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Bovine Microvascular Endothelial Cells Immortalized With Human Telomerase

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Abstract   Primary cultures of bovine microvascular endothelial cells (BME) isolated from the adrenal cortex, are commonly used to study vascular endothelium, but have a limited life span. To circumvent these limitations, we have immortalized BME cells with either simian virus 40 (SV40) or with a retrovirus containing the coding region of human telomerase reverse transcriptase (hTERT), and have investigated whether the clonal populations obtained, maintain differentiated properties characteristic of microvascular endothelium. Immortalized cells were characterized for maintenance of typical endothelial morphology, marker expression, and functional characteristics including uptake of Acetylated low-density lipoprotein (Ac-LDL), capillary-like tube formation in three-dimensional collagen gels, as well as metalloproteinase (MMP) and plasminogen activator (PA)-mediated extracellular proteolysis. Whilst immortalization of BME cells with SV40 was associated with loss of endothelial-specific properties, hTERT–BME exhibited an endothelial phenotype similar to that of wild-type endothelial cells. Specifically, they showed a typical cobblestone morphology, were contact-inhibited, expressed endothelial cell-specific markers (e.g., CD31, vWF) and both fibroblast growth factor receptor 1 (FGFR-1) and vascular endothelial growth factor receptor-2 (VEGFR-2). In addition, they expressed receptors for LDL. Importantly, when grown on collagen gels, hTERT–BME cells underwent MMP-dependent tube-like structure formation in response to VEGFR-2 activation. In a collagen gel sandwich assay, hTERT–BME formed tubular structures in the absence of exogenously added angiogenic cytokines. Sustained tube formation was induced by VEGF-A alone or in combination with FGF-2. From 17 sub-clones that displayed a non-transformed phenotype, a high proliferative capacity and tubulogenic properties in three-dimensional collagen gels, we isolated two distinct subpopulations that display a highly specific response to VEGF-A or to FGF-2. We have generated hTERT–BME cells that maintain endothelial-specific properties and function and have isolated clones that respond differentially to VEGF-A or FGF-2. These immortalized cell lines will facilitate the study of endothelial cell biology. J. Cell. Biochem. 98: 267–286, 2006.   © 2006 Wiley-Liss, Inc.

Key words: angiogenesis; endothelial cells with extended life span; human telomerase; VEGF-A; FGF-2

The vascular endothelium consists of highly-ordered monolayers of quiescent non-migrating cells, which can be induced to migrate and replicate in a number of physiological and pathological settings. This occurs for example during angiogenesis in which new capillary blood vessels are formed from pre-existing vessels in response to angiogenic stimuli. During this process, microvascular endothelial cells locally degrade their basement membrane, and subsequently invade the surrounding interstitial extracellular matrix (ECM) within which they form a capillary sprout. The sprout develops into a functional vessel after formation of a lumen [D'Amore and Thompson, 1987; Zetter, 1988; Carmeliet, 2000]. Central to these events is the endothelial cell's ability to modulate its interactions with the ECM. This is achieved by integral membrane proteins including integrins, which provide a link between the ECM and the cytoskeleton, as well as by extracellular

proteinases and their inhibitors, which mediate focal degradation of the ECM [Pepper, 2001a,b]. The most extensively studied extracellular proteolytic enzymes involved in angiogenesis are the plasminogen activators (PAs) and metalloproteinases (MMPs). Urokinase-type PA (uPA) and tissue-type PA (tPA) are serine-proteinases that convert the widely-distributed zymogen plasminogen into plasmin. Plasmin, in turn, is capable of hydrolyzing most ECM proteins, directly or through the activation of certain MMPs [Andreasen et al., 2000].

Angiogenesis is regulated by the net balance between molecules which have positive or negative regulatory activity on endothelial cells. Positive regulators include the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2) families of cytokines and their cognate tyrosine kinase receptors [Dvorak et al., 1999; Ferrara, 1999; Dow and deVere White, 2000]. Both families increase endothelial cell proliferation, migration, extracellular proteolytic activity, and invasion of three-dimensional matrices in which they form capillary-like tubular structures. Endogenous negative regulators of angiogenesis have also been described, and these include members of the thrombospondin (TSP) family [Pepper, 1997a; de Fraipont et al., 2001].

Much of our understanding of the role of ECs in these events has been made possible by the development of techniques for the isolation and culture of ECs from different types of vessels. However, primary EC cultures have a number of disadvantages including a relatively short life span, heterogeneity both within and between different isolates and the possibility of fibroblast and/or smooth muscle cell contamination, all of which result in batch-to-batch variability in functional assays. These limitations have prompted many laboratories to search for immortalization methods suitable for these cells.

Efforts to extend the life span of ECs have focused on ectopic expression of viral oncogenes [Dubois et al., 1991; Durieu-Trautmann et al., 1991; Schwartz et al., 1991; Ades et al., 1992; Fontijn et al., 1995; Candal et al., 1996; Cajero-Juarez et al., 2002] or spontaneous transformation [Cockerill et al., 1994], addition of exogenous growth factors, and provision of supportive matrix components [Bicknell and Harris, 1996]. Immortal EC generated by viral or spontaneous transformation, however, frequently lose endothelial-specific differentiation properties and thus differ considerably from their normal counterparts.

Another way to bypass senescence is the ectopic expression of the human telomerase reverse transcriptase (hTERT). The ends of vertebrate chromosomes contain large tracts of a repeated hexameric sequence, TTAGGG, known as telomeres. Their function is to protect chromosomes by preventing fusion, recombination, and degradation. Normal somatic cells progressively lose telomeric repeats with each successive cell division. Cancer and immortalized cells counter the telomeric loss by expressing an RNA-dependent DNA polymerase known as telomerase [Kim et al., 1994]. Most human non-mitotic somatic cells do not express TERT but do contain all other enzymatic components necessary for telomere extension. Introduction of TERT into these cells leads to reconstitution of telomerase activity. This approach has been successful for immortalizing fibroblasts, retinal pigment epithelial cells [Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999], human dermal microvascular cells (HDMEC) [Yang et al., 1999; Gagnon et al., 2002; Nisato et al., 2004], and also bovine capillary endothelial cells [Veitonmaki et al., 2003], and can be achieved without converting the cells to a transformed phenotype.

In the work presented herein, we describe the immortalization of BME cells either by injection of simian virus SV40 large antigen DNA, or by transduction with hTERT. Immortalized BME were characterized on the basis of endothelial-specific morphology, marker expression, and function including acetylated-LDL uptake, capillary-like tube formation in three dimensional collagen gels, and MMP and PA-mediated extracellular proteolysis. In addition, we report the isolation of clonal cell subpopulations that respond differentially to individual angiogenic cytokines (VEGF-A and FGF-2).

MATERIALS AND METHODS

Reagents

Recombinant FGF-2 was kindly provided by Dr P. Sarmientos (Farmitalia Carlo Erba, Milano, Italy). Recombinant human vascular endothelial growth factor (VEGF-A165) was purchased from Peprotech, Inc. (Rocky Hill, NJ). Recombinant human VEGF-C (ΔNΔC) was

provided by Dr. K. Alitalo (Biomedicum, Helsinki). Transforming growth factor-beta 1 (TGF-β1) purified from human platelets was obtained from R&D Systems, Inc. (Minneapolis). The synthetic MMP inhibitor BB94 and the related inactive isomer BB1268 were kindly provided by Dr. P. Brown (British Biotech Pharmaceuticals Ltd., Oxford, UK). Neutralizing mAbs against human or mouse VEGFR-2 (KDR/Flk-1) (clones p1C11 and DC101, respectively) were provided by ImClone Systems (New York). Type I collagen was extracted from rat tail tendons as described [Montesano and Orci, 1985]. Fluorescently-labeled Acetylated low-density lipoprotein (Ac-LDL) (Dil-Ac-LDL) was purchased from Paesel and Lorei, Frankfurt, Germany).

Cells

Clonally-derived bovine microvascular endothelial (BME) cells from the adrenal cortex [Furie et al., 1984], kindly provided by Dr. M.B. Furie and S.C. Silverstein (Columbia University, NY), were cultured in minimal essential medium alpha-modification (α-MEM; Gibco BRL, Life Technologies, Basel, Switzerland), 15% heat-inactivated donor calf serum (DCS; Gibco), penicillin (110 U/ml), and streptomycin (110 μg/ml).

SV40 Transformation

BME cells were immortalized by intranuclear injection of simian virus 40 (SV40) DNA as previously described [Garcia et al., 1986]. One out of three microinjected cultures could be propagated for several passages beyond the normal life span of BME cells, but subsequently entered a "crisis" period characteristic of most SV40-immortalized cells [Manfredi and Prives, 1994]. During this period, most of the cells died. A small percentage of cells, however, escaped crisis; they resumed vigorous proliferation and could be serially subcultivated without showing signs of senescence. Since post-crisis cells were morphologically heterogeneous, they were cloned by limiting dilution, yielding five homogeneous subpopulations. Integration of SV40 DNA into the genome of the immortalized clones was assessed as previously described [Garcia et al., 1991]. Expression of intranuclear large T antigen was evaluated by immunofluorescence using a mouse monoclonal antibody (Oncogene Science, Inc., Manhasset, NY). Production of von Willebrand factor was assessed by indirect immunofluorescence using mouse monoclonal antibodies to human factor VIII-related antigen (Dako Diagnostics AG, Zug, Switzerland).

Cloning of Human Telomerase and Transduction of BME With hTERT

A full length hTERT cDNA cloned from human immortalized lymphocytes was cloned into pBABE-Hygro expression vectors as described [Morgenstern and Land, 1990]. BME were infected with MMLV-virus pBabe-hTERT-Hygro and pools of cells were selected with hygromycin B. All viral stocks were prepared in the Laboratory of Dr. B. Thorens (Institute of Pharmacology and Toxicology, University of Lausanne). Infection of the primary cells with viral stock was performed under regular BSL-2 laboratory containment. A pool of cells infected with the pBabe-Hygro-hTERT#1009 virus was designated BME–hTERT cells and was characterized as being resistant to hygromycin. Clones were obtained by limiting dilution and were sub-cultured in gelatin-coated flasks as described [Pepper et al., 1992b].

RT-PCR for Telomerase Transcripts

Total cellular RNA was obtained using a standard protocol and RT-PCR was performed as described [Macfarlane and Dahle, 1993]. The forward and reverse primer sequences for telomerase, chosen in order not to react with endogenous telomerase and to be specific for MMLV hTERT, were as follow: forward, 5'-CTCTCCCCCTTGAACCTCCTCTTTC; reverse, 5'-AGGACACCTGGCGGAAGGAG with a 350-bp expected band. For glyceraldehyde-3-phosphate dehydrogenase, primers were chosen in a similar sequence between human and bovine GAPDH and were as follow: forward, 5'-GCCAT-CACTGCCACCCAGAAGAC-3'; reverse, 5'-GA-GCTTGACAAAGTGGTCGTTGAGG with a 368-bp expected band. cDNAs (20 ng) were amplified for 40 cycles using 53°C for annealing, 2.5 Units/reaction hot start TaqDNA Polymerase (Invitrogen, Carlsbad, CA), 0.5 μM primers, 5% DMSO, 200 μM of each dNTP. PCR products were electrophoresed in a 2% agarose/ethidium bromide gel and photographed under UV light.

Telomeric Repeat Amplification Protocol (TRAP) Assay

Telomerase assays were performed using the TRAP-EZE telomerase detection kit (Intergen, Purchase, NY) on 10^6 cells. Each cell extract was

diluted 1/10 and 1/100 and was incubated with master mix for 30 min at 25°C, then for 5 min at 95°C. Annealing was performed at 50°C for 40 cycles. The TRAP reaction products were separated on a 10% non-denaturing polyacrylamide gel.

RNA Extraction, Northern Blot Analysis

Total cellular RNA was extracted from confluent BME, hTERT−BME, and SV40-BME cell monolayers using Trizol reagent (Life Technologies, Paisley, Scotland) according to the manufacturer's instructions. RNAs were denatured with glyoxal, electrophoresed in 1% agarose gels (5 μg RNA per lane), and transferred overnight onto nylon membranes (Hybond-N, Amersham International plc, Buckinghamshire, UK). Membranes were baked for 3 h at 80°C, and were stained with methylene blue to assess 18S and 28S ribosomal RNA integrity. Hybridization was performed with the following ^{32}P-labeled cRNA riboprobes: bovine uPA, uPAR, PAI-1 [Pepper and Montesano, 1990; Pepper et al., 1990, 1993a], VEGFR-2 [Mandriota et al., 1996], and CD31 or human tPA, vWF [Bonthron et al., 1986], or FGFR-1 [Mandriota et al., 1996]. As an internal control for determination of the amount of RNA loaded, the filters were hybridized simultaneously with a ^{32}P-labeled bovine P0 ribosomal protein cRNA probe [Pepper et al., 1998] or human β-actin as previously described [Tenan et al., 2000]. The intensity of the signals was quantitated with a densitometric scanning apparatus (Molecular Dynamics, CA) by using ImageQuant software, and normalized relative to the internal control.

Acetylated LDL

Uptake of Ac-LDL was evaluated by incubation of living cells in medium containing 10 μg/ml fluorescently-labeled Ac-LDL (Dil-Ac-LDL) for 4 h at 37°C [Voyta et al., 1984]. Coverslips were fixed in 3% formaldehyde, mounted in glycerol, and examined for fluorescence under an inverted Zeiss microscopy equipped with rhodamine filters.

In Vitro Angiogenesis Assay

To evaluate the ability of BME and hTERT−BME cells to form tubular structures in vitro, cells were grown in three-dimensional matrices under each of the following conditions: (1) sandwiched between two collagen layers [Montesano et al., 1983]; (2) grown to confluence on the surface of a collagen gel [Montesano et al., 1986]; or (3) suspended within a three-dimensional fibrin gel [Montesano et al., 1990]. SV40-immortalized BME were grown in three-dimensional fibrin gels in the presence of aprotinin (Trasylol, 200 KIU units/ml) as previously described [Montesano et al., 1990]. Cells were seeded onto collagen gels in 16-mm tissue culture wells (Nunc) at 2×10^5 cells/ml for the invasion assay, at 3×10^5 cells/ml in the suspension assay, and at a concentration of $0.5-1 \times 10^6$ cells/35-mm well in the sandwich assay in medium containing 5% DCS. For sandwich assays, cells were treated after collagen polymerization, while for cells seeded onto collagen gels, treatment began only after the cells had reached confluence (3–5 days). Antagonists were added to the cells 2 h before cytokine on the first day of treatment, and media, cytokines and antagonists were renewed after 2–3 days. In the cell suspension or sandwich assays, cells were cultured without treatment for 14 days. Alternatively, they were treated with either VEGF-A (30 ng/ml), VEGF-C (30 ng/ml), or FGF-2 (10 ng/ml) alone; or with FGF-2 in combination with VEGF-A or VEGF-C as previously described [Montesano et al., 1986; Pepper et al., 1992a]. In the collagen gel invasion assay, BME and BME−hTERT cells were treated with the neutralizing anti-VEGFR-2 antibody p1C11 (20 μg/ml) or the isotype-specific irrelevant antibody DC101 (20 μg/ml) in the presence or absence of VEGF (100 ng/ml), or with the synthetic MMP inhibitor BB94 (10 μM) or its related inactive isomer BB1268 in the presence or absence of FGF-2 (10 ng/ml). Seventeen clones derived from the parental hTERT−BME line were treated for 4 days with FGF-2 (10 ng/ml), 7 days with VEGF (30 ng/ml), or 2 days with both cytokines in combination. Cultures were photographed using a Nikon Diaphot TMD inverted photomicroscope (Nikon, Tokyo, Japan) at the times indicated. Quantitation was performed as described [Pepper et al., 1992a]. Results are shown as the mean additive sprout length $\pm$ SEM (in μm) for at least three experiments per condition. Mean values were compared using the Student's unpaired t-test, and a significant value was taken as $P < 0.05$.

Proliferation Assay

hTERT−BME-derived clones were seeded into gelatin-coated 12-well plates (Costar,

Cambridge, MA) at $3-10 \times 10^4$ cells per well in α-MEM with 5% DCS. Medium was renewed after 2 days. Cells were harvested after an additional day using trypsin and were counted for 1 min using a FACScan Analyser (Becton Dickinson) and CellQuest software. Results are shown as the mean ± SEM of duplicate wells from two independent experiments.

Zymography and Reverse Zymography

Confluent monolayers of BME and hTERT–BME cells in 35-mm tissue culture dishes were washed twice with serum-free α-MEM. Subsequently, 1.5 ml serum-free medium containing 200 Kallikrein Inhibitory Units/ml of Trasylol (Bayer AG, Zurich, Switzerland) was added. For analysis of PAs and PAI-1, BME cells were treated with increasing concentrations of VEGF-A (1–100 ng/ml) for 15 h. Cell extracts and culture supernatants were harvested, and analyzed by zymography and reverse zymography as previously described [Vassalli et al., 1984; Pepper and Montesano, 1990].

RESULTS

BME Cells Immortalized With SV40

Intranuclear injection of SV40 DNA into BME cells allowed the establishment of several clonal populations of immortalized cells. Maintenance of contact-mediated inhibition of cell proliferation and absence of foci or multiple cell layers in post-confluent cultures indicated that the cells were non-transformed. When suspended in three-dimensional collagen gels, SV40-immortalized BME cells formed thin cell cords apparently devoid of a central lumen (data

not shown). When suspended in fibrin gels, some clones of SV40-immortalized BME cells formed irregularly shaped colonies, while other clones formed branching cords, either spontaneously (e.g., clone E6C1B7, Fig. 1A), or in response to FGF-2 (e.g., clone E6I5C, Fig. 1B). Examination of semi-thin sections showed that the cords occasionally enclosed lumen-like spaces (data not shown). Collectively, these findings suggested that SV40-immortalized BME cells had only partially retained the ability to form tube-like structures in three-dimensional matrices. In addition, the lack of mRNA expression (Fig. 3F) and of immunostaining for von Willebrand factor (data not shown) and the inability to significantly take up Ac-LDL (Fig. 3C) indicated that cell immortalization was associated with loss of endothelial-specific properties.

hTERT Expression Extends the Life Span of BME Cells

In light of the previous finding that ectopic expression of the *hTERT* gene can efficiently reconstitute telomerase activity [Yang et al., 1999], extend the life-span, and maintain the morphological and functional properties of several cell types, we decided to use this approach to immortalize BME cells. BME cells transduced with telomerase maintained a steady proliferation rate for 40–45 passages. Whereas non-transduced BME cells became senescent after 20–22 passages and exhibited a highly spread morphology with the occurrence of giant cells (Fig. 2A left), all hTERT–BME resembled young primary ECs in that they exhibited a cobblestone morphology and high
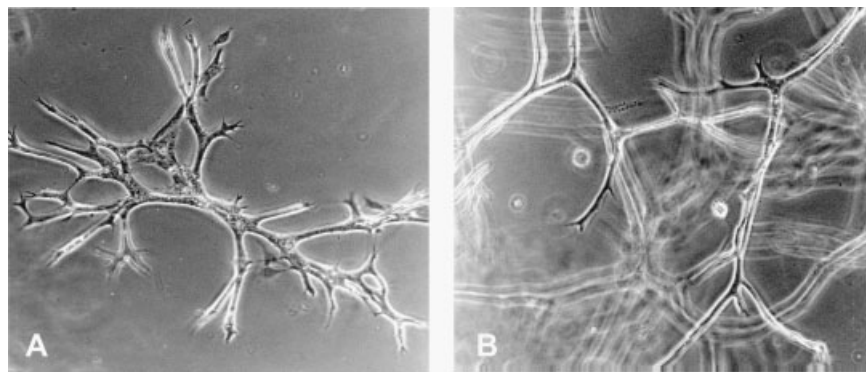

Fig. 1. Morphogenetic properties of SV40-immortalized BME cells. Cells from clone E6C1B7 grown in a fibrin gel for 9 days (**A**) and cells from clone E6I5C grown in a fibrin gel in the presence of 10 ng/ml FGF-2 for 12 days (**B**) have formed thin branching cords in which lumina are not detectable by phase contrast microscopy. Magnification, 125×.
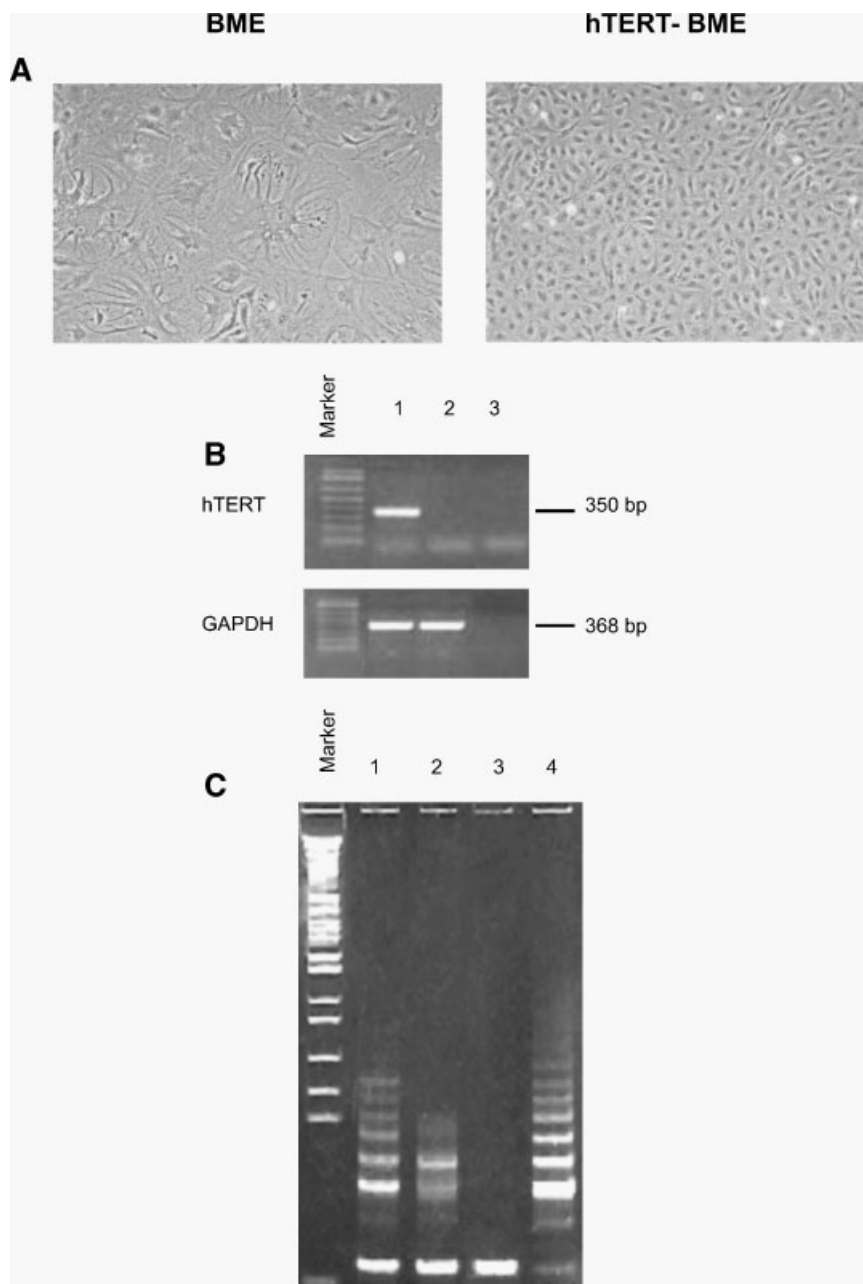
Fig. 2. Endothelial life span is increased by hTERT. **A:** Phase contrast photomicrographs of senescent parental BME cells at late passage (**left**) and hTERT-immortalized BME cells (**right**) which display the typical cobblestone morphology. Magnification, 40×. **B:** RNA extracted from cells was analyzed by RT-PCR and samples were run on a 1.5% agarose gel. The expected 350-bp band for hTERT or 368 bp for GAPDH are indicated. Marker (1-kb ladder, Invitrogen), hTERT–BME cells, passage 9 (**lane 1**), wild-type BME cells (negative control, **lane 2**), H_2O control (**lane 3**). **C:** Cell extracts were prepared and a TRAP assay was performed using the Intergen TRAPeze kit. Samples were run on an 8% acrylamide gel. Marker kit control, hTERT–BME cells (**lane 1**), wild-type BME cells (**lane 2**), H_2O control (**lane 3**), mdx 12 hTERT fibroblasts (positive control, **lane 4**).

saturation densities (Fig. 2A right). In complete medium, the hTERT-transduced cells grew to approximately the same densities as the young primary ECs and remained contact-inhibited. hTERT–BME cells were analyzed for expression of the transduced *hTERT* gene by RT-PCR. Infected cells were found to express the *hTERT* cDNA (Fig. 2B). To test whether the telomerase gene transferred into the cells was active, we performed a TRAP assay on the BME or hTERT–BME cells (Fig. 2C). We could clearly detect TRAP activity in hTERT–BME cells after cultivation for several passages. BME cells had no detectable TRAP activity.
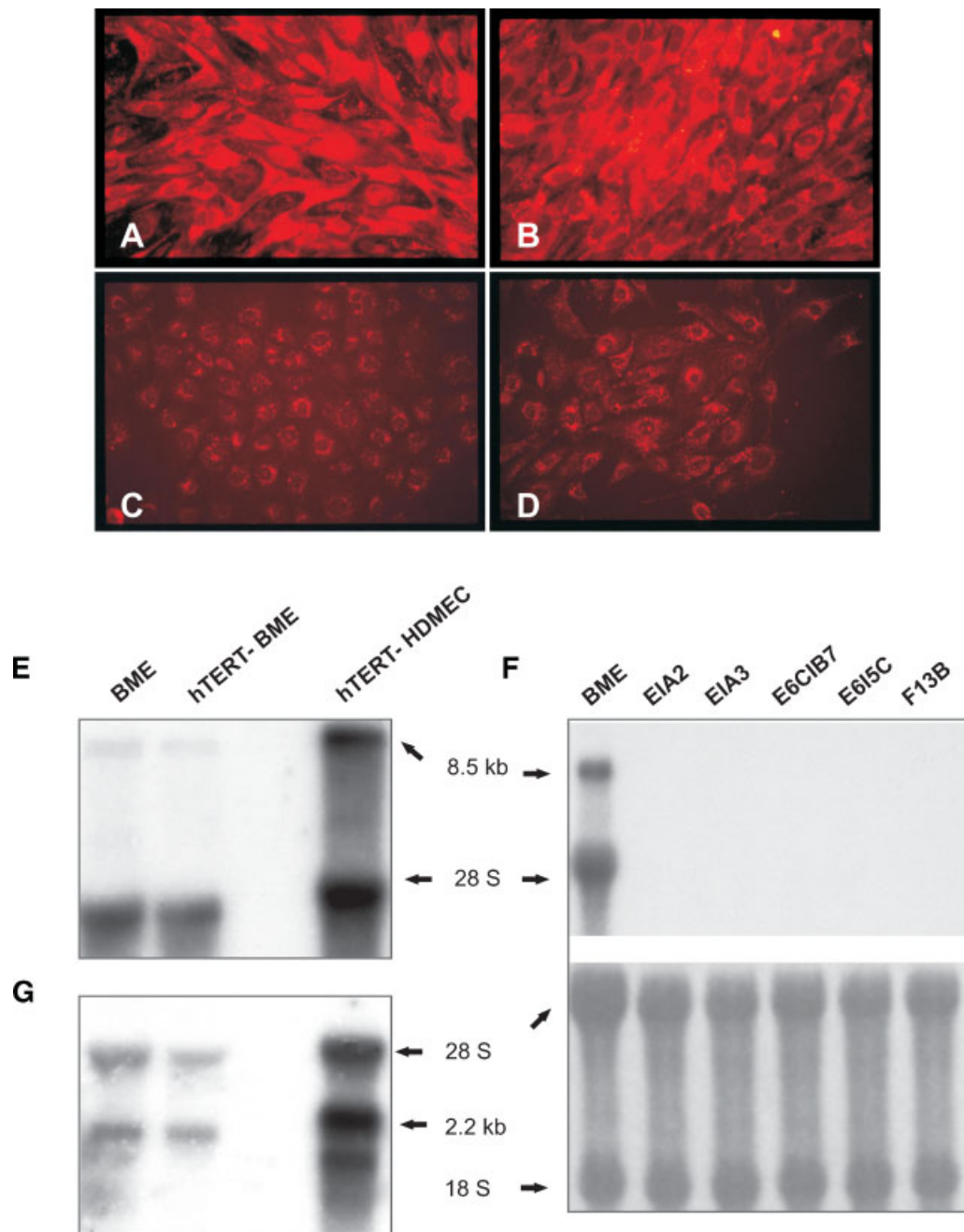
Fig. 3. hTERT- but not SV40-immortalized BME cells retain endothelial characteristics in vitro. Uptake of fluorescent DiI-acetylated LDL by (**A**) parental BME; (**B**) hTERT–BME; (**C**) SV40 immortalized cells (clone F13B8); and (**D**) SV40 immortalized cells (clone EIA2). (Magnification 40×). Cells were photographed using an inverted Zeiss fluorescence photomicroscope equipped with rhodamine filters. Similar results were obtained in three separate experiments. **E–G**: Northern blot analysis of total cellular RNA from BME, hTERT–BME, and five sub-clones of SV40 immortalized cells (10 μg/lane). hTERT–HDMEC were used as a control. Replicate filters were probed with human vWF (E–F), or bovine CD31 [^{32}P]-labeled cRNA probes (G). Filters were stained with methylene blue to reveal 28S and 18S ribosomal RNAs.

hTERT–BME Cells Retain Endothelial Characteristics

Constitutive expression of von Willebrandt factor (vWF) and CD31 (PECAM-1) are re-garded as key markers that distinguish ECs from other cell types both in vitro and in vivo [Albelda et al., 1990; Cines et al., 1998]. Similarly, binding and uptake of Ac-LDL and the formation of tube-like structures in

response to cytokines and matricellular signals also define important EC functions maintained in vitro by primary ECs that have not lost their differentiated phenotype [Bicknell and Harris, 1996]. mRNA was harvested from confluent monolayers of BME and hTERT−BME cells and Northern blot hybridization was performed with ^{32}P-labeled human vWF and bovine CD31 riboprobes. Replicate filters were stained with methylene blue to assess for RNA integrity and uniformity of loading. Total RNA from hTERT−BME showed expression of vWF (Fig. 3E) and CD31 (Fig. 3G) at similar levels to those seen in parental cells. In contrast, none of the five SV40-transfected clones expressed mRNA for vWF. Furthermore, there was no difference in Ac-LDL uptake between parental (Fig. 3A) and hTERT− (Fig. 3B) BME cell lines, demonstrating that hTERT-transduced cells retained these same endothelial features as parental cells.

Angiogenic Properties of hTERT−BME and BME Cells

Previous reports have shown that when BME cells are grown between two layers of collagen, they re-organize rapidly to form capillary-like tubular structures [Montesano et al., 1983]. The collagen sandwich procedure, which recapitulates the resolution phase of angiogenesis, has three effects on wild-type endothelial cells: inhibition of proliferation, induction of capillary-like tube formation (Fig. 4A), and induction of endothelial cell apoptosis [Satake et al., 1998]. In the present study, we examined whether hTERT−BME exhibit the same properties in this assay. When sandwiched between two layers of collagen, refringent lumina were visible in BME cells after 2 days by phase contrast microscopy (Fig. 4A) as previously shown [Cavallaro et al., 2000]. However, in the absence of angiogenic factors, cell survival and formation of tube-like structures was dramatically impaired starting at day 4 (Fig. 4A, left). In contrast, hTERT−BME cell survival and tube formation were maintained for at least 14 days (Fig. 4A, right). When suspended for 7 days in three-dimensional collagen gels, BME cells formed rare and short tube-like structures (Fig. 4B, left), while hTERT promoted cell survival and formed a more complex network of tube-like structure enclosing a patent lumen (Fig. 4B, right).

Effect of Angiogenic Factors on BME and hTERT−BME Tube-Like Capillary Morphogenesis and Survival In vitro

Previous reports have shown that exogenous VEGF-A increases tube formation and survival of BME cells in contrast to FGF-2 that has no significant effect on BME cell reorganization or survival in a sandwich assay [Tille and Pepper, 2002]. We comparatively assessed the effects of individual or combined cytokines [Montesano et al., 1986; Pepper et al., 1992a] on BME and hTERT−BME cell survival and tube formation in a collagen sandwich assay. To this end, cells were treated with VEGF-A (30 ng/ml), VEGF-C (30 ng/ml), or FGF-2 (10 ng/ml) alone; or with FGF-2 in combination with VEGF-A or VEGF-C for 14 days as previously described [Montesano et al., 1986; Pepper et al., 1992a]. As shown in Figure 5A,B, VEGF-C and FGF-2 alone had very little effect on BME cell survival over the 14-day assay period, whereas VEGF-A increased cell network formation and survival. When VEGF-A or VEGF-C were co-added with FGF-2, this markedly accelerated tube formation compared with VEGF-C or VEGF-A alone. The effect elicited by co-addition of FGF-2 and VEGF-A was greater than additive, indicating a synergistic activity of the two cytokines. Kinetic analysis in hTERT−BME cells revealed that all angiogenic factors alone or in combination induced a comparable increase in tube-like structures over the 7-day assay period. Involution occurred at day 10−14 with FGF-2 and VEGF-C alone, whereas VEGF-A induced a sustained invasive response. Co-addition of either VEGF-A or VEGF-C and FGF-2 markedly accelerated tube formation. The synergism induced by co-addition of FGF-2 and VEGF-A was greater than that induced by FGF-2 and VEGF-C. These results demonstrate that in contrast to BME cells, hTERT−BME cells survived over a 14-day period in the absence of exogenously added angiogenic cytokines, but that addition of angiogenic cytokines increased formation of anastomosing tubes during the same period.

In Vitro Angiogenesis Is Mediated Via VEGFR-2 in hTERT−BME

We have previously shown that BME cells express VEGFR-1, -2, and FGFR-1 [Pepper et al., 1992a, 1993b, 1998; Mandriota et al., 1996] and that the in vitro angiogenic activities of VEGF-C
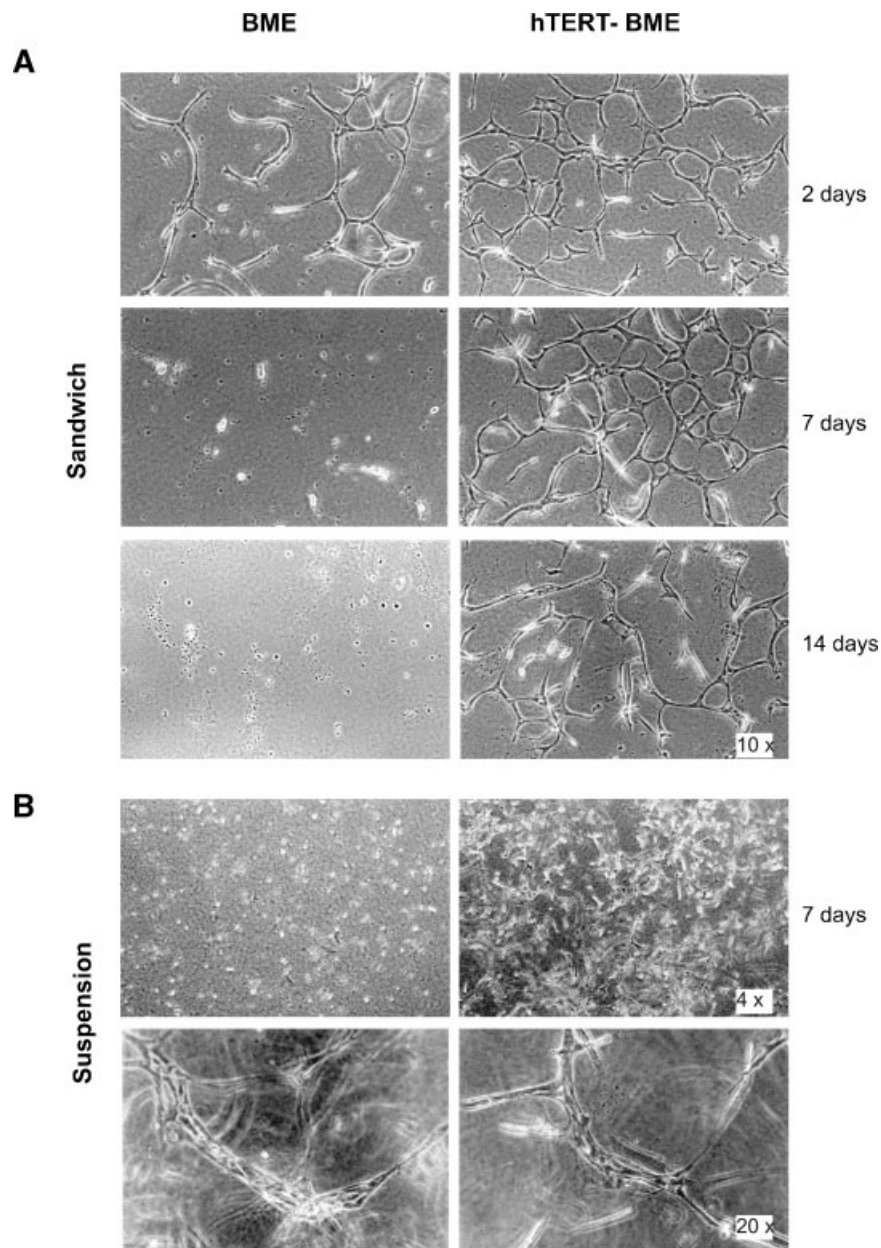
Fig. 4. Kinetics of spontaneous tube formation by hTERT–BME and BME cells in collagen gels. Cells were either sandwiched between two collagen gels (**A**) or seeded in suspension as isolated single cells in a collagen gel (**B**). Cells were left untreated for 14 days and photographed at the indicated time points using a Nikon Diaphot TMD inverted photomicroscope.

and VEGF-A are mediated by VEGFR-2 [Tille et al., 2003]. In order to determine whether immortalized BME cells conserved cell surface expression of VEGFR-2, we cultured hTERT–BME on the surface of a three-dimensional collagen gel and stimulated them with VEGF-A alone (Fig. 6B) or in combination with neutralizing antibodies to human VEGFR-2 (p1C11) (Fig. 6D). An antibody to mouse VEGFR-2 (clone DC101) that we have pre-

viously shown does not prevent bovine VEGFR-2-mediated tube formation [Tille et al., 2003] was used as negative control (Fig. 6C). The resulting capillary-like tubular structures were viewed by phase-contrast microscopy and quantitated. VEGF-A-induced hTERT–BME cell invasion was inhibited by p1C11 whereas DC101 had no effect. The inhibition was dose-dependent with a maximal 96% inhibition at 20 μg/ml (Fig. 6E) demonstrating that hTERT–BME
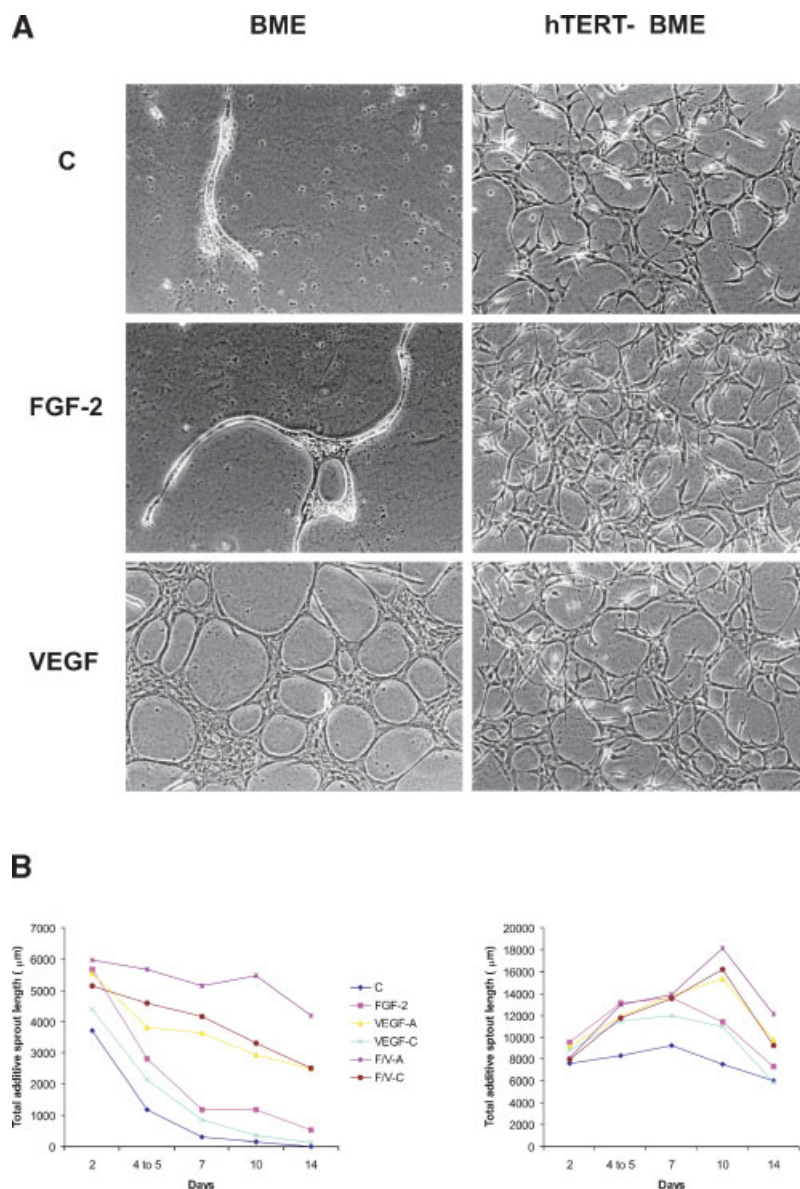
Fig. 5. Kinetics of cytokine-induced tube formation by hTERT–BME and BME cells in collagen gels. **A**: BME and hTERT–BME cells sandwiched between two layers of collagen were treated with FGF-2 (10 ng/ml) or VEGF (30 ng/ml) for 14 days. **B**: Cytokines were added to BME or hTERT–BME cells sandwiched between two layers of collagen, either alone or in combination as indicated: 10 ng/ml FGF-2 (F), 30 ng/ml VEGF-A (V), 30 ng/ml VEGF-C (V–C). Medium was renewed every 2–3 days and cultures were photographed using a Nikon Diaphot TMD inverted photomicroscope. The total additive length of the resulting capillary-like structure (in µm ± SEM) was measured at the indicated time points. Results are from at least three separate experiments per condition.

retained VEGFR-2 surface expression and signaling properties.

Effect of hTERT on MMP Activity

During invasion and tube formation, endothelial cells degrade their basement membrane, proliferate, and migrate into the surrounding collagen-rich matrix. These processes require the co-ordinated activities of many different molecules, including MMPs. MMPs are a family of zinc-containing endopeptidases that degrade various components of the ECM [Overall and Lopez-Otin, 2002]. To determine whether the MMP system might also be necessary for the formation of capillary-like tubular structures by hTERT–BME cells, the cells were cultured on the surface of a three-dimensional collagen gel and stimulated with FGF-2 (10 ng/ml) alone (Fig. 7B) or in combination with BB94 (Fig. 7D) or its inactive isomer BB1268 (10 µM) (Fig. 7C).
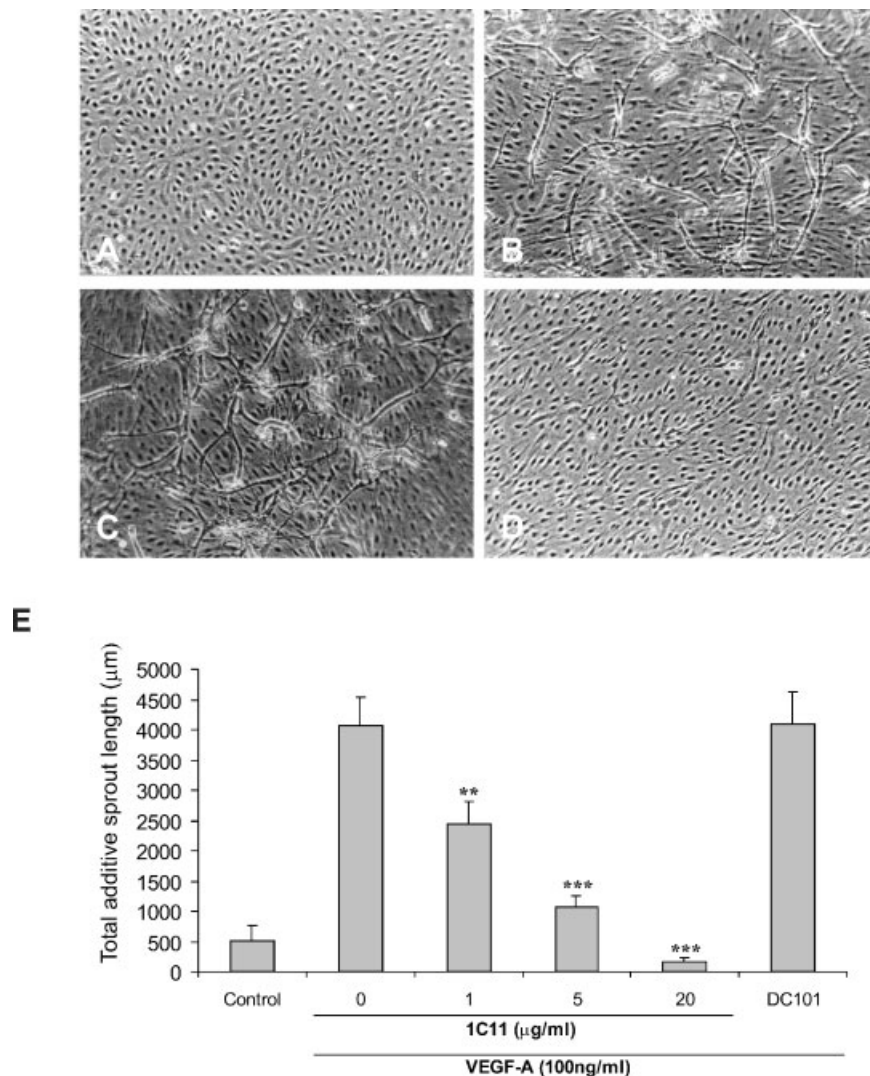
Fig. 6. Involvement of VEGFR-2 in VEGF-A-induced in vitro angiogenesis of hTERT–BME cells. Confluent monolayers of hTERT–BME cells on three-dimensional collagen gels were treated for 4 days with VEGF-A (100 ng/ml) alone or in combination with either a neutralizing (p1C11) anti-VEGFR-2 or an irrelevant (DC101) antibody at the indicated concentrations. Phase contrast view of (**A**) an untreated monolayer; (**B**) cells treated with VEGF-A alone; (**C**) cells treated with VEGF-A in combination with DC101 (20 µg/ml); (**D**) cells treated with VEGF-A in combination with p1C11 (20 µg ml). Representative fields from one of at least three experiments per condition are shown. Quantitative assessment of the effect of 1C11 used at the indicated concentrations on VEGF-A (100 ng/ml)-induced in vitro angiogenesis (**E**). Results are shown as the mean total additive sprout length ± SEM (in µm) from three randomly selected photographic fields per experiment; at least three experiments were performed per condition. *$P < 0.005$, **$P < 0.001$, ***$P < 0.0001$ when compared with values in the absence of the antibody (Student's unpaired t test).

FGF-2-induced hTERT–BME cell invasion was inhibited by BB94 whereas BB1268 had no effect. The inhibition was dose-dependent with a maximal 85% inhibition at 1 µM (Fig. 7E).

Effect of hTERT on the Plasminogen Activator System

Angiogenesis is dependent on finely-regulated extracellular proteolytic activity [Pepper et al., 1996] and the PA/plasmin system has been shown to play an essential role in endothelial cell migration, invasion, and morphogenesis. Angiogenic growth factors have been shown to affect both the expression and the activity of most components of this system, for example, FGF-2 induces uPA while VEGF-A induces uPA and tPA in BME cells [Cavallaro et al., 2001]. We, therefore, comparatively assessed the effect of FGF-2 on mRNA expression, and VEGF-A on the activity of components
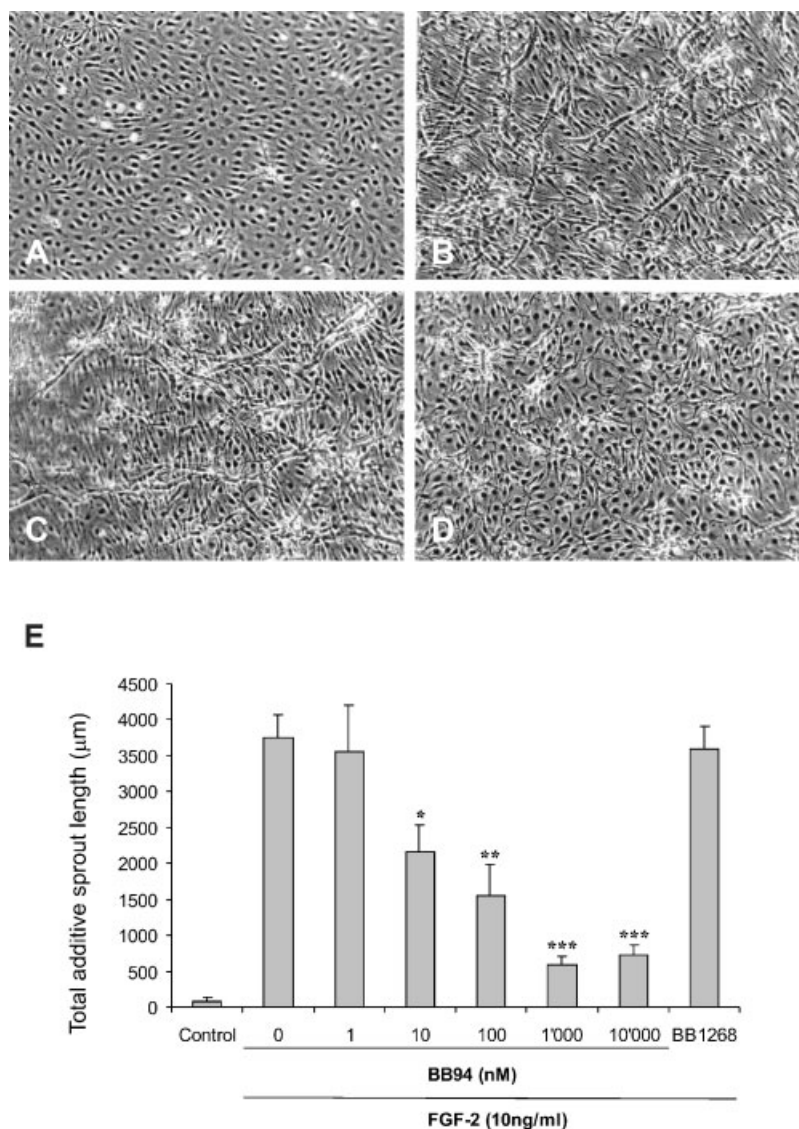
Fig. 7. Formation of capillary-like tubular structures in a collagen matrix depends on MMP activity. hTERT–BME cultured on the surface of three-dimensional collagen gels were treated 4 days with FGF-2 (10 ng/ml) in the presence or absence of BB94 at the indicated concentrations, or its inactive isomer BB1268 (10 μM). **A**: Untreated monolayers; (**B**) cells treated with FGF-2 alone; (**C**) cells treated with FGF-2 in combination with BB1268; (**D**) cells treated with FGF-2 in combination with BB94. Quantitation of BB94 inhibition of FGF-2 induced in vitro angiogenesis (**E**). Results are shown as the mean total additive sprout length $\pm$ SEM (in μm) from three randomly selected photographic fields per experiment. Results are from at least three separate experiments per condition. Mean values were compared using Student's unpaired t-test as described in Figure 6.

of the PA/plasmin system in BME and hTERT–BME cells. Confluent monolayers of BME and hTERT–BME cells were exposed to FGF-2 at increasing concentrations for 15 h, and the effect on uPA, uPAR, tPA, and PAI-1 mRNA expression was assessed by Northern blot hybridization (Fig. 8A). In agreement with previous studies [Cavallaro et al., 2001], FGF-2 increased uPA, uPAR, and PAI-1 expression in BME cells in a dose-dependant manner, while a

minimal effect was observed on tPA expression (Fig. 8A, left). In hTERT–BME cells, FGF-2 stimulated uPA and uPAR expression at lower doses than in BME cells, and tPA was significantly increased. However, the effect of FGF-2 on PAI-1 expression was no longer observed (Fig. 8A, right).

PA activity in response to VEGF-A was assessed in cell extracts (Fig. 8B, upper panel) and culture supernatants (Fig. 8B, lower panel)
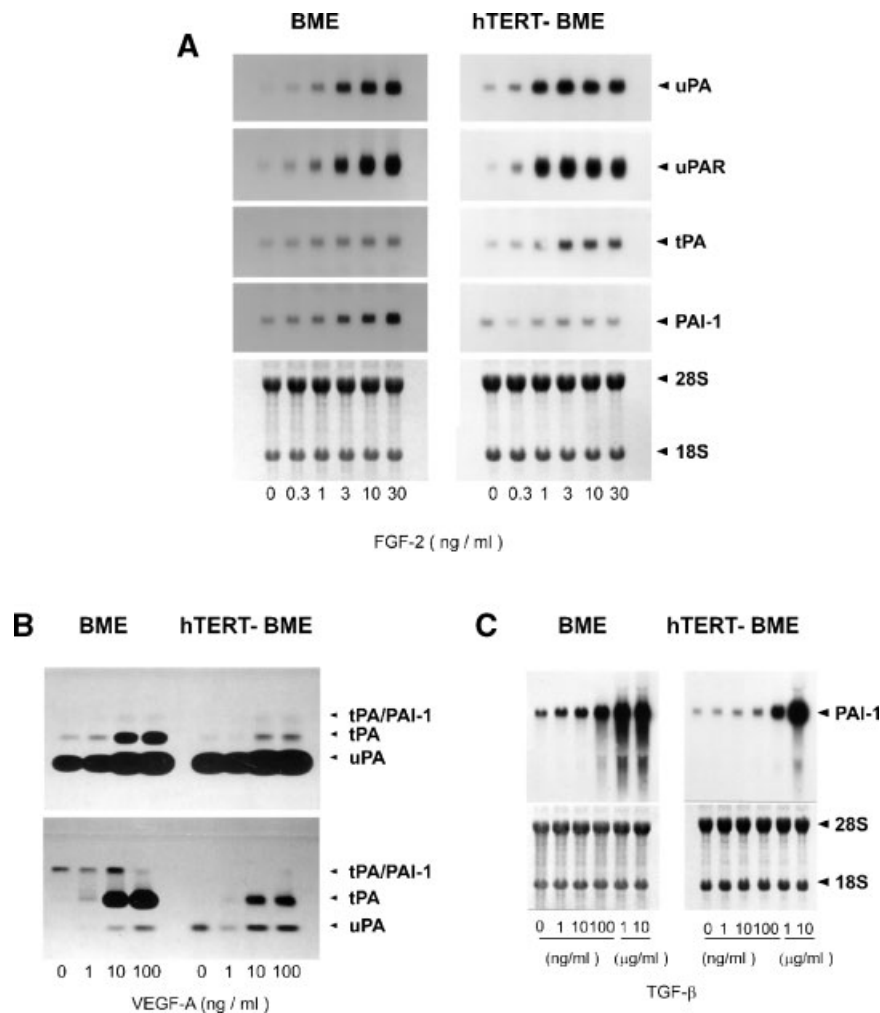
Fig. 8. Effect of FGF-2, VEGF-A, and TGF-β1 on plasminogen activator (PA) and PA inhibitor-1 (PAI-1) expression and activity in BME and hTERT–BME. Confluent monolayers of BME or hTERT–BME cells were treated with the indicated concentrations of FGF-2, VEGF-A, or TGF-β1 (**A–C**). Total cellular RNA was extracted and assessed by Northern blot. Cell extracts and conditioned media were analyzed by zymography. A: Northern blot filters containing 5 µg/lane of total RNA from BME or hTERT–BME cells treated for 15 h with the indicated concentrations of FGF-2 were hybridized with [^{32}P]-labeled bovine uPA, bovine uPAR, human tPA, and bovine PAI-1 cRNA probes. Replicate filters were stained with methylene blue to assess RNA integrity and uniformity of loading. B: Cell extract (**upper panel**) and culture supernatant (**lower panel**) from BME and hTERT–BME cells treated for 15 h with the indicated concentrations of VEGF-A were analyzed by zymography. C: Northern blot analysis (5 µg total RNA per lane) of PAI-1 mRNA expression in BME cells treated for 15 h with the indicated concentrations of TGF-β1.

by zymography. In accordance with previous reports [Cavallaro et al., 2001], VEGF-A increased uPA activity in cell extracts and to a lesser extent in culture supernatants (Fig. 8B, left) of BME cells. More strikingly, VEGF increased tPA activity in cell extracts, and also in supernatants in which tPA was detected in both free and complexed form (Fig. 8B, left). In hTERT–BME cells, high uPA activity in cellular extracts and basal activity in the supernatant were observed with minimal dose-dependent variations (Fig. 8B, right). In contrast, the response of hTERT–BME cells to VEGF-A in terms of tPA production (only detected in its free form in both cell extract and culture supernatant) was markedly less than in BME cells.

TGF-β1 is an anti-proliferative agent. It has previously been reported to have a biphasic effect on endothelial cell invasion of collagen gels [Pepper et al., 1993b; Pepper, 1997b] and to increase both u-PA and PAI-1 mRNA expression in BME cells [Pepper et al., 1990]. We wished to determine whether hTERT might

alter the response of BME cells to TGF-β1. When added to confluent BME cell monolayers, TGF-β1 induced a dose-dependent increase in PAI-1 mRNA, with 100 pg/ml, 1 ng/ml, and 10 ng/ml producing the most intense signal (Fig. 8C, left). In hTERT–BME cells, PAI-1 mRNA levels were increased to a lesser extent (Fig. 8C, right), suggesting that the response to TGF-β1 is reduced in these cells.

Cloning of hTERT–BME Cells

When seeded at low density, parental hTERT–BME cells appeared to be morphologically homogeneous. To assess the possible occurrence of functionally different cell subpopulations, we set out to clone parental hTERT–BME. Out of 20 clones obtained by limiting dilution, 17 formed a regular, contact inhibited monolayer and grew to approximately the same density as young BMEs, suggesting that all these clones had a non-transformed phenotype (data not shown). All 17 hTERT–BME clones were analyzed for the relative expression of the transduced *hTERT* gene by RT-PCR (Fig. 9A) and the signal was normalized relative to 18S RNA. Transduced cells were found to express variable levels of hTERT with higher levels in clones 1–6. We further assessed whether the level of hTERT expression correlated with the proliferation rate in untreated hTERT–BME clones. The proliferation rate was variable but was independent of the level of hTERT expression (Fig. 9B). To evaluate the ability of hTERT–BME clones to generate three-dimensional tube-like structures, we used a collagen gel invasion assay, in which parental hTERT–BME have been shown to penetrate the underlying matrix and to form tubular structures in response to angiogenic cytokines (Fig. 9C). Out of the 17 clones tested, 14 spontaneously invaded the collagen gel to a variable extent independently of the level of hTERT expression. To assess whether hTERT–BME clones had retained their morphogenetic response to angiogenic factors, we assessed the effects of individual or combined cytokines on cell survival and tube-like formation in a collagen gel invasion assay. hTERT–BME clones were treated with FGF-2 in combination with VEGF-A for 2 days (Fig. 10A), FGF-2 (10 ng/ml) for 4 days (Fig. 10B), or VEGF-A (30 ng/ml) for 7 days (Fig. 10C). Total additive length of the resulting capillary-like structures was measured. Of the 17 clones tested, all clones demonstrated a strong response to both cytokines combined (Fig. 10A), 5 clones demonstrated a

strong response to FGF-2 (4, 6, 15,16, and 17) (Fig. 10B), and 4 to VEGF-A (6, 7, 11, and 16) (Fig. 10C). To evaluate whether the intensity of the response induced by the individual cytokine might correlate with the level of expression of VEGFR-2 and FGFR-1, tyrosine kinase receptors for the cytokines tested, we assessed levels of receptor mRNA expression by northern blot (Fig. 10D). All clones expressed both VEGFR-2 and FGF-2, albeit at variable levels. There was no correlation between the intensity of the response induced by the individual cytokines, the level of tyrosine kinase receptor expression, or the level of hTERT expression.

DISCUSSION

The main objectives of this study were to (a) generate bovine endothelial cell lines with an extended lifespan, which maintain their morphogenetic properties and (b) to obtain independent subpopulations that respond differentially to individual angiogenic cytokines. In the work presented herein, we have immortalized BME cells either by nuclear injection of SV40 large T antigen DNA, or by transduction with hTERT.

The present investigations and several previous studies have demonstrated that SV40-transfected ECs undergo a growth crisis after an extended period of proliferation. This is characteristic of most SV40-immortalized cells [Manfredi and Prives, 1994]. In our studies, most of the BME cells died during this period. A small percentage of cells escaped crisis, resumed vigorous proliferation and could be serially subcultivated without showing signs of senescence. Gimbrone et al., have similarly reported outgrowth of a presumably immortal clone from one out of six transformed EC cultures that went into crisis [Gimbrone and Fareed, 1976]. Retroviral infection of parental BME cells with hTERT enabled these cells to escape crisis without any proliferative lag or reduction in growth rate. hTERT-transduced BME cells have been grown to 45 passages, have shown no signs of growth arrest or cell death, and have maintained a phenotype similar to the wild type cells from which they were derived.

Other than effects on replicative lifespan, constitutive expression of hTERT alone did not confer malignant changes. hTERT-transfected BME cells displayed a cobblestone morphology typical of EC, maintenance of contact-mediated
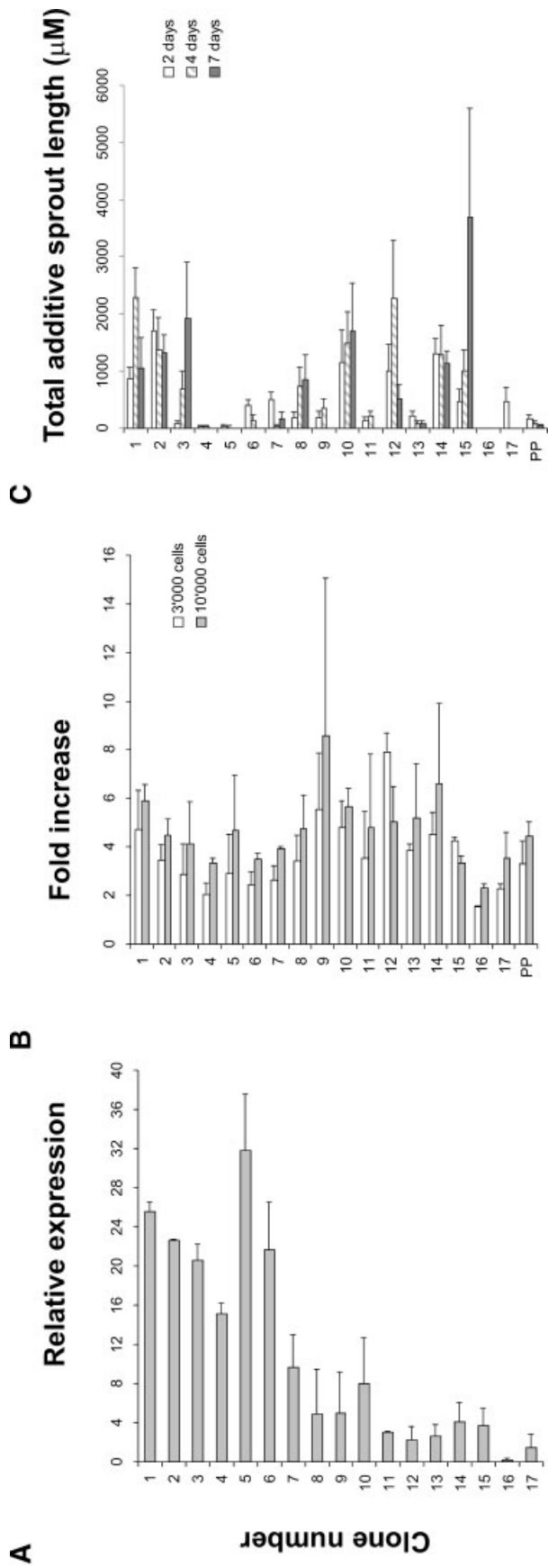
Fig. 9. Relative expression of telomerase, cell proliferation, and spontaneous tube formation in clones isolated from parental hTERT–BME. **A**: hTERT expression. RNA extracted from individual clones was analyzed by RT-PCR and samples were run on a 1.5% agarose gel. The signal was normalized relative to 18S RNA. Results represent the mean from two separate experiments. **B**: Proliferation assay. Clones were seeded at 3 or 10×10^4 cells/well in 12-well plates and counted after 4 days using a FACScan Analyser (Becton Dickinson) and CellQuest software. Results are shown as the mean ± SEM of duplicate wells from two independent experiments. **C**: Clones were cultured on the top of three-dimensional collagen gels in the absence of angiogenic cytokine. Cultures were photographed using a Nikon Diaphot TMD inverted photomicroscope (Nikon, Tokyo, Japan) at the times indicated. Results are shown as the mean additive sprout length ± SEM (in micrometer) for at least three experiments per condition.
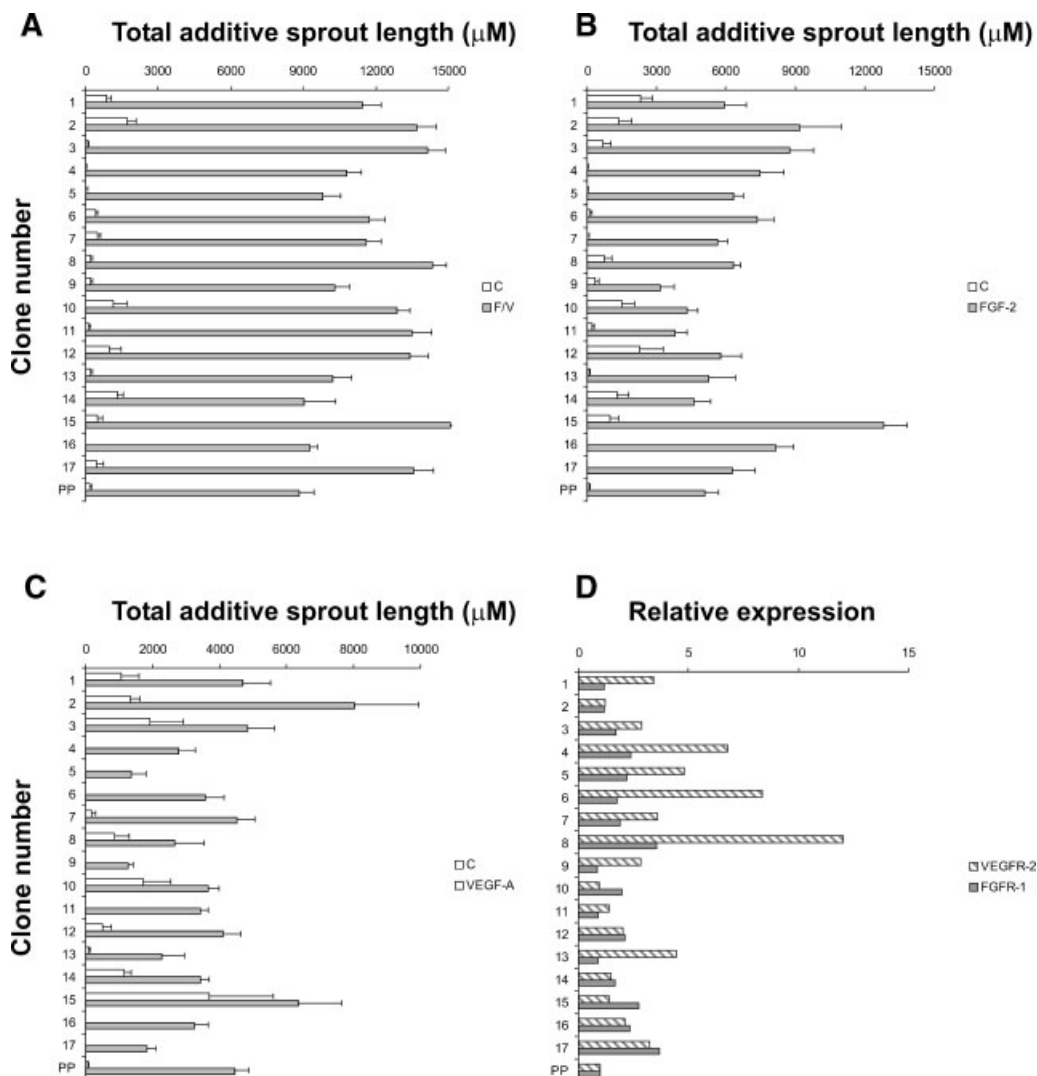
Fig. 10. FGF-2 or VEGF-A-induced tubulogenesis and relative levels of VEGFR-2 and FGFR-1 expression in hTERT−BME clones. hTERT−BME cells were seeded on the surface of collagen gels and were left untreated or treated (**A**) for 2 days with FGF-2 and VEGF-A in combination; (**B**) for 4 days with FGF-2; (**C**) for 7 days with VEGF-A. Results are shown as the mean additive sprout length ± SEM (in μm) for at least three experiments per condition.

D: Expression of FGFR-1 or VEGFR-2 mRNA in hTERT−BME clones. Purified ^{32}P-labeled human FGFR-1 and bovine VEGFR-2 cRNA probes were used for Northern blot analysis. P0 ribosomal phosphoprotein was used as an internal control. The intensity of the signals was quantitated with a densitometric scanning apparatus (Molecular Dynamics, CA) by using ImageQuant software, and normalized relative to P0.

inhibition of cell proliferation, and absence of foci or multiple cell layers in post-confluent cultures, indicating that the cells are not transformed. In addition, transfected cells stably express typical endothelial cell markers including PECAM-1 (CD31) and von Willebrand factor. Similarly, there were no differences in Ac-LDL uptake between parental and hTERT−BME cell lines. Although SV40-BME cells retained normal EC morphology and grew at a normal rate, these cells exhibited an extended lifespan but lost expression of endothelial cell markers such as von Willebrand

factor. In addition, Ac-LDL uptake was reduced in SV40-transformed BME cells.

When grown in three-dimensional matrices that promote the formation of capillary-like tubes by human umbilical vein endothelial cells, bovine micro- and macrovascular cells, and transformed murine endothelial cells [Montesano et al., 1983, 1986, 1990], SV40 BME cells only partially retained the ability to form tube-like structures. Some cells formed thin cell cords apparently devoid of a central lumen. Some clones of SV40-immortalized BME cells formed irregularly shaped colonies when suspended in

fibrin gels, while other clones formed branching cords, either spontaneously or in response to FGF-2 (Fig. 1). In contrast to SV40-BME, hTERT−BME cells maintained the capacity to organize into tube-like structures resembling capillary blood vessels, as they were able to form extensively branched cords when cultured between two layers of collagen (Fig. 4A) or when suspended in three-dimensional collagen gels (Fig. 4B). Moreover, expression of hTERT promoted cell survival, and slightly increased formation of tube-like structures enclosing patent lumina in the absence of angiogenic factors. The robust induction of hTERT−BME tubulogenesis by VEGF-A and the synergistic effect of FGF-2 with VEGF-A or VEGF-C are in accordance with other studies performed on primary bovine ECs [Pepper et al., 1992a, 1998; Cavallaro et al., 2001].

BME cells express many molecules that have been shown to mediate angiogenesis such as VEGFR-1, VEGFR-2, FGFR-1 [Pepper et al., 1995; Pepper and Mandriota, 1998], MMPs, and members of the PA family of proteases [Montesano and Orci, 1985; Pepper et al., 1990, 1998; Mandriota and Pepper, 1997; Cavallaro et al., 2001]. We demonstrate that hTERT−BME express both VEGFR-2 and FGFR-1. To assess the functional activity of VEGFR-2, we used a well-characterized model of in vitro angiogenesis, in which quiescent endothelial cells can be stimulated to invade a three-dimensional collagen gel within which they form capillary-like tubes [Montesano and Orci, 1985]. Our data with VEGFR-2 neutralizing antibodies (P1C11) demonstrate that the effect of VEGF-A on invasion was mediated by VEGFR-2 (Fig. 6). hTERT−BME cells also retained their dependence on MMP activity for in vitro angiogenesis, since addition of BB94 inhibited FGF-2-induced invasion (Fig. 7).

Protease activity is necessary for cell migration and morphogenesis whereas protease inhibitors play an important role by protecting the ECM from inappropriate destruction [Pepper and Montesano, 1990]. With respect to the PA/plasmin system, when compared to parental BME cells, hTERT−BME cells expressed higher basal levels of uPA and uPAR, and lower levels of PAI-1. In agreement with previous data [Pepper et al., 1990; Cavallaro et al., 2001], FGF-2 induced expression of uPA and its receptor and concomitantly increased PAI-1 in BME cells (Fig. 8A). In hTERT−BME cells,

FGF-2 also increased uPA and uPAR (hTERT−BME appeared to be more sensitive to FGF-2 than BME cells), but failed to induce PAI-1. With regard to VEGF-A, which has previously been reported to increase uPA and tPA in BME cells [Cavallaro et al., 2001], hTERT reduced the sensitivity to this cytokine particularly with regard to induction of PAI-1. Finally, the response of BME cells to TGF-β1 in terms of PAI-1 expression appeared to have been blunted by hTERT. Taken together, these results demonstrate that hTERT alters the proteolytic balance of BME cells, both with respect to basal and cytokine-induced levels of protease and inhibitor expression. Despite these alterations, hTERT−BME cells maintained their invasive and morphogenetic properties in a manner that is virtually indistinguishable morphologically from parental BME cells.

hTERT conferred a high cloning efficiency as from 20 clones generated, 17 large and dense colonies were generated that displayed a non-transformed phenotype. All clones retained their proliferative capacity (Fig. 9B) and invasion properties (Fig. 9C) in 3D collagen gels that were independent of the level of hTERT expression (Fig. 9A). When seeded on the top of collagen gels, tube-like structures were formed with a robust response to FGF-2 in combination with VEGF-A, that is in line with other published reports [Pepper et al., 1998] (Fig. 10). Among the 17 clones generated, we identified distinct subpopulations that display a highly specific response to VEGF-A (clone 6, 7, 11, and 16) or to FGF-2 (4, 6, 15,16, and 17), which was independent of the level of expression hTERT or cell surface expression of VEGFR-2 and FGFR-1. Our experience over many years is that there is frequently a large discrepancy between mRNA levels and receptor activity and/or protein expression. We presume that this is due to many possible points of post-transcriptional regulation including translational efficiency, and mRNA and protein stability.

In conclusion, we have generated endothelial cells with an extended life span and have isolated distinct subpopulations that display a highly specific response to the angiogenic cytokines VEGF-A and FGF-2. Given their ability to generate branching capillary-like tubes in collagen gels, these clones may provide a useful tool to investigate the factors that modulate the angiogenic process. Furthermore,

the availability of clonal immortalized bovine endothelial cell lines will facilitate the study of other important pathophysiological processes, such as arteriosclerosis and thrombosis, and should provide a useful tool for screening libraries of potential stimulators or inhibitors of angiogenesis.

ACKNOWLEDGMENTS

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