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Establishment of Immortalized Laryngeal Epithelial Cells Transfected with Bmi1

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Abstract

Primary laryngeal epithelial cells are essential to exploring the mechanisms of laryngeal and voice disorders; however, they are difficult to study and apply because of their limited life span. The purpose of this study was to develop a stable and reliable *in vitro* model for the comprehensive study of the pathogenesis of laryngeal and voice diseases. The pLVTHM-Bmi1 plasmid was constructed and used to immortalize primary laryngeal epithelial cells by lentiviral infection. The expressions of Bmi1, human telomerase reverse transcriptase (hTERT), p53, and pRB pathway proteins were detected by western blotting. Functional characteristics of the immortalized cell lines were verified by cell senescence β-galactosidase staining, 5-ethynyl-2′-deoxyuridine cell proliferation test, and flow cytometry. We successfully introduced Bmi into human subglottic (hSG) cells and human ventricle (hV) cells. Both the human immortalized subglottic Bmi1 (hSG-Bmi1) cell line and the human immortalized ventricle Bmi1 (hV-Bmi1) cell line maintained normal epithelial morphology and divided successfully after more than 20 culture passages. As Bmi1 was overexpressed in these cells, the expression of human telomerase reverse transcriptase (hTERT) and phosphorylated Rb increased while p16 and p21 decreased. Following Bmi1-mediated immortalization, cell senescence decreased significantly, and cell proliferation was accelerated. Tumor formation was not observed for hSG, hV, or hSG-Bmi1, and hV-Bmi1 cells in nude mice. hSG-Bmi1 cells dominated by stratified squamous epithelium and hV-Bmi1 cells dominated by columnar cells were established. The new cell lines lay a foundation for the study of the pathogenic mechanisms of laryngeal and voice diseases.

Keywords

laryngeal epithelial cells, immortal, Bmi1

Introduction

Laryngeal and voice diseases are progressively becoming more common and can have severe impacts on the quality of life of patients[1,2]. Although laryngeal and voice disorders are associated with a variety of pathogenic factors, such as alcohol, smoking, human papillomavirus (HPV), and laryngopharyngeal reflux (LPR)[3–5], the exact mechanism governing pathogenesis is not fully understood. Moreover, the incidence of different pathogenic factors is not the same for the entire laryngeal cavity. HPV mainly infects the vocal cords, ventricular folds, and epiglottic larynx, but an infection in the subglottic region is rare[6]. In contrast, the subglottic mucosa was the most susceptible to LPR injury and the posterior commissure the least[7]. Therefore, to explore the different pathogenic factors in laryngeal and voice disorders comprehensively, an analysis of the different laryngeal regions is necessary.

In our previous study, we identified and cultured laryngeal epithelial cells from different areas of the laryngeal cavity successfully, namely, the postcricoid region, the laryngeal surface of the epiglottis, the subglottis, and the

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ventricular zone[8]. Primary cells can maintain the basic properties of in vivo cells, thus allowing for the study of molecular biological mechanisms to improve treatment. However, primary cells undergo a limited number of cell divisions in culture before they enter a nondividing state known as "senescence," thus adding some limitations for cell research[9]. We also found that primary laryngeal epithelial cells from the fourth generation became largely senescent, cell volume increased, morphological changes occurred, cell proliferation was attenuated, and cellular death increased. This means that it would be difficult to obtain enough cells for basic research on laryngeal and voice diseases.

On the other hand, senescent cells display different expression patterns of important genes such as those encoding p15, p16, p18, p19, p21, p27, and p57[10,11]. This leads to contradictory results between experiments conducted with different batches of cells, even without stimulation. Therefore, to further study the mechanism of laryngeal and voice disorders, we aimed to construct a laryngeal immortalized epithelial cell line and establish an improved cell model.

The first two immortalized laryngeal epithelial cell lines were established by the exogenous introduction of a lentivirus-encoding HPV E6/E7[12] or simian virus 40 (SV40)[13]. The cells immortalized by these methods have proven to be reliable due to their stable genetic background[14,15]. However, the direct action of exogenous oncogenic viruses and their indirect effect on creating aneuploid cells influence cellular behavior, such as limiting the cell's ability to differentiate, among other abnormalities. Fulcher[16] found that air–liquid interface (ALI) cultures of HPV E6/E7 immortalized bronchial epithelial cells were relatively poor at forming tight hydrostatic and electrical resistance barriers. When grown in an ALI, bronchial cells immortalized by SV40 formed a multilayered hyperplastic and abnormal epithelium that resembled carcinoma in situ. Douillard-Guilloux[17] found that immortalized myoblasts transfected with SV40 lost their ability to differentiate into myotubes. Thus, viral oncogene-independent approaches for cell immortalization have been sought, including the expression of Bmi1.

The Bmi1-encoding gene is an important part of the multi-comb gene family (a key epigenetic regulator) and involved in the proliferation, differentiation, and senescence of cells[18]. A variety of immortalized human epithelial cells[19–21] and non-epithelial cells[22,23] have been constructed using the Bmi1 gene. Furthermore, primary cells immortalized by Bmi1 can avoid molecular changes induced by HPV E6/E7 and SV40 viral oncogenes[24] and maintain its differentiation ability[25]. Therefore, the purpose of our study was to introduce Bmi1 into subglottic cells dominated by stratified squamous epithelium and ventricular zone epithelial cells dominated by columnar cells to develop an immortalized laryngeal epithelial cell line that will provide a more accurate cell model for mechanistic studies of laryngeal and voice disorders.

Materials and Methods

Cell Culture

This study was approved by the Southern Medical University Nanfang Hospital Institutional Review Board (No. NFEC-201607-K2-01). Specimens of subglottic and ventricular fold tissue were obtained during laryngeal surgery. Primary laryngeal epithelial cells were cultured and passaged, as previously described[4]. In brief, minced tissue was dissociated with dispase II (Sigma-Aldrich, St Louis, MO, USA) and successively treated with trypsin (Life Technologies, Carlsbad, CA, USA). Cells were cultured in serum-free keratinocyte media KGM™-2 (LONZA, Basel, Switzerland). A 293FT cell line (Cell Bank of Chinese Academy of Science in Shanghai, China) was cultured in Dulbecco's modified Eagle medium/high glucose medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco).

Construction of Plasmids and Stable Transductions

To obtain a laryngeal immortalized epithelial cell line overexpressing Bmi1, pre-Bmi1 was subcloned into the pLVTHM lentiviral vector; the plasmid was named plVTHM/Bmi1 and contained both the green fluorescent protein (GFP) and puromycin resistance markers. 293FT cells (1.5×10^6 cells/well) were cultured in 10-cm culture plates, and when they reached approximately 50% confluency, they were cotransfected with a mixture of 16 μg plVTHM/Bmi1, 12 μg psPAX2, and 6 μg pMD2.G in 17 μl Lipofectamine 3000™ (Invitrogen, Carlsbad, CA, USA) along with 1,000 ml of Opti-MEM (Gibco). The medium was changed after 8 h. After 72 h, viral supernatants were collected and used to infect primary subglottic and ventricular fold epithelial cells directly. Viral solution and KBM-2 medium (1:1) were added to petri dishes containing cells for 5 h, followed by virus-free KBM-2 medium for 5 h; this process was repeated three times. The transduced cells were selected by detecting GFP-expressing cells by flow cytometry (BD Bioscience, San Diego, CA, USA) and observed under a fluorescent microscope.

Extraction of Total RNA and Quantitative Reverse Transcription-Polymerase Chain Reaction (PCR)

Total RNA was extracted from cells using RNAiso Plus (Takara, Shiga, Japan) and reverse transcribed into complementary DNA (cDNA) using an All-in-One first-strand cDNA synthesis kit (GeneCopoeia Inc., Rockville, MD, USA). The expression of Bmi1 and control β-actin was determined by PCR amplification using the SYBR green method (GeneCopoeia Inc.) (primers of Bmi1 and β-actin are listed in Table 1).

Table 1. Targets of Bmi1 and β-Catenin Predicted by Bioinformatic Prediction.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Bmi1	TGGACTGACAAATGC TGGAGA	GAAGATTGGTGGTT ACCGCTG
β-catenin	GACCAGCTCTCTCTT CAGAACAGA	GTTCTCCCTGGGCA CCAA TA

Western Blot Analysis

Total protein was extracted from cells and measured using a bicinchoninic acid protein assay kit (Life Technologies). Protein samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then were transferred to polyvinylidene difluoride membranes (Millipore Corp, Billerica, MA, USA). Membranes were incubated overnight at 4°C with a primary antibody targeting Bmi1, p16, p21, p53, Rb, c-Myc, and Claudin-1(1:1,000; Cell Signaling Technology, Danvers, MA, USA) and anti-pan cytokeratin (1:1,000; Abcam, Cambridge, MA, USA). An antibody targeting glyceraldehyde 3-phosphate dehydrogenase and tubulin (1:10,000; Kangcheng Inc, Shanghai, China) was used as an internal control.

Cellular Senescence Staining

Detection of senescence was carried out using a senescence-associated β-galactosidase staining kit (Beyotime Biotechnology, Haimen, China). Cells were cultured with the mixture overnight at 37°C in a 24-well plate and analyzed using digital microscopy (Olympus, Tokyo, Japan).

5-Ethynyl-2′-Deoxyuridine (EdU) Proliferation Assay

Cell proliferation was detected using an EdU kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. Images were analyzed using digital microscopy (Olympus), and cells were counted using Image J software.

Cell-cycle Experiment

Cell-cycle distribution was analyzed using a cell-cycle detection kit (Keygentec, Nanjing, China) and flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using the ModFit LT v3.0 software (Verity Software House, Topsham, ME, USA).

Tumorigenesis in Nude Mice

Ten female BALB/C nude mice (4- to 5-week-old) were purchased from the Centre of Laboratory Animals of the Southern Medical University. Animal protocols were approved by the Institutional Animal Care and Use Committee of Southern Medical University. The 10 nude mice were randomly divided into 2 groups (subglottic and ventricular fold). Cells (1×10^6) were injected into the left armpit [human subglottic (hSG) or human subglottic (hSG) and human ventricles (hV) cells], left groin (hSG-Bmi1 or hV-Bmi1 cells), and right groin (Hep-2 laryngeal cancer cells) of each mouse. Tumor size was measured every other day. Twenty-eight days later, tumor formation in the nude mice was photographed by magnetic resonance (MR) and a camera. The experimental animals were sacrificed, and the tumor tissue was dissected.

Statistical Analysis

The SPSS software (version 19.0; IBM, Armonk, NY, USA) was used for statistical analysis. Two-tailed Student's *t*-test was used for comparison of two independent groups.

Results

The subglottic tissue was taken from a 63-year-old male patient who underwent total laryngectomy on August 24, 2018. The ventricular zone tissue was taken from a 52-year-old male patient who underwent total laryngectomy on March 18, 2018. The tissue that was obtained had no canceration as determined by pathological examination. hSG and hV cells were oval-shaped and grew like "paving stone," representing characteristic epithelial cell morphology. The cells were passaged successfully (Fig. 1A). After lentiviral transduction of Bmi1 into hSG and hV cells for 72 h, green fluorescence was observed under the inverted fluorescence microscope. GFP-positive cell lines were obtained by flow cytometry. The fluorescence ratios of hSG and hV cells before sorting were 10.5% and 11.4%, respectively; the purity after sorting was more than 99% (Fig. 1B). Specifically, levels of Bmi1 mRNA in the hSG-Bmi1 and hV-Bmi1 cells increased 28.95-fold and 21-fold, respectively ($P = 0.16$ and $P = 0.15$, Fig. 1C). A western blot analysis confirmed that Bmi1 proteins were expressed in the hSG-Bmi1 and hV-Bmi1 cells, whereas lower levels were detected in hSG and hV cells. The expression of cytokeratin and tight junction proteins Claudin-1 in the hSG-Bmi1 and hV-Bmi1 cells did not change significantly compared with hSG and hV cells (Fig. 1D).

hSG-Bmi1 and hV-Bmi1 cells were expanded and cultured successfully beyond 20 passages. Senescence-associated β-galactosidase staining (SA-β-gal staining) showed that the number of senescent subglottic (hSG-P4) and ventricular zone (hV-P4) epithelial cells after passage 4 were significantly higher than that of hSG-P2 and hV-P2 cells, especially for the hV cells (Fig. 2A). The Edu assay showed that the proliferation rate of hSG cells decreased from 26% in generation P2 to 3% in generation P4 ($P = 0.073$). hV cells also decreased significantly, from 29% in generation P2 to 4% in generation P4 ($P < 0.033$). After introduction of Bmi1, the proliferation rate of hSG-Bmi1-P5 increased to 32%, which was significantly higher than hSG-P4 ($P < 0.001$). There was no significant difference

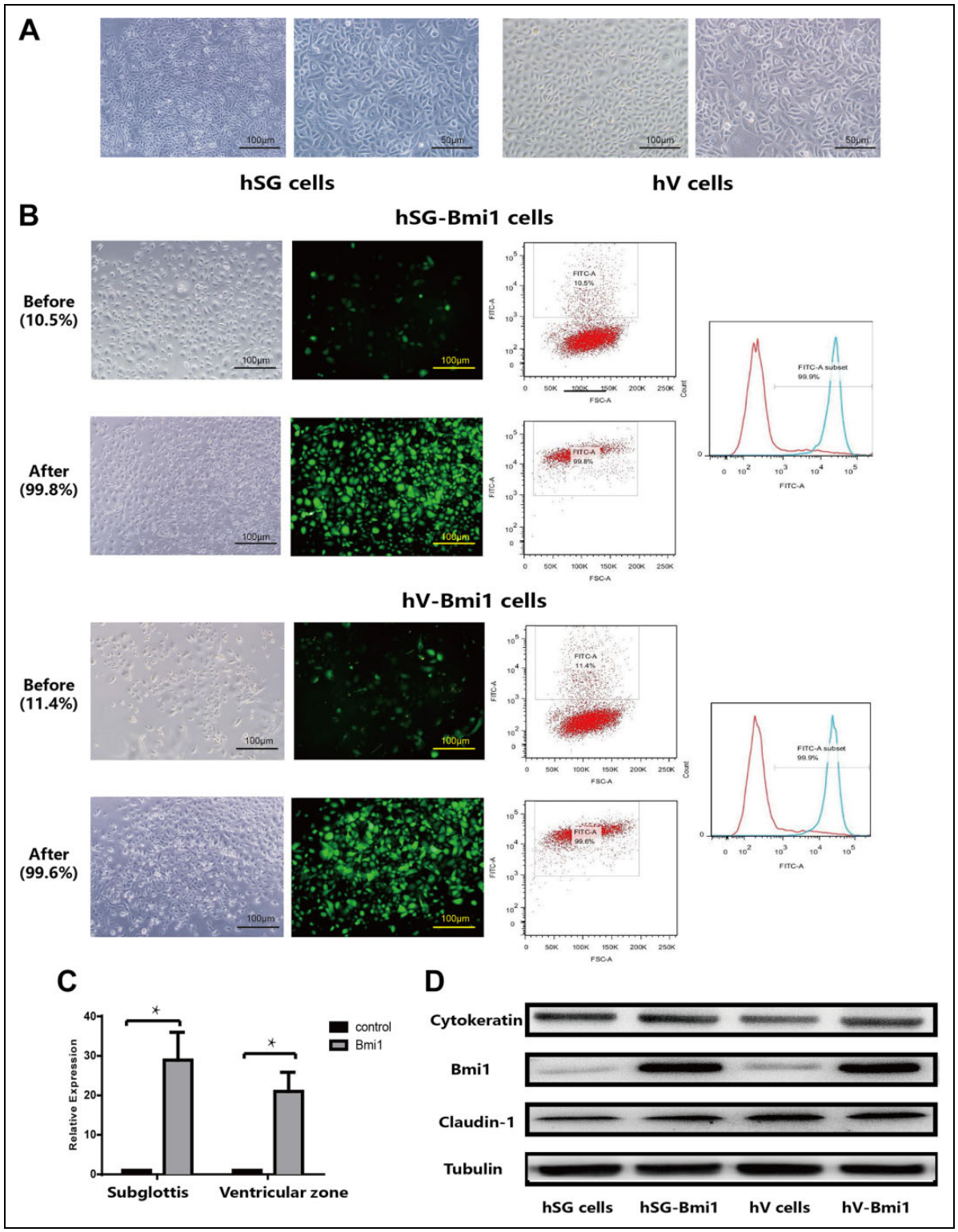

Fig. 1. (A) Morphology of primary subglottic and ventricular fold epithelial cells (magnification, ×100, bar 100 μm, magnification, ×200, bar 50 μm). (B) GFP indicates green fluorescent protein in subglottic and ventricular fold epithelial cells, and flow cytometry sorting after lentivirus infection (magnification, ×100, bar 100 μm). (C) The expression levels of Bmi1 in hSG-Bmi1 and hV-Bmi1 cells using quantitative real-time polymerase chain reaction. (D) Expression of Bmi1, cytokeratin, and Claudin-1 in hSG-Bmi1 and hV-Bmi1 cells analyzed using western blotting.

GFP: green fluorescent protein; hSG: human subglottic; hSG-Bmi1: human immortalized subglottic Bmi1; hV: human ventricle; hV-Bmi1: human immortalized ventricle Bmi1.

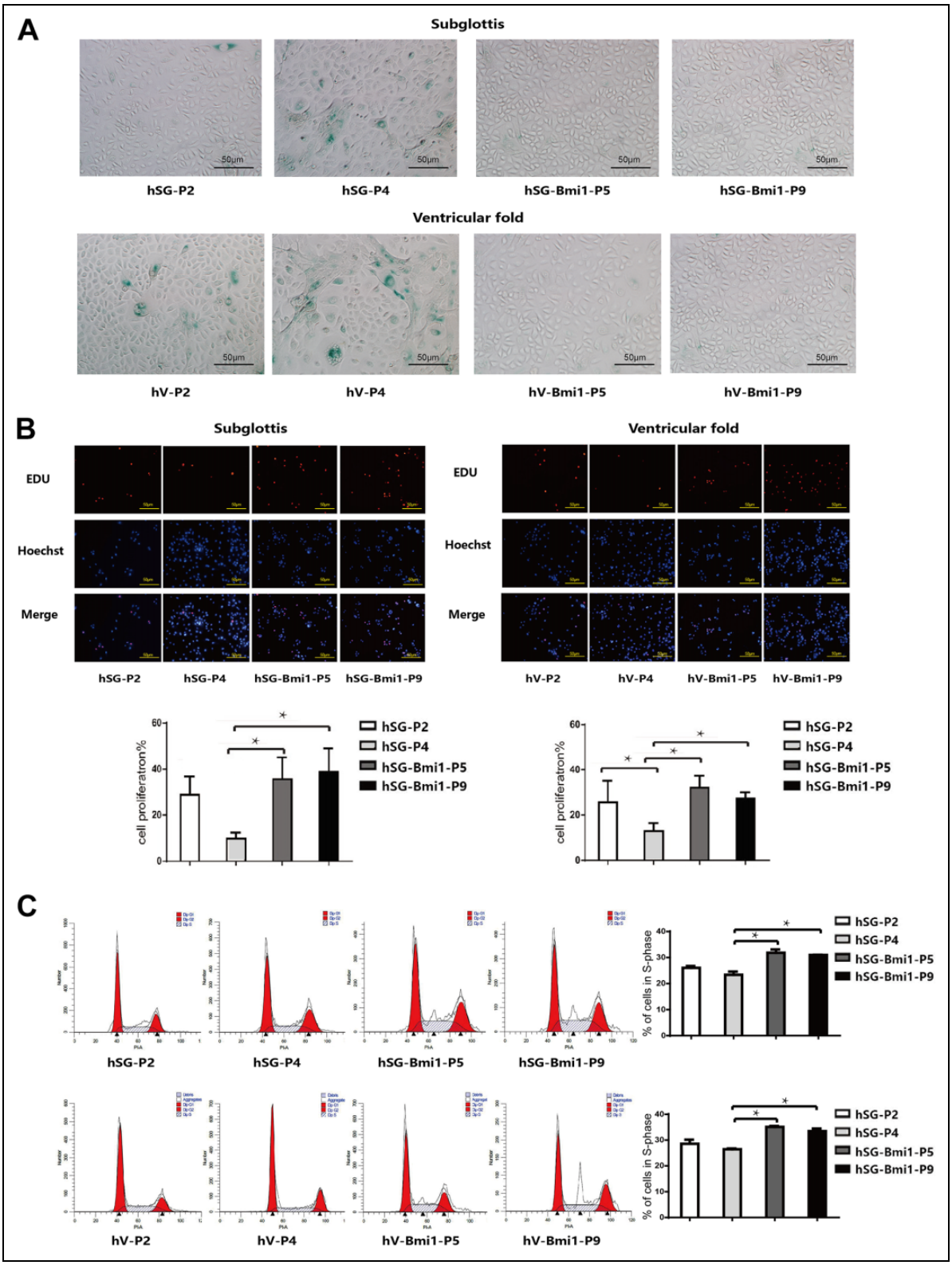

Fig. 2. (A) Effects of Bmi1 overexpression on senescence of subglottic and ventricular fold epithelial cells measured using SA-β-gal staining (magnification, ×200, bar 50 μm). (B) Effects of Bmi1 overexpression on proliferation of subglottic and ventricular fold epithelial cells measured using 5-ethynyl-2′-deoxyuridine assays (magnification, ×200, bar 50 μm). (C) Representative histograms of cell-cycle assays showing the percentage of subglottic and ventricular fold epithelial cells in S phase of Bmi1 overexpression.
EdU: 5-Ethynyl-2'-Deoxyuridine; hSG: human subglottic; hSG-Bmi1: human immortalized subglottic Bmi1; hV: human ventricle; hV-Bmi1: human immortalized ventricle Bmi1.

between hSG-Bmi1-P5 and hSG-Bmi1-P9. The proliferation rate of hV-Bmi1-P5 was 39%, which was significantly higher than that of hV-P4 ($P < 0.001$). The proliferation rate of hV-Bmi1-P9 was 31% (Fig. 2B). Cell-cycle assays showed that the percentage of cells in the S-phase of hSG-P4 and hV-P4 cells (26.44% and 23.37%, respectively) was lower than that of hSG-P2 and hV-P2 cells (28.57% and 26.06%, respectively) ($P = 0.21$ and $P = 0.119$). After transfection with Bmi1, the proportion of S phase cells in hSG-Bmi1-P5 and hV-Bmi1-P5 cells (35.12% and 31.86%, respectively) was significantly higher than that of hSG-P4 and hV-P4 cells ($P = 0.001$ and $P = 0.021$, Fig. 2C).

The expression of human telomerase reverse transcriptase (hTERT) in hSG, hV, hSG-Bmi1-P5, hV-Bmi1-P5, hSG-Bmi1-P9, and hV-Bmi1-P9 cells was detected by western blotting. The expression of hTERT in hSG-Bmi1 and hV-Bmi1 cells was significantly increased (Fig. 3A). The expression of Bmi1 in hSG-Bmi1 and hV-Bmi1 cells was significantly increased and maintained, while expression of p53 did not change significantly; moreover, the expression of p16 and p21 was downregulated. The expression of generation P2 phosphorylated Rb was downregulated in hSG-P4 and hV-P4 cells, and the expression of phosphorylated Rb were increased and maintained stable after Bmi1 transfection (Fig. 3B).

To determine whether Bmi1 could affect tumor growth in vivo, we implanted hSG, hV, hSG-Bmi1, hV-Bmi1, and Hep-2 cells (positive control) subcutaneously in nude mice ($n = 5$ per group). After 5 days, tumor formation was observed in the Hep-2 injection area; the tumor increased in size significantly after 10 days. No cellular masses were found in hSG, hV, hSG-Bmi1, and hV-Bmi1 cells at the injection site. After 28 days, a few tumors in the right inguinal region of nude mice were ruptured. MR imaging showed that only the right groin (Hep-2 cells) had mass formation, and no mass was found in the left armpit (hSG or hV cells) or left groin (hSG-Bmi1 or hV-Bmi1 cells). The mice were sacrificed and dissected further to confirm this. The only tumor that formed was located in the right inguinal region; no other formations were found. The tumor was further stripped and photographed (Fig. 3C).

Discussion

For the first time, two strains of immortalized laryngeal epithelial cells, hSG-Bmi1 and hV-Bmi1, have been successfully constructed by Bmi1 transduction. These cells overcame the lifetime limitation of stably passaging primary cells. While the number of senescent cells was reduced significantly, cell proliferation was increased. As such, they provide a stable, reliable, and efficient cell research model for studying the pathogenic mechanism of laryngeal and voice disorders *in vitro*. The classical method of cell immortalization comes from the "telomere-telomerase" theory. Telomeres are protective structures located at the end of chromosomes. They reduce the chances of producing potential carcinogenic mutations in DNA due to cell division by limiting the number of cell divisions[26]. Telomeres gradually shorten with each round of cell division[27]. When telomeres are shortened to a certain length, they activate DNA damage response pathways and trigger replication senescence[28]. To bypass this, most cancers activate telomerase and extend telomeres by a reverse transcriptional synthesis of DNA to avoid death and gain unlimited proliferation[29]. Telomerase is a ribonucleoprotein complex containing an RNA component and an essential protein catalytic subunit with a reverse transcriptase motif. Human Telomerase Reverse Transcriptase (hTERT) is the only limiting factor of telomerase activity in most normal cells. The upregulation of hTERT expression is the most common means by which cells can replicate indefinitely[30,31]. We found that the expression of hTERT in hSG-Bmi1 and hV-Bmi1 cells was significantly enhanced. Bmi1 may, therefore, induce immortalization of laryngeal epithelial cells by activating telomerase.

Cultures of primary laryngeal epithelial cells are often contaminated by the proliferation of fibroblasts, which leads to culture failure. Although primary laryngeal epithelial cells were purified using KBM-2 medium, there may still be residual laryngeal fibroblasts. When culture conditions are conducive to the growth of fibroblasts, they proliferate in large numbers (supplemental Fig. S1). Fibroblasts can fundamentally be eliminated by transduction of Bmi1. It has been shown that the activation of telomerase by Bmi1 is cell type-specific. Bmi1 can only activate telomerase in epithelial-derived cells and does not induce telomerase activity in fibroblasts[20]. In our experiment, hSG-Bmi1 and hV-Bmi1 did not reproduce the growth of laryngeal fibroblasts.

The composition of laryngeal epithelial mucosa is complex and changeable. The vocal cords and the postcricoid area are dominated by stratified squamous epithelium. The subglottic and laryngeal surface of the epiglottic areas transitions from pseudostratified ciliated columnar epithelium to the scaly epithelium; nonetheless, the main cell type is columnar cell[32]. The incidence of different laryngeal and voice disorders varies in different anatomical areas of the laryngeal cavity due to histological composition and embryonic origin[7,33,34]. HPV infects basal cells of the stratified squamous epithelium through micro epithelial trauma but rarely does the infection enter through the columnar epithelium[35]. In addition, Bulmer[7] proposed that the squamous epithelial membrane can bind to mucus to form mucin and exfoliated cuticles to protect the laryngeal mucosa; therefore, the postcricoid region is less vulnerable to LPR than the subglottic mucosa. To elucidate the pathogenesis of different laryngeal inflammatory and malignant diseases in the laryngeal epithelium, it is necessary to study the subregions of the laryngeal cavity. Therefore, unlike previous studies, which immortalized cells from a single part of the larynx, we selected subglottic epithelial cells dominated by columnar cells and ventricular zone epithelial cells

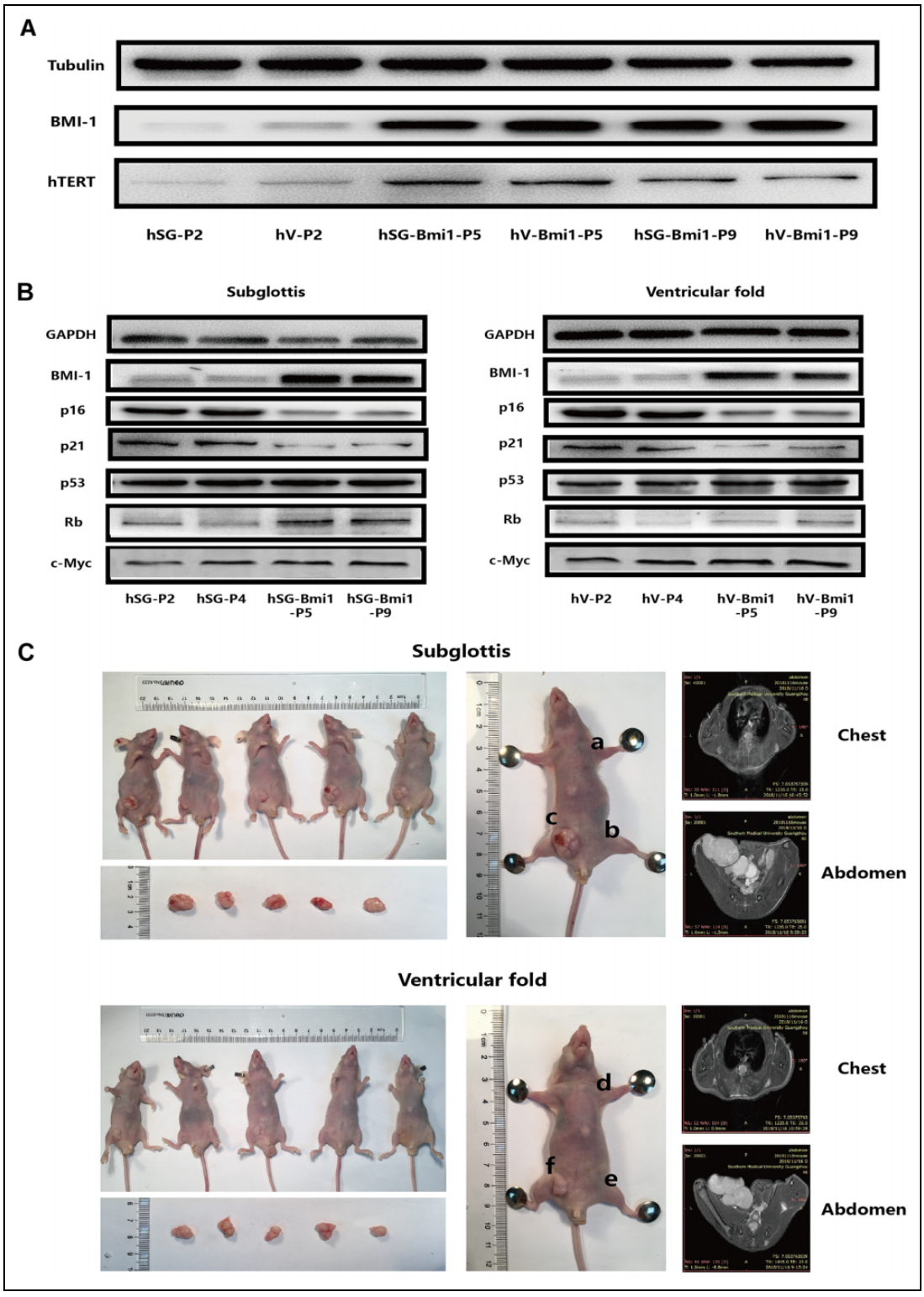

Fig. 3. (A) Expression of hTERT in hSG-Bmi1 and hV-Bmi1 cells using western blotting. (B) The expression level of the Bmi1 pathway protein in western blotting. (C) The effect of overexpression of Bmi1 on tumorigenesis of subglottic and ventricular fold epithelial cells (a. hSG cells; b. hSG-Bmi1 cells; c. Hep-2 cell lines; d. hV cells; e. hV-Bmi1 cells; f. Hep-2 cell lines).
hSG: human subglottic; hSG-Bmi1: human immortalized subglottic Bmi1; hTERT: human telomerase reverse transcriptase; hV: human ventricle; hV-Bmi1: human immortalized ventricle Bmi1.

dominated by squamous cells to establish immortalized laryngeal epithelial cell lines for follow-up studies of laryngeal and voice disorders.

Furthermore, we explored the senescence and proliferation of hSG-Bmi1 and hV-Bmi1 cells. After transduction of Bmi1, the senescence of laryngeal epithelial cells decreased significantly, and the proliferation of laryngeal epithelial cells was accelerated while the proportion of S-phase cells increased. We believe that the introduction of Bmi1 may promote DNA synthesis, cell-cycle differentiation from the G1 to the S phase, and cell proliferation by reducing the formation of senescent cells and enhancing cell activity. The cell cycle depends on four-cycle checkpoints, G1/S transition, S-phase checkpoint, G2/M transition, and mitotic spindle checkpoint, in which the transition from the G1 to the S phase is controlled by the p16-Rb pathway[36,37]. Furthermore, the effect of stress-induced senescence induced by the p16/Rb pathway was detected by western blotting. The expression of p16 increased, and phosphorylated Rb decreased as the number of passages increased, suggesting that the p16/Rb senescence pathway was activated. The activation of the p16/Rb pathway may be due to the loss of dependent tissue structure, cell fragility of primary laryngeal epithelial cells, and the abnormal levels of nutrients, growth factors, and oxygen *in vitro*, which led to cell stress and the induction of senescence and cellular death[38]. After transfection of Bmi1, hSG-Bmi1 and hV-Bmi1 cells may upregulate telomerase activity, block the expression of p16, enhance Rb, significantly reduce the formation of senescent cells, promote the differentiation of laryngeal epithelial cells from the G1 to the S phase, and accelerate the growth and proliferation of laryngeal epithelial cells. Moreover, we found that after transfection of Bmi1, laryngeal epithelial cells retained their epithelial and tight junction characteristics.

Because Bmi1 is an oncogene, we were concerned about whether it can induce malignant transformation of laryngeal epithelial cells after Bmi1 transduction. Therefore, we observed the potential for tumor formation of hSG-Bmi1 and hV-Bmi1 cells and the primary laryngeal epithelial cells in nude mice. We found that there was no tumor formation in any area apart from where Hep-2 cells were implanted, suggesting that primary laryngeal epithelial cells and laryngeal epithelial immortalized cells had no tumorigenic ability. Indeed, this was consistent with what was observed in previous literature. Zhang et al.[22] transduced human placenta-derived mesenchymal cells with Bmi1 not forming any foci *in vitro*, and cell division stopped after they reached confluence. This may be because that the overexpression of Bmi1 does not eliminate DNA damage checkpoints[20]. Although the Bmi1 gene is an oncogene, which is highly expressed in many malignant tumors and acts in coordination with other carcinogenic factors to promote the malignancy, primary laryngeal epithelial cells transfected with Bmi1 alone do not have the characteristics of tumor formation in vivo and could be clearly distinguished from laryngeal cancer cells.

Conclusion

In summary, primary laryngeal epithelial cells grow slowly and cannot be passaged continuously; so we established and verified two types of immortalized laryngeal epithelial cells by activating telomerase activity in primary laryngeal epithelial cells via transduction of Bmi1. Due to differences in pathogenic resistance between laryngeal subregions, using hSG-Bmi1 and hV-Bmi1 cells to explore the mechanism of laryngeal and voice diseases is more comprehensive, and has broad applications.

Acknowledgments

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Ethical Approval

Ethical approval to report this case was obtained from Southern Medical University Nanfang Hospital Institutional Review Board (No. NFEC-201607-K2-01).

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Nanfang Hospital Animal Ethic Committee approved protocols (No. NFYY-2018-95).

Statement of Informed Consent

Informed consent was obtained from all individual participants included in the study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

References

1. Naunheim MR, Goldberg L, Dai JB, Rubinstein BJ, Courey MS. Measuring the impact of dysphonia on quality of life using health state preferences. Laryngoscope. 2020;130(4): E177–E182.
2. Branski Ryan C, Sabrina C-K, Pusic A, Cano Stefan J, Klassen A, Mener D, Patel S, Kraus DH. Measuring quality of life in dysphonic patients: a systematic review of content

development in patient reported outcomes measures. J Voice. 2010;24(2):193–198.
3. Derkay CS, Wiatrak B. Recurrent respiratory papillomatosis: a review. Laryngoscope. 2008;118(7):1236–1247.
4. Wang L, Tan JJ, Wu T, Zhang R, Wu JN, Zeng FF, Liu YL, Han XY, Li YF, Li XP. Association between laryngeal pepsin levels and the presence of vocal fold polyps. Otolaryngol Head Neck Surg. 2017;156(1):144–151.
5. Tan JJ, Wang L, Mo TT, Wang J, Wang MG, Li XP. Pepsin promotes IL-8 signaling-induced epithelial–mesenchymal transition in laryngeal carcinoma. Cancer Cell Int. 2019; 19(Mar 20):64.
6. Wang HK, Duffy AA, Broker TR, Chow LT. Robust production and passaging of infectious HPV in squamous epithelium of primary humankeratinocytes. Genes Dev. 2009;23(2): 181–194.
7. Bulmer DM, Ali MS, Brownlee IA, Dettmar PW, Pearson JP. Laryngeal mucosa: its susceptibility to damage by acid and pepsin. Laryngoscope. 2010;120(4):777–782.
8. Mo TT, Tan JJ, Wang MG, Dai YF, Liu X, Li XP. Optimized generation of primary human epithelial cells from larynx and hypopharynx: a site-specific epithelial model for reflux research. Cell Transplant. 2019;28(5):630–637.
9. Shay JW, Wright WE. Hayflick, his limit, and cellular ageing. Nat Rev Mol Cell Biol. 2000;1(1):72–76.
10. Luo C, Zhou S, Zhou Z, Liu Y, Yang L, Liu J, Zhang Y, Li H, Liu Y, Hou FF, Zhou L. Wnt9a promotes renal fibrosis by accelerating cellular senescence in tubular epithelial cells. J Am Soc Nephrol. 2018;29(4):1238–1256.
11. Pei XH, Xiong Y. Biochemical and cellular mechanisms of mammalian CDK inhibitors: a few unresolved issues. Oncogene. 2005;24(17):2787–2795.
12. Lee SH, Samuels T, Bock JM, Blumin JH, Johnston N. Establishment of an immortalized laryngeal posterior commissure cell line as a tool for reflux research. Laryngoscope. 2015; 125(2):E73–E77.
13. Powell J, Verdon B, Wilson JA, Simpson AJ, Pearson J, Ward C. Establishment of an immortalized human subglottic epithelial cell line. Laryngoscope. 2019;129(11):2640–2645.
14. Olyslaegers DA, Desmarets LM, Dedeurwaerder A, Dewerchin HL, Nauwynck HJ. Generation and characterization of feline arterial and venous endothelial cell lines for the study of the vascular endothelium. BMC Vet Res. 2013;9:170.
15. Wu LA, Feng J, Wang L, Mu YD, Baker A, Donly KJ, Gluhak-Heinrich J, Harris SE, MacDougall M, Chen S. Immortalized mouse floxed Bmp2 dental papilla mesenchymal cell lines preserve odontoblastic phenotype and respond to BMP2. J Cell Physiol. 2010;225(1):132–139.
16. Fulcher ML, Gabriel SE, Olsen JC, Tatreau JR, Gentzsch M, Livanos E, Saavedra MT, Salmon P, Randell SH. Novel human bronchial epithelial cell lines for cystic fibrosis research. Am J Physiol Lung Cell Mol Physiol. 2009;296(1):L82–L91.
17. Douillard-Guilloux G, Mouly V, Caillaud C, Richard E. Immortalization of murine muscle cells from lysosomal alpha-glucosidase deficient mice: a new tool to study pathophysiology and assess therapeutic strategies for Pompe disease. Biochem Biophys Res Commun. 2009;388(2): 333–338.
18. Haupt Y, Alexander WS, Barri G, Klinken SP, Adams JM. Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice. Cell. 1991;65(5):753–763.
19. Song LB, Zeng MS, Liao WT, Zhang L, Mo HY, Liu WL, Shao JY, Wu QL, Li MZ, Xia YF, Fu LW, et al. Bmi-1 is a novel molecular marker of nasopharyngeal carcinoma progression and immortalizesprimary human nasopharyngeal epithelial cells. Cancer Res. 2006;66(12):6225–6232.
20. Dimri GP, Martinez JL, Jacobs JJ, Keblusek P, Itahana K, Van Lohuizen M, Campisi J, Wazer DE, Band V. The Bmi1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. Cancer Res. 2002;62(16): 4736–4745.
21. Kim RH, Kang MK, Shin KH, Oo ZM, Han T, Baluda MA, Park NH. Bmi1 cooperates with human papillomavirus type 16 E6 to immortalize normal human oralkeratinocytes. Exp Cell Res. 2007;313(3):462–472.
22. Zhang X, Soda Y, Takahashi K, Bai Y, Mitsuru A, Igura K, Satoh H, Yamaguchi S, Tani K, Tojo A, Takahashi TA. Successful immortalization of mesenchymal progenitor cells derived from human placenta and the differentiation abilities of immortalized cells. Biochem Biophys Res Commun. 2006; 351(4):853–859.
23. Tátrai P, Szepesi Á, Matula Z, Szigeti A, Buchan G, Mádi A, Uher F, Német K. Combined introduction of Bmi1 and hTERT immortalizes human adipose tissue-derived stromal cells with low risk of transformation. Biochem Biophys Res Commun. 2012;422(1):28–35.
24. Kong QL, Guan S, Guo BH, Zeng MS. Pre-malignant nasopharyngeal epithelial cell models. Ai Zheng. 2009;28(10): 1012–1015.
25. Cudré-Mauroux C, Occhiodoro T, König S, Salmon P, Bernheim L, Trono D. Lentivector-mediated transfer of Bmi-1 and telomerase in muscle satellite cells yields a duchennemyoblast cell line with long-term genotypic and phenotypic stability. Hum Gene Ther. 2003;14(16): 1525–1533.
26. Samassekou O, Gadji M, Drouin R, Yan J. Sizing the ends: normal length of human telomeres. Ann Anat. 2010;192(5): 284–291.
27. Houghtaling BR, Cuttonaro L, Chang W, Smith S. A dynamic molecular link between the telomere length regulator TRF1 and the chromosome end protector TRF2. Curr Biol. 2004; 14(18):1621–1631.
28. von Zglinicki T, Saretzki G, Ladhoff J, d'Adda di Fagagna F, Jackson SP. Human cell senescence as a DNA damage response. Mech Ageing Dev. 2005;126(1):111–117.
29. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer. 1997;33(5):787–791.
30. Belgiovine C, Chiodi I, Mondello C.Telomerase cellular immortalization and neoplastic transformation. Multiple

functions of a multifaceted complex. Genome Res. 2008; 122(3–4):255–262.
31. Shay JW, Wrisht WE. Senescence and immortalization: role of telomeres and telomerase. Carcinogenesis. 2005;26(5):867–874.
32. Tucker J, Vidic B, Tucker GF, Stead J. Survey of the development of laryngeal epithelium. Ann Otol Rhinol Laryngol. 1976;85(5 Suppl 30 PT 2):1–16.
33. De Stefani E, Boffetta P, Deneo-Pellegrini H, Brennan P, Correa P, Oreggia F, Ronco AL, Mendilaharsu M. Supraglottic and glottic carcinomas: epidemiologically distinct entities? Int J Cancer. 2004;112(6):1065–1071.
34. Johnston N, Knight J, Dettmar PW, Lively MO, Koufman J. Pepsin and carbonic anhydrase isoenzyme III as diagnostic markers for laryngopharyngeal reflux disease. Laryngoscope. 2004;114(12):2129–2134.
35. Herfs M, Hubert P, Moutschen M, Delvenne P. Mucosal junctions: open doors to HPV and HIV infections? Trends Microbiol. 2011;19(3):114–120.
36. Ruas M, Peters G. The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta. 1998;1378(2): 115–177.
37. Rayess H, Wang MB, Srivatsan ES. Cellular senescence and tumor suppressor gene p16. Int J Cancer. 2012;130(8): 1715–1725.
38. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. Genes Dev. 2010;24(22):2463–2479.