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Comparison and improvement in primary airway fibroblast culture across different mammalian species

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Abstract

This study aims to establish rabbit, rat and human models of primary airway fibroblasts, improve existing culture methods of human, and provide alternatives to benign airway stenosis *in vitro*. We used conventional «tissue adherent method» to culture airway primary fibroblasts extracted from New Zealand rabbits, Sprague-Dawley (SD) rats and human subjects. To improve quality of this culture, we combined the «tissue adherent method» with « trypsinization", and compared the success rate of the two approaches. Cultures were examined using an inverted microscope, following hematoxylin-eosin and immunohistochemical staining. The different species were identified based on the total number of chromosomes. We successfully cultured primary airway fibroblasts isolated from three species. Human airway primary fibroblasts are more difficult to culture. The efficiency of culture is low, when using the «tissue adherent method». However, the rate of successful culture is improved when combined with the "trypsinization", and by using the «serum adherent, organizing tablet» technology. In conclusion, we demonstrate that primary airway fibroblasts from three mammalian species can be cultured successfully *in vitro*, for a reliable cellular model of benign airway stenosis. Culturing human primary airway fibroblasts is technically more challenging than the other two species. It is necessary to improve it.

Key words: Primary airway fibroblasts, serum adherent, tissue tablet, benign airway stenosis.

Introduction

Airway granulation is one of the factors responsible for delayed healing of benign airway stenosis. Currently, tracheal endoscopic intervention is the most common method of treating this condition, but it often leads to hyperplasia. Airway granulation, followed by repeated illness, makes benign airway stenosis clinically intractable (1-4). Therefore, there is an urgent need to explore new and alternative methods of treatment. Because in the majority of cases, the narrowing of airways is caused by excessive proliferation of granulation tissue, which is mostly composed of fibroblasts, one possible solution could involve suppressing the proliferation of these fibroblasts. Elucidating the mechanism underlying the pathological expansion of fibroblasts could be key to developing an effective therapy against benign airway stenosis. To do so, it is necessary to establish reliable and robust primary cultures of airway fibroblasts. These could then be used to develop cellular models of benign airway narrowing for *in vitro* experiments (5,6).

Fibroblasts have strong regenerative capacity, and hence can be cultured *in vitro*. While airway fibroblasts are common in animals such as horses and mice (7,8), in humans they occur in small numbers. Human fibroblast cultures have been established from skin, gums, lungs, heart and other organs (9-13), and cultured in single adherent organization (14). In the present study, primary airway fibroblasts from rabbits, SD rats and humans were cultured and compared. It is known that the lower the animals in the evolutionary tree, the stronger their ability to regenerate. Amphibian limb regeneration occurs flawlessly to restore lost tissues, while Mammalian wound often results in scarring (15). Hence, the regenerative capacity in humans is weak . This, in combination with limited patient tissue availability and the presence of airway infections in some patients, makes the culturing of human primary airway fibroblasts more difficult than analogous animal cells.

In this study, primary airway fibroblasts isolated from New Zealand rabbits and SD rats, were cultured using the conventional «tissue adherent method». In addition, human primary airway fibroblastswere digested enzymatically, and cultured using the tissue adherent method, and the «serum adherent, organizing tablet» technology. The aim of this study is to improve the success rate of *in vitro* culture of human primary airway fibroblasts and establish a robust cellular model of the same.

Primary cell cultures usually refer to cells within the first tenpassages, and exclude cryopreserved cells. Consequently, the absolute number of cells that can be used in an experiment is limited. Synchronized culture of animal cells is relatively easier and, therefore, can be used to optimize experimental conditions for human cellular models, including the duration and dosage of drug exposure. In addition, in the absence of suitable clinical samples to establish primary human airway fibroblast models, animal cells can be used as an alternative.

Materials and methods

Instruments and reagents

Clean bench (AIR TECH), cell incubator (SANYO), centrifuge (Shanghai medical device company), micro pipette (Eppendorf, Germany), inverted micro-

scope and imaging system (OLYMPUS Japan, Model - U-LH 100HG, Shanghai Instrument Factory). High glucose DMEM (Hyclone, USA), 25% trypsin -EDTA, fetal bovine serum (FBS) (Gibco Australia), Penicillin-Streptomycin (American MP Biomedicals), moxifloxacin (Bayer, Germany). Immunohistochemical primary antibodies:Vimentin (Maixin, mouse anti-pig monoclonal antibody, Catalogue number: MAB-0178), Cytokeratin (broad spectrum) (CK (Pan), (Maixin, mouse anti-human monoclonal antibody, Catalogue number: MAB-0671); corresponding secondary antibodies; Ready to use immunohistochemical Elivision plus kit, (Maixin, product number: KIT-9901).

Cell culture

Three month-old female rabbits were anesthetized with 10% chloral hydrate and propofol, the skin in the neck region was disinfected and prepared for surgery. An incision of approximately 5cm length was made along the neck, and the different layers of the skin were separated in order to expose the trachea. Tracheal tissue of approx 1 cm length was dissected and placed in sterile saline solution at 0-4°C, and immediately transferred to a clean tube. Next, the tracheal tissue was rinsed twice with saline and moxifloxacin alternatively, cut into 1-2 mm^2 pieces using sterile forceps and ophthalmic scissors. The tissue pieces were digested for 3 min with trypsin-EDTA and placed at the bottom of a six-well plate. The culture media of 20% FBS in high glucose DMEM was added slowly just enough to cover the tissue, so that the tissue blocks do not float away, since this will lead to failure of inoculation(Figure 1A). The medium was replaced gently every 3-5 days, while keeping track of the tissue and making sure that the tissue did not float. After 7-10 days of culture, primary cells could be seen growing out of tissue blocks. After 20 days the outgrowing cells were passaged by digesting using Trypsin-EDTA. Tracheal tissue from adult male SD rats was isolated, cultured and passaged in the same way as described above.

The study protocol was approved by the IRB of Second Affiliated Hospital of Fujian Medical University (approval No. 2015-007). With accepting unanimously by Second Affiliated Hospital of Fujian Medical University ethics committee. After obtaining informed consent, patients diagnosed with benign airway stenosis (due to endobronchial tuberculosis, airway foreign body, or following after intubation granulation) were subjected to a sterile biopsy procedure where their granulation tissue was extracted under bronchoscopy, placed in sterile saline solution at 0-4°C and immediately transferred to a clean tube. (Usually , as a treatment ,these granulations lead to tracheobronchial stenosis were gained by small biopsy forceps to discard) .Between April and October 2014, 35 patients were subjected to a strict screening procedure to determine if they qualified for extraction of granulation tissue for this study. Of these, 13 were deemed unqualified because of either necrotic membrane, blood clots or polluted tissue (inoculation in infection within 24-48 h). Of the remaining 22 samples that qualified for this study, two were cultured in two separate dishes due to their large size, a total of 24 times. Samples from eight patients were cultured using the conventional "tissue adherent method" (Figure 1A), in a

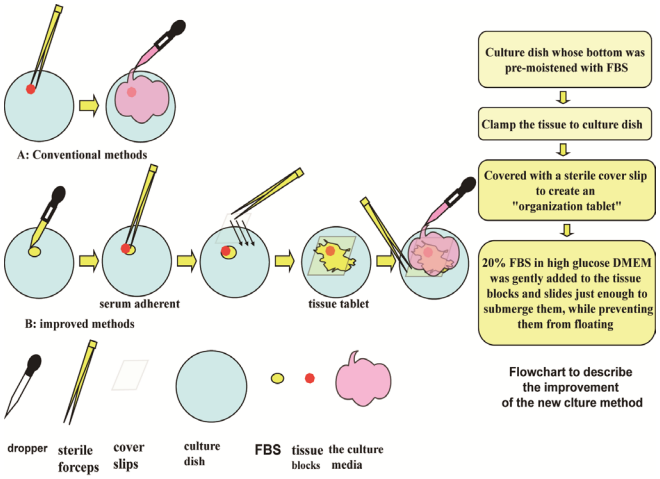

Figure 1. Flowchart to describe the improvement of the new clture method compare with the conventional method.

manner similar to the New Zealand rabbits and SD rats. Ten patient samples were cultured using a combination of «tissue adherent» and "trypsinization" methods. Details of the modified culture method are as follows: The granulation tissue was rinse twice with saline and moxifloxacin alternatively in order to remove as much blood as possible. It was then digested for 3 min with trypsin-EDTA, sterile forceps were used to clamp the tissue to a six-well plate whose bottom was pre-moistened with FBS and covered with a sterile cover slip to create an «organization tablet». The culture medium of 20% FBS in high glucose DMEM was gently added to the tissue blocks and slides just enough to submerge them, while preventing them from floating to avoid failure of inoculation (Figure 1B). The culture medium was replaced every 3-5 days, while keeping track of the tissue blocks and slides. After 10-14 days, primary cells could be seen growing out of the tissue blocks. These cells were adherent and grew on the bottom of the dish and cover slips. Slides were removed after 20-30 days in culture, and placed on another dish where they continued to grow for 5-7 days. The outgrowing cells were digested using trypsin-EDTA and cultured in medium containing 20% FBS in high glucose DMEM, which was also used to terminate the digestion. The cells thus detached were either left to grow in the same culture dish or transferred to a new dish. At a confluency greater than 80%, cells were passaged into 25 cm^2 flasks and cultured further. Passages 5-8 (P5-P8; not higher than P10) were selected for further experiments.

H&E staining

Cells from passage 3-4 were isolated from a 10 mm culture dish, placed on a sterile glass slide, allowed to grow out. The slides covered with cells were stained with hematoxylin and eosin (H&E) and the morphology of the cells was observed under the microscope.

Cryopreservation and recovery

Cells from passages 3-4 (P3-P4) were used for cryopreservation; they could be stored in liquid nitrogen for upto 1 year, and for less than 6 months at -80°C. When needed, they were thawed and plated for culture.

Purification and characterization of cells

In order to purify the primary airway cultures, P3 cells were cultured in medium containing 10-15% FBS.

While the fibroblasts in this population had a growth advantage, the proportion of other cells such as epithelial cells gradually reduced and eventually they disappeared from culture leading to a pure population of fibroblasts. The latter can also be purified by with trypsin using a method called "differential digestion".

Immunocytochemical staining to identify vimentin and cytokeratin. Tablets with outgrowing cells at P5-P6 were washed thrice with PBS, dried naturally for 3-4h, immersed in PBS for 30 min, and covered with non- immunogenic animal serum for 15 min. Next, they were washed thrice with PBS for 3 min per wash, and incubated with the primary antibodies, mouse anti-pig vimentin and rabbit anti-human keratin (Cytokeratin), overnight at 4°C. The negative control samples were incubated with non-immune serum only. The following day, samples were washed thrice with PBS for 3 min per wash, incubated with the appropriate secondary antibodies at room temperature (RT) for 15 min, washed thrice for 3 min each in PBS, and gently dried. Finally, to develop the antibody reaction, the samples were covered with DAB substrate for 15 min followed by hematoxylin for 15 s, and observed and imaged under the microscope .

Chromosomal characterization of different species

Healthy cultures of airway primary fibroblasts were treated with colchicine (to arrest cells in metaphase) at a final concentration of 2-3 μg/mL, and allowed to grow for 4-6 hours before terminating the culture. Next, they were digested with Trypsin-EDTA for 3-5 min, the reaction was terminated using culture medium, and the entire cell suspension was centrifuged. The cell pellet as resuspended in 0.2-0.3 mL pre-warmed (at 37°C) hypotonic solution (0.075 M KCl), pipetted and incubated in a water bath set at 37°C for 15 min. Next, the swollen cells were pre-fixed in 1.5 mL of a fixative comprised of methanol and acetic acid in a 3: 1 ratio. They were mixed gently and incubated in a water bath set at 37°C for 15 min. These fixed cells were centrifuged, and to the cell pellet thus obtained, 8ml fixative was added, gently mixed and incubated in the water bath at 37°C for 10 min. The cells were again centrifuged at RT, the supernatant was discarded leaving the cell pellet in approximately 0.5 mL secondary fixative. To this pellet, three to four drops of fresh fixative was added and resuspended. The suspension was then dropped onto a slide (3 drops per slide, and 1-2 drops per piece). After air drying, the morphology of the chromosomes was observed and their numbers were quantified under the microscope.

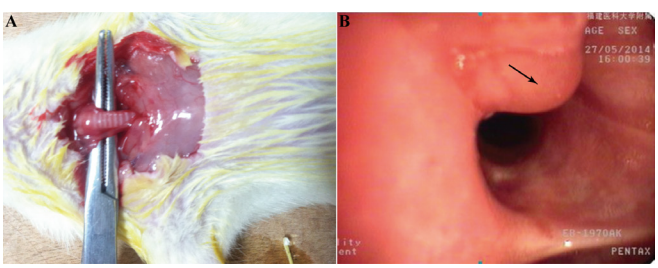

Figure 2. Primary fibroblast culture from tissues derived from the airway. (A) SD rat trachea. (B) granulation tissue from benign airway stenosis bronchoscopy. Arrow indicates granulation tissue.

Results

Isolation of primary fibroblast

In order to improve the primary airway fibroblast, we firstly isolated the primary primary fibroblast. In this study, the primary fibroblast isolated from the tissues derived from the airway. The isolated process was illustrated as the Figure 2A and 2B. In this way, the primary airway fibroblast was successfully established.

Outgrowth of primary airway fibroblasts and cell culture

In order to observe the primary airway fibroblast culture across different mammalian species, the outgrowth of primary airway fibroblast from inoculated tissue blocks of the New Zealand rabbits (Figure 3A), SD rats (Figure 3B) and human were examined (Figure 3C,D). From the Figure, we found that the outgrowth form of primary airway fibroblast in New Zealand rabbits and SD rats was similar.

In this study, the passaging and prolonged culture of primary airway fibroblasts isolated from New Zealand rabbits (Figure 4A), SD rabbits (Figure 4B) and human (Figure 4C,D), were also examined. Especially, for the human primary airway fibroblasts, which were isolated from two different types of benign airway stenosis. The results indicated taht the primary airway fibroblast cultures could be successfully passaged and maintained for a prolonged duration of time (Figure 4A to D).

H&E staining and immunocytochemical staining of passaged and purified

We performed HE and immunohistochemical staining of passaged and purified primary airway fibroblast cultures (Figure 5A to C, Figure 6A to C). Our assay shows that human primary airway fibroblasts that are vimentin-positive contain uniform brown granular particles in the cytoplasm. The cytokeratin- negative cells contained no brown particles. We ruled out the possibility that some cells in culture could be epithelial cells, because these are cytokeratin-positive, and we did not

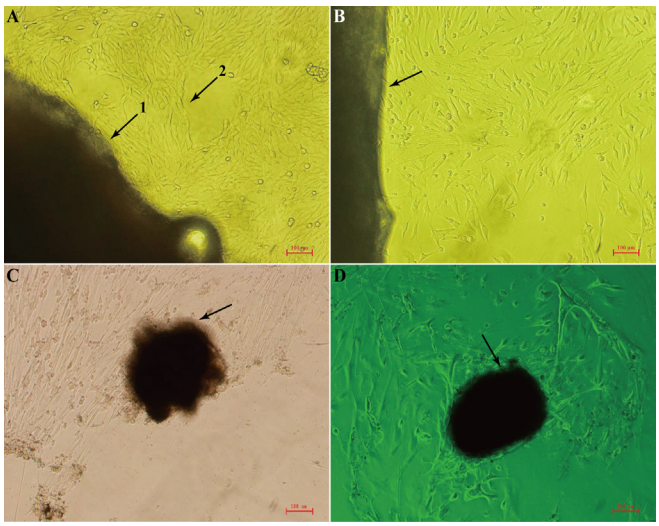

Figure 3. Outgrowth of primary airway fibroblasts from tissue blocks following inoculation. (A) ×100 New Zealand rabbits (arrow 1: Inoculation of the organization block; arrow 2: Growing edge of the cells migrating out of the tissue block). (B) ×100 SD rats (arrow indicates Inoculation of the organization block). (C and D) ×100 Human tissue. arrow indicates inoculated tissue blocks.

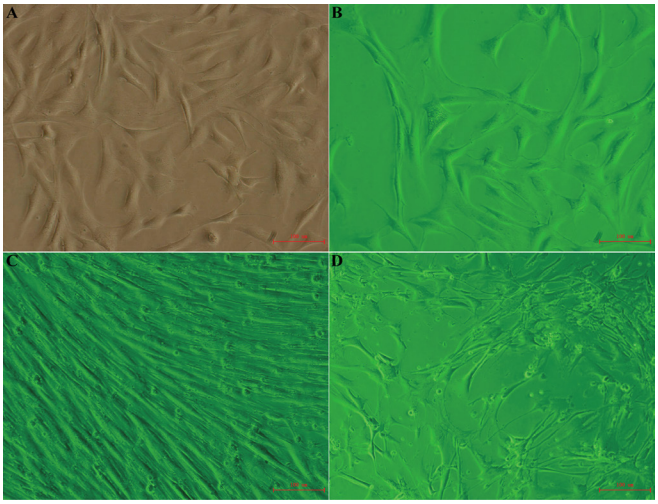

Figure 4. Passaging and prolonged culture of primary airway fibroblasts. (A) ×200 New Zealand rabbits. (B) ×200 SD rats; (C) ×200 human tissue from endotracheal intubation in patients diagnosed with benign airway stenosis, Shi xx, F, 44 y; (D) ×200 human tissue from endobronchial TB (tuberculosis) patients with benign airway stenosis, Shu xx, F, 36 y.

detect any cytokeratin staining in our cultures. Therefore, we successfully identified the airway cells in our cultures as predominantly primary fibroblasts, that can be further purified by passaging.

Chromosomal analysis of different species

The Chromosome analysis can provide a "birds eye view" of an individual's genetic information. Therefore, we observed the genetic information in different species, including New Zealand rabbits (Figure 7A), SD rats (Figure 7B) and human (Figure 7C), by using the Chromosomal analysis. The result showed that the chromosome shape was different in three different species. Also, the chromosome number in human (46 chromosomes) was more compared to the New Zealand rabbits (44 chromosomes) and the SD rats (42 chromosomes). The above features are known to be distinctive of the genera.

Conventional and improved methods of culturing human airway fibroblasts

In this study, we analyzed the conventional methods and the improved methods of culturing human airway fibroblasts (Figure 8A to D). The improved method of culture was selected from Huang xx, M, 78y, patients with bronchial foreign body (Figure 8A). The conventional method of culture of samples was obtained from Liu xx, M, 40y, a patient diagnosed with bronchial foreign body (Figure 8B). The spiral growth in improved method culture was also selected from Huang xx, M, 78y (Figure 8C). The conventional method of culture was also selected from patients with bronchial foreign body (Figure 8D).

Comparison of success rate of primary airway fibroblast cultures

When tracheal tissue from New Zealand rabbits and SD rats was cultured using the conventional method, all cells survived and we obtained a 100% success rate. When the granulation tissue from human airway was cultured in a similar manner, in 13 cultures of samples obtained from eight patients, we successfully derived fibroblast cultures four times, and failed to do so the remaining nine times, providing a success rate

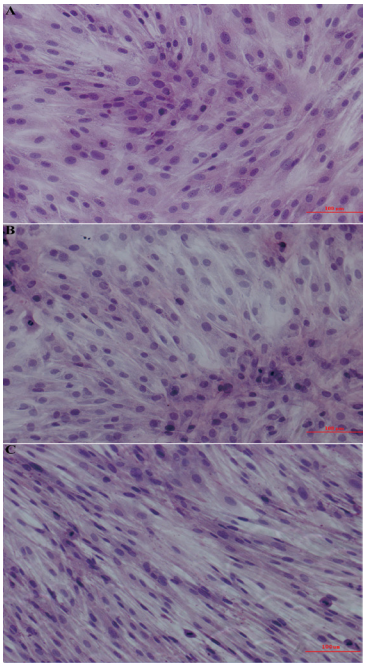

Figure 5. H&E staining of passaged and purified primary airway fibroblast cultures. (A) New Zealand rabbit. (B) SD rats. (C) Human tissue. Figure 5. H&E staining of passaged and purified primary airway fibroblast cultures. (A) ×200 New Zealand rabbit. (B) ×200 SD rats. (C) ×200 Human tissue.

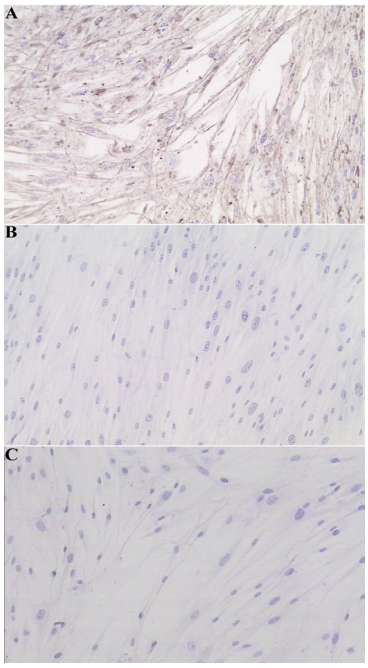

Figure 6. Immunocytochemical staining of human primary airway fibroblast. (A) ×200 Vimentin-positive; (B) ×200 negative control for vimentin. (C) ×200 cytokeratin-negative.

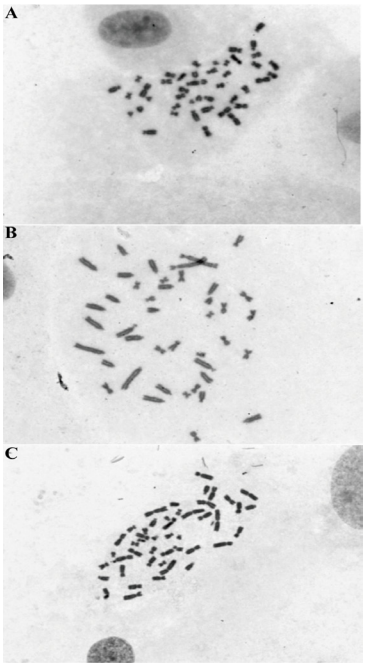

Figure 7. Chromosomal analysis of different species. (A) New Zealand rabbit: 44 chromosomes. (B) SD rats: 42 chromosomes. (C) Human: 46 chromosomes.

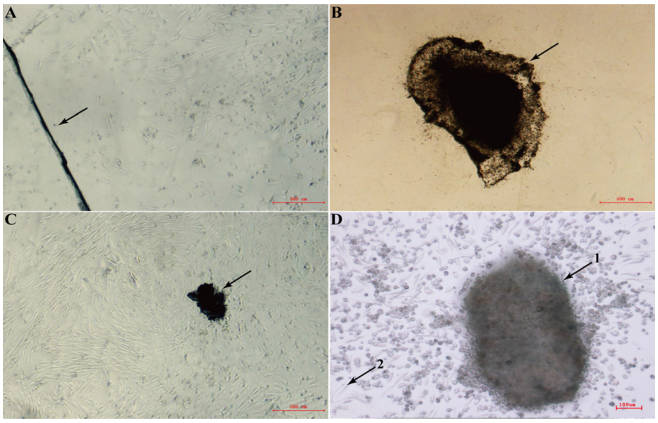

Figure 8. Conventional and improved methods of culturing human airway fibroblasts. (A) ×200 Improved method culture of samples from Huang xx, M, 78y, patients with bronchial foreign body (arrow indicates the edge of the tablet slides). (B) ×200 Conventional method of culture of samples obtained from Liu xx, M, 40y, a patient diagnosed with bronchial foreign body. Arrow indicates the direction of organization- note that outgrowing cells cannot be seen, suggesting failure of culture. (C) ×200 Spiral growth in improved method culture of sample from Huang xx, a patient with bronchial foreign body (arrow indicates pancreatic enzyme digestion of organization). (D) ×100 Conventional method of culture of samples from patients with bronchial foreign body (1 arrow indicates the direction of organization, 2 arrow indicates the edge of outgrowing cells).

of 30.8%. When cultured using the new improved method, in 11 cultures of samples obtained from ten patients, we obtained a success rate of 100%. Moreover, in case of two patients (NO.3 and NO.12), while the conventional method failed, we could successfully derive primary airway fibroblast culture using the new improved method. On the other hand, two patients (NO.4 and NO.10) whose granulation tissue was large and was divided into 2 small dishes for culture, both methods resulted in the successful derivation of airway fibroblast culture. In case of one patient (NO.8), out of three attempts by the conventional methods, only one succeeded, resulting in a success rate of 33.3%. However, when using the improved method, the first trial resulted in successful derivation of culture, indicating a success rate of 100%. We compared the efficiency of the two methods using Fisher's exact probability calculation, which gave a P value ≤ 0.01. This suggests that our new improved method for obtaining primary airway fibroblast culture is better than the conventional method (Table 1).

Discussion

Airway granulation is one of the leading causes of delayed healing during benign airway stenosis. Treatment options for this condition include surgery, medication and intervention. Currently, endoscopic tracheal intervention is the main method of choice for treating benign airway stenosis. However, this approach often leads to unwanted proliferation causing hyperplasia and poor efficacy in some patients (2-4). Recent years have shown a clearly upward trend in the incidence of benign airway stenosis (endobronchial tuberculosis, following scar formation in the endotracheal intubation airway leading to proliferation and granulation). Therefore, inhibition of airway granulation could be explored as a novel strategy to treat benign airway stenosis. If successful, this approach will have high clinical significance. The main component of the hyperplasia and granulation tissue found in benign airway stenosis is fibroblasts. Therefore, the first step towards identifying a therapeutic strategy would be to culture airway primary fibroblasts *in vitro* and establish a reliable and robust cellular model that can facilitate the investigation of the best ways to effectively suppress airway granulation and unchecked proliferation of fibroblasts. Such a model will also allow the study of the mechanisms underlying disease pathology and help find an effective therapy against intractable benign airway stenosis (5,6,16).

Primary cultures usually refer to passages 1 to 10 following tissue extraction and establishment of a dissociated cell culture. While cultures at P1 to P4 are used for purification and cryopreservation, P5 to P8 cultures are used for experimental study. This limits the number of cells available for experimentation. In addition, obtaining human tissue samples is difficult due to reasons such as the number of cases, patient cooperation, and whether the specimen satisfies all the qualification criteria for use in experiments. Furthermore, because the proliferative capacity of human airway fibroblasts is weak relative to other species, it is extremely challenging to culture these cells. In this study, samples from 21 patients were used to establish 35 cultures, while 13 samples failed to qualify for the experiments we conducted. Using the conventional culture method, we obtained a success rate of only 30.7% during the first passage, while requiring 30-40 days *in vitro*. Establishment of a cellular model with this method is time-consuming and labor intensive. Cellular models from animal tissue (New Zealand rabbits, SD rats) was relatively easier to establish suing the conventional method, giving a success rate of 100%, with the early batches requiring only 20 days *in vitro*. Animal cells could be used as an alternative to establish cellular models of benign airway stenosis where there are no proper clinical cases to support the study or where conditions are restricted. However, if clinical cases are insufficient, then animal cell models could be used to complement human studies, especially for optimizing experimental conditions, since the availability of human cells is limited.

During evolution, regeneration has served as a self-defense mechanism. In general, lower animals have stronger regenerative capacity than higher animals, since the former have relatively poor structural and functional organization, and are usually more susceptible to injury. Since humans have a strong defense capability, their regenerative capacity is weaker than lower mammals (rabbits, SD rats) (13). Regeneration in the trachea is also weaker than in the skin, because of which, culturing of primary human airway fibroblasts is extremely difficult. However, fibroblasts possess strong regenerative potential when damaged and during tissue injury. This process involves cell division, proliferation and the formation of fibrous tissue with repair function. Therefore, culturing of primary human airway fibroblasts should be feasible.

In this study, we successfully cultured airway primary fibroblasts obtained from animals (rabbits, SD rats) *in vitro* using the conventional method, with a success rate of 100%. This suggests that animal cell models are

Table 1. Comparison of the success rate of primary airway fibroblast cultures derived from different mammalian species and established using the conventional inoculation method. The success rate of cultivating primary human airway fibroblasts using the conventional versus the improved method is also compared.

Species		Conventional method		Improved method		Remarks		
		Success	Failure	Success	Failure	Age	Gender	Health
New Zealand rabbit		6	0			3 m	Female	Healthy
Total		6	0					
Success rate		100%						
SD rat		6	0			6 m	Male	Healthy
total		6	0					
Success rate		100%						
	1		2			27y	F	Endobronchial tuberculosis
	2		2			21y	M	Airway stenosis after endotracheal intubation
	3		2	1		46y	F	Airway stenosis after endotracheal intubation
	4	1		2		22y	M	Endobronchial tuberculosis
	5			1		49y	F	Bronchial foreign body
	6	1				40y	M	Bronchial foreign body
Human	7			1		78y	M	Bronchial foreign body
	8	1	2	1		36y	F	Endobronchial tuberculosis
	9			1		37y	F	Endobronchial tuberculosis
	10	1		1		49y	M	Endobronchial tuberculosis
	11			1		42y	F	Bronchial foreign body
	12		1	1		41y	F	Endobronchial tuberculosis
	13			1		22y	F	Endobronchial tuberculosis
Total		4	9	11	0			
Success rate		30.8%		100%				

easier to create and use in experiments in general laboratory conditions. Using the same procedure, we found that primary culture of human airway fibroblasts could only be performed at a much reduced success rate of 30.7%. In higher mammals such as humans, the lower the regenerative capacity of itself, the lower the survival rate of cells *in vitro*. Coupled with the difficulty of obtaining airway granulation cells from a relatively small amount of tissue, weak proliferative capacity, frequent respiratory infection in the patients enrolled, and sample contamination despite the use of aseptic technique, human airway fibroblast culture is challenging. A total of 35 specimens were obtained from 21 patients. After excluding 13 unqualified specimens, only 22 specimens were analyzed in this study. We next asked how could one improve the efficiency of primary human airway fibroblast culture *in vitro*? In this study, we explored the merits of an improved method, and found the success rate to be 100%. The sharp increase in success rate could be due to the following reasons: First, the «serum adherent» approach was used to improve culture method; specifically, a drop of fetal bovine serum (FBS) was applied to the culture dish before trypsin-EDTA mediated digestion of the tissue was placed into the droplet. This process allows digested tissue to adhere easily, and for the fibroblasts to grow along the walls of the tissue culture dish. However, high FBS concentration can inhibit cell growth. Therefore, it should be replaced after the cells attach to the wall. Secondly, in the conventional method, inoculation and replacement of tissue culture medium always led to the floating of the tissue block leading to failure of inoculation. In the improved method, we adopted an organization tablet approach where we placed sterile coverslips on the tissue blocks, thereby applying buoyancy antagonistic to gravity and thus preventing the floating of tissue blocks. Such drawn organizations often consisted of multiple layers of cells from inside-out, including airway epithelial cells, airway fibroblasts and microvascular endothelial cells. The location of airway fibroblasts attached to the bottom of the dish was convenient for the cells to grow and migrate out, thus increasing the success rate of culture. In fact, we found it difficult to distinguish between the different organizational levels. In contrast, in the conventional inoculation method where often adherent epithelial cells are located at the bottom, the organization is not conducive for fibroblasts to outgrow and migrate out of the tissue blocks. In the improve method, short-term digestion of the inoculated tissue block and the coverslip tablet creates two adherent surfaces, making it convenient for fibroblasts to grow out, migrate, attach and proliferate.

Finally, in the improved method, following the initial digestion and passage, we removed the coverslip and placed it in another 3.5cm dish and cultured it separately, followed subsequently by digestion and passaging. Because during fibroblast cell division and reproduction, intensive cell-cell contact leads to inhibition, no passaging may be needed, but a significant number of the fibroblasts fail to divide despite attachment, resulting in an uneven distribution of cells. Enzymatic digestion ensures that the cells can be redistributed so they can continue to divide and proliferate. This leads to a high percentage of fusion, so that when passaged, cells tend to enter the logarithmic growth phase, and further culturing is relatively easy.

In conclusion, it is possible to establish primary airway fibroblast cultures *in vitro*. To determine ways to inhibit airway granulation and repeated proliferation and to study the mechanism of refractory benign airway stenosis, it is essential to build a good cellular model.

Although the requirement for primary animal airway fibroblast culture is not high, it is simpler to create and can be used as either an alternative to or to complement human models. In thus study, we show that while the conventional method yielded low success rate of primary culture of human airway fibroblasts, the modified improved method not only markedly improved the success rate, but is also technically easy to perform and simple to adopt. Despite this, culturing of fibroblasts from human airway by inoculation cultivation method is time consuming and laborious, suffers from several shortcomings, and needs to be further improved. This is especially needed for patients diagnosed with endobronchial tuberculosis, in cases where it is easy to obtain necrotic tissue, where cell growth is relatively slow after inoculation and the activity is poor.

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