Original Research

Overexpression of CXCR4 in tracheal epithelial cells promotes their proliferation and migration to a stromal cell-derived factor-1 gradient

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Abstract

Tracheal reconstruction has been an important issue in clinic, but it is limited for the ability of epithelial regeneration. Several reports have shown that stromal cell-derived factor-1 (SDF-1) and chemokine receptor CXCR4 play an important role in cell proliferation and migration of multiple cell types. But there is no report of SDF-1 and CXCR4 in tracheal cells. In this paper, the rat tracheal epithelial cells covered with cilium were isolated and cultured using two enzyme digestions, and CXCR4 lentivirus was constructed and infected to the tracheal cells successfully. The results showed that the expression of CXCR4 which was covered on cellular membrane majorly was low in normal cells, and the cell proliferation was increased accompanied with the increase in SDF-1 concentration. The cell proliferation, migration and intracellular free calcium were increased significantly in CXCR4 lentivirus infected groups in a dose-dependent manner, and these effects could be inhibited after CXCR4 inhibitor AMD3100 treated because the expression of CXCR4 was decreased. Our findings indicate that the activation of CXCR4 may promote tracheal cell proliferation and migration to the sites of airway injury where SDF-1 is regulated.

Keywords: rat tracheal epithelial cells, CXCR4, SDF-1

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Introduction

It is well known that reconstruction of tracheal defects is essential following trauma or oncological surgery.[1] Direct end-to-end anastomosis is currently the most effective way to treat patients with tracheal defects.[2,3] However, this procedure can be only performed to allow for closure of defects up to 6 cm at maximum.[4] If a tracheal segment >6 cm long needs to be resected, direct anastomosis is impossible and leads to the fistula. Therefore, the important problem clinicians encountered is to find an ideal tracheal implant that could maintain a patency lumen and normal physiological function.[5–7]

Tissue engineering is an interdisciplinary branch that integrates the knowledge of biomaterial science and cell culture. It has great potential to offer tissue substitutes that resemble normal tissue architecture. Most importantly, it could overcome the shortage of reliable tissues for transplantation and the immune reactions that result from allograft.[8] Such approach has been performed for reconstruction of tracheal defects in recent years by using chondrocyte,[9] epithelial cells[10–12] and mesenchymal stem cells.[13] However, tissue-engineered trachea is not a simple tube transplantation process, but should ensure the trachea substitute to has complete ciliated epithelium coverage and maintain the real respiratory tract epithelial function. Adams *et al.*[14] report an inverse correlation between epithelial coverage and degree of airway obliteration. Thus, it is essential to induce the epithelial cells to the inner surface of trachea substitute *in vitro.* Cytokine or chemokine which regulates diverse cellular reactions, affects cell proliferation, migration, extracellular matrix synthesis and release could encourage the repair and regeneration of damaged tissues to solve this problem.

Stromal cell-derived factor-1 (SDF-1) is a member of chemokine CXC subfamily originally isolated from murine bone marrow stromal cells[15] and it can cause the cytoskeleton re-modeling, endothelial cells adhesion and migration, meanwhile, mediate a variety of specific signals to produce physiological and pathological effects through interaction with the GTP-binding protein (G-protein)-coupled 7-transmembrane chemokine receptor CXCR4.[16] SDF-1 has been indicated to be up-regulated in ischemic brain tissue,[17,18] myocardial tissue[19,20] and kidneys,[21,22] and acts as a potent chemoattractant to recruit circulating or residing CXCR4-expressing hematopoietic stem cells and mesenchymal stem cells to lesions.[23] Cells with high

differentiation and proliferation potential can act as candidates to repair and regenerate the destructive tissues. Nevertheless, the role of SDF-1/CXCR4 axis in reconstruction of tracheal defects has never been reported. In this study, we aimed to research the effect of CXCR4 and SDF-1 on the tracheal epithelial cells proliferation, migration and intracellular free calcium releasing by constructing CXCR4 lentiviral vector and infecting tracheal epithelial cells. This research may provide some rationales and methods to solve the problem of epithelial regeneration in the tracheal tissue engineering.

Materials and methods

Isolation and culture of rat tracheal epithelial cells

The tracheas were excised from the respiratory tract immediately following their removal from the rats and placed into phosphate-buffered saline (PBS) solution. The tracheas were then washed with PBS containing 100 U/mL penicillin and 100 μg/mL streptomycin for three times. The tracheas were filled with proteinase solution (0.1% dispase) at 4°C for 18 h, then turned to 0.25% dispase and incubated at 37°C with 5% CO_2 for 10 min. Following this incubation, the culture medium supplemented with 10% fetal bovine serum (FBS) was added and centrifuged at $800 \times g$ for seven minutes. The cells were suspended in keratinocyte serum free medium (K-SFM) culture medium containing 5% FBS, and incubated at 37°C under a 5% CO_2 humidified atmosphere.

Plasmid construction

CXCR4 gene was first amplified from template plasmid obtained from OriGene Technology (Rockville, MD, USA) and TA cloned into pGEM-T vector (Promega, Madrid, Spain). Recombinant plasmids were identified by sequencing and digestion with *Age*I and *Eco*RI enzymes. Subsequently, the CXCR4 gene was cloned into lentiviral vector FUGW (Promega) which carries a green fluorescent protein (GFP) reporter gene. Human kidney 293T-cells (2×10^5) were seeded on 10 cm plates and cultured overnight at 37°C followed by transfection with 12 μg of FUGW, 6 μg of VSVG, 6 μg of RSV-REV and 6 μg of pMD1 g/RRE using Lipofectamine 2000. Virus supernatants were collected 48 h after transfection and stored at −80°C for the following experiments.

DNA transfection

Rat tracheal epithelial (RTE) cells were plated in a six-well cluster dish at a density of 4×10^5 cells/mL and cultured in 1640 medium with 10% FBS. After 24 h, the diluted lenti-CXCR4 or empty lentiviral vector supernatant was added to each well and incubated for another 24 h at 37°C.

RT-PCR analysis

Total RNA was isolated from non-transfected (normal control group), empty lentiviral vector transfected (Blank control group) and CXCR4 lentivirus transfected RTE cells (lenti-CXCR4 group) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the reverse transcription was performed using a RT reagent Kit (TaKaRa, Dalian, China). The realtime experiments were conducted on an iQ5 Multicolor RealTime PCR Detection System (Bio-Rad, Hercules, CA, USA) using a SYBR Green Realtime PCR Master Mix (TaKaRa). The PCR reactions consisted of three minutes at 95°C followed by 35 cycles of at 95°C for 30 s, at 60°C for 30 s and at 72°C for 30 s. The data were calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the individual internal control level. The primer sequences for CXCR4 and GAPDH were as follows:

GAPDH-F: CAAGGTCATCCATGACAACTTTG
GAPDH-R: GTCCACCACCCTGTTGCTGTAG
CXCR4-F: TACACCGAGGAAATGGGCTCA
CXCR4-R: AGATGATGGAGTAGATGGTGGG

Immunoblotting analysis

The cells in Blank control group and lenti-CXCR4 group were lysed in cells lysis buffer, then the proteins were separated on 12% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane. After blocking with 5% non-fat dry milk for two hours, the membranes were immunoblotted with primary CXCR4 antibody (1:100; Boshide, Wuhan, China) overnight at 4°C, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (1:80; Boshide) for two hours. Immunoreactive bands were detected by the method of enhanced chemiluminescence.

Immunofluorescence analysis

The cells in Blank control group and lenti-CXCR4 group were seeded onto 96-well plate at a density of 2×10^4 cells/per well and fixed in methanol/acetone (7/3) at −20°C for 10 min. After blocking with 5% non-fat dry milk for 30 min, the cells were immunoblotted with CXCR4 antibody (1:100; Boshide) for one hour and then incubated with FITC labeled secondary antibody (1:80; Boshide) for one hour. Nuclei were stained with DAPI (4,6-diamino-2-phenylindole, St Louis, MO, USA). The fluorescence expression was detected using fluorescence microscope.

MTT [3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay

The RTE cells in normal control group and lenti-CXCR4 group were plated in 96-well culture plates (1×10^4 cell/well) and incubated overnight. SDF-1 diluted to various concentrations from 0.1 to 1000 nmol/L was applied to the cells for 48 h, 20 μL MTT was added into the plates, and then cells were incubated for four hours at 37°C. After the medium was removed, 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the resulting purple formazan and absorbance was read at 490 nm using a microplate reader.

Transendothelial migration

In vitro analysis of migration was performed on transwell microporous membranes, which separate the upper and lower chambers in six-well tissue culture plates. The normal or lenti-CXCR4 transfected RTE cell suspensions (5×10^4 cells/100 μL) were added to the upper chambers. Different final concentrations of SDF-1 (0, 1, 3, 10, 30, 100 and 300 nmol/L) or AMD3100 (0, 0.005, 0.05, 0.5, 5, 50, 500 and 5000 nmol/L) were placed in the lower chambers. The cells were allowed to migrate for 24 h at 37°C. The non-migrated cells were removed from the upper surface by scraping with a cotton swab. After incubation, the filter was fixed and stained with crystal violet. All of the cells that had migrated from the upper to the lower side of the filter were counted under a light microscope at a magnification of $\times$400.

Measurement of intracellular free calcium mobilization

The intracellular free calcium mobilization was measured using the fluorescent calcium indicator, Fluo-4. The SDF-1/AMD3100 treated cells were incubated in Hanks' balanced salt solution containing Fluo-4 for 30 min at 37°C, then washed with PBS for three times and analyzed using flow cytometry.

Statistical analysis

All experiments were performed three times and the differences between the treated and control cells were analyzed by Student's *t*-test. The data were expressed as means $\pm$ SD of three independent experiments and $P < 0.05$ was considered significant.

Results

CXCR4 expression in RTE cells

RT-PCR and Western blotting were performed to explore the expression of CXCR4 in non-transfected (normal control group), empty lentiviral vector transfected (Blank control group) and CXCR4 lentivirus transfected (lenti-CXCR4 group) RTE cells. As shown in Figure 1, the expression of CXCR4 was low in normal and Blank control group, and it increased significantly in CXCR4 lentivirus transfected RTE cells at mRNA and protein levels ($P < 0.05$). Immunofluorescence analysis showed that a number of colonies were GFP-positive and a significant part of the green fluorescence corresponding to CXCR4 was observed on the cell membrane. In addition, the CXCR4 lentivirus transfected RTE cells showed stronger green fluorescence than the Blank control (Figure 2), suggesting the lenti-CXCR4 transfection was more efficient.

SDF-1 induces cell proliferation

The cell survival was evaluated using MTT assay. The absorbance values were gradually increased in normal control group and lenti-CXCR4 group after treatment with SDF-1 and this cell proliferation effect behaved in a dose-dependent manner (Figure 3). When the SDF-1 concentration increased to 100 and 1000 nmol/L, the absorbance values were 0.445 ± 0.060 and 0.519 ± 0.095 in lenti-CXCR4 group which were significantly higher than the low concentration treatment (0.1 mmol/L, 0.223 ± 0.021 and 1 mmol/L, 0.283 ± 0.045; $P < 0.05$). Although the absorbance values were higher in lenti-CXCR4 group compared with normal control group under the same SDF-1 concentration, however, there was no significant difference between them ($P > 0.05$).

SDF-1 affects the cell migration

The SDF-1-induced migration was evaluated on transwell microporous membranes *in vitro*. The results indicated that only few cells were migrated into the lower chamber in normal control group (34.8 ± 2.3) and there was no significant difference along with the SDF-1 concentration increased. However, the cells in lenti-CXCR4 group moving from upper chamber to lower chamber were increased following the SDF-1 intervention, and behaved in a dose-dependent manner obviously (Figures 4a and c). When the SDF-1 concentration increased to 100 and 300 nmol/L, the migration cells were 89.8 ± 4.7 and 100.0 ± 4.8, which were significantly higher than normal control group (34.8 ± 2.3), 0 mmol/L SDF-1 concentration group (32.8 ± 2.2) and the low concentration treatment (30 mmol/L, 62.4 ± 5.0; $P < 0.05$).

To confirm if the increased migration resulted from SDF-1 in RTE cells is dependent upon its receptor CXCR4, we

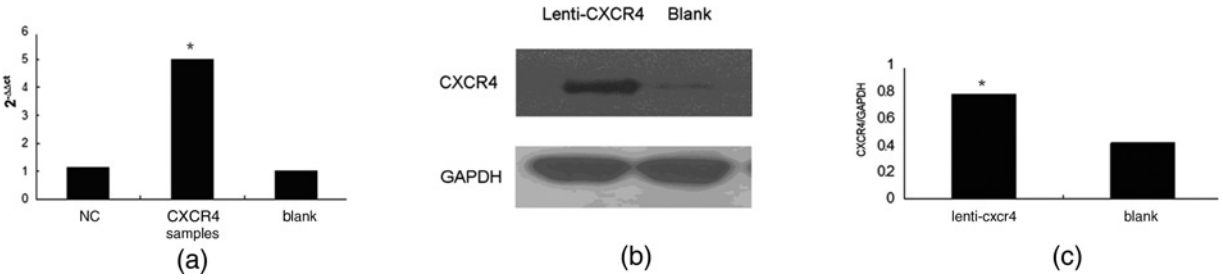

Figure 1 Chemokine receptor (CXCR4) expression in normal tracheal epithelial cells (normal control, NC), empty lentiviral vector transfected tracheal epithelial cells (blank group) and CXCR4 lentivirus transfected tracheal epithelial cells (Lenti-CXCR4 group). (a) The relative quantity of CXCR4 at mRNA level. (b) The immunoblotting results of CXCR4 at protein level. (c) The relative quantity of CXCR4 at protein level. $^*P < 0.05$ compared with NC and blank group

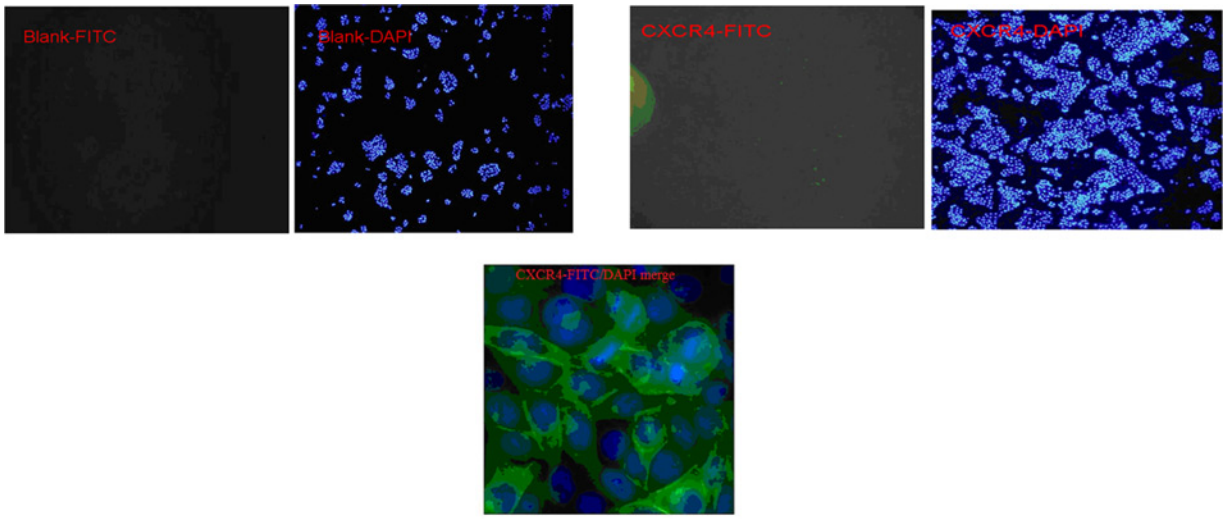

Figure 2 Empty lentiviral vector transfected tracheal epithelial cells (blank) and CXCR4 lentivirus transfected cells (CXCR4) are stained with FITC (CXCR4, green) and DAPI (nucleus, blue). (A color version of this figure is available in the online journal)

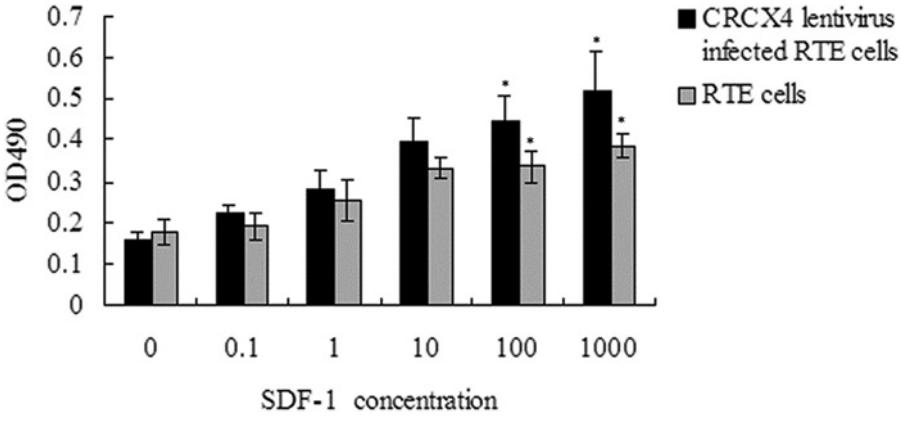

Figure 3 Comparison of stromal cell-derived factor-1 (SDF-1)-induced cell proliferation in normal tracheal epithelial cells and CXCR4 lentivirus transfected cells. The data are representative of three independent experiments. $^{*}P < 0.05$ compared with 0 mmol/L SDF-1 concentration and low SDF-1 concentration (0.1 and 1 mmol/L)

treated the cells with AMD3100, a CXCR4 inhibitor, to block the function of CXCR4 prior to the addition of SDF-1. As shown in Figures 4b and d, AMD3100 inhibited the migration in a dose-dependent manner. When the AMD3100 concentration increased to 50 nmol/L, the migration cells were 31.4 ± 2.6, which was significantly lower than 0 mmol/L AMD3100 concentration group (86.4 ± 2.4).

SDF-1 induces intracellular free calcium mobilization

Intracellular free calcium was measured using the fluorescent calcium indicator, Fluo-4. SDF-1 could increase the release of intracellular free calcium, especially when the concentration was more than 10 nmol/L (Figure 5a). Meanwhile, AMD3100, a CXCR4 inhibitor, was used to block the function of CXCR4 prior to the addition of SDF-1, the results showed that AMD3100 could inhibit the release of intracellular free calcium at 50–100 nmol/L oppositely (Figure 5b).

Discussions

Recruitment of cells to damaged tissue and regeneration of the tissue is a complex multi-step process. It involves sensing the signal from the remote injured tissue that calls for the release of these cells from their storage niche into circulation, homing of circulating cells to the target tissues, and *in situ* proliferation and differentiation of these cells into matured, functional cells.[24] Recent studies indicate cytokines and/or chemokines (such as SDF-1) are up-regulated under injury conditions and direct cells mobilization, trafficking, and homing through interaction with their acceptors.[25] In this study, we simulated the tissue damage by a serial of SDF-1 concentration gradient. Lentiviral vectors, derived from human immunodeficiency virus, have been

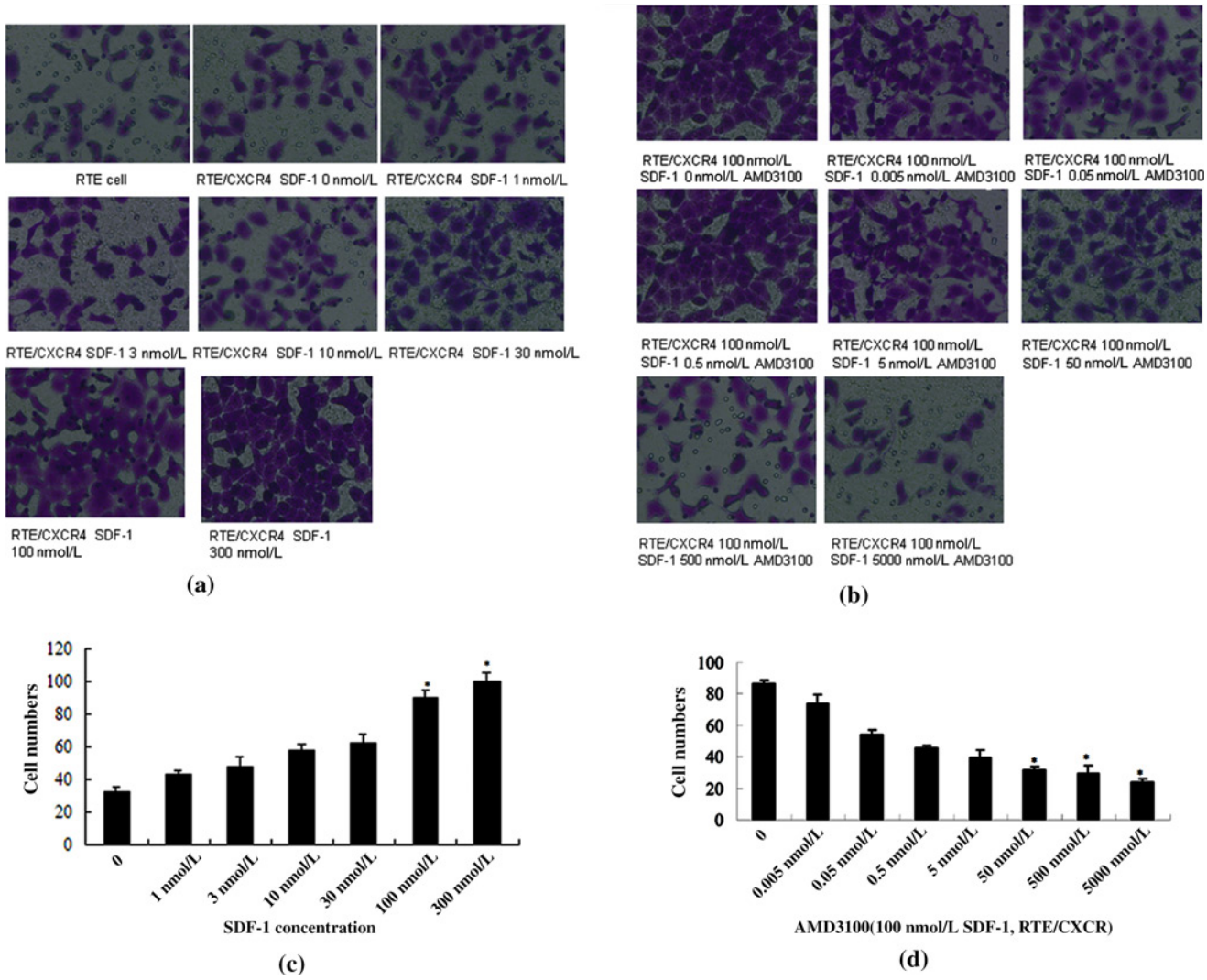

Figure 4 Tracheal epithelial cells are migrated to an stromal cell-derived factor-1 (SDF-1) gradient. The CXCR4 inhibitor AMD3100 inhibits this SDF-1-induced migration. (a) Representative staining of SDF-1 treated groups at 0, 1, 3, 10, 30, 100 and 300 nmol/L. (b) Representative staining of AMD3100 treated groups at 0, 0.005, 0.05, 5, 50, 500 and 5000 nmol/L. (c) The number of migration cells of SDF-1 treated groups is quantified by counting the cells from 10 random fields. (d) The number of migration cells of AMD3100 treated groups is quantified by counting the cells from 10 random fields. The data are representative of three independent experiments. * (c) $P < 0.05$ compared with 0 mmol/L SDF-1 concentration and low SDF-1 concentration (1–30 mmol/L). * (d) $P < 0.05$ compared with 0 mmol/L SDF-1 concentration and low AMD3100 concentration (0.005–5 mmol/L). (A color version of this figure is available in the online journal)

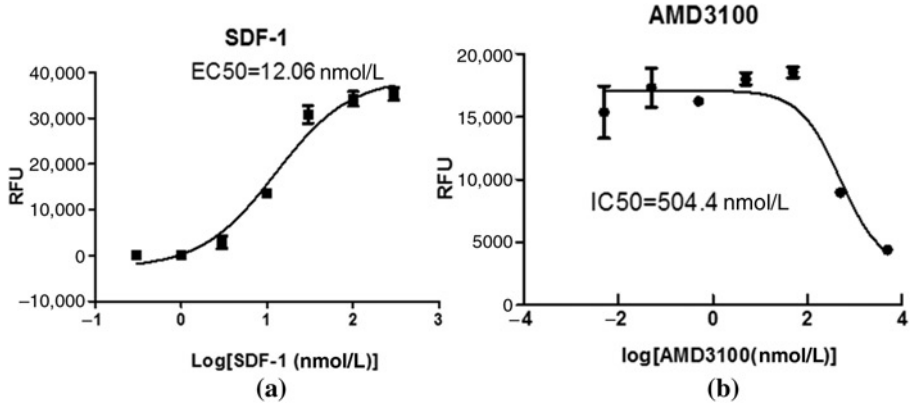

Figure 5 Stromal cell-derived factor-1 (SDF-1) treatment increases the release of intracellular free calcium. The CXCR4 inhibitor AMD3100 inhibits the release of SDF-1-induced intracellular free calcium. (a) The SDF-1-induced intracellular free calcium delivery is quantified and EC_{50} is calculated. (b) The AMD3100-induced intracellular free calcium delivery after SDF-1 stimulated is quantified and IC_{50} is calculated. The data are representative of three independent experiments

proven to be highly efficient, versatile and safe vehicles for gene transfer.[26] The FUGW lentivirus used in the present study also offers significant advantages because of its predicted biosafety.[27,28] Thus, CXCR4 gene that is considered to be a new surface marker expressed on several types of stem cells,[29] was transfected into lentiviral vectors to improve the tracheal stem cell function. As expected, the expression of CXCR4 which was covered on cellular membrane majorly was low in normal tracheal cells, and up-regulated in CXCR4 lentivirus transfected RTE cells at mRNA and protein levels. These results led to increased cell proliferation ability in CXCR4 lentivirus transfected group. Interestingly, cell proliferation was enhanced along with the increase in SDF-1 concentration. To investigate the function of CXCR4/SDF-1 signaling in cell movement, the transwell microporous membranes were used.[30–32] We found a significant induction of migration of tracheal epithelial cells by SDF-1 and the migration behaved in a dose-dependent manner, which was in accordance with previous studies.[33,34] The cell migration function of SDF-1 could be inhibited by AMD3100, a neutralizing CXCR4 antagonist. These findings indicate that SDF-1/CXCR4 axis can govern the directional chemotaxis of tracheal endothelial cells to the injury tissue.

Calcium ion is an important signal substance that could deliver extracellular signals into cells.[35] The concentration of calcium ion is more than 10^5 nM/L in mitochondrion and endoplasmic reticulum. It releases into cytoplasm to regulate cell movement, muscle contraction, cell proliferation, cell differentiation and other physiological functions which are related to cell migration and movement.[36] The guanine nucleotide-binding regulatory protein α-subunit, Gα16, is primarily expressed in hematopoietic cells and interacts with a large number of seven-membrane span receptors including chemoattractant receptors, such as CXCR4. Thus, Gα16 protein can be activated when the CXCR4 is activated, which subsequently activates phospholipase C and mediates phosphoinositide hydrolysis to produce diacylglycerol and inositol triphosphate that could interact with IP3 receptor and cause the release of intracellular calcium.[37] Determination of intracellular calcium is used to detect the activation of CXCR4. In our research, the intracellular calcium was increased significantly after SDF-1 treatment and behaved in an obvious dose-dependent manner. AMD3100 showed an inhibitory action markedly, especially when the concentration was more than 50 nmol/L. These results indicate that the activation of CXCR4 is correlated with the cell proliferation and migration, and the CXCR4 inhibitor AMD3100 can suppress these effects on the RTE cells.

To date, chemokines and their receptors have been largely studied for their roles in directing migration and activation. CXCR4 and SDF-1 have been shown to mediate the chemotaxis of hematopoietic progenitor cells,[38] lymphocytes,[39] and monocytes[40] and others. Our research demonstrates that CXCR4 can mediate the migration of RTE cells to an SDF-1 gradient. Alternatively, the research of internal calcium ion, an important signal substance, proves the idea that the SDF-1 and CXCR4 pathway may directly deliver essential proliferation and differentiation signals to cells that can stimulate cell proliferation and migration. Based on the results, RTE cell transfected with CXCR4 lentivirus cultured *in vitro* can be used as a suitable trachea substitute for tracheal tissue engineering. These results could provide expedient evidence for the exploitation of tracheal surrogates.

Author contributions: WS designed the experiments, JL and XY performed and interpreted the experiments, and WS was involved in writing the manuscript.

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