Comparison of miRNA profiling during airway epithelial repair in undifferentiated and differentiated cells *in vitro*

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Abstract

Respiratory epithelium is a highly integrated structure that efficiently protects lungs from extrinsic irritants thanks to rapid repair of the wound. The repair is a complex process that requires coordinated expression of networks of genes. Plausible regulators of this process are microRNAs. We investigated if global miRNA silencing influences the epithelial repair and if changes in miRNA expression profile during repair are similar between two bronchial epithelial cell cultures: differentiated and undifferentiated cells.

Two bronchial cell types were used:16HBE14o- and NHBE. Transfection was performed with siRNAs against Drosha and Dicer. For miRNA profiling, non-transfected cells were cultured until confluent and harvested for RNA isolation at baseline (cells before wounding) and at different time post-wounding (8, 16, 24 and 48 hours). MicroRNA expression profiling was performed using TaqMan Array Human MicroRNA Card A. Target prediction was done in miRNA body map, and pathway analysis using DAVID.

Cells with downregulated Drosha and Dicer demonstrated a significantly delayed wound repair in comparison to control in both cell lines. MiRNA expression profiling revealed that ten miRNAs exhibited significant changes over time after cell injury. These genes showed a similar expression pattern in both cell lines. The predicted targets of these miRNAs were then clustered by pathway analysis into six biological groups related to wound repair.

Silencing of global miRNA expression confirmed that miRNAs are crucial for airway epithelial repair. Moreover, epithelial cells of two different origins demonstrated some similarities in miRNA expression pattern during wound repair independent of differentiation state.

Key words: airway epithelium, wound repair, miRNA, 16HBE14o- cells, NHBE cells

Short title: MiRNA in airway epithelial repair

Background

The contact between inhaled air and the cellular environment of the respiratory system requires effective mechanisms that protect human lungs from extrinsic irritants. The airway epithelium plays a key role in this process. The epithelial cells cover the airways starting from the nasal cavity, through the trachea and branches into bronchi and single bronchioles (Crystal et al. 2008). The main types of cells forming its structure are: goblet, club, ciliated and basal cells and their proportion in the epithelium may vary depending on their location in the respiratory tract (Soleas et al. 2012).

Under physiological conditions epithelium acts as a passive and active barrier that efficiently protects the respiratory system (Kale and Arora 2013; McLellan et al. 2015). Any possible damages of the respiratory epithelium is immediately repaired in the process comprising several steps. At the lesion site, extracellular matrix (ECM) is formed and this stage is crucial for proliferating and migrating cells to adhere to ECM surface, cover the damage and restore respiratory epithelium function (Sacco et al. 2004). However, some of the irritants may lead to acute (e.g. viral infections) or chronic inflammation (e.g. asthma) in the airways resulting in epithelium damage and aberrant repair (remodeling) (Lambrecht and Hammad 2014).

Models widely used for epithelial repair studies are based on cells cultured *in vitro*. There are many possibilities including undifferentiated cell lines such as 16HBE14o-, Calu-3 and BEAS-2B or primary cells grown in air-liquid interface culture (ALI) that develop cilia and thus better mimic the situation observed *in vivo* in the airways (Berube et al. 2010). The primary cells used in many studies are commercially available normal human bronchial epithelial cells (NHBE) (Davis et al. 2015). These cells are cultured in Air-Liquid Interface (ALI) model in which cells grow on the permeable surface of the inserts. The submerged culture is maintained until full confluency and then cells are fed from the bottom only and exposed to the air. This enables their differentiation and formation of cells typical for respiratory epithelium *in vivo*

(e.g. ciliated cells). Moreover, the cells secrete mucus and form multilayer structure which makes NHBE cells an appropriate model to study the biology of airway epithelium. However, the culture of these cells is time consuming and expensive (Berube et al. 2010; Lin et al. 2007).

Promising alternative for primary cultures may be immortalized cell lines such as 16HBE14o- cell line derived from human bronchial epithelium and transformed with SV40 large T-antigen (Manford et al. 2005). This cell line has the ability to form tight junctions and create a polarized epithelial layer *in vitro* (Forbes et al. 2003). It was successfully used in the previous functional studies on the repair of injured epithelium which confirms it may be employed as an *in vitro* respiratory epithelium model (Adam et al. 2007).

Despite many *in vitro* and *in vivo* models that mimic the function of respiratory epithelium, there are still many unresolved issues regarding epithelial repair (Coraux et al. 2005). One possible mechanism of this process may be post-transcriptional gene regulation by microRNAs. MicroRNAs are single-stranded, short (22 nt), non-coding RNAs that silence the expression of target genes by binding to their mRNA 3'UTR region. The functional, mature miRNA formation is controlled by two enzyme complexes (RNase III proteins) (Cai et al. 2009; Ha and Kim 2014). First enzyme, Drosha, is responsible for the processing of primary miRNA (pri-miRNA) transcript, resulting in ~70 nt double-stranded pre-miRNA. Further processing involves its transport from the nucleus to the cytoplasm where the second enzyme, Dicer cuts off one RNA strand thus forming single-stranded, functional mature miRNA(He and Hannon 2004). The number of all genes regulated by miRNAs was estimated for approximately 30% (Cheng and Li 2008). Therefore, we hypothesized that miRNA may be a potent regulator of airway epithelial repair.

The aim of this paper was to compare the miRNA expression profiles during epithelial repair in two bronchial cell lines, differentiated cells (NHBE) and undifferentiated cells (16HBE14o-). Our purpose was to investigate whether phenotypically different epithelial cells

show similar miRNAs expression profile during wound repair and if the same miRNAs are involved in airway epithelial repair in both cell lines.

Materials and methods

Cell culture and wounding assays

16HBE14o- cells

The 16HBE14o- bronchial epithelial cell line was cultured under standard conditions (Adam et al. 2007). For the wounding assay cells were seeded onto 6-well plates at the initial density of $3x10^5$ cells and cultured until confluent. Forty eight hours after reaching full confluence cells were damaged by scraping off the monolayer with a P200 Gilson pipette tip. After that, the medium and cell debris were removed and 2 ml of fresh serum-containing medium was added to the remaining cells. At least 3 points of reference per well of a 6-well plate were used for miRNA profiling analysis.

NHBE cells

Cells were grown on $75cm^2$ flasks in bronchial epithelium growth which consists of BEBM with BEGM Single Quot Kit Supplements & Growth Factors, Lonza) for approximately a week. Then the cells were subcultured onto collagen-coated transwell inserts ($3x10^5$cells on 1.2 cm^2) (Costar, Corning) in a 12 well culture plate in ALI (1:1 BEBM:DMEM 3.5g/L D-glucose with SingleQuots) medium until confluent. After that, the cells were exposed to an air-liquid interface by adding ALI medium supplemented with 100 nM retinoic acid (Sigma-Aldrich) only to the basolateral chamber. Medium was changed three times per week and any liquid or mucus that appeared on the apical surface was removed. Cilia were observed 3-4 weeks post transition to ALI. Wounding assays on ciliated cells were performed as described above, followed by miRNA profiling.

Transfection of cells

Cells were subcultured into 12-well plates at the initial density of 3x105 cells. At 70-80% confluence for 16HBE14o-and full confluence for NHBE, both were transfected with Lipofectamine 3000 (ThermoFisher Scientific) according to manufacturer's protocol: lipofectamine reagent and siRNA were diluted separately in OptiMEM medium (ThermoFisher Scientific), then incubated together for 5 minutes and used for transfection in standard culture medium. The best Lipofectamine/siRNA ratio was optimized experimentally and included 3µl of Lipofectamine 3000 reagent and 15 pmol of each fluorescently labelled siRNA(Qiagen) (RNASE3L_3 FlexiTube for Drosha andHs_DICER1_11 FlexiTube for Dicer). As a negative control, we used 3µl of Lipofectamine 3000 reagent and 30 pmol of All Stars Negative Control (Qiagen). The medium was changed the next day. The cells were allowed to grow for 48 hours until wounding assays and time lapse experiments were performed.

Time lapse microscopy

Time lapse images were captured at 15-minutes intervals on a Leica DM IRB phase-contrast inverted microscope (Leica; Milton Keynes, UK) in a chamber maintained at 36 ± 1°C and 5%CO_2 atmosphere. The images were collected with a cooled Hamamatsu ORCA digital camera (Hamamatsu Photonics, Welwyn Garden City, UK) connected to a computer running Cell^P software (Olympus, London, UK) over 48-hours (until complete wound closure). For quantitative analysis of time lapse serial images ImageJ software (Schneider et al. 2012) was used.

MicroRNA profiling

RNA isolation from non-transfected cells was performed with use of miRCURY RNA Isolation Kit - Cell & Plant (Exiqon). Samples were collected in three biological repeats at the following time points: baseline (cells before wounding), 8, 16, 24 and 48 hours after wounding. Isolation was performed according to the manufacturer's protocol. Samples were frozen at -70°C for further use in microarray experiment.

MicroRNA expression profiling of two cell lines was performed for each time point using TaqMan Array Human MicroRNA Card A v.2.0 (ThermoFisher Scientific) containing 378 mature human microRNAs in a TaqMan Low Density Array format (TLDA). Complementary DNA was generated with the use of Megaplex Primer Pools (Human Pools A v2.1, Thermo Fisher Scientific). Real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific), according to the manufacturer's protocol. MiRNA expression datasets were compared between baseline and each time point using DataAssist software v.3.01 (Applied Biosystems). The comparative CT method (Schmittgen and Livak 2008) was used for calculating relative quantification of gene expression after outliers removal and data normalization based on the endogenous control gene expression (U6 snRNA-001973). The detailed description can be found in the previous work (Szczepankiewicz et al. 2013). Two-way ANOVA was used to compare dCt values between two cell lines. Statistical analysis was done in Statistica package.

To confirm if selected miRNAs followed similar expression profile over time in different cell lines, we performed cluster analysis using STEM algorithm (Short Time series Expression Miner) software (Ernst and Bar-Joseph 2006). Stem assigns genes form the input list to the model profile that most closely matches the gene's expression profile as determined by the correlation coefficient. The model profiles are selected by the software by random allocation, independent of the data, the algorithm then determines which profiles have a statistically significant higher number of genes assigned using a permutation test. It then uses standard hypothesis testing to determine which model profile has significantly more genes assigned as compared to the average number of genes assigned to the model profile in the permutation runs.

Target genes and pathways prediction

To identify biological pathways for miRNAs showing similar expression profiles for both cell lines, we performed pathway enrichment analysis. The potential target mRNAs for miRNAsthat showed similar changes upon repair process were identified using miRNA BodyMap tool available at: http://www.mirnabodymap.org. This tool gives the integrated results from several prediction algorithms: DIANA, PITA, TargetScan, RNA22 (3UTR), RNA22 (5UTR), TargetScan_cons, MicroCosm, miRDB, RNA22 (5UTR), TarBase and miRecords. To minimize the target prediction noise, only target genes predicted by five or more prediction algorithms from those mentioned above were included.

The list with predicted target genes was then analysed with use of The Database for Annotation, Visualization and Integrated Discovery (DAVID) v.6.7 (Huang da et al. 2009a; Huang da et al. 2009b) to identify pathways(D. 2001; Kanehisa et al. 2004), functional-related gene groups and biological themes, particularly GO terms(Ashburner et al. 2000), in which the analysed sets of target genes were statistically the most overrepresented (enriched). Enrichment score (ES) reflects the degree to which a set of genes is overrepresented at the extremes (top or bottom) of the entire ranked list of genes and is presented as the maximum deviation from zero encountered in the random search (weighted Kolmogorov–Smirnov-like statistic). The magnitude of ES depends on the correlation of the gene with the phenotype. Fold enrichment value is described as a ratio of the two proportions showing how many of input genes is involved in the process/pathway in relation to the background information (number of genes involved in this process from all genes in human genome).

Results

Global miRNA silencing delays epithelial wound repair

Transfection of 16HBE14o- and NHBE cells showed statistically significant differences between cells transfected siRNAs against Drosha and Dicer as compared to the negative control ($p=0.004$ for NHBE and 0.003 for 16HBE cells). Inhibition of global miRNA expression

resulted in delayed repair in both epithelial cells lines. For 16HBE14o- cells, we observed delayed, but completed wound closure, for NHBE cells, only 20% of wounded area was repaired within the observation time (48 hours) (Figure 1).

Several miRNAs revealed common expression pattern during repair in both cell types

Profiling analysis showed some similarities in expression profiles of several miRNAs between 16HBE14o- and NHBE cells. On this basis, we selected a group of ten miRNA genes which expression was changing for at least 1.5 dCt value between any of analysed time points during wound repair and which expression was not significantly different between the two cell types except for one miRNA gene (supplementary table S1). Eight out of ten miRNA genes demonstrated similar pattern: relative mRNA levels normalized to U6 snRNA were increasing up to 8 or 16 hours post-injury and then they were decreasing. However, we have also found miRNAs (hsa-miR-23b and hsa-miR-424) that presented a different pattern: 8 hours after wounding the relative mRNA levels normalized to U6 snRNA were increased, followed by the decrease later during repair. The expression pattern for both cell lines was further confirmed by STEM cluster analysis which showed that profile 8 (model profile that most closely matches gene's expression profile as calculated by the correlation coefficient) was common for NHBE and 16HBE cells. The results were shown on Figure 2.

Predicted pathways for common miRNAs involved in repair

With the use of miRNA body map database we identified 78 genes possible target genes for selected 10 miRNAs. We then divided those target genes into two groups based on miRNA expression data (miRNAs downregulated and upregulated 8 hours post wounding) and analysed them using the Database for Annotation, Visualization and Integrated Discovery (DAVID). Using the highest classification stringency and enrichment score above the value of one, we found five different biological clusters for miRNAs that showed decreased expression 8 hours after wounding (table 1). For the two other miRNAs with increased expression 8 hours after

wounding, one cluster was found (table 2). However, these clusters were not significant after FDR correction.

Discussion

The main observation of our study is that airway epithelial cells, independent of their phenotype, share several miRNA genes involved in repair processes. These genes show similar expression pattern for both epithelial cell types: undifferentiated growing in polarized monolayer (16HBE14o-) as well as differentiated primary cells growing in ALI cultures.

This is of particular importance from technical point of view as primary cell cultures are considered the best model of *in vitro* studies on airway epithelial function (Villenave et al. 2012). Nevertheless, their maintenance is difficult, time consuming, expensive and highly dependent on the source of obtained cells. For this reason, an alternative research model may be immortalized cell lines (such as 16HBE14o-) that are more feasible to culture (Lin et al. 2007). Although these cells are not fully differentiated, previous studies showed that 16HBE14o- cell line can be a promising alternative to primary NHBE in some experimental models e.g. in virus infection studies (Liu et al. 2013).

Our observation that global miRNAs silencing significantly delayed the wound closure in analysed cells confirmed that miRNA are an important regulator of repair process in airway epithelial cells. We observed that in 16HBE14o- cells the wound has closed faster than in case of NHBE. The possible explanation is associated with technical issues regarding cells growing in ALI cultures: they are seeded on collagen that is usually removed together with cells during wounding assay. This requires more time for the NHBE cells to cover the damage and grow on the porous surface (insert) without collagen. What is more, some of the cells are ciliated, so the proliferation relies on the smaller number of basal cells. Nevertheless, we found that miRNAs are crucial in wound repair process in both cell types.

In order to identify the specific miRNA genes involved in airway epithelial repair independent of cell phenotype, we compared miRNA expression profiles in two different cell types: undifferentiated 16HBE14o- grown in monolayer and ciliated NHBE cultured in air-liquid interphase (ALI). Our analysis showed that 10 miRNA genes demonstrated similar expression pattern in both cell types which suggests that they are involved in basic repair processes common for both cell types and independent of the differentiation status. These miRNAs are plausible regulators of 78 target genes involved in several biological pathways/GO terms associated for example withregulation of actin cytoskeleton, focal adhesion, tight and gap junction and cytokine-cytokine receptor interaction.

Previous studies regarding the role of miRNA in airway epithelial repair are limited. The most recent report on the related subject showed that expression of miR-19a is increased in severe asthma; functional experiments showed this miRNA enhances cell proliferation in severe asthma phenotype with aberrant epithelial repair (Haj-Salem et al. 2015).

The time points analysed in this study represent different stages of epithelial repair depending on the cell type. Our previous experiments showed that for 16HBE14o- cells 8 hours following wounding about 50% of wounded area is repaired and 16 hours post-wounding wound closure is completed (Szczepankiewicz et al. 2013) whereas NHBE cells show 50% of wound area repaired after 16 to 24 hours and wound closure is completed by 48 hours (data not published), mainly due to technical reasons as described above. Similar observations regarding differences in wound closure time in different cell types were reported previously (Perrio et al. 2007). Although the time required for repair completion was different in both cell types, we assumed that the same molecular changes underlying repair process occur in cells upon the same stimulus (wounding). Wounded cells communicate with others and mediate signals to induce apoptosis, migration and transcription of genes responsible for proliferation (Lampugnani 1999; Sacco et al. 2004; Zahm et al. 1997).

As it was shown previously, at the beginning of repair process wound closure was due to cell migration and not proliferation(Howat et al. 2002) thus we hypothesized that at 8 hours gene expression of miRNAs regulating cell motion and migration will be downregulated. The pathway analysis confirmed that assumption showing significant enrichment score for gene ontology terms associated, among others, with cell motion. However, with the determined cut off values (enrichment score above number of one and the highest classification of stringency) we did not find any GO terms and pathways directly related to wound repair (as presented in table 1).

On the contrary, two miRNAs that showed increased expression at the beginning of wound repair (at 8 hours post-wounding) showed the highest enrichment score for genes involved in e.g. cytoskeleton organization and non-bounded membrane organelle.

In conclusion, our data demonstrates that miRNAs are important regulators of wound repair in airway epithelial cell lines regardless of their differentiation state. Moreover, we have observed similar expression profile for several miRNA genes during repair of the epithelial cells of different phenotypes.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Fig. 1. Changes in the progress of wound repair for NHBE (figure a) and 16HBE (figure b). The normal line – cells transfected with Drosha/Dicer siRNA, dotted line – cells transfected with negative control (two-way ANOVA).

Fig.2. Changes in expression profiles of selected miRNAs at 8, 16, 24 and 48 hours post-injury for NHBE (top) and 16HBE14o- (bottom). The profile analysis in STEM was based on dCt values (gene expression normalized to U6-snRNA).

Tables

Table 1. Five different clusters predicted for miRNAs which exhibited decline in their expression 8 hours after wounding

Term	p-Value (uncorrected)	Fold Enrichment	Genes
Annotation Cluster 1 Enrichment Score: 1.53			
GO:0048858~cell projection morphogenesis	0.01	5.21	PLXNA3, ONECUT2, ISL1, MYH10, NUMBL
GO:0032990~cell part morphogenesis	0.02	4.99	PLXNA3, ONECUT2, ISL1, MYH10, NUMBL
GO:0000902~cell morphogenesis	0.05	3.58	PLXNA3, ONECUT2, ISL1, MYH10, NUMBL
GO:0032989~cellular component morphogenesis	0.07	3.21	PLXNA3, ONECUT2, ISL1, MYH10, NUMBL
Annotation Cluster 2 Enrichment Score: 1.53			
GO:0045893~positive regulation of transcription, DNA-dependent	0.01	3.75	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1
GO:0051254~positive regulation of RNA metabolic process	0.01	3.71	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1
GO:0045941~positive regulation of transcription	0.02	3.17	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1
GO:0010628~positive regulation of gene expression	0.02	3.08	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.03	2.86	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1
GO:0051173~positive regulation of nitrogen compound metabolic process	0.04	2.77	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1
GO:0010557~positive regulation of macromolecule biosynthetic process	0.04	2.73	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1
GO:0031328~positive regulation of cellular biosynthetic process	0.05	2.61	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1

GO:0009891~positive regulation of biosynthetic process	0.05	2.57	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1
GO:0010604~positive regulation of macromolecule metabolic process	0.11	2.08	ATF7IP, ONECUT2, TEAD3, PDX1, ISL1, SRF, HMGA1
Annotation Cluster 3 Enrichment Score: 1.4			
GO:0006915~apoptosis	0.03	2.97	MEF2D, PACS2, FKBP8, BBC3, CYFIP2, MAPK7, SIAH2
GO:0012501~programmed celldeath	0.03	2.92	MEF2D, PACS2, FKBP8, BBC3, CYFIP2, MAPK7, SIAH2
GO:0008219~cell death	0.06	2.49	MEF2D, PACS2, FKBP8, BBC3, CYFIP2, MAPK7, SIAH2
GO:0016265~death	0.06	2.47	MEF2D, PACS2, FKBP8, BBC3, CYFIP2, MAPK7, SIAH2
Annotation Cluster 4 Enrichment Score: 1.39			
GO:0030900~forebrain development	0.02	6.72	PLXNA3, ISL1, MYH10, NUMBL
GO:0007409~axonogenesis	0.04	5.29	PLXNA3, ISL1, MYH10, NUMBL
GO:0048667~cell morphogenesis involved in neuron differentiation	0.05	4.89	PLXNA3, ISL1, MYH10, NUMBL
GO:0048812~neuron projection morphogenesis	0.05	4.79	PLXNA3, ISL1, MYH10, NUMBL
GO:0000904~cell morphogenesis involved in differentiation	0.07	4.18	PLXNA3, ISL1, MYH10, NUMBL
Annotation Cluster 5 Enrichment Score: 1.2			
GO:0070013~intracellular organelle lumen	0.05	1.80	ATF7IP, MEF2D, PACS2, GCDH, YY1, TRPC4AP, MAPK7, TEAD3, LRCH4, SRF, GTF2B, HMGA1
GO:0043233~organelle lumen	0.06	1.76	ATF7IP, MEF2D, PACS2, GCDH, YY1, TRPC4AP, MAPK7, TEAD3, LRCH4, SRF, GTF2B, HMGA1
GO:0031974~membrane-enclosed lumen	0.07	1.72	ATF7IP, MEF2D, PACS2, GCDH, YY1, TRPC4AP, MAPK7, TEAD3, LRCH4, SRF, GTF2B, HMGA1

Table 2. Cluster predicted for miRNAs which exhibited upregulated expression 8 hours after wounding

Term	p-Value (uncorrected)	Fold Enrichment	Genes
Annotation Cluster 1 Enrichment Score: 1.25			
IPR001478:PDZ/DHR/GLGF	0.003	33.542	SHROOM3, PTPN3, DLG2
SM00228:PDZ	0.004	26.114	SHROOM3, PTPN3, DLG2
GO:0005856~cytoskeleton	0.21	3.085	SHROOM3, PTPN3, DLG2
GO:0043228~non-membrane-bounded organelle	0.51	1.641	SHROOM3, PTPN3, DLG2
GO:0043232~intracellular non-membrane-bounded organelle	0.51	1.641	SHROOM3, PTPN3, DLG2