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Evaluation of the Effect of RhoB Inhibition on Epithelial to Mesenchymal Transition Properties in NSCLC A549 Cells

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Abstract: Epithelial to mesenchymal transition (EMT) is a naturally occurring process during embryonic development and wound healing that is implicated in cancer progression when aberrantly reactivated. EMT has also been linked to chemotherapy resistance, anti-apoptosis, migration, and invasion of cells. It is thought to endow cancer cells with the ability to self-renew and mesenchymal properties that promote dissemination and metastasis. RhoB, a protein that binds GTP, regulates cellular processes such as cell survival, tumorigenesis, angiogenesis, migration, and metastasis. RhoB down-regulation is correlated to higher degrees of tumor progression and invasiveness. The loss of RhoB during cancer progression is thought to induce EMT by regulating the expression of certain EMT-associated transcription factors. This study aims to investigate the possible relationship between RhoB expression and the EMT characteristics of non-small cell lung cancer cells, such as in vitro migration proprieties. RhoB siRNA or All Stars siRNA negative control was transfected into A549 cells to determine whether RhoB depletion could promote EMT properties. The wound-healing assay was used for evaluating cell migration. The results demonstrate that the knockdown of RhoB promoted the migration ability of A549 cells. The current study may help to better understand the role of RhoB in EMT.

Keywords: EMT, RhoB siRNA, Migration, Wound healing assay.

Introduction

Lung cancer is a malignant disease that often does not show obvious symptoms until its advanced stages. In the late 20th century, lung cancer has become the leading cause of cancer-related deaths worldwide (Molina et al., 2008), with 1.76 million deaths in 2018, according to WHO (World Health Organization, 2018). Lung cancer accounts for 31% of cancer-related deaths in women and approximately 26% of cancer-related deaths in men (Viani et al., 2012). The 5-year survival rate of lung cancer patients is 36-73% (Padda et al., 2014). Lung cancer is divided into four main types: squamous cell carcinoma, adenocarcinoma, large cell carcinoma, which are included in the non-small cell lung cancer, and small-cell carcinoma. (Travis et al., 2012). Non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancer cases. Although new therapies have been developed, the 5-year survival rate of patients with NSCLC is as low as 15% (Gao et al., 2014).

Epithelial- to- mesenchymal transition (EMT) is a reversible mechanism in which epithelial cells lose characteristic epithelial proprieties while simultaneously gaining mesenchymal stem cell characteristics (Acloque et al.,2009; Maier et al.,2010; Stone et al.,2016; Skrypek et al., 2017). Epithelial-mesenchymal transition plays a role in normal physiological functions such as embryonic development during embryogenesis as part of tissue remodeling and during wound healing, but can also be recapitulated by cancers (Hay,1968; Thiery 2003; Lee at al.,2006; Thiery et al.,2006; Visvader and Lindeman,2008; Micalizzi et al.,2010). It contributes to pathological conditions such as fibrosis and tumorigenesis (Aroeira et al.,2007; Angadi and Kale, 2015; Stone et al.,2016). Cancer cells undergo a change in phenotype to make them more mobile and lose

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polarity (Angadi and Kale, 2015). EMT allows cancer cells to be more invasive and metastasize ("walk out" of the primary tumor) and contributes to chemoresistance (Thiery et al., 2002; Thiery et al., 2006; Christiansen and Rajasekaran., 2006). Once metastatic cells reach a secondary site, they must regain the proliferative characteristics of epithelial cells to form a secondary tumor (Acloque et al.,2009; Stone et al.,2016; Skrypek et al., 2017).

Migration is a key process that enables cells to modify and reach their proper location in a given environment to perform their function (Te Boekhorst et al., 2016). This phenomenon plays an important role in various processes in multicellular organisms such as gastrulation, embryogenesis, nervous system development, tissue homeostasis, and trafficking of immune cells. However, if cell migration is deregulated, it may lead to many pathological processes, including inflammation and cancer metastasis (Charras and Sahai, 2014; Mayor and Etienne-Manneville,2016; Van Helvert et al., 2018). In the development and progression of cancer, metastasis occurs as tumor cells migrate through the circulatory and lymphatic systems from the primary tumor, invade the basement membranes and endothelial walls and eventually colonize distant organs (Friedl and Wolf, 2003; Friedl and Alexander, 2011).

RhoB, a Rho GTPase, is different from other Rho family members in terms of various properties such as intracellular localization, short half-life at both mRNA and protein levels in cells, and isoprenylation of the -COOH terminal (Adamson et al., 1992). Several studies have reported that in many cancer types, RhoB expression is downregulated, while RhoA and RhoC expressions are upregulated (Chen et al., 2000; Abraham, 2001; Kamai et al., 2001; Forget et al., 2002; Adnane et al., 2002; Kamai et al., 2003; Horiuchi et al., 2003; Jiang et al., 2004; Mazieres et al., 2004; Mazières et al., 2005; Sato et al., 2007; Zhou et al., 2011). As the tumor progresses towards advanced invasive carcinoma, RhoB remains localized in the cytoplasm, loses its ability to translocate to the nucleus, and its expression decreases dramatically in the middle and poorly differentiated regions of the tumor (Adnane et al., 2002).

Increasing evidence suggests a relationship between EMT induction and RhoB downregulation (Bousquet et al., 2009; Vega et al., 2015; Bousquet et al., 2016; Calvayrac et al., 2017). Data from various studies have shown that RhoB inhibition is accompanied by cytoskeletal rearrangements such as modulation of the cytoskeleton of actin and vimentin and altering the expression of vinculin and cadherins, which are the main features of the EMT process (Bousquet et al., 2009). During cancer progression, RhoB loss causes EMT through overexpression of mRNA levels of SLUG but not SNAIL transcription factor, and a decrease in E-cadherin mRNA and protein levels (Bousquet et al., 2009; Vega et al., 2015; Bousquet et al., 2016; Calvayrac et al., 2017). This suggests that RhoB downregulation has an effect only on specific EMT-inducing transcription factors (Shih and Yang., 2011; Baldwin et al., 2014; Wang et al., 2016).

This study aims to investigate the contribution of RhoB expression to the in vitro EMT properties of non-small cell lung cancer cells, such as cell migration.

Materials and Methods:

Cell Culture:

A549 cells were grown in RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C and 5% CO_2 in a humidified incubator. Cells were seeded in 25 cm^2 tissue culture flasks and passaged when 80% confluence was reached.

Transfection with RhoB siRNA

Before transfection, 6×10^4 cells were seeded in 24-well plates in an appropriate culture medium containing serum and antibiotics. In the short time until transfection, the cells were incubated under normal growth conditions (typically 37°C and 5% CO_2). RhoB siRNA or AllStars siRNA negative control (used as RhoB siRNA negative control) was diluted in Opti-MEM I medium without serum. To dilute HiPerfect, Opti-MEM I was added and gently mixed by inverting the tube 2-3 times. Diluted HiPerFect was added to the diluted RhoB siRNA and mixed by vortexing. RhoB siRNA or RhoB siRNA negative control (Qiagen) were allowed to form transfection complexes with HiperFect (Qiagen) for 20 minutes at room temperature (15−25°C) in serum-free Opti-MEM I (Invitrogen) at 25 nM final concentration according to the manufacturer's instructions. Then,

transfection complexes were added drop-wise onto A549 cells. The plate was swirled gently to ensure uniform distribution of the transfection complexes. The cells were incubated for 24 hours with the transfection complexes under their normal growth conditions. RhoB knockdown was monitored 24 h after transfection. Transfected cells were harvested for total RNA isolation. The relative expression of RhoB was evaluated by qRT-PCR.

RT-PCR:

Total RNA was isolated from cells using the miRNeasy Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription reaction was carried out using the High-Capacity RNA to cDNA ™ kit (Applied Biosystems). SYBR green-based real-time RT-PCR was carried out using the Power SYBR® Green PCR Master Mix kit (Applied Biosystems) to measure the expression of RhoB in cells by the StepOnePlus Real-Time PCR System (Applied Biosystems). GAPDH was used as an endogenous control.

Scratch Wound Healing Assay:

A549 cells transfected with RhoB siRNA or AllStars siRNA negative control were seeded at a density of $2x10^5$ cells/well in 24-well plates. After the cells reached 90-100% confluence, they were serum starved overnight in media before starting the experiment. The confluent cell monolayer was then wounded by scraping the monolayer using a 10µl pipette to generate scratch wounds and washed with PBS twice to eliminate cell debris. Then, cells were allowed to migrate for 72 hours in the 1% FBS RPMI-1640 medium at 37°C. Wound closure or cell migration images were photographed when the scrape wound was introduced at 0 h and 24, 48, and 72 h after initial wounding. Six locations in each transfection group were visualized and photographed using a 10X objective lens under a Nikon ECLIPSE TS100 phase-contrast inverted microscope equipped with a DS-Fi1 Camera. The migration areas were measured by calculating the difference between the wound area at the indicated time points and the initial wound area using the Image J- NIH (U. S. National Institutes of Health, Bethesda, MA, USA).

Statistical Analysis:

All data are expressed as mean ± SD. Student's *t*-test for the comparison between groups was performed using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, California USA). Those with a *p*-value equal to 0.05 or less were considered statistically significant.

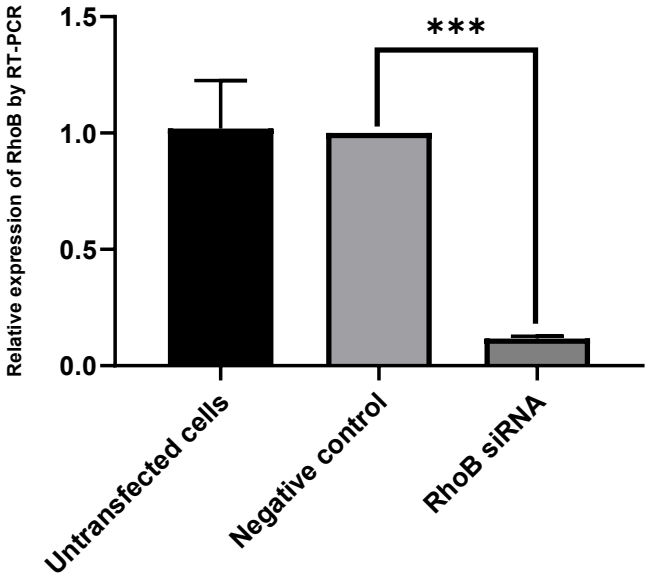

Figure 1. Relative expression of RhoB siRNA by RT-PCR. (*** indicates $p<0.001$).

Results and Discussion

RT-PCR:

To explore whether RhoB siRNA can decrease the expression of RhoB, A549 cells were transfected with RhoB siRNA or RhoB siRNA negative control (AllStars siRNA negative control) at a final concentration of 25 nM for 24 h, then the relative expression of RhoB was measured by qRT-PCR. The relative expression of RhoB was 0.12 ± 0.01 in A549 cells transfected with RhoB siRNA, which was significantly decreased, as compared to 1.00 ± 0.00 in A549 cells transfected with RhoB siRNA negative control cell group (p< 0.001; Fig.1) when analyzed by RT-PCR. The results suggest that RhoB siRNA could decrease RhoB expression in A549 cells.

Scratch Wound Healing Assay:

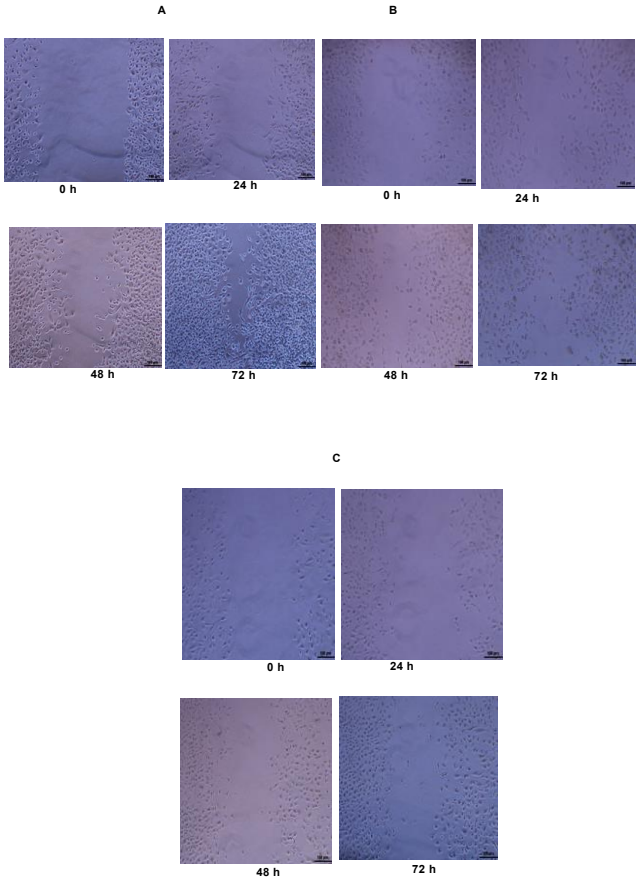

Figure 2. Phase-contrast photographs of the cultures taken at 0 h and at the indicated time intervals. Scale bar= 100 µm.

To explore the effects of RhoB siRNA on the migration of A549 cells, an in vitro scratch assay was performed on A549 cells transfected with RhoB siRNA (Fig. 2A) or RhoB siRNA negative control (Fig. 2B) and untransfected cells (Fig. 2C) and images were taken at 0h, and 24,48 and 72 h incubation times after initial wound scratching using a phase-contrast microscope. The migration rate was calculated by measuring the total distance that A549 cells migrated towards the center of the wound from the edge of the wound.

Wound closure% at different time points was represented as the percentage of wounded area at time 0. The capacity of wound healing in A549 cells transfected with RhoB siRNA was significantly enhanced compared to control cells at 24 h (p= 0.013; Fig. 3A), at 48 h (p=0.030; Fig. 3B), and at 72 h (p= 0.025; Fig. 3C) after scratching, suggesting that RhoB siRNA could promote cell migration in A549 cells, which further emphasize the function of RhoB in the migration and metastasis of non-small cell lung cancer cells.

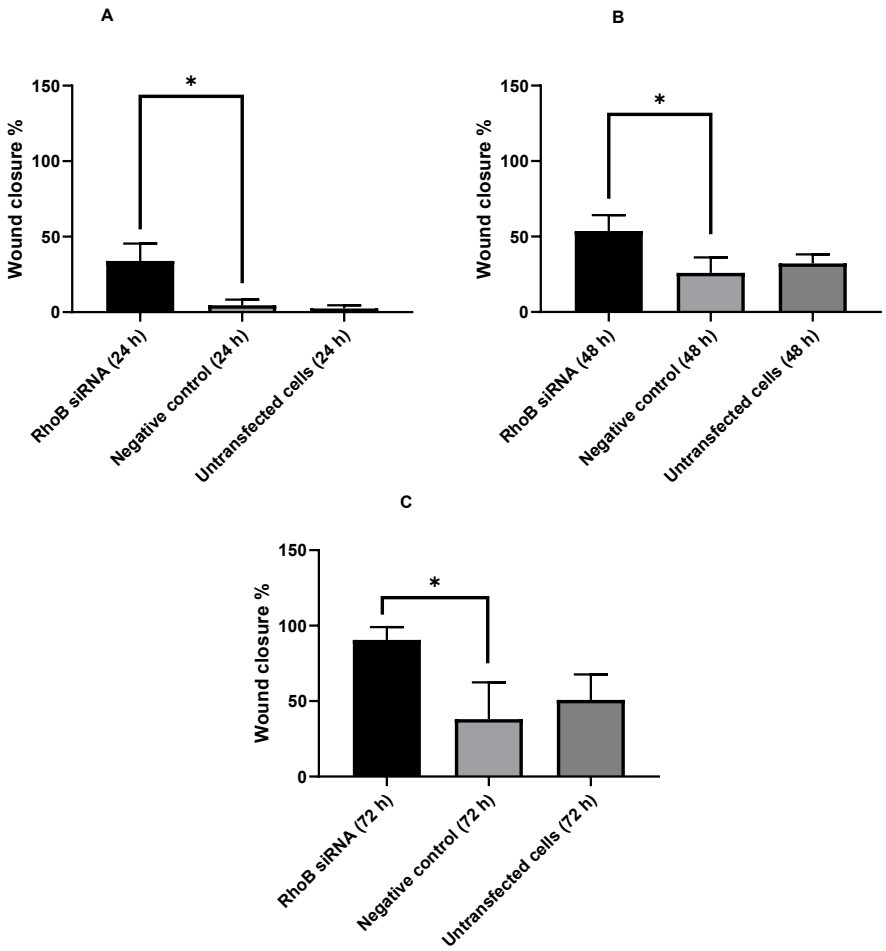

Figure 3. Analysis of the wound area closure of A549 cells at different time points. (* indicates $p < 0.05$).

Discussion and Conclusion:

RhoB, a protein that binds GTP, is involved in regulating many cellular processes such as apoptosis, tumorigenesis, angiogenesis, migration, and metastasis (Howe and Addison,2012). RhoB down-regulation is correlated to higher degrees of lung tumor progression and invasiveness (Adnane et al.,2002; Wang et al.,2003; Mazieres et al.,2004; Sato et al.,2007; Chen et al.,2016). The loss of RhoB during cancer progression is thought to induce EMT by regulating the expression of certain EMT-inducing transcription factors (Shih and Yang., 2011; Baldwin et al., 2014; Wang et al., 2016). This study aimed to investigate the relationship between RhoB expression and the in vitro EMT functional properties of non-small cell lung cancer cells, such as cell motility and migration. For this purpose, RhoB siRNA or RhoB siRNA negative control was transfected into A549 cells to determine whether RhoB depletion could promote EMT properties. The scratch wound-healing assay was used for evaluating cell migration. Results revealed that RhoB knockdown significantly enhanced the migration of NSCLC A549 cells. Our results are in accordance with previous studies, reporting that low expression of RhoB promoted EMT, migration, and invasion (Bousquet et al.,2009; Ma et al.,2019). Taken together, these results indicate that RhoB plays an important role as a tumor suppressor in tumorigenesis, cancer cell motility, EMT, and metastasis in NSCLC and could be a potential target for cancer treatment.

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