Novel Isolation and Biochemical Characterization of Immortalized Fibroblasts for Tissue Engineering Vocal Fold Lamina Propria

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Tissue regeneration of the vocal fold lamina propria extracellular matrix (ECM) will be facilitated by the use of suitable vocal fold fibroblast (VFF) cell lines in appropriate model systems. Primary human VFFs (hVFFs) were steadily transduced by a retroviral vector containing human telomerase reverse transcriptase (*hTERT*) gene; immortalized cells grew and divided vigorously for more than 120 days. Biochemical characterization of the six transduced lines included, at different time points, expression of *hTERT*, telomerase activity, telomere lengths, and transcript levels of ECM constituents. Telomere lengths of the transfected lines were elongated and stable. Gene expression levels of collagen I α 1, collagen I α 2, collagen VI α 3, elastin, and fibronectin were measured between the transduced cell clones and the primary hVFFs to verify transcription. Absence of inter- and intraspecies contamination was confirmed with DNA fingerprinting and karyotype analysis. Cell morphology, growth, and transcription expression were examined on 2D scaffolds—collagen, fibronectin, and hyaluronic acid. Immortalized hVFFs demonstrated normal attachment and spread on 2D scaffolds. Collagen I α 1, collagen I α 2, collagen VI α 3, elastin, and fibronectin transcript expression was measured from immortalized hVFFs, for all surfaces. This is the first report of immortalization and biochemical characterization of hVFFs, providing a novel and invaluable tool for tissue regeneration applications in the larynx.

Introduction

VOICE DISORDERS AFFECT an estimated 3–9% of Americans yearly.¹ The impact of voice disorders and vocal fold disease on quality of life cannot be overestimated.² Vocal fold scarring is the single greatest cause of poor voice after vocal fold injury.³ Vocal fold scarring may cause a deformity of the vocal fold edge, a disruption of the viscoelastic-layered structure of the lamina propria, and an increase in stiffness of the vibratory structure and glottic incompetence. Vocal fold scarring has been suggested by Hirano⁴ as one of the major voice problems awaiting improvement in the future. The foremost reason for the inability to adequately treat these dysphonias is that present surgical options do not adequately address the extracellular matrix (ECM) biomechanical tissue properties and do not mimic the complex composition of the ECM. Successful development of new therapeutic regenerative interventions for vocal fold disease, including scarring, and an improved understanding of molecular development, pathogenesis, and biological features of the vocal fold lamina propria ECM depend on the availability of reproducible *in vitro* cell culture models of human origin. With specific regard to tis-

sue engineering, the topic of cell sourcing, cell tissue/characterization was recently recognized as a critically important focus area needing attention if progress is to be made in the field.⁵ More specific needs for success include availability of distinct cell sources for tissue regeneration, cell characterization, and development of universal donor cell lines.⁵ Thus, a reproducible, characterized source of vocal fold fibroblasts (VFFs) of human origin would have far-reaching implications, and it will have a significant impact on the field of tissue engineering for the vocal fold lamina propria.

The VFF is the key cell in the vocal fold lamina propria that not only plays an important role in supporting, but also contributes to healing of damaged vocal folds by producing the vast majority of ECM proteins, such as elastin, collagen, and fibronectin. Primary cell cultures of VFFs from ECM isolated from various animal sources have served as useful models for studying the mechanisms controlling wound injury and repair mechanisms.^{6,7} The use of primary cell culture of VFFs of human origin has been problematical because the source of tissue—normal vocal fold from live donors—is virtually impossible to obtain, and if obtained, insufficient numbers of cells are more often than not cultivated. Under current *in vitro*

conditions, human VFFs (hVFFs) possess a relatively short replicative life span and the insufficiency of hVFF lines hinders *in vitro* studies. Further, the limited proliferation capacity of hVFF cultures severely limits their ability to be genetically modified. After a series of population doublings, primary cells enter a state where they no longer divide, called replicative senescence, marked by distinct change in cell morphology, gene expression, and metabolism.

To overcome the cell growth-arrest barrier, many researchers have successfully produced immortalized cells by applying different methods such as carcinogenic agents,^{8,9} radiation,¹⁰ and transfecting viral oncogenes,^{11,12} including simian virus, human papilloma virus, and Epstein-Barr virus.^{13–16} But these immortalized cells in culture experience loss of DNA damage response, increased genomic instability, and, in some cases, tumorigenicity. Recently, the expression of human telomerase reverse transcriptase (*hTERT*), the enzyme responsible for elongating or maintaining telomere length by adding to its end tandem TTAGGG repeats via its endogenous RNA template,¹⁷ has been implicated as an important step in the immortalization process.¹⁸ Telomerase, which consists of the catalytic protein subunit, *hTERT* and the human RNA component of telomerase (*hTR*), and several associated proteins, has been primarily associated with maintaining the integrity of cellular DNA telomeres in normal cells.^{19,20} Telomerase activity is correlated with the expression of *hTERT*, but not with that of *hTR*.^{21,22} *hTR* is constitutively expressed, while *hTERT* is almost universally absent in most normal somatic cells²³ whose telomerase is repressed and whose telomere is progressively shortened, leading to limited proliferative life span.²⁴ Reconstitution of telomerase activity, achieved by transducing exogenous *hTERT* gene in normal human diploid cells, has been shown to enable different types of somatic cells to extend life span or immortalize. Support for such a phenomenon comes from Bodnar *et al.*,²⁵ who demonstrated that the induction of telomerase into normal retinal pigment epithelial cells and BJ foreskin fibroblasts can extend their endogenous telomeres and life span.

To date, there are no reports of immortalization of hVFFs by any methodology. In the current study, we immortalize hVFFs by transduction with the *hTERT* gene to prevent cellular senescence during isolation and expansion. We further biochemically characterize these immortalized cells and confirm absence of inter- and intraspecies contamination. Lastly, to substantiate applicability for tissue regeneration, immortalized cell behavior and transcript expression are measured in response to the presence of 2D scaffolds—collagen gel, fibronectin, and hyaluronic acid.

Materials and Methods

Cells and culture conditions

Two hVFF primary lines were established from primary tissue samples harvested from 21–59-year-old donors (one male and one female). In each case the vocal fold lamina propria was removed from an excised larynx within 4 h of death. Care was taken to not include underlying muscle. The vocal folds were judged to be normal without any evidence of disease by the attending surgeon. Donors did not have a history of smoking or laryngeal surgery. Tissue was resected and immediately placed in sterile phosphate-buffered saline.

The research protocol was conducted with approval by the Institutional Review Board of the University of Wisconsin–Madison.

Surgically resected vocal fold lamina propria tissue was cut into small pieces and suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.01 mg/mL streptomycin sulfate, and 1× NEAA (all from Sigma, St. Louis, MO). Cells were grown on uncoated plastic tissue culture dishes at 37°C in 5% CO₂–humidified atmosphere. After 14 days, the adherent confluent hVFFs were trypsinized and passaged. Fibroblast categorization and identification has previously been reported with this culture methodology.²⁶ Briefly, we employed a subtractive immunohistochemical methodology to identify our cultured hVFFs using specific antibodies to cells that could potentially contaminating hVFF lineages—skeletal muscle, endothelial, and epithelial cells. hVFFs are identified by ruling out these cells of other lineage that are found in vocal fold tissue. These cells were considered as population doubling level 0 (0 PDs) and were further subcultured in the same medium as described above. Medium was changed twice a week. Cell growth curves were determined by quadruplicate cell counts of trypsinized cells daily. To determine cell PDs, cells were subcultured until they reached a post-mitotic stage. PDs of the cells were calculated at the end of each passage by $2^N = (Cf/Ci)$, where *N* denotes PD, *Cf* denotes the total cell number harvested at the end of a passage, and *Ci* denotes the total cell number of attached cells at seeding. Growth counts were completed in triplicate.

Retroviral vectors, transduction, selection, and cloning

Immortalized hVFF lines were established by transfection of primary hVFF with the defective retrovirus expressing *hTERT* and neomycin resistance. The retrovirus was generated from plasmid pBABE-neo-*hTERT* (plasmid 1774; Addgene, Cambridge, MA), which contains the simian virus 40 promoter and *hTERT* cDNA, as well as the long terminal repeat promoter driving expression of the neomycin-resistant gene, which allows for selection of transduced cells in neomycin-containing medium. pBABE-neo (plasmid 1767; Addgene) was used as a control.

RetroPack PT67 packaging cell line (Clontech, Mountain View, CA) was transfected with pBABE-neo-*hTERT* and pBABE-neo in the presence of Clonfectin (ratio of DNA to Clonfectin, 1:2) (Clontech) and then followed by selection with 500 µg/mL G418 (Sigma) for 7 days. Two weeks after transfection, surviving clones were isolated and assayed by NIH 3T3 cells to determine viral titers. The clones with the highest titers ($5\text{--}10 \times 10^6$ CFU/mL) were selected, and their supernatants were collected, filtered through a 0.45 µm syringe filter, and used to transduce hVFFs.

Two active proliferating independent strains of hVFFs (second passage, approximately PD 8) were seeded into six-well plates at a density of 2×10^5 cells/well. When cells reached about 30–40% confluence, the medium was removed and the cells were washed twice with serum-free Dulbecco's modified Eagle's medium. In presence of polybrene (4–8 µg/mL), a total of 2 mL retroviral suspension was added to the cells and incubated for 24 h at 37°C, and then 2 mL conditional medium was added. The next infection was completed 20 h later with the same procedure, and repeated

TABLE 1. PRIMER SEQUENCES AND PRODUCTS OF REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Gene	GenBank #	Forward Primer	Reverse Primer	Size of PCR product
Collagen I α 1	NM_000088	5'-AATGGTGCTCTGGTATTGCTGGT-3'	5'-ACCAGTGTCTCCTTTGCTGCCA-3'	141 bp
Collagen I α 2	NM_000089	5'-AACAAATAAGCCATCACGCCTGCC-3'	5'-TGAAACAGACTGGGCCAATGTC CA-3'	101 bp
Collagen VI α 3	NM_057167	5'-GAAGAAGGAGTCAGCCATCG-3'	5'-ATTCGAGAGAACTGCCTGGA-3'	189 bp
Elastin	NM_000501	5'-AAGCAGCAGCAAAGTTCGGT-3'	5'-ACTAAGCCTGCAGCAGCTCCATA-3'	187 bp
Fibronectin	NM_002026	5'-ACCTACGGATGACTCGTGCTTGA-3'	5'-CAAAGCCTAAGCACTGGCACAACA-3'	116 bp
β -Actin	NM_001101	5'-ACGTTGCTATCCAGGCTGTGCTAT-3'	5'-CTCGTGAGGATCTTCATGAGGT AGT-3'	188 bp
<i>hTERT</i>	NM_198255	5'-AAGAGCCACGTCTCTACCTTCACA-3'	5'-TGGCACATGAAGCGTAGGAAGA-3'	167 bp

two to four times. To select cells transduced with the pBABE-neo-*hTERT* or pBABE-neo, cells were incubated in culture medium containing 300 μ g/mL G418 (Sigma) for 2–3 weeks. One G418-resistant cell clone was obtained using a limited dilution method. Thirty-one G418-resistant clones from 21T hVFFs and 21 G418-resistant clones from 59T hVFFs were obtained; among them, six clones transduced with the retrovirus expressed *hTERT* gene, designed A2, A8, A10, E6, E7, E10, and four clones infected with the retrovirus without *hTERT* gene, named B1, B3, F1, and F5, were selected for further investigation. Experiments were carried out for 120–130 days.

Detection of transcription of genes by RT-PCR

When the primary hVFFs and transduced cell lines reached subconfluence, total cellular RNA was isolated using an RNA extraction kit (RNeasy Mini Kit; Qiagen, Valencia, CA), according to manufacturer's instructions. First-strand cDNA was synthesized from 1 μ g of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen). mRNA level was quantified by real-time PCR^{27,28} using a LightCycler System (Roche, Indianapolis, IN), with amplification of β -actin as control. mRNA from the cDNA sample was amplified with specific primer pairs for *hTERT*, fibronectin, elastin, collagen I α 1, collagen I α 2, collagen VI α 3, and β -actin. The primer sequences, GenBank access number, and expected PCR product sizes are listed in Table 1. Amplification was carried out for 45 cycles at 95°C for 10 s, 55°C for 5 s, and 72°C for 8 s in a 20 μ L reaction mixture containing 2 μ L cDNA, 0.5 μ M of each primer, 2–3 mM MgCl₂ (dependent on the target gene), dNTPs, and Tag DNA polymerase from LightCycler FastStart DNA Master SYBR Green I (Roche) by the LightCycler 1.5 System. Exact amplification efficiencies of target and reference genes were assessed by LightCycler software before any calculation of normalized gene expression was completed. The specificity of every pair of primers was confirmed by melting curves. Figure 1 demonstrates the single peak and band size for each gene product. In all of our real-time PCR experiments, the standard curve method was used for quantification of gene expression. Results were shown by the mRNA concentration (ng/ μ L), normalized by housekeeping gene, β -actin mRNA (ng/ μ L). Each sample was tested in triplicate and repeated more than two times with similar results.

Telomerase activity assay

Telomerase activity was measured using a PCR-based telomeric repeat amplification protocol (TRAP assay). TRAP assays were performed using a Telomerase PCR ELISAplus Kit (Roche) according to manufacturer's protocol. This assay is based upon the specific amplification of telomerase-mediated elongation products combined with nonradioactive detection using an ELISA protocol and is a highly sensitive approach to the semi-quantitative detection of telomerase activity. Briefly, 2×10^6 hVFFs and transduced hVFF lines were isolated. Cells were lysed, and the lysates were adjusted to 0.5 μ g protein/ μ L. Negative controls were obtained by heat inactivation of telomerase at 85°C for 10 min. Telomeric repeat amplification was performed using 2 μ L lysate. The elongation of the primers by telomerase took place at 25°C (30 min). Telomerase was inactivated by two cycles at 94°C (5 min). These elongation products, as well as the internal standard (IS) included in the reaction vessel, were amplified by PCR. The control group contained a positive control template DNA with the same sequence as a telomerase product with eight telomeric repeats (TS8). The PCR conditions were 30 $\times$ 30 s/94°C, 30 s/50°C, and 90 s/72°C, followed by primer elongation at 72°C for 10 min. The PCR products were split into two aliquots, denatured, and hybridized separately to digoxigenin (DIG)-labeled detection probes, specific for the telomeric repeats and for the IS. The resulting product was immobilized via the biotin-labeled primer to a streptavidin-coated microtiter plate. Immobilized PCR product was detected with anti-DIG peroxidase using 3,3',5,5'-tetramethylbenzidine as a substrate. Color development was stopped after 15 min. The absorbance of the samples was measured at 450 nm using a

FIG. 1. Electrophoresis of real-time PCR primers. PCR product size of each gene matched the predicted sizes as in Table 1.

microtiter plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Samples were considered to be telomerase positive if the difference in absorbance ($\Delta A = A_S - A_{S,0}$) was higher than the twofold background activity ($2 A_{S,0}$). Relative telomerase activities (RTA) with different samples in an experiment were obtained using the following formula: $RTA = [(A_S - A_{S,0})/A_{S,IS}]/[(A_{TS8} - A_{TS8,0})/A_{TS8,IS}] \times 100$, where A_S is the absorbance of sample; $A_{S,0}$, the absorbance of heat-treated sample; $A_{S,IS}$, the absorbance of IS of the sample; A_{TS8} , the absorbance of control template (TS8); $A_{TS8,0}$, the absorbance of lysis buffer; and $A_{TS8,IS}$, the absorbance of IS of the control template (TS8). These experiments were independently repeated in triplicate.

Telomere Length Analysis

Telomere length was determined by Southern blot analysis of telomere restriction fragments using the TeloTAGGG Telomere Length Assay kit (Roche). Genomic DNA was extracted from primary 21T, 59T hVFFs, and the immortalized clone cells, A2, A8, A10, E6, E7, E10, and transfected control cells (B1, B3, F1, and F5) at different time points, using DNA extraction Kit (Roche) according to manufacture's instructions. Two micrograms of genomic DNA was digested with the restriction enzymes *HinfI* and *RsaI*, and the fragmented DNA was then electrophoresed through a 0.8% agarose gel in 1× TAE buffer at 5V/cm for 4h. The gel was then twice denaturated in 1.5 M NaCl and 0.5 M NaOH buffer for 15 min at room temperature, and twice neutralized in 3 M NaCl and 0.5 M Tris-HCl, pH 7.5, for 15 min, and then transferred to positively charged nylon membrane using capillary transfer with 20× SSC transfer buffers for overnight. The transferred DNA was fixed by UV-crosslinking (120 mJ/cm²) and hybridized to a DIG-labeled probe, specific for telomeric repeats. The *hTERT* signals were detected by a highly sensitive chemiluminescence substrate. The average telomere length

was determined by comparing the signals relative to a molecular weight standard according to the formula, mean terminal restriction fragment (TRF) = $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is the chemiluminescent signal and L_i is the length of TRF at position i .²⁹ Telomere length analysis was completed in triplicate.

Genetic analysis

To authenticate and detect any potential intra- or inter-species cross contamination of the primary and immortalized hVFF lines, DNA fingerprinting was performed on all of the cell lines (A2, A8, A10, B1, B3, E6, E7, E10, F1, and F5) according to standard techniques using Powerplex 16 kit (Promega, Madison, WI) by Cell Line Genetics LLC (Madison, WI). The DNA fingerprint for each cell line was compared with that of every other cell line from the same parental strain. Karyotyping analysis was performed on a combination of 20–24 G-banded metaphase cells harvested from the primary hVFF and transduced hVFF lines by Cell Line Genetics LLC.

Cell characterization and transcript expression on 2D scaffolds

Immortalized hVFFs (A8, passage 7, 5×10^5) were seeded into the wells of six-well plate, which were then seeded onto

FIG. 2. *hTERT* gene expression levels of transduced hVFF lines by RT-PCR. *hTERT* mRNA data were normalized by housekeeping gene β -actin. (A) *hTERT* gene mRNA level of *hTERT*-transduced hVFF lines and control vector-transduced cell lines at passage 7. (B) *hTERT* gene mRNA of *hTERT*-transduced hVFF lines at passages 7 and 17.

FIG. 3. Expression of exogenous telomerase activity in two independent parental normal hVFFs (21T and 59T hVFF) and their transduced cell lines. The treatment of the cells was described in Materials and Methods section. One of three independent TRAP assays is shown. (A) Quantification telomeric repeat amplification protocol assay of parental (21T and 59T), *hTERT*-transduced clones (A2, A8, A10, E6, E7, and E10), and vector-transduced (B1, B3, F1, and F5). Cellular extracts at passage 7 were tested for telomerase activity. Telomerase activity levels were normalized to the level of the internal control amplification products. (B) Telomeric repeat amplification protocol assay of *hTERT*-transduced hVFF cell clones at passages 7, 13, and 17.

collagen gel (Vitrogen, San Diego, CA), fibronectin (BD Biosciences, Sparks, MD), hyaluronan-Extracel (Glycosan, Salt Lake City, UT), and untreated polystyrene. Cells were harvested on days 1, 3, 5, and 7 after starting the cultures. Cells were washed three times with phosphate-buffered saline, collected for RNA extraction, and prepared for real-time PCR as described above.

Results

Transduction and selection of hVFFs

After retroviral infection of two active proliferating independent strains of hVFFs (21T and 59T), transduced cells were selected with G418 for 3 weeks, while G418-treated, nontransduced primary cells died after 2 weeks. Thirty-one G418-resistant colonies of 21T and 21 colonies of 59T were cloned, and among them 10 clones (A2, A8, A10, B1, B3, E6, E7, E10, F1, and F5) were further characterized.

hTERT gene expression

After cloning of transduced cells by limited dilution, we selected 10 clones (A2, A8, A10, B1, B3, E6, E7, E10, F1, and F5), which were subcultured and continued to proliferate. The presence of *hTERT* mRNA was confirmed at passage 7. RT-PCR (real-time PCR) revealed that all *hTERT*-transduced cell clones (A2, A8, A10, E6, E7, and E10) expressed very high amounts of *hTERT* mRNA, whereas virtually no message was detectable in control vector-transduced cell clones (B1, B3, F1, and F5) (Fig. 2A). These results demonstrate that the *hTERT* gene was integrated into the genomic DNA of clones A2, A8, A10, E6, E7, and E10, and transcribed into mRNA. At passage 17, repeating the same experiments showed that *hTERT* mRNA remained at consistently high levels (Fig. 2B).

Telomerase activity

Ten transduced clones and two parental hVFFs were tested for telomerase activity by the telomeric repeat

FIG. 4. Southern blot (telomere length assay) of transduced cell lines and parental hVFFs. Genomic DNA was isolated from *hTERT*-transduced populations, control, and uninfected parental lines at passages 8 and 18, and then hybridized with a telomeric probe to visualize the TRF. The first lane shows the molecular weight marker in kb. (A) At passage 8, telomere length of the cell lines established from 21T and 59T strains and telomeres of *hTERT*-positive clones (A2, A8, A10, E6, E7, and E10) were 19–20 kb, much longer than that *hTERT*-negative clones (B1, B3, F1, and F5) and parental cells (21T and 59T) whose range was 7.0–8.0 kb. The broad band of *hTERT*-negative and parental cells indicates wide variability of telomere length, while *hTERT*-transduced cells are more uniform. (B) Telomere length of *hTERT*-transduced cell lines and parental hVFFs at passages 8 and 18. Comparing the parental 21T and 59T, all of *hTERT*-positive cell lines maintained stable telomere length at 19–20 kb until passage 18.

amplification protocol assay. At passage 7, six *hTERT*-transduced clones (A2, A8, A10, E6, E7, and E10) showed high telomerase activity, but no activity in the heat-treated cell extracts, control clones (B1, B3, F1, and F5), and non-transduced parental cells (21T and 59T) (Fig. 3A). Further, telomerase activity was repeatedly measured for the cell clones at passages 13 and 17 demonstrating stable activity over 120 days (Fig. 3B). Overall, these results indicate that *hTERT*-transduced cell clones possessed stable telomerase activity.

Telomere length analysis

To determine if *hTERT*-reconstituted telomerase acts on its normal chromosomal substrate, we isolated genomic DNA from the parental cells, the control vector-transduced cells, and cells transfected with an *hTERT* retrovirus at passages 8 and 18. DNA was analyzed by Southern blots probed with a telomere-specific probe to visualize the TRFs that include the telomeres. At passage 8, the average telomere size of the *hTERT*-transduced cell clones was in the range of 19–20 kb and considerably longer than that of parental 21T and 59T strains (7.0 and 8.4 kb, respectively). The four clones transduced with an empty vector showed a wide variability of telomere lengths, with a peak of approximately 7.0–7.4 kb

(Fig. 4A). The TRF length of parental cells (21T and 59T) was substantially longer at an early passage (P8) than at a late passage (P18), reflecting the loss of telomeric DNA sustained during extended passaging in culture (Fig. 4B). Once TRF reaches a length of about 4 kb, telomeres may no longer protect chromosome ends, which in turn may lead to the genomic instability and cell death associated with crisis.²⁵ TRF was already shortened to 6.1–7.0 kb in our parent cells 21T and 59T at passage 18. In contrast, the *hTERT* expressing cells maintained telomere length at a size of 19.0–20.0 kb at passage 18 (Fig. 4B). These cells continually proliferated, with no evidence of entering crisis (Figs. 5B and 6B). These results indicate that ectopic expression of *hTERT* had elongated and stabilized the telomeres.

Morphological and growth characterization of clonal cells

The morphology of the transduced cell clones was a typical spindle shape, similar to the original hVFFs in growth phase and confluence (Fig. 7). Immortalized cells showed growth saturation as they reached confluency (data not shown). The growth rate of the transduced cell clones from 21T hVFFs was determined by daily cell counts of trypsinized cells (Fig. 5A). We compared the growth rates of *hTERT*-transduced cell lines

FIG. 5. Growth curves of transduced cell clones established from 21T hVFFs. (A) Growth curves of *hTERT*-positive cells (A2, A8, and A10) and control cells (B1 and B3). (B) PDs of *hTERT*-transduced single cell clones (A2, A8, and A10) and control vector-transduced cell lines (B1 and B3) are shown over 120 days of culture. X-axis indicates incubation days, and Y-axis indicates the number of population doublings.

FIG. 6. Growth curves of transduced cell clones established from 59T hVFFs. (A) Growth curves of *hTERT*-positive cells (E6, E7, and E10) and control cells (F1 and F5). (B) PDs of *hTERT*-transduced single-cell clones (E6, E7, and E10) and control vector-transduced cell lines (F1 and F5) are shown over 120 days of culture. X-axis indicates incubation days, and Y-axis indicates the number of population doublings.

FIG. 7. Morphologic appearance of parental hVFF cells, *hTERT*-transduced single clones (A2 and E6), and control empty vector-transduced single clones (B1 and F1) in growth phase.

(A2, A8, and A10) with those of the control cell lines (B1 and B3) at passage 8 (Fig. 5A). We found that transducing *hTERT* gene into hVFFs induced higher proliferation rates. Long-term population doublings of the transduced 21T cell lines (Fig. 5B) depicted that *hTERT*-negative cells (B1 and B3) ceased dividing on days 40–50 of culture (PD 30–35). By contrast, the growth rates of the *hTERT*-positive cell clones (A2, A8, and A10) were higher than those of B1 and B3 clones, and showed stable proliferation and divided vigorously beyond 120 days (PD 60–70). Similar results were also found in transduced cell clones established from 59T hVFFs (Fig. 6A, 6B). All *hTERT*-transduced cells (E6, E7, and E10) showed higher proliferation rates than the control clones (F1 and F5). These data demonstrate that there were no signs of senescence of *hTERT*-transduced cell clones in culture for 120 days.

Functional characterization of the immortalized cell clones

Because measurable transcript levels of collagen, fibronectin, and elastin represent the function of hVFFs, we examined the expression of the genes for collagen $\alpha 1$, collagen $\alpha 2$, collagen $\text{VI}\alpha 3$, fibronectin, and elastin between the transduced hVFF lines and their parental strain hVFFs using RT-PCR analysis (Fig. 8). The expression of these genes in each sample was normalized to β -actin expression. These results suggest that the transduced hVFF lines expressed the same genes as the respective parental hVFFs.

Genetic analysis of the clonal cells

DNA fingerprinting of the transduced cell lines (A2, A8, A10, B1, B3, E6, E7, E10, F1, and F5) demonstrated that they were from two cell strains. Table 2 shows the DNA fingerprints of A2, A8, A10, B1, and B3 cell lines, with matching loci. DNA fingerprinting loci patterns for E6, E7, E10, F1, and F5 matched each other, demonstrating that they were all from the same strain (Table 3). No variation

was observed in the independent cell clones from the parental strains (21T and 59T). Loci measured did not match DNA fingerprinting patterns of any cell lines published in ATCC or the NIH Website. Cytogenetic analysis demonstrated that all of the parental primary lines and transduced hVFF lines had normal human karyotypes (Fig. 9A, 9B). Lack of intra- and interspecies cross contamination was confirmed.

Cell characterization and transcript expression on 2D scaffolds

Immortalized A8 clone cells and primary 21T were seeded on 2D scaffolds and cultured for up to 7 days to test cell attachment, spread, and transcript expression. Immortalized A8 cells adopted a spindle-shaped morphology and spread uniformly on the Extracel and collagen gel scaffolds up to 7 days, comparable with primary hVFFs, and the cells grown on fibronectin and polystyrene surfaces (data not shown). Gene expression for collagen $\alpha 1$, collagen $\alpha 2$, collagen $\text{VI}\alpha 3$, fibronectin, and elastin for immortalized A8 hVFFs is displayed across days for scaffolds assessed in Figure 10. Immortalized cells synthesized and secreted all the aforementioned genes, which have been implicated in ECM production of the vocal fold lamina propria, across all time points and conditions.

Discussion

An ideal cell source for use in vocal fold lamina propria ECM engineering paradigms should be readily available, show robust proliferation, and possess the potential to synthesize the ECM. To date, candidate cells are unavailable commercially, and normal VFF (hVFF) primary lines are very difficult to acquire. Further, because primary cells reach senescence after a limited number of population doubling, researchers frequently need to reestablish fresh cultures from explanted tissue—a tedious process, one made more

FIG. 8. Expression of collagen I α -1 (**A**), collagen I α -2 (**B**), collagen VI α -3 (**C**), fibronectin (**D**), and elastin (**E**) genes in *hTERT*-transduced cell clones (A2, A8, A10, E6, E7, and E10), control vector-transduced cell clones (B1, B3, F1, and F5), and parental hVFFs (21T and 59T). mRNA levels (ng/ μ L) were normalized by housekeeping gene β -actin.

difficult in laryngology research due to the paucity of tissue available. To employ the same material throughout a research project, researchers need primary cells with an extended replicative capacity or immortalized cells. Such an established cell line provides an almost unlimited supply of cells with similar genotype and phenotype, allowing them to be used for complex, continuous long-term studies and interlaboratory comparisons. Our long-term goal in developing hVFF-immortalized lines is to provide a tool for

mechanistic studies of vocal fold lamina propria ECM development and regeneration.

Under current conditions, hVFFs have limited proliferation and dividing capacity. Cellular senescence occurs *in vitro* secondary to the loss of telomeric DNA with passage progression.³⁰ Telomeres, which are specialized structures at the ends of eukaryotic chromosomes, play a role in chromosome protection, positioning, replication, and meiosis^{31,32} and are thought to control entry into senescence. Human telomeres

TABLE 2. DNA FINGERPRINTS FOR A2, A8, A10, B1, AND B3 CELL LINES

Name	Amel	vWA	D8S1179	TPOX	FGA	D3S1358	THO1	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta D
A2	X,Y	14	13,14	8,11	22,24	15,16	6,9,3	29,30	10,21	13,14	11,12	12	8,10	9,12	12,13	10,12
A8	X,Y	14	13,14	8,11	22,24	15,16	6,9,3	29,30	10,21	13,14	11,12	12	8,10	9,12	12,13	10,12
A10	X,Y	14	13,14	8,11	22,24	15,16	6,9,3	29,30	10,21	13,14	11,12	12	8,10	9,12	12,13	10,12
B1	X,Y	14	13,14	8,11	22,24	15,16	6,9,3	29,30	10,21	13,14	11,12	12	8,10	9,12	12,13	10,12
B3	X,Y	14	13,14	8,11	22,24	15,16	6,9,3	29,30	10,21	13,14	11,12	12	8,10	9,12	12,13	10,12

TABLE 3. DNA FINGERPRINTS FOR E6, E7, E10, F1, AND F5 CELL LINES

Name	Amel	vWA	D8S1179	TPOX	FGA	D3S1358	THO1	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta D
E6	X	17	10,13	8	22,25	14,18	6	29,31,2	15,19	11,13	11,12	13	11	11,13	10,11	9,13
E7	X	17	10,13	8	22,25	14,18	6	29,31,2	15,19	11,13	11,12	13	11	11,13	10,11	9,13
E10	X	17	10,13	8	22,25	14,18	6	29,31,2	15,19	11,13	11,12	13	11	11,13	10,11	9,13
F1	X	17	10,13	8	22,25	14,18	6	29,31,2	15,19	11,13	11,12	13	11	11,13	10,11	9,13
F5	X	17	10,13	8	22,25	14,18	6	29,31,2	15,19	11,13	11,12	13	11	11,13	10,11	9,13

FIG. 9. Cytogenetic analysis of immortalized hVFF cell lines, showing all transduced fibroblast lines with normal human karyotypes. (A) Karyotype of a cell from 21T male donor (passage 5). (B) Karyotype of a cell from 59T female donor (passage 5).

consist of repeats of the TTAGGG/CCCTAA sequence at the chromosome ends, and these repeats are synthesized by the ribonucleoprotein enzyme, telomerase.³³ In the present study, we have established six hVFF lines immortalized by transducing the catalytic subunit of telomerase, *hTERT* gene, into primary hVFFs, resulting in increased telomerase activity, elongated telomere length, and the extension of life span. Control vector-transduced cell clones showed no telomerase activity and senesced after 2 months. Whereas *hTERT*-transduced cells have been maintained in culture for more than 4 months. They continued to divide rapidly; the maximum PD measured was PD 70. They do have the potential to divide forever. These outcomes provide sufficient evidence that the hVFFs obtain immortalization by transduction with ectopic *hTERT*.

HVFFs produce ECM proteins and play an essential role in the development, differentiation, maintenance, and repair of the vocal fold lamina propria. In the six hVFF clones im-

mortalized by transduction with *hTERT* genes, mRNA expression of fibroblast functional genes, collagen I α 1, collagen I α 2, collagen VI α 3, fibronectin, and elastin, was confirmed. Further functional significance includes the findings that the *hTERT*-transduced hVFF clones possessed the typical fibroblast spindle shape concomitantly with close to double growth rates than the primary hVFFs. Karyotyping demonstrated genetic stability across the clone lines, and DNA fingerprinting indicated absence of contamination. Taken together, these findings signify that the immortalized hVFF clones have stable genotypes and retained critical phenotypic markers of the original primary hVFFs as distinguished by morphologic appearance, gene expression, and chromosome karyotyping.

A variety of scaffolds have demonstrated promising results in the regeneration of injured vocal fold tissue.^{34–38} The findings of the present study demonstrate that the immortalized hVFFs are able to attach and spread on a variety of

FIG. 10. Expression of collagen I α -1, collagen I α -2, collagen VI α -3, fibronectin, and elastin genes in hTERT immortalized cell clones, A8 hVFFs on Extracel, collagen, fibronectin, and plastic surfaces. mRNA levels (ng/ μ L) were normalized by housekeeping gene β -actin.

constructs. Cell synthesis and transcript expression for ECM constituents that are involved maintaining the structure and function of the lamina propria were measured across 7 days on four surfaces. Differing patterns of expression over time between surfaces further support the functional aptitude of the cell lines.

In conclusion, we have created six immortal hVFF lines by transducing *hTERT* into primary VFFs. Such *hTERT*-immortalized clones (retrovirus-mediated *hTERT* gene transfer) are the first established and characterized immortalized hVFF lines to be reported in the literature. They will provide powerful tools to study tissue regeneration of the vocal fold lamina propria ECM. Using genetically modified hVFFs in complementary cell-based assays, *in vitro* coculture models, and *in vivo* models will allow detailed mechanistic studies of specific genetic and molecular pathways. Knowledge from these types of investigations will influence and advance therapeutic options for vocal fold ECM regeneration.

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