Accepted Manuscript

Title: MicroRNA-328 is involved in wound repair process in human bronchial epithelial cells

Authors: Beata Narożna, Wojciech Langwinski, Claire Jackson, Peter Lackie, John W. Holloway, Aleksandra Szczepankiewicz

PII: S1569-9048(17)30095-2
DOI: http://dx.doi.org/doi:10.1016/j.resp.2017.03.010
Reference: RESPNB 2790

To appear in: Respiratory Physiology & Neurobiology

Received date: 21-7-2016
Revised date: 6-3-2017
Accepted date: 24-3-2017

Please cite this article as: Narożna, Beata, Langwinski, Wojciech, Jackson, Claire, Lackie, Peter, Holloway, John W., Szczepankiewicz, Aleksandra, MicroRNA-328 is involved in wound repair process in human bronchial epithelial cells. Respiratory Physiology and Neurobiology http://dx.doi.org/10.1016/j.resp.2017.03.010

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
MicroRNA-328 is involved in wound repair process in human bronchial epithelial cells

Running title: MicroRNA 328 modifies airway epithelial repair

Beata Narożna, MSc\textsuperscript{a}, Wojciech Langwinski, MSc\textsuperscript{a}, Claire Jackson, PhD\textsuperscript{b}, Peter Lackie, PhD\textsuperscript{b}, John W. Holloway, Prof.\textsuperscript{c}, Aleksandra Szczepankiewicz, PhD\textsuperscript{a}\textsuperscript{*}

\textsuperscript{a}Laboratory of Molecular and Cell Biology, Department of Pediatric Pulmonology, Allergy and Clinical Immunology, Poznan University of Medical Sciences, 27/33 Szpitalna St., 60-572 Poznan, Poland

\textsuperscript{b}Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK

\textsuperscript{c}Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, UK

\textsuperscript{*}Corresponding author:
Aleksandra Szczepankiewicz, PhD
Laboratory of Molecular and Cell Biology, Department of Pediatric Pulmonology, Allergy and Clinical Immunology, Poznan University of Medical Sciences, Poland
27/33 Szpitalna St., 60-572 Poznan, Poland; Tel. +48-618491311, fax. +48-61-8480111, e-mail: alszczep@ump.edu.pl
Highlights

- Global miRNA silencing delays the repair of bronchial epithelial cells *in vitro*
- MiRNA-328 is a potent regulator of epithelial wound repair
- MiRNA-411, miR-609, miR-342 and miR-888 do not seem to influence the repair process
- MiRNA-328 is predicted to be involved in the regulation of actin cytoskeleton pathway

Abstract

Our aim was to investigate the role of microRNA on epithelial wound repair by global microRNA silencing. We have also analysed the influence of five miRNAs (miR-328, miR-342, miR-411, miR-609, miR-888, previously identified) on wound repair in 16HBE14o-bronchial epithelial cell line. Cells were transfected with siRNAs against human DROSHA and DICER1 or miRNA mimics or inhibitors. Wounding assays were performed and the cells were observed using time-lapse microscopy. The area of damage was calculated at chosen time points, followed by data analysis. Cells with silenced global miRNA expression showed a significantly slower repair rate compared to the control cells (p=0.001). For miR-328, we observed significantly delayed repair in cells transfected with the inhibitor compared to control (p=0.02). Global microRNA silencing significantly decreased the repair rate of airway epithelial cells in vitro, indicating an important role of miRNA in the regulation of wound repair and that miR-328, possibly involved in actin pathway, may be a potent modifier of this process.

**Keywords:** epithelial cells, wound repair, miRNA, gene silencing, time-lapse microscopy, pathway analysis
1. Introduction

The airway epithelium provides the first line of defence against potentially damaging environmental agents and facilitates the clearance of particles deposited in the airways. It is also responsible for the integration of innate and adaptive immune responses [1, 2]. Due to the presence of tight junctions, under normal conditions, airway epithelium constitutes a tightly regulated, almost impermeable mechanical barrier [3]. It is also a secretory tissue that produces lipid mediators, growth factors, bronchoconstrictor peptides, cytokines and chemokines [4].

Injury to airway epithelium results in the loss of its structural integrity and function [5]. Studies in animals have shown that small injuries of the epithelium are rapidly repaired, due to migration, spreading and proliferation of cells close to the wound [6]. Once the barrier is repaired, the differentiated characteristics are then restored. However, if the injury is either severe or continuous, the repair process is disturbed, resulting in increased permeability for environmental allergens, pathogens, and toxic substances [7]. Chronic inflammation of the airways leads to aberrant repair and functional and structural changes in the airway epithelium. This process underlies the pathogenesis of many respiratory diseases, including asthma, one of the most common chronic airway diseases [8]. Asthma is characterized by chronic inflammation of the airways followed by functional and structural changes of the respiratory tract that may lead to airflow obstruction and bronchial hyperreactivity.

The key steps in the wound repair process are cell dedifferentiation, migration, spreading of the cells to close the wound, proliferation, and differentiation. These processes require coordinated expression of many genes [9]. A key regulators that control coordinated gene expression are microRNAs (miRNAs), small, single-stranded, noncoding RNAs. Individual miRNA may have hundreds of target mRNAs and each mRNA may be regulated by several different miRNAs. The effect of each miRNA on gene expression is wide-reaching...
and they form non-linear gene networks with target mRNAs. The expression of Dicer, the enzyme responsible for the processing of miRNA precursors, is essential for lung morphogenesis [10]. Moreover, the expression of miRNAs has been shown to differ during lung development [11]. Furthermore, transgenic over-expression of the miR-17-92 cluster (present in lung cancer) in the airway epithelium promotes proliferation and inhibits differentiation of lung epithelial progenitor cells [12].

We have previously shown altered expression of miRNAs during the repair of airway epithelium in vitro, suggesting that miRNAs may be important regulators of this process [13]. To confirm this, we aimed to perform global microRNA silencing using inhibitors of DROSHA and DICER proteins that are responsible for the processing of miRNA precursors. Then, we have selected five miRNAs showing the most significantly altered expression at different stages of wound regeneration in airway epithelium (miR-328, miR-342, miR-411, miR-609, miR-888) to investigate their influence on the repair rate.

2. Materials and Methods

2.1 Cell culture

The 16HBE14o- bronchial epithelial cell line was grown in MEM with Earle’s salts (Thermo Fisher Scientific), supplemented with 10% FBS (Life Technologies, UK), 20 mM L-glutamine, 10 U/ml of penicillin, 10 µg/ml of streptomycin sulphate and 0.2% nystatin. Cells were maintained at 37°C in a 5% CO₂ incubator.

2.2 Selection of miRNAs

Our previous work on microRNA profiling during airway epithelial wound repair [13] has revealed at least 10 potential miRNAs that could regulate this process. Over 10-fold changes in expression were observed at 5 different time points of repair in epithelial cells following wounding: 4 hours (25% of wound area is covered by cells), 8 hours (50% of wound area is covered by cells), 16 hours (wound area is completely covered by cells) and 24
hours (repair completed) in comparison to the baseline (cells before wounding). Out of these 10 miRNAs, we selected 5 genes showing the most significant expression changes over time; 3 genes were upregulated (miR-609, miR-411, miR-328) and 2 were downregulated (miR-888 and miR-342-5p). Then, we used 3 microRNA inhibitors that silenced the upregulated miRNAs, so that we could observe the effect on wound repair. For the downregulated miRNAs, we have applied mimic miRNAs to study whether their presence during epithelial repair had any effect on this process.

2.3 Transfection

Epithelial cells were seeded at a density of 1x10^5 cells per well on 12-well plates. At 70-80% confluence, cells were transfected with Lipofectamine 3000 (Thermo Fisher Scientific) in Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific), according to the manufacturer’s protocol. Each experiment included three biological replicates for all siRNAs, miRNAs mimics and inhibitors.

For global microRNA silencing experiments, the cells were transfected with 25 pmol of negative control (AllStars Negative siRNA Control, Qiagen) or co-transfected with 12.5 pmol of siRNA against DROSHA (Hs_RNASE3L_3 FlexiTube siRNA, Qiagen) and 12.5 pmol of siRNA against DICER (Hs_DICER1_11 FlexiTube siRNA, Qiagen), all fluorescently labelled.

To study the effect of specific miRNA on the epithelial repair, cells were transfected either with fluorescently labelled 90 pmol of negative control (Thermo Fisher Scientific), 90 pmol of mimic (in the case of miR-342 and miR-888) or inhibitor (for miR-328, miR-411 and miR-609) (Thermo Fisher Scientific).

Optimization of transfection experiments was performed by running multiple tests with different concentrations of lipofectamine and miRNA mimic/inhibitors and siRNAs.
Transfection efficiency for each experiment was assessed as a percentage of cells showing fluorescent signal under the microscope and varied between 50-70%.

2.4 Wounding assays

Scratch assay is a commonly used technique [14-17] to create in vitro wound repair model used in this study and our previous report [13]. It is based on the approach that cell signals are propagated by mechanical stimulation, such as Ca^{2+} waves [18]. Moreover, one of the major advantages of this method, compared to other techniques used to wound the cells (e.g. burning, freezing, etc.), is that it does not damage the plastic plates on which the epithelium grows.

Forty-eight hours after reaching confluence, the cells were damaged with single line wounding pattern line using a P200 Gilson pipette tip. Medium and cell debris were removed, and new medium was added.

2.5 Time-lapse microscopy

Time-lapse images (4x magnification) were captured by a Hamamatsu ORCA digital camera at 15-minute intervals for 30 hours on Leica DM IRB phase-control inverted microscope in chamber maintained at 36±1°C and 5% CO_{2} atmosphere. The rate of repair over time in treated and untreated cells was assessed using ImageJ software. The areas of damage were measured at each time point in the cultures. At least three fields of view were counted for each sample. The area of repair was calculated by subtracting the area of damage at each time point from the original area of damage at time 0.

2.6 Statistical analysis

Significant differences in rate or time-course of wound healing in comparison to negative control were assessed using paired t-test and two-way ANOVA after data transformation to normal distribution. All data are represented by mean ± SD. Statistically significant P value was below 0.05. Calculations were carried out in Statistica v.10.
2.7 Target genes and pathways prediction

Since miR-328 showed a significant influence on the rate of wound repair, we performed the pathway enrichment analysis. Using miRNA BodyMap (available at http://www.mirnabodymap.org), a tool based on several prediction algorithms (mirBase, TargetScan, miRDB, MicroCosm, DIANA, TarBase, PITA, RNA22, miRecords), we identified the best-predicted target genes. To minimize the target prediction noise, only genes predicted by four or more prediction algorithms were included. To identify BioCarta & KEGG pathways [19, 20], the list of potential targets was analysed with the use of The Database for Annotation, Visualization and Integrated Discovery (DAVID) v.6.7 [21, 22].

3. Results

3.1 Global microRNA silencing

The process of wound repair for cells with inhibited global miRNA expression and control cells was shown in figure 1. Cells transfected with siRNAs directed against DROSHA and DICER showed significantly delayed repair of the wound as compared to negative control cells (p=0.001) (figure 2). The significant differences were observed at 2, 4, 8 and 12 hours post-wounding.

3.2 Transfection of selected miRNAs with antagomirs or mimic miRNA

Transfection experiments involving miR-342, miR-411, miR-609 and miR-888 inhibitors have not shown significant differences between cells transfected with either inhibitor or mimic and cells transfected with scramble negative control (p=0.999, p=0.882, p=0.999 and p=1.000, respectively), as shown in figure 3. Transfection of cells with miR-328 inhibitor resulted in significantly delayed repair in comparison to cells transfected with negative control (p=0.02) (figures 4 and 5). The significant differences were observed at 4, 8, 12 and 16 hours post-wounding. The experiment was repeated to verify the results (p=0.02).

3.3 Target pathways
A list of 1486 target genes, predicted to be regulated by miR-328, was analysed with the DAVID annotation tool to identify the most enriched pathways involved in epithelial wound repair. Several pathways were found significantly enriched (specifically associated with the target gene list), however, after correction for multiple testing, only the actin-cytoskeleton pathway remained significant (enrichment score 1.9; p=0.042) (table 1).

4. Discussion

The main finding of this study is that altered global microRNA expression significantly influences the rate of epithelial wound repair in vitro and that inhibition of miR-328 impedes the repair process.

Our previous results showed altered expression of numerous miRNAs during epithelial repair [13], suggesting their potential role in this process. In this study, we found that downregulation of DICER and DROSHA, enzymes involved in miRNA maturation, significantly delays repair process in bronchial epithelial cells, thus confirming that microRNAs play a crucial role in wound regeneration process.

Moreover, we have observed significant involvement of one microRNA, miR-328, in the epithelial repair, whereas little or no effect was observed for the other four miRNAs (miR-342, miR-411, miR-609, miR-888). Our study showed that downregulating the expression miR-328 gene by transfecting the cells with its inhibitor significantly delayed repair process as compared to the control cells.

To our knowledge, there are no previous findings on the function of miR-328 in the wound repair of airway epithelium. Published data on the possible function of this miRNA showed that it inhibits the proliferation of human melanoma cells via regulating the expression of one of its target genes, TGFβ2 (transforming growth factor), a cytokine regulating cell adhesion, proliferation, differentiation and migration [23]. In the study by Luo et al., upregulated expression of miR-328 was found to promote migration and invasion of
hepatocellular carcinoma cells [24]. This effect was correlated with decreased expression of protein tyrosine phosphatase (*PTPRJ*) gene, another possible target of miR-328, involved in cells growth, differentiation and mitotic cycle [25]. Downregulation of miR-328 followed by CD44 overexpression in gastric cancer leads to acquired resistance to reactive oxygen species (ROS); this has been implicated to play a role in gastric cancer pathogenesis [26].

Further studies reported that this microRNA is involved in regulating the expression of genes associated with transport across the membranes [27], cell-cell interactions, cell adhesion and migration [28], and calcium-dependent processes, such as cell motility, cell division and cell death [29]. These studies are in line with our findings and suggest that miR-328 may represent a potent modifier of the complex process of wound repair.

Moreover, pathway analysis of predicted target genes for miR-328 has confirmed its involvement in the regulation of two pathways: regulation of actin cytoskeleton and CXCR4, that are responsible, among others, for cell migration, focal adhesion and adherence junction formation, processes that are important in wound repair [30, 31].

For the other four analysed miRNAs (miR-411, miR-609, miR-342-5p and miR-888) we did not observe their significant influence on wound repair, despite changes in expression during epithelial repair in vitro that we discovered previously [13]. Studies on their possible function showed that overexpression of miR-411 inhibited proliferation of chondrocytes [32], but promoted the proliferation and growth of hepatocellular carcinoma cells [33]. Another miRNA, miR-342-5p, was previously reported to play a role in the tumorigenesis in breast cancer [34, 35] and colon cancer [36]. To our knowledge, there are no previous reports on the possible function of miR-609 and miR-888. The results of our experiment suggest that the role of these four selected miRNAs may not be crucial in wound repair.

Our results have shown that miRNAs play an important role in the repair process of bronchial epithelium. There is emerging evidence that microRNA might modulate the
processes of respiratory diseases, such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and lung cancer [37]. The aberrant wound repair process can lead to airway remodelling, which, in turn, underlies many respiratory diseases. MicroRNAs also control the expression of numerous genes responsible for ROS synthesis (NOX2, POX) and ROS elimination via redox-active signaling pathways (MAPK, NF-κB) [38]. Vice versa, ROS may also influence miRNA genes affecting methylation, biogenesis and oxidative damage-induced mutations. For example, miR-15a/16 deficiency led to apoptosis of lung epithelium after oxidative stress [39], whereas reduced microRNA-34a upon oxidative stress decreased the expression of sirtuin-1 and sirtuin-6 in bronchial epithelium and thus accelerated cellular senescence [40]. Reactive oxygen species were shown to mediate the thickening of the bronchial walls and tissue fibrosis in CF [41]. They have been also described in the pathogenesis of allergic asthma, where the contact with environmental antigens results in ROS overproduction, and, in turn, might amplify the hyperresponsiveness and the inflammation in the bronchi [42]. The role of oxidative stress and ROS was also observed in COPD, as they stimulate cytokines release and contribute to the damage of the diaphragm [43]. Therefore, it is possible that miRNAs expression may be also influenced by reactive oxygen species, leading to aberrant repair in different respiratory diseases.

Among limitations we should consider the fact that we analysed only partial (50-70%) silencing of DROSHA/DICER genes, however this enabled us to observe their effect on wound repair. In conjunction with the transfection efficiency, it might explain the seemingly small (around 10%) decrease in the wound repair rate at the early time points. Nevertheless, the results remain significantly different from the control cells.

In conclusion, impaired epithelial repair resulting from downregulated expression of DROSHA and DICER proteins indicates that miRNAs play an important role in regulating wound repair of airway epithelium. Moreover, we report here that the inhibition of miR-328
delays epithelial wound repair rate in vitro. Further experiments are warranted to identify the exact function of this microRNA.

**Funding:** This study was supported by the Polish National Science Centre grant no. 2011/01/M/NZ3/02906.

**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Acknowledgements**

BN was a recipient of an EAACI Research Fellowship 2015.

We thank the personnel from the Biomedical Imaging Unit and Primary Ciliary Dyskinesia (PCD) Diagnostic and Research Team, University of Southampton for the assistance and technical support.
References


miR-328 targeting CD44 in cancer cells and promote redox adaptation. *Carcinogenesis* 2014, **35**:1003-1011.


42. Jiang L, Diaz PT, Best TM, Stimpfli JN, He F, Zuo L: *Molecular characterization of redox mechanisms in allergic asthma.* *Ann Allergy Asthma Immunol* 2014, **113**:137-142.
Figure legends

Figure 1 Representative images of different stages of the airway epithelial wound repair for DROSHA/DICER inhibitor and control cells (T0 – immediately after wounding, T4 – 4 hrs after wounding, T8 – 8 hrs after wounding, T16 – 16 hrs after wounding, T24 – 24 hrs after wounding)

Figure 2 Comparison of wound repair rate at different time points for cells transfected with siRNAs against DROSHA/DICER (dotted line) versus cells transfected with negative siRNA control (normal line) (two-way ANOVA); bars represent standard deviations (n=3); asterisks show significant differences between time points
**Figure 3** Comparison of wound repair rate at different time points for cells transfected with four miRNA modifiers (dotted line) versus cells transfected with negative inhibitor (normal line): a. miR-342 mimic, b. miR-411 inhibitor, c. miR-609 inhibitor, d. miR-888 mimic (two-way ANOVA); bars represent standard deviations (n=3)
**Figure 4** Representative images of different stages of the airway epithelial wound repair for miR-328 inhibitor and control cells (T0 – immediately after wounding, T4 – 4 hrs after wounding, T8 – 8 hrs after wounding, T16 – 16 hrs after wounding, T24 – 24 hrs after wounding)

![Representative images of different stages of the airway epithelial wound repair](image)

**Figure 5** Comparison of wound repair rate at different time points for cells transfected with miR-328 inhibitor (dotted line) versus cells transfected with negative control inhibitor (normal line) (two-way ANOVA) (n=3); asterisks show significant differences between time points

![Comparison of wound repair rate at different time points](image)
Tables

Table 1. The results of pathway analysis of predicted target genes for miR-328 in DAVID software (bolded p value indicates significance after multiple testing correction with Benjamini-Hochberg correction)

<table>
<thead>
<tr>
<th>Category</th>
<th>Pathway</th>
<th>Enrichment score</th>
<th>No. of genes</th>
<th>% of gene list</th>
<th>P</th>
<th>p corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocarta</td>
<td>CXCR4 Signaling Pathway</td>
<td>2.8</td>
<td>7</td>
<td>0.5</td>
<td>0.029</td>
<td>1.000</td>
</tr>
<tr>
<td>Biocarta</td>
<td>Roles of beta-arrestin-dependent Recruitment of Src Kinases in GPCR Signaling</td>
<td>3.1</td>
<td>6</td>
<td>0.4</td>
<td>0.035</td>
<td>0.990</td>
</tr>
<tr>
<td>Kegg</td>
<td>Regulation of actin cytoskeleton</td>
<td>1.9</td>
<td>35</td>
<td>2.4</td>
<td>0.0002</td>
<td>0.042</td>
</tr>
<tr>
<td>Kegg</td>
<td>Pathways in cancer</td>
<td>1.5</td>
<td>43</td>
<td>2.9</td>
<td>0.004</td>
<td>0.300</td>
</tr>
<tr>
<td>Kegg</td>
<td>Focal adhesion</td>
<td>1.7</td>
<td>29</td>
<td>2</td>
<td>0.006</td>
<td>0.290</td>
</tr>
<tr>
<td>Kegg</td>
<td>Axon guidance</td>
<td>1.8</td>
<td>20</td>
<td>1.3</td>
<td>0.012</td>
<td>0.420</td>
</tr>
<tr>
<td>Kegg</td>
<td>Renal cell carcinoma</td>
<td>2.2</td>
<td>13</td>
<td>0.9</td>
<td>0.014</td>
<td>0.390</td>
</tr>
<tr>
<td>Kegg</td>
<td>Pancreatic cancer</td>
<td>2.1</td>
<td>13</td>
<td>0.9</td>
<td>0.017</td>
<td>0.400</td>
</tr>
<tr>
<td>Kegg</td>
<td>Insulin signaling pathway</td>
<td>1.7</td>
<td>20</td>
<td>1.3</td>
<td>0.019</td>
<td>0.390</td>
</tr>
<tr>
<td>Kegg</td>
<td>Phosphatidylinositol signaling system</td>
<td>2.1</td>
<td>13</td>
<td>0.9</td>
<td>0.021</td>
<td>0.380</td>
</tr>
<tr>
<td>Kegg</td>
<td>Acute myeloid leukemia</td>
<td>2.2</td>
<td>11</td>
<td>0.7</td>
<td>0.023</td>
<td>0.370</td>
</tr>
<tr>
<td>Kegg</td>
<td>mTOR signaling pathway</td>
<td>2.3</td>
<td>10</td>
<td>0.7</td>
<td>0.029</td>
<td>0.410</td>
</tr>
<tr>
<td>Kegg</td>
<td>Neurotrophin signaling pathway</td>
<td>1.7</td>
<td>18</td>
<td>1.2</td>
<td>0.032</td>
<td>0.410</td>
</tr>
<tr>
<td>Kegg</td>
<td>Glioma</td>
<td>2.1</td>
<td>11</td>
<td>0.7</td>
<td>0.038</td>
<td>0.440</td>
</tr>
<tr>
<td>Kegg</td>
<td>T cell receptor signaling pathway</td>
<td>1.7</td>
<td>16</td>
<td>1.1</td>
<td>0.038</td>
<td>0.420</td>
</tr>
<tr>
<td>Kegg</td>
<td>Calcium signaling pathway</td>
<td>1.5</td>
<td>23</td>
<td>1.5</td>
<td>0.041</td>
<td>0.420</td>
</tr>
</tbody>
</table>