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MicroRNA-146a modulates human bronchial epithelial cell survival in response to the cytokine-induced apoptosis ☆

Xiangde Liu *, Amy Nelson, Xingqi Wang, Nobuhiro Kanaji, Miok Kim, Tadashi Sato, Masanori Nakanishi, YingJi Li, Jianhong Sun, Joel Michalski, Amol Patil, Hesham Basma, Stephen I. Rennard

Pulmonary, Critical Care, Sleep and Allergy Medicine, Department of Internal Medicine, University of Nebraska Medical Center, 985885 Nebraska Medical Center, Omaha, NE 68198-5885, USA

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ABSTRACT

MicroRNA plays an important role in cell differentiation, proliferation and cell death. The current study found that miRNA-146a was up-regulated in human bronchial epithelial cells (HBECs) in response to stimulation by TGF-ß1 plus cytomix (a mixture of IL-1ß, IFN-γ and TNF-α). TGF-ß1 plus cytomix (TCM) induced apoptosis in HBECs (3.4 ± 0.6% of control vs 83.1 ± 4.0% of TCM treated cells, $p < 0.01$), and this was significantly blocked by the miRNA-146a mimic (8.8 ± 1.5%, $p < 0.01$). In contrast, a miRNA-146a inhibitor had only a modest effect on cell survival but appeared to augment the induction of epithelial-mesenchymal transition (EMT) in response to the cytokines. The MicroRNA-146a mimic appears to modulate HBEC survival through a mechanism of up-regulating Bcl-XL and STAT3 phosphorylation, and by this mechanism it could contribute to tissue repair and remodeling.

The inflammatory microenvironment is associated with initiation and progression of various lung diseases including asthma, emphysema and lung cancer [1–3]. In the airways, many cells including inflammatory cells and resident cells produce inflammatory mediators [4–9]. Among the mediators commonly increased in airway inflammation are IL-1β, TNF-α, IL-6, IL-8 and TGF-ß1 [10–14]. While it has been reported that these inflammatory cytokines regulate various functions of lung structural cells [5,14–16], less is known about the potential roles of these inflammatory cytokines in modulating epithelial-mesenchymal transition (EMT) and apoptosis in human bronchial epithelial cells (HBECs). In this regard, recent studies have demonstrated that miRNAs play important roles in regulating a variety of cell functions [17,18].

Mature microRNAs (miRNAs) are expressed at varying levels in a tissue-specific manner. A number of studies indicate that miRNAs are involved in tissue development, cell differentiation and apoptosis, and thus, miRNAs have been considered as targets for blocking disease pathogenesis [17–19]. To date, more than 850 human miRNAs have been predicted and over 450 human miRNAs have been identified [http://microrna.sanger.ac.uk], but only a few miRNAs are specifically expressed in lung tissue and a limited number of miRNAs have been reported that might be involved in the development of lung diseases including lung cancer, asthma and fibrosis [20–25]. In this regard, miR-200c modulates E-cadherin expression in cells undergoing EMT [22,26], and over-expression of the *let-7* miRNA in A549 cells inhibits cell growth [20] while introduction of miRNA-146a into cervical cancer cells promotes cell proliferation [27].

The current study was, therefore, designed to investigate the potential role of miRNA-146a in regulating HBEC survival and proliferation in response toTGF-ß1 plus cytomix (a mixtures of IL-1ß, TNF-α and IFN-γ), which we have previously demonstrated induce EMT in the A549 cells [28]. Here, we report that HBECs underwent either EMT or apoptosis in response to the cytokine stimulation, and that miRNA-146a was significantly up-regulated in the cells exposed to the cytokines. Introduction of a miRNA-146a mimic not only protected HBECs from cytokine-induced apoptosis, but also promoted cell proliferation. Modulation of HBEC survival and proliferation by miRNA-146a may be mediated by STAT3 signaling in that STAT3 is highly phosphorylated (Tyr 705) in the cells transfected a miRNA-146a mimic followed by cytokine stimulation.

Materials and methods

Cell culture and cytokine treatment. HBECs were acquired from bronchial biopsies using a previously published method with modifications [29]. Primary HBECs were cultured in V30 (type I collagen) coated 60mm dishes using Small Airway Growth Medium (SAGM, Clonetics, Lonza, Switzerland). Cells were fed every 2–3

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* Corresponding author. Fax: +1 402 559 4878.
E-mail address: xdliu@unmc.edu (X. Liu).

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days and passaged once a week. Passages 3–7 were used for the current study. Except for evaluating concentration dependence, cells were treated with 2 ng/ml TGF-ß1 plus 2.5 ng/ml IL-1ß, 5 ng/ml TNF-α and 5 ng/ml IFN-γ in SAGM.

MicroRNA microarray assay. Three strains of primary HBECs were treated with TCM for 24 h. MicroRNAs were extracted using miRNA Isolation Kit following the manufacturer's instructions (Ambion, Applied Biosystem, Austin, TX). MicroRNA expression was profiled using *mir*Vana™ miRNA labeling kit and *mir*Vana™ miRNA Probe Set 1564V2 (Ambion, Applied Biosystem, Austin, TX) following the manufacturer's instructions.

Real time RT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and 10ng of total RNA was reverse transcribed using a miRNA reverse transcription kit (Cat #: 4366596, Applied Biosystem, Foster City, CA). Real time PCR was conducted in a total volume of 20 μL using ABI Prism 7500 (Applied Biosystem, Foster City). The rRNA control kit (Applied Biosystem, Foster City, CA) was used as an internal control.

Transfection of a mimic or an inhibitor of miRNA-146a. HBECs were plated at a density of 2×10^5 cells/well (6-well plate) in SAGM without antibiotics. The next day, cells were transfected with 100 nM negative control miRNA (Cat #: CN-001000-01), 100 nM miRNA-146a mimic (Cat #: C-300630-03) or 100 nM miRNA-146a inhibitor (Cat #: IH-300630-05, Dharmacon, Lafayette, CO) using lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 6 h transfection in Opti-MEM medium (Invitrogen, Carlsbad, CA), cells were cultured in SAGM overnight before cytokine exposure.

Proliferation assay. Following the transfection of a miRNA-146a mimic or inhibitor, the cells were treated with TCM for 2 days in SAGM. Cells were then trypsinized and plated at a density of 2×10^4 cells/well (24-well plate). Cells were fed every 2 days and cell number was counted with a Coulter Counter.

Immunoblots. Cell lysate was extracted and 10 μg of total protein was subjected to electrophoresis. After transferring and blocking, it was reacted with primary antibodies at 4 °C overnight. Target proteins were subsequently detected and quantified using horseradish peroxidase conjugated IgG with the ECL plus and Typhoon Scanner (Amersham Pharmacia Biotech, Buckinghamshire, England).

LIVE/DEAD cytotoxicity/viability assay. Cell viability was evaluated using the LIVE/DEAD Kit (Molecular Probe, Invitrogen, Carlsbad, CA). Cells were observed and photographed under fluorescence microscope with green or red filters. The presence of nucleic acid staining by EthD-1 (red) was counted as dead cells, while cells that were green were scored as live cells if the morphology was normal or as apoptotic cells if they were green but manifested condensed nuclei, cell shrinkage and membrane blebs.

Statistical analysis. All quantitative data are expressed as mean ± standard error of the mean. Statistical comparisons of multi-group data were analyzed by analysis of variance (ANOVA) followed by student's *t* test for values that appeared different with the Tukey's (one-way) comparison using PRISM4 software. $p < 0.05$ was considered significant.

Results

Induction of EMT and apoptosis by TCM in HBECs

We have previously reported that A549 cells underwent EMT in response to TCM [28]. In the current study, we further investigated the effect of TCM on HBECs. Similar to the A549 cells, EMT was also induced in HBECs in response to TCM (Fig 1). Interestingly, however, not only EMT, but also apoptosis was induced by the cytokines in a concentration dependent manner (Fig 1). While surviving cells underwent EMT that was characterized by induction of a spindle shape and loss of cell–cell contact, majority of cells underwent apoptosis characterized by cell shrinkage and membrane bleb formation (Fig. 1D–F, indicated by arrows). However, few cells' nuclei were positively stained in red (Fig. 1G–I), indicating cytokines did not induce necrosis. The EMT phenomenon was further confirmed by the disappearance of E-cadherin expression and increased expression of vimentin after 48 h of cytokine treatment (data not shown).

Up-regulation of miRNA-146a by the inflammatory cytokines in HBECs

MicroRNA expression in response to the cytokine stimulation was assessed by microarray in three different strains of primary HBECs. As shown in Fig. 2A, miRNA-146a was significantly up-regulated by the cytokines, an average of 3.8 ± 0.2-fold was observed in all three strains of cytokine-treated vs non-treated cells. This alteration was further confirmed by real time RT-PCR assay (Fig. 2B).

MicroRNA-146a protection of HBECs from cytokine-induced apoptosis

HBECs were transfected a mimic or an inhibitor of miRNA-146a followed by TCM treatment. In response to the TCM-stimulation, the majority of cells transfected with a negative control miRNA underwent apoptosis as evidenced by morphologic observation (Fig. 3A) and by quantification of apoptotic cell numbers following LIVE/DEAD staining (Fig. 3B). However, in the cells transfected with the miRNA-146a mimic, induction of apoptosis by the cytokines was significantly blocked (Fig. 3A and B), 83.1 ± 4.0% of negative control miRNA transfected cells vs 8.8 ± 1.5% of miRNA-146a mimic transfected cells (Fig. 3B, $p < 0.01$). Interestingly, while the miRNA-146a mimic remarkably blocked apoptosis, it had no apparent effect on EMT (Fig. 3A and B). In contrast, the inhibitor of miRNA-146a resulted in augmented EMT induction and had a much more modest effect on blockade of apoptosis-induced by the cytokines (Fig. 3A and B).

Effect of miRNA-146a mimic on Bcl-XL expression and STAT3 phosphorylation

We have reported that Bcl-XL, NF-kB and STAT3 play an important role in modulating HBEC survival in response to cigarette smoke extract [30,31]. The current study found that, in response to the stimulation of TCM, Bcl-XL level was significantly suppressed in the cells transfected with negative control miRNA but not in the cells transfected with the miRNA-146a mimic (Fig. 4A). In contrast, Bax expression in the cells was not significantly affected by the cytokines although it was slightly stimulated by the miRNA-146a mimic (Fig. 4A). Furthermore, STAT3 was not affected by the miRNA-146a mimic alone. TCM significantly inhibited expression and phosphorylation of STAT3 in the cells transfected with a control miRNA. In contrast, STAT3 remained highly expressed and phosphorylated (Tyr 705) in the cells transfected with the miRNA-146a mimic and exposed to the cytokines (Fig. 4B). Consistent with the level of STAT3 phosphorylation, the proliferation rate was highest in the cells transfected with the miRNA-146a mimic followed by stimulation with inflammatory cytokines (Fig. 4C). Interestingly, neither p65 nor p50 phosphorylation was affected by the miRNA-146a mimic (data not shown).

Discussion

The current study was destined to determine if miRNA-146a might play a role in regulating cell survival following cytokine stimulation of HBECs. We have demonstrated that HBECs undergo

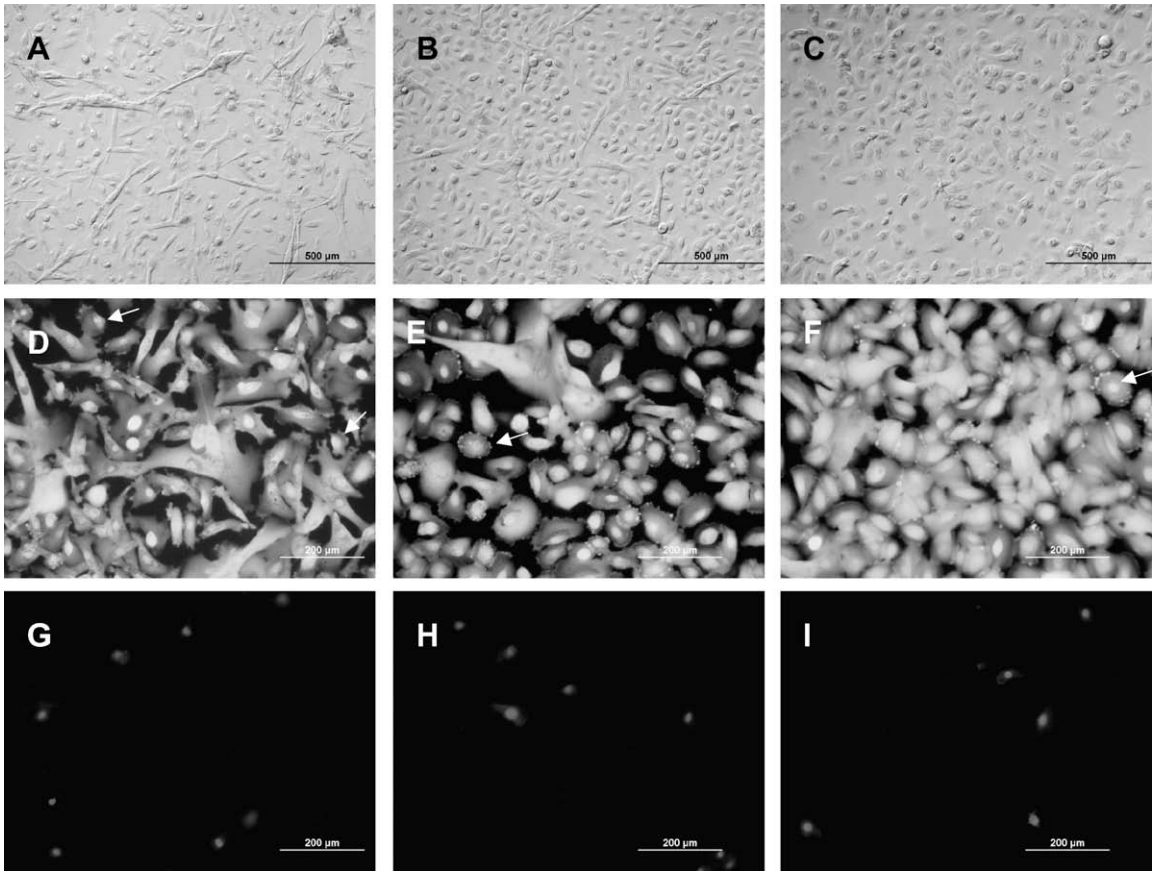

Fig. 1. Induction of EMT and apoptosis by TCM in HBECs. HBECs were treated with TCM for 48 h. EMT and apoptosis were observed under phase contrast microscopy (A–C) and by LIVE/DEAD assay (D–I). (D–F) Alive cells under green filter. (G–I) Dead cells under red filter. (A, D and G) Cells treated with 2 ng/ml TGF-ß1 + 2.5 ng/ml IL-1ß + 5 ng/ml TNF-α + 5 ng/ml IFN-γ. (B, E and H) Cells treated with 0.5 ng/ml TGF-ß1 + 0.625 ng/ml IL-1ß + 1.25 ng/ml TNF-α + 1.25 ng/ml IFN-γ. (C, F and I) Cells in medium only. Scale bars: 500 μm for (A)–(C); 200 μm for (D)–(I). Arrows: examples of apoptosis with cell shrinkage but without nucleic acid staining.

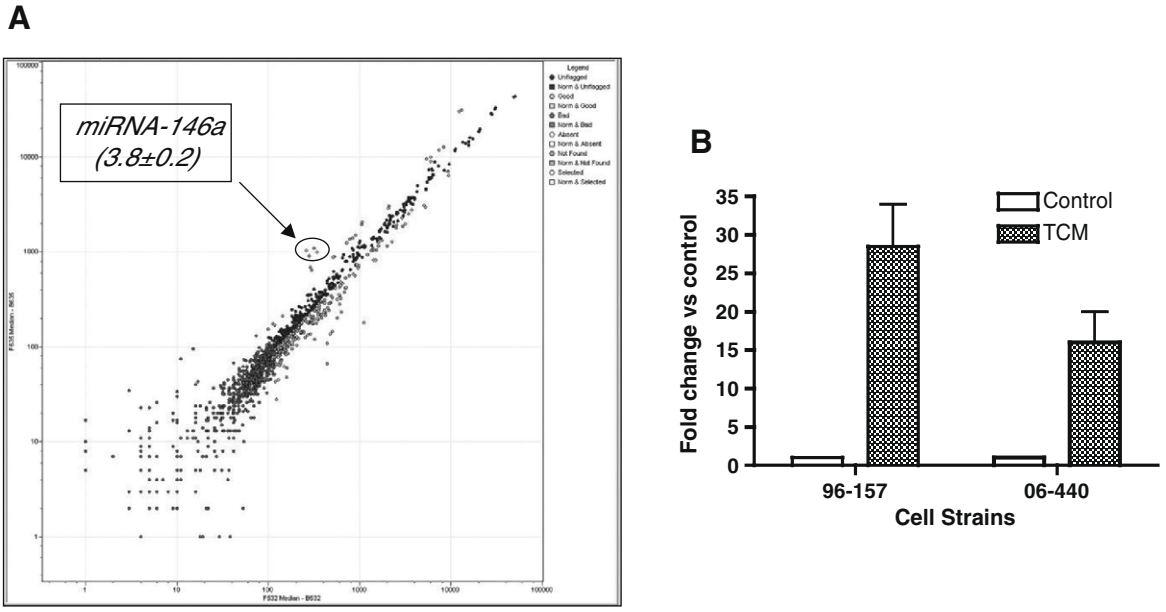

Fig. 2. MicroRNA-146a expression in HBECs. HBECs were treated with TCM for 24 h. (A) miRNA expression by microarray. Four dots in the circle indicate one representative of three strains tested (an average of 3.8 ± 0.2-fold of control in all three strains). Vertical axis: cells treated with TCM; horizontal axis: cells in medium only. (B) Quantification of miRNA-146a expression by real time RT-PCR. Data presented was from two strains of the HBECs. Vertical axis: fold change compared to control; horizontal axis: cell strains. Control: medium only; TCM: TGF-ß1 plus cytomix.

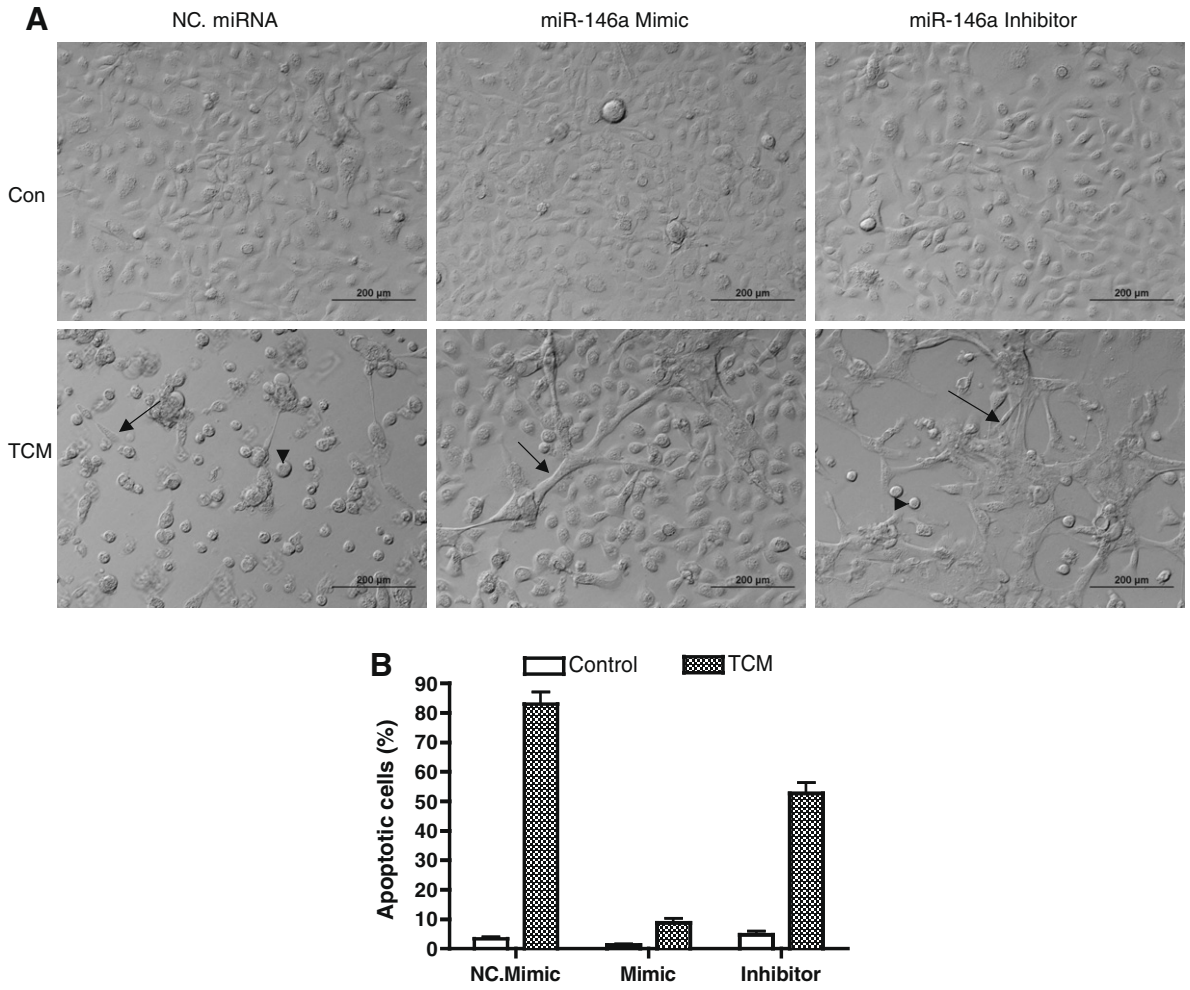

Fig. 3. Introduction of a miRNA-146a mimic protected HBECs from apoptosis. Following transfection, the cells were treated with or without TCM for 72 h. (A) Cell morphology and viability under phase contrast microscope. Con: medium only. TCM: TGF-ß1 plus cytomix. Scale bar = 200 μm. Arrow-heads: indicate examples of apoptotic cells. Arrows: indicate examples of the cells undergone EMT. (B) Percentage of apoptotic cells (%). Cells were stained with LIVE/DEAD kit and apoptotic cells were counted under high power field (400×). Vertical axis: percent of apoptotic cells (%); horizontal axis: transfection of miRNA-146a mimic or inhibitor. Open bar: cells in medium only; TCM: cells treated with TGF-ß1 plus cytomix.

both apoptosis and EMT in response to TCM, and that expression of miRNA-146a is up-regulated in HBECs exposed to TCM. Introduction of a miRNA-146a mimic into HBECs protected cells from cytokine-induced apoptosis, but had no apparent effect on induction of EMT. HBECs transfected with the miRNA-146a mimic had a higher level of Bcl-XL expression and STAT3 phosphorylation following subsequent cytokine stimulation, suggesting that miRNA-146a protects HBECs from apoptosis by modulating STAT3 signaling and anti-apoptotic protein expression. Interestingly, a miRNA-146a inhibitor also modestly increased cell survival and appeared to increase the induction of EMT. Together, these results support a role for miRNA-146a in modulating the survival, and possibly the differentiation, of HBECs in the presence of TGF-ß1, IL-1ß, TNF-α and IFN-γ.

In response to airway insults, cytokines and growth factors are released from the inflammatory and structural cells. These cytokines including IL-1ß, TNF-α and IFN-γ, and growth factors such as TGF-ß are considered to play an important role in airway remodeling. Here, we report that exposure of HBECs to TCM resulted in two different fates. First, these cytokines could induce in a population of the HBECs to undergo EMT. Second, the cytokines could also induce HBECs to undergo apoptosis. The factors that regulate which of the possible disparate cell fates following TCM-stimulation remain to be fully determined. The current study, however, defines miRNA-146a as one intracellular mediator that plays a role in this process.

Accumulating studies have demonstrated that miRNAs are involved in regulating variety of cell functions [18,32,33]. For instance, miRNA-146a expression is altered in the inflammatory milieu and contribute to variety of cell functions [18,34–37]. MicroRNA-146a was up-regulated in cervical cancer cells and fibroblasts from rheumatoid arthritis [27,34,35]. Further, miRNA-146a was up-regulated by TNF-α and IL-1ß in rheumatoid arthritis synovial fibroblasts [35] and introduction of miRNA-146a into a cervical cancer cell line promoted cell proliferation [27]. Consistently, here, we report that miRNA-146a was up-regulated by TCM in HBECs, and introduction of a miRNA-146a mimic not only protected HBECs from apoptosis, but also promoted cell proliferation, suggesting miRNA-146a is involved in regulating cell survival and proliferation in HBECs. Interestingly, a miRNA146a inhibitor also modestly reduced apoptosis but apparent enhancement of EMT in response to TCM by morphologic criteria. An effect of miRNA146a on EMT and its mechanism, which was not the major focus of the current study, remains to be further studied.

Several signal transduction pathways are involved in regulating cell survival or apoptosis [38–40]. We have reported that STAT3

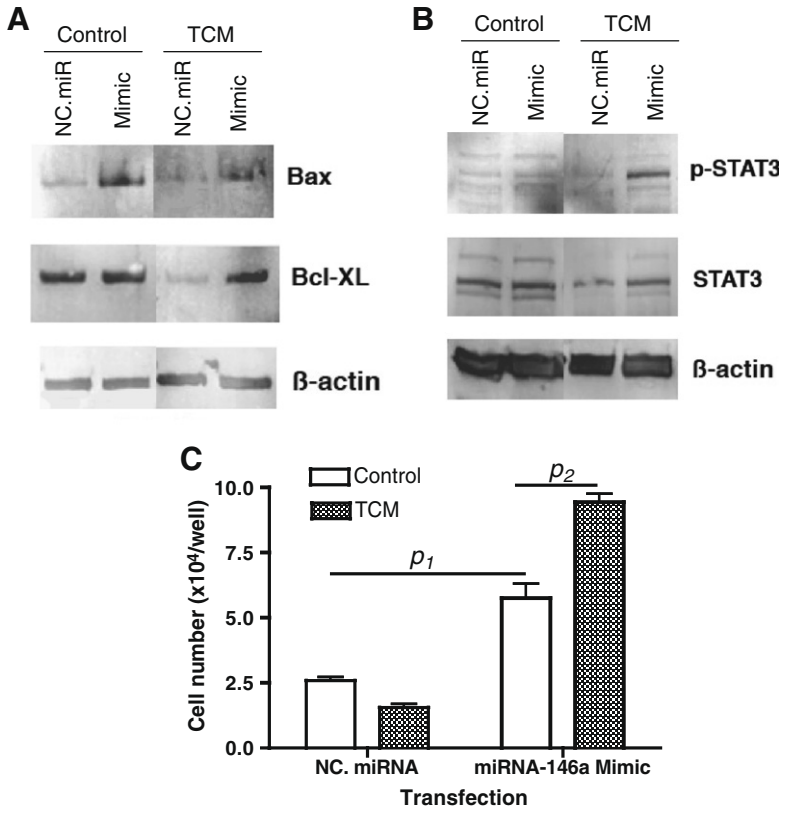

Fig. 4. Alteration of Bcl-XL expression and STAT3 phosphorylation in HBECs. Following transfection, HBECs were treated with TCM for 48 h. Cell lysates were subjected to immunobloting. (A) Expression of Bax and Bcl-XL. (B) STAT3 expression and activation (phospho-tyr 705). (C) Cell proliferation assay. In parallel to A and B, cells were also plated into 24-well plates and allowed to grow. Cell number was counted with a Coulter Counter. Data presented was cell number on day 6. Vertical axis: cell number per well; horizontal axis: cells transfected with negative control miRNA (NC. miRNA) or miRNA-146a mimic. Open bar (Con): cells in medium only; hatched bar (TCM): cells treated with cytokines for 48 h. $p1 < 0.01$; $p2 < 0.01$.

and NF-kB pathways are required for HBECs to survive following cigarette smoke-induced DNA damage and repair [30,31]. In the current study, therefore, the effect of the miRNA-146a mimic on STAT3 and p65/p50 phosphorylation was assessed by immunoblotting. While neither p65 nor p50 was altered by miRNA-146a, STAT3 was highly phosphorylated in the cells transfected with the miRNA-146a mimic and exposed to the cytokines, indicating STAT3 may be involved in mediating miRNA-146a modulation of HBEC survival. Furthermore, in the presence of the cytokines, Bcl-XL was significantly down-regulated in HBECs transfected with the negative control miRNA but not in the cells transfected with the miRNA-146a mimic. These results suggest that blockade of HBEC apoptosis by miRNA-146a may be mediated by STAT3 signaling through regulating Bcl-XL expression.

Taken together, the current study demonstrates that HBECs undergo either EMT or apoptosis in response to TCM. MicroRNA-146a is up-regulated in the cells exposed to the cytokines and the miRNA-146a mimic inhibits cells from undergoing apoptosis. Blockade of HBEC apoptosis by miRNA-146a may be mediated by STAT3 signaling pathway, which regulates the anti-apoptotic protein Bcl-XL. By this mechanism, miRNA-146a may play an important role in tissue remodeling in the milieu of airway inflammation.

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