Brain and Retinal Vascular Endothelial Cells with Extended Life Span Established by Ectopic Expression of Telomerase

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Purpose. To study blood–retina barrier (BRB) regulation, we sought to establish neuronal microvascular endothelial cells (ECs) with expanded life span by ectopic expression of the human telomerase gene (hTERT).

Methods. Primary cultures of human brain and bovine retinal microvascular endothelial cells (HBECs and BRECs, respectively) were transfected with the catalytic component of human telomerase human telomerase reverse transcriptase (hTERT), and colonies were selected with puromycin. The endothelial origin of these cells was confirmed by immunocytochemistry. Reconstituted basement membrane matrix and three-dimensional collagen gel were used to induce the formation of tubulelike structures. To assess endothelial permeability, ECs were cultured on the upper chamber of migration assay membrane filters, with or without astrocyte coculture. Transepithelial electrical resistance (TER) was measured using a voltohmmeter.

Results. Both bovine retinal and human brain microvascular ECs expressing hTERT resembled young primary ECs in their morphology and growth response after more than 100 population doublings. Both bovine and human hTERT cells expressed von Willebrand factor, a key marker distinguishing ECs from other cell types; formed angiogenic webs in reconstituted basement membrane matrix; and, in a VEGF-dependent manner, formed tubule-like structures in three-dimensional collagen gel. Coculture of both types of cells with astrocytes resulted in a decrease in EC permeability, as assessed by TER. VEGF induced the breakdown of the HBEC monolayer barrier, and astrocytes in coculture appeared to attenuate the effects of VEGF.

Conclusions. Ectopic expression of hTERT enables adult HBECs to bypass the first mortality checkpoint but not the second mortality checkpoint, allowing generation of neuronal ECs with extended, but not indefinite life span. (*Invest Ophthalmol Vis Sci.* 2003;44:3219–3225) DOI:10.1167/iovs.02-0852

Diabetes mellitus, which affects millions of people all over the world, produces significant ocular morbidity.[1] Retinal edema, hemorrhage, ischemia, microaneurysms, and neovascularization characterize diabetic retinopathy. One of the earliest indications of diabetic retinopathy is altered capillary permeability.[2,3] It is now clear that any treatment to preserve full vision in diabetes must be instituted before capillary instability, incompetence, and closure occur.[3] This requires a better understanding of the inner blood–retina barrier (BRB) and the response of its component cells to the changes in microenvironment. Microvascular endothelial cells (ECs) in neuronal tissue such as brain and retina play an essential role in the blood–brain/retina barrier, regulation of blood flow, angiogenesis, inflammation, and metastasis. A wealth of information has been collected using cultured retinal vascular ECs, especially those from bovine retina.[4–6] However, normal mammalian microvascular ECs, similar to other somatic cells, undergo finite in vitro cell division before reaching irreversible growth arrest, known as replicative senescence.[7] This problem represents a major obstacle to the study of microvascular pathobiology. Therefore, extending the life span of ECs without phenotypic and hemodynamic changes is highly desirable for in vitro angiogenesis and for studying vascular permeability.

Components regulating the finite life span include the mitotic "clock" that counts the number of cell divisions and entry into the postmitotic state.[8] Telomeres are short repetitive DNA sequences (TTAGGG) at the ends of chromosomes and are synthesized by telomerase. They function to protect chromosomes from degradation, fusion, and recombination. In human cells, telomere length is progressively shortened with each cell division as a result of the inability of the DNA polymerase complex to replicate the very 5′ end of the lagging strand.[9] Telomerase is a ribonucleoprotein that synthesizes and directs the telomeric repeats onto the 3′ end of existing telomeres, using its RNA component as a template. Telomerase activity has been shown to be specifically expressed in immortal cell lines, cancer cells, and germ cells.[10,11] It is absent, however, in adult somatic cells. Studies of telomeres and telomerase suggest that critical telomere shortening is associated with the onset of cellular senescence and that telomere length may function as a "mitotic clock" to sense cell aging and, eventually, signal replicative senescence or programmed cell death.[12] Therefore, ectopic expression of hTERT may bypass replicative senescence.

Expression of the catalytic component of telomerase has been reported to impart replicative immortality in several primary cultured cells, including fibroblast and retinal pigment epithelial cells, without converting in a neoplastic transformation phenomenon.[13] Extension of EC life span has been achieved by ectopic expression of viral oncogenes or spontaneous transformation.[14] However, these cell lines present important genomic modifications that usually induce phenotypic alterations and loss of critical differentiated EC functions. Yang et al.[15] reported immortalization of both human large vessel and microvascular ECs by ectopic expression of hTERT, which preserved their differentiated and functional phenotype and maintained their angiogenic potential in vitro. Furthermore, they and others have shown that introduction of telomerase into normal human ECs in vitro does not lead to abnormal

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growth patterns, cell transformation, or genomic instability. Functionally, hTERT-immortalized human dermal microvascular ECs form stable tubules when cocultured in the presence of human glioma cells in vitro[16] and form microvascular structures when subcutaneously implanted in severe combined immunodeficiency mice.[17] To date, to our knowledge, there have been no neuronal microvascular ECs with an extended life span that have been generated by using hTERT expression. These cells are useful for blood–brain/retina barrier studies in vitro.

In the present study, we established hTERT-expressing human brain and bovine retinal vascular ECs that showed an extended life span. They also possessed normal EC morphology and specific neuronal tissue–derived EC function, including tube and barrier formation in response to angiogenic factor stimulation. These cells should be valuable tools for in vitro studies on the blood–brain/retina barrier and angiogenesis.

Materials and Methods

Cell Culture

Human brain vascular endothelial cells (HBECs) were isolated from a 49-year-old white male with no known neuronal diseases and provided at passage 2 (500,000 cells) as a gift by Clonetics (BioWhittaker, Walkersville, MD). Bovine retinal vascular endothelial cells (BRECs) from VEC Technologies, Inc. (Rensselaer, NY) were isolated from a pool of bovine retinas. Cells were cultured in endothelial basic medium (EBM) or endothelial growth medium (EGM, which is EBM supplemented with growth factors and 5% serum; BioWhittaker). Primary cultures of rat cerebral astrocytes (RCAs) were obtained from the cerebra of 2- to 3-day-old Holtzman Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) by the method of McCarthy and de Vellis.[18,19] Cerebra were removed from newborn pups and immersed in saline solution (138 mM NaCl, 5.4 mM KCl, 1.1 mM Na_2HPO_4, 22 mM glucose, and 0.9 mM $CaCl_2$) at 4°C. Tissue was mechanically disrupted and resuspended in culture medium (DMEM-Ham's F-12 medium; DMEM/F-12, 1:1, vol/vol) containing 5% fetal calf serum, 5% horse serum, and 1% penicillin–streptomycin. Suspended cells were filtered through cell strainers, and cells in the filtrate were plated at high density in 75-cm^2 flasks and grown in complete culture medium in an atmosphere of 5% CO_2-95% O_2 at 37°C. On day 10, cell cultures were shaken for 18 hours in an atmosphere of 5% CO_2-95% O_2 at 37°C (250 rpm, stroke diameter = 1.5 inches) to remove contaminating oligodendrocytes and neurons. After shaking, astrocytes were recovered by trypsin-EDTA and replaced at one third of their confluent density. On reaching confluence, astrocytes were plated for astrocyte-EC coculture or plated in 75-cm^2 flasks for collecting conditioned media.

Gene Transduction

Gene transfer was achieved by retrovirus-mediated gene transfer. pLPCX-hTERT was obtained through Clontech/Geron (Palo Alto, CA) with a material transfer agreement. The NIH-3T3–derived cell line PT67 was used as viral packaging cells to produce virus, and the expression of hTERT for the retroviral vector was driven by the Moloney murine leukemia virus 5′ long terminal repeat promoter. Conditioned medium was collected from the PT67 packaging cell line stably transfected with pLPCX-hTERT and was mixed with EGM (1:1). This mixture was supplemented with a transfection reagent (8 μg/mL; Polybrene; Sigma-Aldrich, St. Louis, MO) and used to infect primary HBECs (passage 4) and BRECs (passage 5) cultured at 30% to 50% density. EGM containing puromycin (2 μg/mL) was used to select transfected cells. The selected cells were then maintained in EGM.

Telomeric Repeat Amplification Protocol Assay

Telomerase activity from the hTERT transgene or endogenous telomerase was assayed by the modified PCR-based method with a telomeric repeat amplification protocol (TRAP) kit (TRAPeze Telomerase detection kit; Intergen, Purchase, NY). TS primer (5′-AATCCGTCGAGCAGAGTT-3′), cell extract or heat-inactivated cell extract, and PCR reagents were incubated at 30°C for 30 minutes and then subjected to 33 PCR cycles of 94°C for 30 seconds and 59°C for 30 seconds. The resultant PCR products were separated on 12.5% 19:1 acrylamide/bisacrylamide gels. The PCR products were stained with (SYBR Green; Applied Biosystems, Foster City, CA) and images were obtained with a digital camera (EDAS290; Eastman Kodak, Rochester, NY).

Tube Formation in Matrigel and Three-Dimensional Collagen

Reconstituted basement membrane matrix (Matrigel; BD Pharmingen, San Diego, CA) was placed on ice and allowed to thaw overnight at 4°C. Matrigel (350 μL) was layered onto each well of a prechilled 12-well plate and the plate was placed in an incubator at 37°C for 30 minutes until solidified. hTERT-HBECs or hTERT-BRECs (3.5×10^5 cells in 500 μL EBM or EGM) were seeded onto the matrix and incubated at 37°C in a 95% air and 5% CO_2 environment for 96 hours. Plates were photographed using an inverted microscope (Humphrey Zeiss, San Leandro, CA) equipped with a digital camera (Spot Camera, Diagnostic Instruments, Sterling Heights, MI). Three-dimensional (3-D) collagen gels containing hTERT-HBECs were prepared as described by Yang et al.[20] Briefly, collagen gels were formed by mixing 4 volumes of ice-cold gelatin solution (10× M199, H_2O, 0.53 M $NaHCO_3$, 200 mM L-glutamine, type I collagen, 0.1 N NaOH; 100:27.7:50:10:750:62.5 by volume) with 1 volume of cell suspension (9×10^6 cells/mL in cells in EBM). Gel–cell suspension (300 μL) was plated in one well of a 12-well plate. The mixture was allowed to solidify 1 hour at 37°C in a 95% air and 5% CO_2 incubator. The gel was then overlaid with 500 μL EGM or EBM, with or without VEGF supplement. Development of capillary-like networks and tubular structures were evaluated at 48 hours. At the end of the culture period, the gels were fixed in 4% paraformaldehyde and stained with rhodamine phalloidin (5 U/mL) and 4′,6′-diamino-2-phenylindole (DAPI; 4 μM) to visualize the actin cytoskeleton and nuclei, respectively. Digital images were taken with a fluorescence microscope (Humphrey Zeiss) equipped with a digital camera (Spot; Diagnostic Instruments).

Astrocyte-Endothelial Coculture and Transendothelial Electrical Resistance Measurement

Cell migration assay membrane filters (Transwell; Costar, Corning, NY; clear, 0.3 μm, 12-mm diameter, tissue culture treated polyester membrane) were set in a 12-well plate upside down. The EGTA-lifted astrocytes from confluent culture were transferred onto insert membranes (5:1 ratio in culture area). The unattached cells after 24 hours were removed. When confluence, examined by inverted microscope, was reached (approximately 1 to 2 days after plating), the inserts were turned over and 3×10^5 ECs were plated onto the upper surface of each insert. Coculture medium was EGM. When the ECs reached confluence (approximately 5 days after plating), the transendothelial electrical resistance (TER) was measured in a tissue-resistance measurement chamber (Endohm; World Precision Instruments, Inc., Sarasota, FL). Resistance of blank filters was subtracted from experimental filters before final TER values (ohms per square centimeter) were calculated. All experiments were performed in triplicate. The results were expressed as means ± SE of three experiments. Probabilities were determined by ANOVA.

Immunocytochemistry

Cells were grown on fibronectin-collagen–coated chamber slides for immunostaining with rabbit polyclonal anti-human von Willebrand factor (vWF; Dako, Carpinteria, CA, 1:500 dilution), mouse monoclonal anti-pig α-smooth muscle actin (1:400 dilution) and anti-pig glial fibrillary acidic protein (GFAP, 1:800 dilution; both from Sigma-Aldrich, and

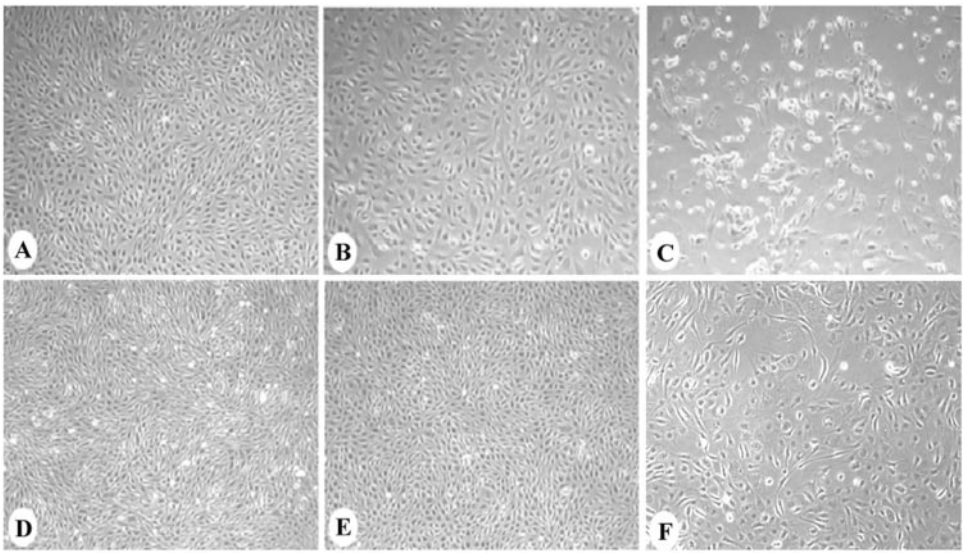

FIGURE 1. Cell morphology of continuously passaged parental and hTERT-HDMECs as a function of passage number. Phase-contrast images were taken from early parental HBECs (**A–C**) and BRECs (**D–F**) at passage 7 (**A**, **D**), hTERT-HBECs and hTERT-BRECs at passage 28 (**B**, **E**), or late parental HBECs and BRECs at passage 14 (**C**, **F**).

cross-reacting with human antigens), or on the filter inserts for immunostaining with rabbit polyclonal anti-ZO-1 (1:100 dilution; Zymed, S. San Francisco, CA). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% normal goat serum and 2% BSA, and then stained with the designated primary antibody overnight at 4°C. Secondary antibody was either FITC or Texas red conjugated anti-mouse (1:100 dilution) or rabbit (1:200 dilution) IgG and incubated with samples for 1 hour at room temperature. Nuclei were counterstained with 4′,6′-diamino-2-phenylindole (DAPI) contained in the mounting medium (Vector Laboratories, Burlingame, CA).

Statistics

Each experiment was repeated at least three times. Results were expressed as mean ± SE. Statistical significance was determined by one-way or two-way ANOVA (Sigma Stat; SSPS Science, Chicago, IL). $P < 0.01$ was considered significant (marked ** in figures). $P < 0.05$ but greater than 0.01 was also considered significant (marked * in figures).

RESULTS

Effect of hTERT Expression on Retinal ECs

Yang et al.[15] reported that ectopic expression of recombinant hTERT can efficiently reconstitute telomerase and extend the life span of five different types of human ECs. In the present study, we expressed hTERT in HBECs and BRECs by retroviral transduction. Among 20 hTERT-HBEC clones selected, 2 were cultured continually and maintained a similar proliferation rate for 36 passages. Whereas parental HBECs became senescent after 13 to 14 passages and exhibited a typical flattened morphology (Fig. 1C, compare with 1A), all hTERT-HBECs and hTERT-BRECs resembled young primary ECs that exhibited a cobblestone morphology and high saturation densities (Figs. 1B and 1E, compared with Figs. 1A and 1D). In complete medium (EGM), the hTERT-transduced cells grew to approximately the same densities as the young primary ECs and remained density inhibited. However, the hTERT-HBECs showed signs of senescence, after 36 passages. The cells proliferated at a slower rate, and the cultures reached lower saturation densities than the earlier-passage cells. Therefore, ectopic expression of hTERT in HBECs extended the HBEC replicative life span to a limited number of passages. The hTERT-BRECs have been continuously cultured to date for more 35 passages after selection with similar proliferation rate.

We assessed telomerase activity in HBECs by the TRAP assay (Fig. 2). The characteristic DNA ladder of the TRAP assay, which is indicative of telomerase activity, was observed in early-passage cells (P7) and became more apparent in HBECs transduced with hTERT (passage 28), but was less apparent in late-passage parental HBECs (passage 14).

Constitutive expression of vWF and CD31 is characteristic of ECs.[21] Immunocytochemistry studies using anti-vWF antibody showed strong expression of vWF in most hTERT-transfected or transduced cells and a similar staining pattern compared with their parental primary cells (Fig. 3). There was no specific staining observed when antibodies against α-smooth muscle actin, a marker for smooth muscle cells and pericytes,

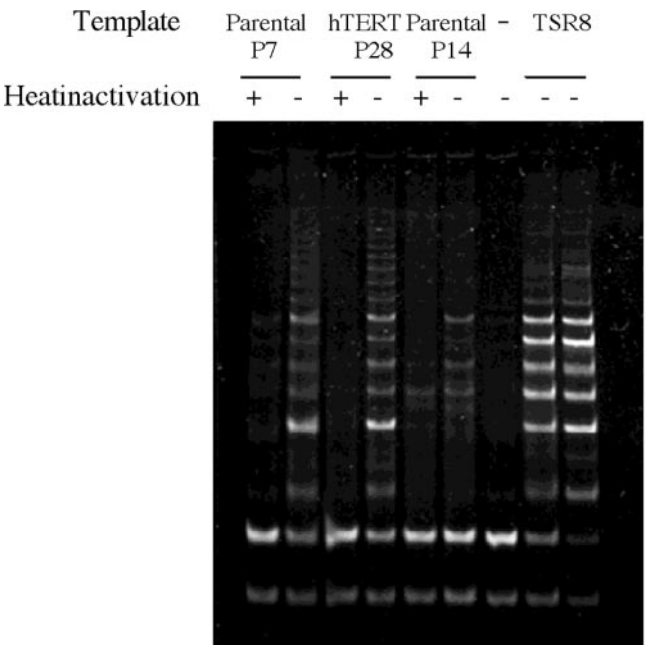

FIGURE 2. Telomerase activity in HBECs and BRECs after hTERT expression. Telomerase activity was measured by TRAP assay. PCR samples without template or with TSR8 template served as negative and positive controls, respectively.

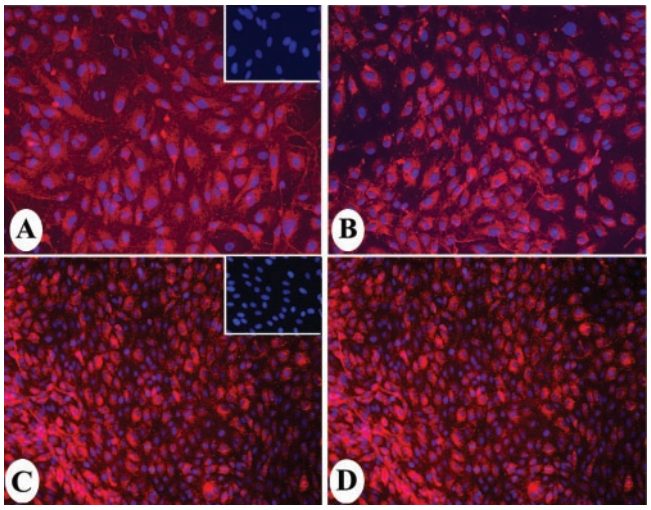

FIGURE 3. Expression of EC-specific marker by telomerase-expressing HBECs (**A**, **B**) and BRECs (**C**, **D**). Parental (**A**, **C**) and hTERT-expressing HBECs (**B**) or HBECs (**D**) were stained with anti-vWF and DAPI. *Insets*: negative controls without the first antibody. Note that parental and hTERT-expressing cells showed similar staining.

and GFAP, a marker for glial cells and astrocytes, were used (data not shown). Therefore, the hTERT-transduced HBECs and BRECs appeared to be ECs.

Angiogenic Characteristics of hTERT-HBECs and hTERT-BRECs

In response to matrix signals, ECs form tubulelike networks with a branching morphology. We first tested the ability of hTERT-HBECs and hTERT-BRECs to respond to Matrigel, which mimics the physical and functional properties of basement membrane.[14] Both ECs started to align themselves within 2 hours of culture on Matrigel and formed weblike structures within 4 hours. By 18 hours, the tubelike structures were apparent and extensive and covered most areas of the tissue culture dishes in a weblike form (Fig. 4). The tubelike structures were maintained for an additional 24 hours in HBECs and 72 hours in BRECs. Tube formation and maintenance were observed in both normal—which contains bFGF (0–0.1 pg/mL), epidermal growth factor (EGF; 0.5–1.3 ng/mL), insulinlike growth factor (IGF-1; 15.6 ng/mL), platelet-derived growth factor (PDGF; 12 pg/mL), nerve growth factor (NGF; $<$0.2 ng/mL) and TGF-β (2.3 ng/mL)—and growth factor–reduced Matrigel, which has less EGF ($<$0.5 ng/mL), PDGF ($<$5 pg/mL), IGF-1 (5 ng/mL), and TGF-β (1.7 ng/mL), with growth factor–free EBM or EGM, suggesting that angiogenic growth factors are not required for tube formation in the Matrigel.

ECs also differentiated in a 3-D collagen gel model and formed tubelike structures in response to angiogenic growth factors.[22] Figure 5 shows the requirement of VEGF for tubular morphogenesis of hTERT-transfected HBECs in collagen gel. No tube formation was observed when EBM was used. However, when VEGF (20 ng/mL) was added to the EBM or when EGM was used, HBECs aggregated and elongated to form tubelike structures. Similarly, bFGF in EGM also induced tube formation of HBECs in collagen gel (data not shown). Unlike previous reports, we found that HGF is not required for VEGF or bFGF induction of endothelial tube formation. Figure 6 shows a quantitative analysis of the mean tube length. These data demonstrate that hTERT-transduced HBECs preserve the differentiated phenotype and respond to extracellular matrix signals and angiogenic growth factor as well as primary ECs.

Behavior of hTERT-HBECs and hTERT-BRECs on Membrane Inserts

HBEC and BREC cells cultured on porous membrane inserts formed a polarized monolayer with barrier properties that could be assessed by measurement of TER, the most frequently used method to evaluate paracellular permeability.[23] TER was measured using a TER system (Endohm-snap; World Precision Instruments), which had a low background (18 $\Omega.cm^2$ for blank membrane insert), and gave consistent TER values for each sample. HBREC monolayers possessed a TER of 18 $\pm$ 0.57 Ω/cm^2, whereas RCAs had almost no resistance (4.5 $\pm$ 1.5 Ω/cm^2; Fig. 7A). To investigate whether hTERT-transfected ECs respond to astrocytes in the modulation of tight junction permeability, hTERT-HBECs were cocultured with astrocytes on opposite sides of membrane inserts or on the bottom of culture vessels for 3 to 6 days. Coculture of astrocytes on the opposite side of inserts resulted in a onefold increase in TER (36.3 $\pm$2.7 Ω/cm^2) whereas culture of the same number of astrocytes on the bottom of culture plates resulted in a 30% elevation of TER (Fig. 7A). Coculture of NIH-3T3 cells on filter inserts showed no effects on HBEC TER reading, suggesting the effects observed for astrocytes on endothelial permeability are astrocyte-specific. hTERT-BRECs possessed a TER of approximately 23 $\pm$1.3 Ω/cm^2, which increased to 60.67 $\pm$ 2.97 Ω/cm^2, a 1.25-fold increase, after 7 days of astrocyte coculture (Fig. 7B). Addition of VEGF induced a significant reduction of TER in hTERT-BREC culture, but not in hTERT-BREC–astrocyte coculture.

DISCUSSION

It has been reported that ECs from diverse tissues exhibit differences in growth and morphology. For example, brain vascular ECs demonstrate tight junctions by electron microscopy[24] but adipose microvascular and aortic ECs do not. In the present study, we have described the establishment of human brain and bovine retinal microvascular ECs with extended life span in culture by expression of hTERT, the catalytic subunit of telomerase. These cells continue to proliferate beyond a limit at which normal cells undergo senescence and have two to three times the life span of the parental cells. Consistent with the extended life span, the telomerase activities are higher in these two hTERT-expressing cells than in their parental cells. The hTERT-transduced neuronal microvascular ECs with extended life span undergo tubulogenesis in 3-D collagen culture in a VEGF-dependent manner and form a barrier when cultured on migration assay membrane inserts (Transwell; Corning Costar). Coculture of the hTERT-BRECs with astrocytes induced the formation of a highly impermeable barrier and rendered EC resistant to VEGF-induced barrier breakdown. Finally, hTERT-transduced HBECs, isolated from the brain of a 49-year-

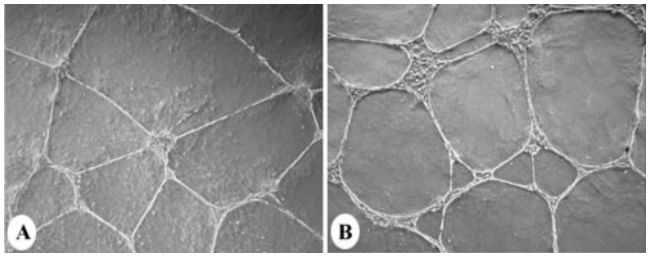

FIGURE 4. Morphogenic responses of hTER-HBECs and hTERT-BRECs exposed to Matrigel. HBECs (**A**) and BRECs (**B**) were overlaid on Matrigel and cultured for up to 96 hours. They were examined at 2, 6, 18, and 24 hours, and every day thereafter by inverted microscope. Micrographs were taken at 18 hours after plating.

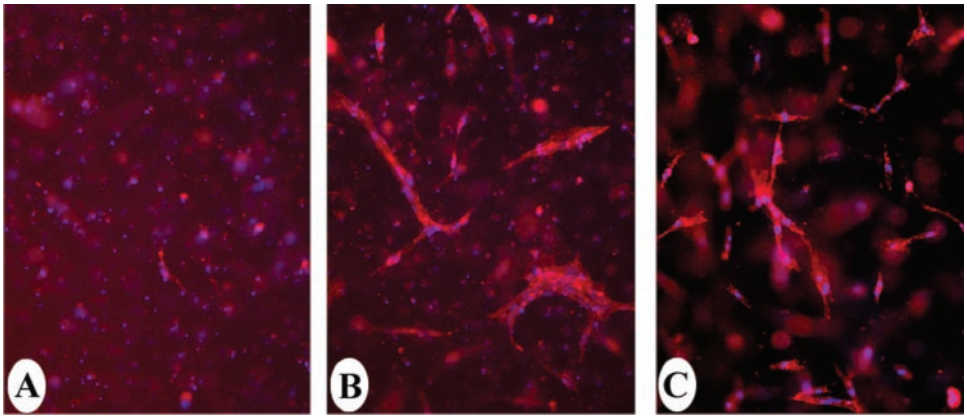

FIGURE 5. Morphogenic responses of hTERT-HBECs and hTERT-BRECs exposed to 3-D collagen gel. hTERT-expressing HBECs were cultured in 3-D collagen gel with (**A**) EBM, (**B**) EBM+VEGF (20 ng/mL), or (**C**) EGM. Forty-eight hours after plating, rhodamine-phalloidin was used to stain actin filaments, and DAPI was used to stain nuclei.

old male, were observed to have an extended but not indefinite life span, suggesting that hTERT expression enabled these adult HBECs to bypass the first mortality checkpoint (M_1) but not the second mortality checkpoint (M_2).[25]

Human ECs, similar to other normal diploid human cells, have a limited life span, and spontaneously become senescent. The establishment of immortalized cell lines, with cells that retain the differentiated phenotype and have an extended life span, has been a growing interest for those focused on in vitro studies with ECs. Many attempts have been made to immortalize cells, including transfection with SV40 T antigen,[26,27] integration with the E6/E7 genes of human papilloma virus,[28] and spontaneous transformation.[29] However, these immortalized cells are prone to a variety of genomic alterations. Recently, it has been reported that the life span of several different types of primary vascular ECs can be extended without affecting the differentiated phenotype by ectopic expression of hTERT.[15,30] Our study represents the first to use neuronal vascular ECs. Furthermore, we used human TERT to extend the life span of the ECs of nonhuman origin. Our analysis of the EC phenotypic pattern revealed that hTERT-HBECs and hTERT-BRECs retain EC-specific characteristics, which include normal cobblestone cell morphology, characteristic immunostaining of EC-specific antigens, such as factor VIII–related antigen, and the capacity to form capillary-like tubules in vitro.

During the process of immortalization, at least two mortality checkpoints in the cell cycle, M_1 and M_2, must be bypassed. Cells that have bypassed M_1 (senescence) have an extended life span, but are not necessarily immortal.[25] Although our hTERT-expressing HBECs show greatly extended life span compared with their parental cells, their growth rate decreased when they reached a certain population-doubling level. Thus, it appears that transducing cells with hTERT enabled the HBECs to overcome M_1, which resulted in an extended life span, but not M_2. Thus, telomerase activity may not be sufficient for immortalization of the HBECs we used. It was previously reported that the life span of primary neonatal human dermal microvascular ECs (HDMECs) could be extended more than 200 population doublings without affecting the differentiated phenotype by ectopic expression of hTERT.[16,17] The reason that the extended life span of HBECs is not as long as that of HDMECs is not clear. A possible explanation is the age of donor. The HBECs we used were isolated from adult brain (49 years old), whereas the HDMECs were neonatal. It has been reported that the mean terminal restriction fragment length of ECs in culture was demonstrated to show an inverse relationship between donor age and telomere length.[11] Thus, it is possible that the ectopic expression of hTERT is not suffi-

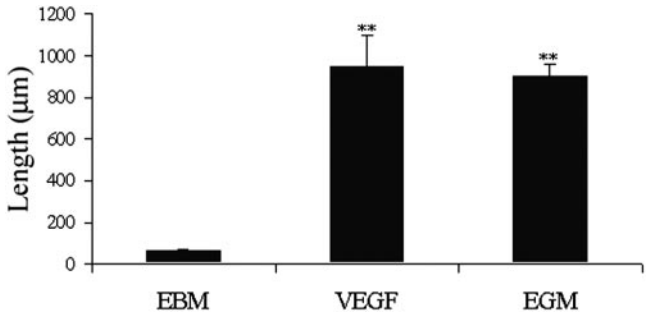

FIGURE 6. Quantitation of tube formation elicited by VEGF and growth medium. The average tube length was calculated as described in the text.

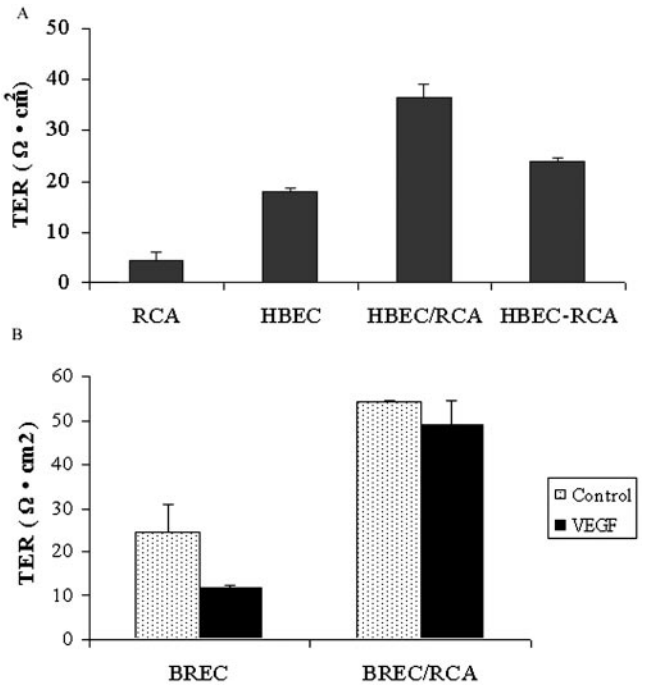

FIGURE 7. Effects of astrocyte coculture and VEGF on paracellular permeability of hTER-HBECs and hTERT-BRECs. hTERT-expressing HBECs (**A**) and BRECs (**B**) on tissue culture-treated filters in EGM alone or with treated RCAs cultured either on the back of filters (HBEC/RCA or BREC/RCA) or on the bottom of wells (HBEC-RCA). The cultured hTERT-expressing BRECs (**B**) with or without astrocyte coculture were also treated with 20 ng/mL VEGF. The TER measurements were taken at day 4 in HBECs and day 7 in BRECs. Results represent an average of three filters per time point and are expressed as mean ± SEM of three independent experiments.

cient to extend the life span of adult ECs with short telomeres to an unlimited passage number. Consistent with this notion, Krump-Konvalinkova et al.[30] recently reported that human pulmonary microvascular ECs isolated from adult donors older than 50 years could be immortalized only after cotransfection of plasmids encoding hTERT and SV40 large T antigen. Another possible explanation for the extended, but limited, life span of hTERT-HBECs is the differentiation state, because recent experiments support organ-specific endothelial differentiation. The neuronal ECs at the site of the blood–brain/retina barrier may consist of adapted cells that are especially resistant to genetic changes because of their location and specialized functions.

We showed that both hTERT-HBECs and hTERT-BRECs are sensitive to Matrigel and form angiogenic webs, suggesting that these cells possess this functional characteristic of ECs. However, we also observed that the Matrigel induced web formation in the presence or absence of angiogenic growth factors and serum. Furthermore, studies have shown that certain cell types other than ECs, such as epithelial cells and dermal fibroblasts can form networks on Matrigel.[31] Thus, tubule formation on Matrigel may not be a suitable model for in vitro tubulogenesis or angiogenesis. However, we found that growth factor-rich EGM is essential for the formation of capillary-like structures in 3-D collagen gel. This need for EGM can be replaced fully by VEGF, suggesting VEGF is necessary and sufficient for hTERT-HBECs to form capillary-like structures in collagen. This is different from the report that HGF is essential for VEGF to promote HUVEC tubulogenesis in 3-D type I collagen gels.[32] The reason for the discrepancy between these two studies is not clear. Nevertheless, the ability of VEGF to support endothelial tube formation in 3D collagen gels presents an opportunity to determine underlying mechanisms that mediate tubulogenesis and angiogenesis in vitro.

ECs from the central nervous system, including the retina, are different from those in the most peripheral capillaries in that there is absence of fenestrations and presence of tight junctions between adjacent ECs. They form a highly impermeable blood–brain/retina barrier.[33] It has been reported that tight junctions and other blood–brain barrier characteristics are maintained in primary brain microvascular EC culture as increased TER.[34–36] However, others believe that maintenance of tight junctions in brain microvascular ECs is dependent on cocultivation with astrocytes.[37] Using membrane filter inserts (Transwell; Corning Costar) and a TER system (Endohm-snap; World Precision Instruments) to measure TER, we showed that hTERT-HBECs and hTERT-BRECs formed a barrier on the filter inserts, as evidenced by measurable TER that is significantly higher than that in cultured fibroblasts or astrocytes. Consistent with previous studies showing that the formation of a highly impermeable barrier of brain and retinal ECs is induced and maintained by astroglial cells,[38–40] in our in vitro study, coculture with astrocytes on these filter inserts (Transwell; Corning Costar) induced an increase in the TER of ECs; however, direct contact of ECs and astrocytes appears to be necessary for the induction of the highly impermeable barrier. That we were able to detect small but significant differences in TER (~6 Ω/cm^2) when noncontact astrocyte coculture was used suggests that the system is sensitive and can be used to detect suitable changes in barrier properties in vitro. Finally, in our study the hTERT-expressing ECs were sensitive to VEGF in inducing an increase in permeability, and astrocytes in contact with ECs appeared to protect the ECs from VEGF-induced barrier breakdown in vitro. Further study is needed to understand the underlying mechanism for this astrocyte-mediated protection.

In summary, the extended life span in human brain and BRECs cells through telomerase expression will allow us to conduct extensive studies of the cell and molecular biology of neuronal tissue-derived ECs and in particular, their role in blood–brain and blood–retina barrier formation and angiogenesis, which otherwise would be impossible because of the quantitative limitation of primary culture of ECs.

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