmin and trypsin (9). In contrast, MMP-2 lacks a serine protease recognition motif (10) and is activated at the cell surface of invasive cells by a multimeric receptor/activation complex consisting of the tissue inhibitor of metalloproteinase 2 (TIMP-2), the membrane type 1 MMP (MT1-MMP), and integrin $\alpha v\beta 3$ (11). It was postulated that association of MMP-2 with this receptor/activator complex occurs in two steps: the C-terminal domain of MMP-2 binds to TIMP2 (12), which, in turn, associates with the membrane-bound MT1-MMP (13–15). Because the active site of MT1-MMP is inhibited by TIMP-2, it was proposed that at least two MT1-MMP molecules are required for MMP-2 processing (14): a TIMP-2/MT1-MMP receptor complex and a TIMP-2-free MT1-MMP processing enzyme that initiates MMP-2 activation by cleaving the N-terminal propeptide. The processing of MMP-2 by MT1-MMP generates the intermediate MMP-2 form that binds in a second step to integrin $\alpha v\beta 3$ via its C terminus (16). Recent evidence suggests that the binding to integrin $\alpha v\beta 3$ contributes to autocatalytic maturation and that $\alpha v\beta 3$-expressing cells can express functionally active MMP-2 on the cell surface (16, 17). As a result of the autocatalytic activity of MMP-2, a 29- to 32-kDa C-terminal hemopexin fragment (PEX) is generated. Both autocatalytically derived and recombinant PEX binds integrin $\alpha v\beta 3$ and blocks cell surface activation of MMP-2 (17, 18) by competing with MMP-2 for binding to integrin $\alpha v\beta 3$ (17). Interestingly, PEX formation depends on the stage of angiogenesis: it accumulates during early stages and reaches maximum levels during later phases of angiogenesis (i.e., vessel maturation) (17). Therefore, it was concluded that PEX acts as an endogenous inhibitor of MMP-2 activation (11, 17).

To test whether the inhibitory effect of PEX on MMP-2 activation could be used for an antiangiogenic gene therapy approach, we used lentiviral vectors (19–21) to deliver and express PEX in different angiogenesis models. We report that lentivirally delivered PEX suppresses angiogenesis in a number of different models.

Materials and Methods

Construction of Viral Vectors. After reverse transcription (Superscript; GIBCO/BRL) of mRNA isolated from mouse embryonic fibroblasts, the MMP-2 N-terminal leader [corresponding to the first 29 aa (22, 23)] and the MMP-2 hemopexin domain (amino acids 441–633) were amplified by PCR (PFU-TAQ; Stratagene) by using the primer pairs H3MMPf/PEXR1 and PEXf2/MMPRXHO, respectively. In addition, a FLAG-tagged construct was cloned by PCR amplification by using the primer pairs H3MMPf/FLAGr and FLAGf/MMPRXHO. The FLAG primers were engineered to encode a FLAG epitope 4 aa downstream of the putative signal sequence site (24) of MMP-2. The amplified products then were cloned into the *Hin*dIII-*Xho*I site of pBluescript (Stratagene). The native (nPEX-LV) or the FLAG-tagged (PEX-LV) version of PEX then was cloned into p156RRLsinPPT (25). As an internal promoter, we used the cytomegalovirus promoter obtained from pcDNA3 (Invitrogen). All constructs were confirmed by sequence analysis. Primer sequences were as follows: H3MMPf, 5′-CCCAAGCTTGCCGCCACCATGGAGGCACGAGTGGCCTGG-3′; PEXR1, 5′-AGCGATGGCGCGGCCCAACAG-3′; PEXf2, 5′-ATCTGCAAACAGGACATTGTC-3′; MMPRXHO, 5′-CCGCTCGAGTCAGCAGCCCAGCCAGTCTG-3′; FLAGR, 5′-GTAGTCGGGCGATGGTGCAGCGATGGCGCGGCCCAA-3′; and FLAGF, 5′-AAGGACGACGATGACAAGATCTGCAAACAGGACATTGTC-3′.

Abbreviations: CAM, chicken chorioallantoic membrane; MMP, matrix metalloproteinase; HUVECs, human umbilical vein endothelial cells; LTR, long terminal repeat; PEX, hemopexin domain of MMP-2; MT1-MMP, membrane type 1 MMP; SIN, self-inactivating; TIMP-2, tissue inhibitor of metalloproteinase 2; bFGF, basic fibroblast growth factor; I.U., infectious units; GFP, green fluorescent protein; VSV-G, G protein of vesicular stomatitis virus.

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Virus Production. Lentiviral vectors were produced by using a four-plasmid, third-generation, Tat-free packaging system (26). The two packaging plasmids (encoding HIV gag, pol, and rev), together with the plasmid coding for vesicular stomatitis virus envelope and the vector itself, were transfected into 293T cells by using the calcium phosphate method. Typically, we transfected 12 15-cm dishes and harvested the virus by collecting the cell culture medium 24, 48, and 72 h after changing the transfection medium to DMEM containing 10% FCS. After filtering the collected medium through 0.45-μm filters, we concentrated the virus by spinning at 68,000 $\times$ *g* for 2 h, followed by a second spin (59,000 $\times$ *g* for 2.5 h at room temperature). The resulting pellet was resuspended in 200 μl of Hanks' buffer. The titer of lentiviral vectors was determined by measuring the amount of HIV p24 gag antigen by ELISA (Alliance; NEN). To calculate the amount of infectious units (I.U.), the p24 titer was correlated to the biological activity of a similar virus carrying a green fluorescent protein (GFP) cassette by using serial dilutions of the GFP virus to transduce 293T cells (1 ng of p24 = 1×10^5 I.U.).

Endothelial Cell Migration and Tube Formation. Endothelial cell migration assays were performed in modified Boyden chambers (Costar), with the upper chamber containing filters of 8.0-μm pore size. The lower surface of the membranes was coated with collagen I (10 μg/ml) for 2 h at 37°C. Early-passage (p3–7) human umbilical vein endothelial cells (HUVECs) (Clonetics, San Diego) were serum-starved in Starvemedia (M199, 0.5% serum) for 2 h and then harvested with trypsin/EDTA. After washing once in migration buffer (DMEM/0.1% BSA), cells were seeded at a density of 50,000 cells per well (in 100 μl of migration buffer) on the filters. Migration was induced by addition of migration buffer containing 30 ng/ml basic fibroblast growth factor (bFGF; Scios, Mountain View, CA) to the lower chamber. After 2 h, the number of migrated cells was quantified by staining the lower surface of the membrane filter with crystal violet (1%). Cells on the upper surface of the membrane were removed by mechanical cleaning and extensive washing with deionized water. Stained cells were counted under a stereomicroscope by using a hemocytometer (8 fields per filter). The invasion assays were carried out as described above with the following modifications. The filter insets (upper chambers) were coated with growth factor-depleted Matrigel (50 μl, 37.5 μg/cm^2) (Becton Dickinson). Filters were dried overnight at 37°C. The Matrigel was reconstituted with 100 μl of DMEM at least 2 h before use. Invasion was quantified after 6 h.

For tube formation assays, growth factor-depleted Matrigel was applied into a 24-well tissue culture plate (400 μl per well). After polymerization of the Matrigel (37°C, 1 h), 2-h serum-starved HUVECs were harvested by using trypsin/EDTA, washed with Assaymedia (M199/1% FBS), and seeded at a density of 10,000 cells per well (final volume, 500 μl) on polymerized Matrigel in the presence or absence of bFGF at 30 ng/ml. The plate was incubated at 37°C for 24 h, and then the medium was aspirated and cells were fixed in neutral buffered 10% formalin. Representative pictures were taken at $\times$10 magnification, and tube length was measured.

Analysis of CD31 and PEX Expression. Analysis of CD31 expression of endothelial cells was performed by staining with anti-CD31 antibody (clone MEC13.3), kindly provided by Annunciata Vecchi (Mario Negri Institute for Pharmacological Research, Milan), plus a secondary antibody labeled with the fluorochrome Alexa 568 (Molecular Probes). After washing and mounting with Slow Fade (Molecular Probes), samples were analyzed by a laser-scanning MRC1024 confocal microscope (Bio-Rad).

For the analysis of PEX and CD31 expression in tumor tissue, snap-frozen tumors were cut into 4-μm sections and then fixed with 4% paraformaldehyde (10 min) followed by permeabilization in 0.1% Triton X-100 (2 min). Unspecific binding was blocked with 5% BSA in PBS before staining. PEX expression was quantified by using the polyclonal Ab Octapeptide (Zymed). CD31 expression was determined by staining with anti-CD31 antibody (clone MEC13.3). Fluorescence was analyzed by using a laser-scanning MRC1024 confocal microscope. The number of blood vessels per field was quantitated with a $\times$20 objective at a minimum of four fields per section and four tumors per condition.

Western blotting of native and FLAG-tagged PEX was carried out with mAbs TV88 (17) and M2 (Sigma), respectively.

Chicken Chorioallantoic Membrane (CAM) Assay. CAM assays were carried out as described (17, 27). After cutting a window in the eggshell, filter discs (5-mm diameter) saturated with bFGF (3 μg/ml) were placed on the CAM. Eight hours later, 10 μl of Hanks' solution (GIBCO/BRL) containing virus was pipetted on the filter discs to infect the CAM. Sixty-four hours later, the CAM underlying the filter disk was excised. Angiogenesis was quantified by counting the blood vessel branch points under a stereomicroscope. The angiogenic index was calculated by subtracting the number of branch points from the branching in the absence of bFGF stimulation.

Tumor-induced angiogenesis was analyzed by growing CS-1 cells (0.5×10^6 cells per egg) for 7 days on the CAMs. After 7 days, the tumors were excised and dissected. Fifty milligrams of these tumors then was seeded on fresh CAMs grown for another 9 days. Thereafter, tumors were harvested, weighed, and processed for histological analysis.

Growth of Human Melanomas in Nude Mice. Six- to eight-week-old female nu/nu nude mice (The Jackson Laboratory) were injected s.c. (using 28 gauge needles) with a single-cell suspension of 3.5×10^6 M21L human melanoma cells per animal. Every 5 days, tumor volume was evaluated by using a caliper. At days 5 and 10 after tumor implantation, we injected 2×10^8 I.U. (in 100 μl of PBS) of lentiviral vectors in the vicinity of the tumors. At day 20, mice were killed. The tumors were weighed, frozen, and subjected to histological analysis.

All data are expressed as mean $\pm$ SEM.

Results

PEX Transduction. To analyze the effect of PEX (17) on angiogenic processes *in vivo*, a C-terminal fragment (amino acids 441–633) of murine MMP-2 (22) was cloned into HIV-based lentiviral vectors (25, 28, 29). To ensure secretion of PEX by the target cells, the signal peptide sequences of MMP-2 were included in the constructs (nPEX-LV) (Fig. 1*A*). In addition, a PEX vector was designed that contains an N-terminal FLAG-epitope tag (PEX-LV, Fig. 1*A*). FLAG epitope-tagged PEX purified from stably expressing 293T cells bound to integrin $\alpha v\beta 3$ and TIMP-2 in a dose-dependent manner (data not shown).

All lentiviral vectors used for this study contain self-inactivating (SIN) mutations in their 3′ long terminal repeat (LTR) (29, 30) and a Rous sarcoma virus promoter in the 5′ LTR (Fig. 1*A*) that allows the use of a Tat-free, third-generation packaging system (26). To increase transduction efficiency, we cloned a central polypurine tract 5′ to the internal promoter, which has been shown to enhance infection of nondividing cells (25, 31). In addition, a posttranscriptional regulatory element of the woodchuck hepatitis virus was included (Fig. 1*A*) that enhances expression of transgenes carried by lentiviral vectors (32). All viruses were pseudotyped with the G protein of the vesicular stomatitis virus (VSV-G) which allows the infection of broad host spectrum and concentration of the virus. Taken together, these modifications of the lentiviral system enhance gene delivery into target cells and increase the efficiency of

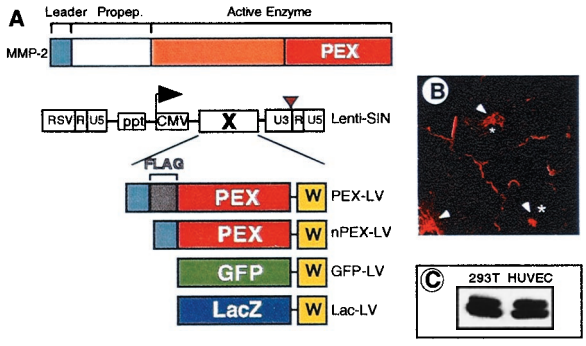

Fig. 1. Lentiviral vectors used for PEX delivery and transduction of endothelial cells with lentiviral vectors. (*A*) Schematic structure of MMP-2 (*Upper*) and lentiviral vectors (*Lower*). The lentiviral vectors contain the following features. The U3 element of the 5′ LTR is replaced by a Rous sarcoma virus promoter (RSV) that drives expression of the vector transcripts in the packaging cells. The 3′ LTR contains a SIN mutation (brown triangle) to ensure self-inactivation in the target cell. Expression of the transgene (X) is driven by the internal cytomegalovirus (CMV) promoter. The difference between nPEX-LV and PEX-LV is the inclusion of a FLAG-tag (gray) in the latter. W, posttranscriptional regulatory element of woodchuck hepatitis virus (yellow); ppt, polypurine tract. (*B*) Analysis of PEX expression by HUVECs transduced with PEX-LV using Abs directed against the FLAG-tag. Arrowheads, Golgi apparatus; *, nucleus. (*C*) Western blot analysis of PEX expression in 293T (left lane) cells stably expressing PEX and HUVECs (right lane) transduced with PEX-LV.

vector production (titers greater than 1×10^{10} units/ml are regularly obtained).

Inhibition of Endothelial Cell Invasion and Tube Formation. HUVECs were transduced with PEX-LV (multiplicity of infection of 50), and PEX expression was analyzed by immunocytochemistry (Fig. 1*B*) and Western blotting (Fig. 1*C*). Interestingly, HUVECs transduced with PEX-LV secreted amounts of PEX almost similar to those secreted by stably transfected 293T cells (Fig. 1*C*).

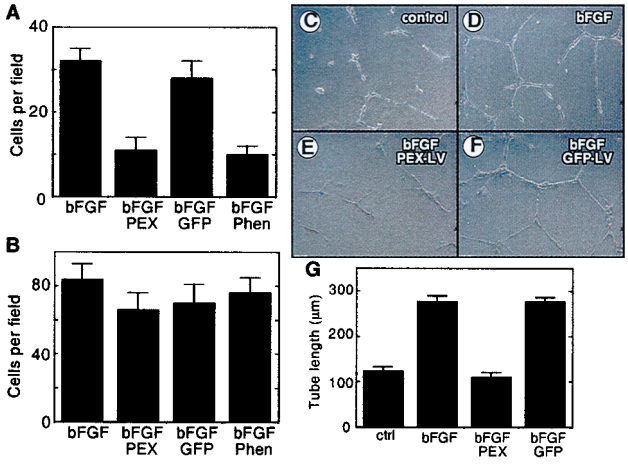

Fig. 2. Effect of PEX on endothelial cell function *in vitro*. (*A* and *B*) bFGF-induced invasion (*A*) and migration (*B*) of HUVECs in modified Boyden chambers. Effect of bFGF in uninfected cells (bFGF), and cells infected with PEX-LV (bFGFPEX) or with GFP-LV (bFGFGFP). bFGFPhen, cells treated with *o*-phenanthroline. (*C–G*) Formation of capillary-like structures of endothelial cells *in vitro*. Within 24 h after seeding on a Matrigel, HUVECs start to form capillary-like structures (*C*). Addition of bFGF enhances this process (*D*) in uninfected HUVECs but not in PEX-expressing cells (*E*). Transduction of HUVECs with Lac-LV (*D*) has no effect on tube formation. (*H*) Summary of the effect of PEX on tube formation. bFGF, untreated control; bFGFPEX, PEX-expressing cells; bFGFGFP, GFP-transduced HUVECs.

Because MMP-2 facilitates endothelial cell migration across the basal lamina, we tested the effect of PEX expression on bFGF-induced invasion (transmigration) of a reconstituted basal lamina in modified Boyden chambers. In this assay (33), cells transmigrate from an upper well through a porous filter coated with Matrigel toward a chemoattractant (i.e., bFGF) in the lower chamber. Addition of bFGF (30 ng/ml) to the lower chamber induced transmigration of HUVECs through the Matrigel (Fig. 2*A*). The bFGF-induced response was reduced by 3-fold in cells transduced with PEX-LV, as compared with control GFP-LV. Incubation of HUVECs with *o*-phenanthroline ($C_{12}H_8N_2$; 100 μg/ml)—a synthetic MMP inhibitor that unspecifically blocks enzymatic activity by chelating metal ions—reduced bFGF-induced invasion to the same level as observed with PEX-LV. To ensure that PEX expression did not interfere with chemotaxis or viability of the transduced cells, bFGF-induced migration was analyzed in the absence of Matrigel (Fig. 2*B*). bFGF (30 ng/ml) induced a chemotactic response in uninfected HUVECs that was not significantly different from cells secreting PEX or cells expressing GFP. Furthermore, *o*-phenanthroline (100 μg/ml) also had no significant effect on HUVEC migration (Fig. 2*B*).

Apart from migration and invasion, tube formation is another important parameter of endothelial function that can be evaluated *in vitro*. HUVECs cultured on a Matrigel rapidly align, extend processes into the matrix, and, finally, form capillary-like structures (34, 35). These structures are composed of polarized cells connected via complex junctions surrounding a central lumen (36). Furthermore, previous studies (37) have shown that MMP-2 is important for the formation of these capillary-like structures. Within 24 h after seeding on Matrigel, HUVECs formed tubular structures (Fig. 2*C*). Addition of bFGF (30

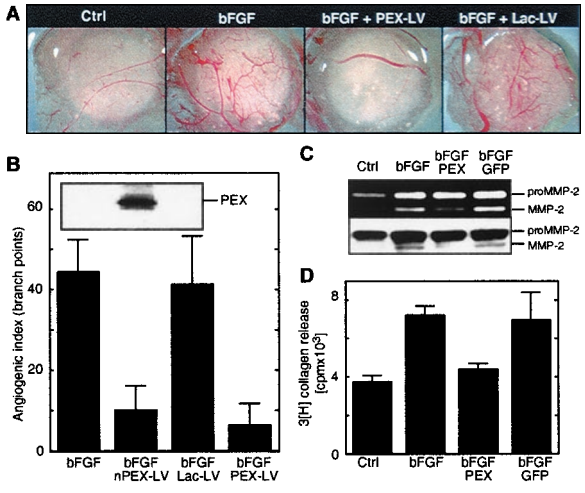

Fig. 3. Effects of PEX on angiogenesis in the chick CAM model. (*A—D*) Virally delivered PEX inhibits bFGF-induced angiogenesis through inhibition of MMP-2 activation. (*A*) Chick CAMs incubated for 72 h with filter disks soaked in PBS (Ctrl), bFGF, bFGF in the presence of PEX lentivirus (bFGF + PEX-LV), or LacZ virus (bFGF + Lac-LV). (*B*) Quantification of bFGF-induced angiogenic response by counting vessel branch points. (*Inset*) Western blot analysis using TV88 antibody to detect PEX expression in CAM lysates (from left to right: bFGF, bFGF + PEX-LV, bFGF + Lac-LV). (*C* and *D*) bFGF-induced MMP-2 activation (gelatin zymography) (*C Upper*) and collagenolytic (*D*) activity in CAM lysates. The same CAM lysates were used both for gelatin zymography and for collagenase assays. The upper and lower bands correspond to the 72-kDa MMP-2 proenzyme (proMMP-2) and the activated MMP-2 (≈62 kDa), respectively. To ensure the presence of the MMP-2 proenzyme in equal amounts, lysates also were analyzed by Western blotting (*C Lower*) by using MMP-2 specific mAb TV88. Ctrl, PBS-treated CAM; FGF, bFGF-treated CAM; bFGFPEX, PEX-expressing CAM treated with bFGF; bFGFGFP, GFP-expressing CAM treated with bFGF.

ng/ml) significantly enhanced tube formation, with an increased number of intercellular contacts and overall complexity of the network (Fig. 2*D*). PEX clearly suppressed the bFGF-induced response (Fig. 2 *E* and *G*), whereas GFP expression did not affect tube formation (Fig. 2*F*). Transduction with PEX-LV had no significant effect on bFGF-induced endothelial cell proliferation during the 24-h incubation period, as compared with cells transduced with GFP-LV or Lac-LV (data not shown).

PEX Inhibits Angiogenesis in the CAM Assay. To study the effect of PEX on angiogenesis *in vivo*, we initially used the CAM assay (38, 39). Incubation of bFGF-soaked filter discs for 72 h on the CAMs of 10-day chicken embryos induced a clearly visible (Fig. 3*A*) angiogenic response that was quantified by counting vessel branch points under a stereomicroscope. Eight hours after placing the filter discs onto the CAMs, the area underlying and surrounding the filter disk was infected topically. The VSV-G pseudotyped lentiviral vectors efficiently transduced the CAM tissue as shown by Western blot analysis (Fig. 3*B Inset*). Lentiviral delivery ($\approx 4 \times 10^8$ I.U.) of native (nPEX-LV) or FLAG-tagged (PEX-LV) PEX reduced the bFGF-induced angiogenesis to one-fourth ($n = 8$) and to one-eighth ($n = 14$), respectively, which was significantly ($P < 0.008$) different from the uninfected controls (Fig. 3*B*). Transduction of the CAMs with Lac-LV (or GFP-LV) did not significantly ($P > 0.4$, $n = 8$) affect the bFGF-induced angiogenic response (Fig. 3 *A* and *B*).

To verify that PEX inhibits MMP-2 activation *in vivo*, lysates of the CAMs used for angiogenesis assays were prepared and analyzed by gelatin zymography and collagen release assays (Fig. 3 *C* and *D*). Activated MMP-2 accumulates in bFGF-treated CAMs as shown by the conversion of the 72-kDa MMP-2 proenzyme to the ≈62-kDa activated form of MMP-2 in the zymogram and Western blot (Fig. 3*C Upper*). Expression of PEX suppressed this bFGF-induced activation of MMP-2, whereas expression of GFP had no significant effect on MMP-2 activation. The presence of equal concentrations of the proenzyme was confirmed by Western blot analysis by using MMP-2-specific Ab TV-88 (17) (Fig. 3*C Lower*). The zymogram did not show other gelatinase activities (e.g., 92-kDa gelatinase) except from those derived from the 72-/62-kDa proteins. Inhibition of MMP-2 activation by PEX was paralleled by the suppression of bFGF-induced collagenolytic activity of the lysates (Fig. 3*D*). These data show that bFGF-induced accumulation of activated MMP-2, which is the predominant gelatinase in this system, is blocked by PEX expression *in vivo*, resulting in a reduced overall collagenolytic activity and suppression of bFGF-induced angiogenesis.

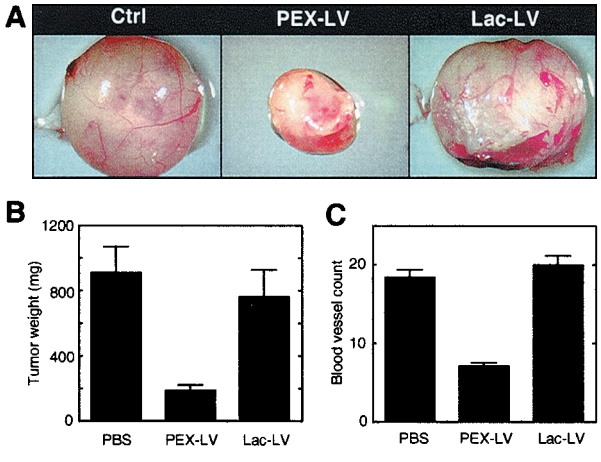

Fig. 4. Lentiviral delivery of PEX inhibits tumor growth (*A* and *B*) and tumor-induced angiogenesis (*C*) in a CAM tumor model using CS-1 hamster melanoma. (*A*) Representative examples of CS-1 tumors treated with PBS, PEX-LV, or a Lac-LV. (*B*) Weight of tumors expressing PEX or LacZ compared with control tumors (PBS). (*C*) Tumor vasculature quantified as vessels per field (×20 magnification).

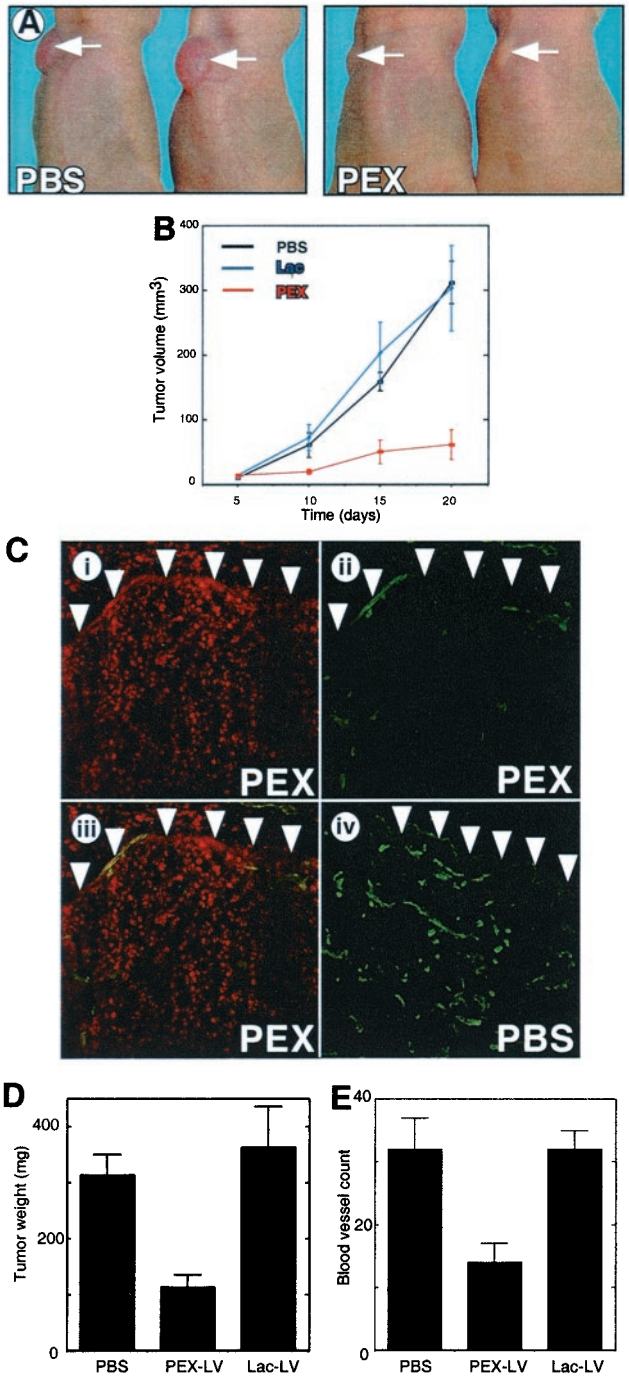

Fig. 5. PEX inhibits human melanoma (M21L) growth in nude mice. (*A*) Two representative examples of nude mice 20 days after tumor implantation injected with PBS (*Left*) or with PEX-LV (*Right*). (*B*) Analysis of tumor volume starting 5 days after tumor implantation. Five days after implanting the tumors, PBS (black), Lac-LV (blue), or PEX-LV (red) was injected around the tumor, $n = 7$, for each group. (*C*) Analysis of PEX expression and vascularization in tumors. Staining with polyclonal Abs against FLAG-tag (*i*) and CD31(PECAM-1) (*ii*) in a section through a tumor treated with PEX-LV. (*iii*) Merger of *i* and *ii*. (*iv*) Representative histological sections of a PBS-treated tumor. The arrows indicate the border of the tumor. (*D* and *E*) Effect of PEX on tumor weight (*D*) and tumor-induced angiogenesis (*E*).

Tumor Suppression by PEX. Next, we used a CAM tumor model to study whether overexpression of PEX would be a feasible approach to suppress tumor-induced angiogenesis. Additionally, this also should serve to test the ability of the lentiviral vector system to deliver a sufficient amount of angiogenesis inhibitor and subsequent suppression of tumor growth *in vivo*. Hamster melanoma cells (CS-1) that do not express detectable amounts of integrin $\alpha v\beta 3$ (40) form solid, well vascularized tumors within 8 days after seeding on CAMs. Fragments (50 mg per egg) of these CS-1 tumors were subcultured on fresh, 9-day-old CAMs. Twenty-four hours later, we topically infected the CAMs surrounding the tumors with 2×10^8 I.U. of PEX-LV or LacZ-LV. Nine days after seeding the tumors, the tumor-bearing CAMs were harvested (Fig. 4*A*). The tumors treated by lentiviral delivery of PEX were clearly smaller and visibly less vascularized than the control tumors (Fig. 4 *A* and *B*). Treatment with PEX-LV ($n = 10$) reduced tumor weight to 21% and 25%, as compared with the PBS-treated ($n = 9$) and Lac-LV-treated ($n = 10$) CAMs, respectively (Fig. 4*B*). Vascularization, as determined by counting vessels in tumor sections, was reduced significantly in the PEX-LV group compared with the Lac-LV tumors (Fig. 4*C*). PEX inhibited tumor growth and vascularization in a dose-dependent manner, as determined by using increasing multiplicities of infection of PEX virus (data not shown), with an almost complete suppression of both parameters at high PEX concentrations ($\approx$15 μg per tumor).

In a more clinically relevant situation, preestablished s.c. human melanomas were treated with PEX lentivirus in a mouse model. In this tumor model, M21L human melanoma cells that do not express integrin $\alpha v\beta 3$ (41) were implanted s.c. in nude mice. Five days after implantation, approximately 20-mm^3 tumors were established (Fig. 5*B*), and PEX-LV or a lentivirus carrying LacZ (4×10^8 I.U.) was injected topically in the vicinity of the tumors. The control animals ($n = 13$) were injected with PBS. Over the next 15 days, the M21L cells grew to well vascularized tumors in the controls (Fig. 5*A Left*), whereas in the PEX-treated animals ($n = 12$), the tumors grew much slower (Fig. 5*B*) and were less vascularized (Fig. 5 *A*, *C*, and *E*). At day 20, mice were killed to assess tumor weight, vascularization, and PEX expression. Treatment with the PEX lentivirus resulted in a 64% reduction of tumor weight (Fig. 5*D*). Analysis of PEX expression with polyclonal Abs to FLAG showed that administration of PEX-LV transduced the outer segment of the tumors (Fig. 5*C*). Interestingly, CD31 (PECAM-1) staining for endothelial cells in histological tumor sections revealed not only a 56% reduction in the number and size of tumor vessels in the PEX-LV group (Fig. 5 *C* and *E*), but also showed a peripheral zone of reduced vascularization that overlapped with the area of highest PEX expression (Fig. 5*C*, *i–iii*), whereas PBS-treated tumors exhibited a large number of vessels especially close to the tumor border (Fig. 5*C*, *iv*).

Discussion

It previously has been shown that purified PEX, a noncatalytic fragment of MMP-2, prevents the binding of MMP-2 to integrin $\alpha v\beta 3$, thereby disrupting angiogenesis (17): we have extended these observations by demonstrating that viral delivery of PEX suppresses neovascularization in different animal models. Expression of PEX in the vicinity of an angiogenic process specifically blocks MMP-2 activation, which is essential for the remodeling of the extracellular matrix. Therefore, angiogenic endothelial cells are likely to be prevented from invasion in the presence of virally produced PEX. These findings demonstrate that lentiviral vectors are an efficient vehicle for delivery of antiangiogenic factors *in vivo*. Retroviral (42, 43) and adenoviral (44, 45) vectors have been used for viral delivery of angiogenic regulators, such as dominant-negative mutants of Flk-1 (42), angiostatin (44), or endostatin (45). Adenoviral vectors can infect dividing and nondividing cells but have some undesirable immunological consequences (19). Additionally, they have to overcome the preexisting host immune response against the incoming viral proteins (46). The retroviral vectors (based on murine leukemia virus) cannot transduce nondividing cells and, hence, can be used only to target dividing cells. Lentiviral vectors can infect both dividing and nondividing cells (28, 47–50). Recent improvements in the safety and transduction efficacy (25, 26, 30–32) make lentiviral vectors a promising tool for *in vivo* gene delivery. Because these lentiviral vectors integrate into the host genome, a long-term expression of the transgene is possible. In addition, the transgene expression can be switched on and off at will by incorporation of inducible elements into the vectors (51). Our results are an important step forward toward modulation of the angiogenic balance (between pro- and antiangiogenic factors) *in vivo*, given the rather short-lived effect of pharmacological regulators of extracellular matrix remodeling such as chemical compounds and purified proteins that presently are available.

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1. Risau, W. (1997) *Nature (London)* **386,** 671–674.
2. Folkman, J. (1995) *Nat. Med.* **1,** 27–31.
3. Kalebic, T., Garbisa, S., Glaser, B. & Liotta, L. A. (1983) *Science* **221,** 281–283.
4. Werb, Z. (1997) *Cell* **91,** 439–442.
5. Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C. S., Bauer, E. A. & Goldberg, G. I. (1988) *J. Biol. Chem.* **263,** 6579–6587.
6. Murphy, G. & Crabbe, T. (1995) *Methods Enzymol.* **248,** 470–484.
7. Pyke, C., Ralfkiaer, E., Huhtala, P., Hurskainen, T., Dano, K. & Tryggvason, K. (1992) *Cancer Res.* **52,** 1336–1341.
8. Brooks, P. C., Stromblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P. & Cheresh, D. A. (1996) *Cell* **85,** 683–693.
9. Nagase, H., Ogata, Y., Suzuki, K., Enghild, J. J. & Salvesen, G. (1991) *Biochem. Soc. Trans.* **19,** 715–718.
10. Okada, Y., Tsuboi, S., Tsuda, Y., Nagamatsu, Y. & Yamamoto, J. (1990) *FEBS Lett.* **272,** 113–116.
11. Silletti, S. & Cheresh, D. (1999) *Fibrinolysis Proteinolysis* **13,** 226–238.
12. Fridman, R., Fuerst, T. R., Bird, R. E., Hoyhtya, M., Oelkuct, M., Kraus, S., Komarek, D., Liotta, L. A., Berman, M. L. & Stetler-Stevenson, W. G. (1992) *J. Biol. Chem.* **267,** 15398–15405.
13. Zucker, S., Drews, M., Conner, C., Foda, H. D., DeClerck, Y. A., Langley, K. E., Bahou, W. F., Docherty, A. J. & Cao, J. (1998) *J. Biol. Chem.* **273,** 1216–1222.
14. Kinoshita, T., Sato, H., Okada, A., Ohuchi, E., Imai, K., Okada, Y. & Seiki, M. (1998) *J. Biol. Chem.* **273,** 16098–16103.
15. Butler, G. S., Butler, M. J., Atkinson, S. J., Will, H., Tamura, T., van Westrum, S. S., Crabbe, T., Clements, J., d'Ortho, M. P. & Murphy, G. (1998) *J. Biol. Chem.* **273,** 871–880.
16. Deryugina, E. I., Bourdon, M. A., Jungwirth, K., Smith, J. W. & Strongin, A. Y. (2000) *Int. J. Cancer* **86,** 15–23.
17. Brooks, P. C., Silletti, S., von Schalscha, T. L., Friedlander, M. & Cheresh, D. A. (1998) *Cell* **92,** 391–400.
18. Ward, R. V., Atkinson, S. J., Reynolds, J. J. & Murphy, G. (1994) *Biochem. J.* **304,** 263–269.
19. Verma, I. M. & Somia, N. (1997) *Nature (London)* **389,** 239–242.
20. Amado, R. G. & Chen, I. S. (1999) *Science* **285,** 674–676.
21. Trono, D. (2000) *Gene Ther.* **7,** 20–23.
22. Reponen, P., Sahlberg, C., Huhtala, P., Hurskainen, T., Thesleff, I. & Tryggvason, K. (1992) *J. Biol. Chem.* **267,** 7856–7862.
23. Aimes, R. T., French, D. L. & Quigley, J. P. (1994) *Biochem. J.* **300,** 729–736.
24. Chinnaiyan, A. M., O'Rourke, K., Tewari, M. & Dixit, V. M. (1995) *Cell* **81,** 505–512.

25. Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M. & Naldini, L. (2000) *Nat. Genet.* **25,** 217–222.
26. Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D. & Naldini, L. (1998) *J. Virol.* **72,** 8463–8471.
27. Brooks, P. C., Clark, R. A. & Cheresh, D. A. (1994) *Science* **264,** 569–571.
28. Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M. & Trono, D. (1996) *Science* **272,** 263–267.
29. Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L. & Trono, D. (1998) *J. Virol.* **72,** 9873–9880.
30. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H. & Verma, I. M. (1998) *J. Virol.* **72,** 8150–8157.
31. Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L. & Charneau, P. (2000) *Cell* **101,** 173–185.
32. Zufferey, R., Donello, J. E., Trono, D. & Hope, T. J. (1999) *J. Virol.* **73,** 2886–2892.
33. Albini, A. (1998) *Pathol. Oncol. Res.* **4,** 230–241.
34. Kubota, Y., Kleinman, H. K., Martin, G. R. & Lawley, T. J. (1988) *J. Cell Biol.* **107,** 1589–1598.
35. Grant, D. S., Tashiro, K., Segui-Real, B., Yamada, Y., Martin, G. R. & Kleinman, H. K. (1989) *Cell* **58,** 933–943.
36. Grant, D. S., Lelkes, P. I., Fukuda, K. & Kleinman, H. K. (1991) *In Vitro Cell Dev. Biol.* **27A,** 327–336.
37. Schnaper, H. W., Grant, D. S., Stetler-Stevenson, W. G., Fridman, R., D'Orazi, G., Murphy, A. N., Bird, R. E., Hoythya, M., Fuerst, T. R., French, D. L., *et al.* (1993) *J. Cell Physiol.* **156,** 235–246.
38. Ausprunk, D. H., Knighton, D. R. & Folkman, J. (1974) *Dev. Biol.* **38,** 237–248.
39. Brooks, P. C., Montgomery, A. M. & Cheresh, D. A. (1999) *Methods Mol. Biol.* **129,** 257–269.
40. Thomas, L., Chan, P. W., Chang, S. & Damsky, C. (1993) *J. Cell Sci.* **105,** 191–201.
41. Cheresh, D. A. & Spiro, R. C. (1987) *J. Biol. Chem.* **262,** 17703–17711.
42. Millauer, B., Shawver, L. K., Plate, K. H., Risau, W. & Ullrich, A. (1994) *Nature (London)* **367,** 576–579.
43. Hurford, R. K., Jr., Dranoff, G., Mulligan, R. C. & Tepper, R. I. (1995) *Nat. Genet.* **10,** 430–435.
44. Griscelli, F., Li, H., Bennaceur-Griscelli, A., Soria, J., Opolon, P., Soria, C., Perricaudet, M., Yeh, P. & Lu, H. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6367–6372.
45. Sauter, B. V., Martinet, O., Zhang, W. J., Mandeli, J. & Woo, S. L. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4802–4807.
46. Kafri, T., Morgan, D., Krahl, T., Sarvetnick, N., Sherman, L. & Verma, I. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 11377–11382.
47. Reiser, J., Harmison, G., Kluepfel-Stahl, S., Brady, R. O., Karlsson, S. & Schubert, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 15266–15271.
48. Poeschla, E., Corbeau, P. & Wong-Staal, F. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11395–11399.
49. Miyoshi, H., Takahashi, M., Gage, F. H. & Verma, I. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 10319–10323.
50. Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M. & Torbett, B. E. (1999) *Science* **283,** 682–686.
51. Kafri, T., von Praag, H., Gage, F. H. & Verma, I. M. (2000) *Mol. Therapies* **1,** 516–521.