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mRNA Levels of Epithelial and Mesenchymal Markers in Lung Epithelial Cell Lines

Ekaterina Sergeevna Karetnikova[1,2], Natalia Jarzebska[1,3],
Roman Nikolaevich Rodionov[3], Elena Rubets[2,3],
Alexander Georgievich Markov*[#2], Peter Markus Spieth*[#1]

Abstract

Background: Epithelial-mesenchymal transition (EMT) is an important physiologic process that determines the outcome of lung tissue healing after injury. Stimuli and molecular cascades inducing EMT lead to up-regulation of the mesenchymal-specific genes in the alveolar epithelial cells and to down-regulation of the genes coding for epithelial markers. Alveolar epithelial cell lines are commonly used as in vitro models to study processes occurring in the lung tissue. The aim of this study is to quantify and compare mRNA expression levels of epithelial and mesenchymal markers in a number of lung epithelial cell lines.
Methods: Lung epithelial cell lines L2, R3/1 and RLE-6TN were cultured. Repeated mRNA isolation, reverse transcription, and quantitative PCR with primers to epithelial (E-cadherin, occludin, and ZO-2) and mesenchymal (α-SMA, collagen III, and vimentin) markers were performed.
Results: First, our study revealed a higher level of epithelial transcripts in the RLE-6TN cell line compared to L2 and R3/1 cells. Secondly, we have found simultaneous mRNA expression of both epithelial (E-cadherin, occludin and ZO-2) and mesenchymal (α-SMA, collagen III and vimentin) markers in all cell lines studied.
Conclusions: Our data indicate that at the transcriptional level the L2, R3/1, and RLE-6TN cell lines are at one of the intermediate stages of EMT, which opens new possibilities for the study of EMT on cell lines. Determination of the direction of changes in epithelial and mesenchymal markers will make it possible to establish the factors responsible for both EMT and reverse mesenchymal-epithelial transition.

Keywords: Cell Line, Epithelial-Mesenchymal Transition, Lung, mRNA.

Introduction

Epithelial-mesenchymal transition (EMT) is an important physiological process that occurs both in normal tissue regeneration after injury and in fibrosis. It is currently believed that proper regulation and constraint of EMT lead to regeneration, while excessive activation and spread of EMT cause tissue fibrosis (1). The molecular mechanisms of EMT that determine the outcome in the form of tissue repair or fibrosis is not fully understood and require further investigation. Epithelial cells undergoing EMT downregulate intercellular contacts, loose the apical-basal polarity, disintegrate attachment with the basal membrane, acquire motility, move into the interstitium, initiate synthesis of intercellular matrix components, and as a result gain a mesenchymal phenotype like fibroblasts and/or

1: Department of Anesthesiology and Critical Care Medicine, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany.
2: Department of General Physiology, Saint-Petersburg State University, Saint-Petersburg, Russia.
3: Division of Angiology, Department of Internal Medicine III, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany.
[#]**The first and the second authors contributed equally to this work**.
***Corresponding author: Alexander Georgievich Markov; Tel: +7 9117968544; E-mail: a.markov@spbu.ru& Peter Markus Spieth; Tel: +49 3514587372; E-mail: Peter.Spieth@ukdd.de.**
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myofibroblasts. The processes that occur during EMT are all caused by changes in protein expression levels. The cells in the process of EMT decrease the expression of proteins typical for epithelium and increase the expression of mesenchymal proteins (2-5). The presence/absence of proteins specific for epithelial or mesenchymal tissues can be used to determine the cell phenotype. Tight junction and adherens junction are protein complexes crucial for epithelial formation and function (6-8). E-cadherin as the essential part of the epithelial adherens junctions is a generally accepted marker of the epithelial phenotype (9, 10). Tight junction proteins equally with E-cadherin are specific markers for the epithelial cell phenotype. Key molecular determinants of tight junctions include two families of integral membrane proteins and a family of adapter proteins, namely Zonula occludens (ZO-1, ZO-2, ZO-3). The integral membrane proteins include the family of claudins (27 members in mammals) and the family of tight junction associated proteins, which includes occludin. ZO proteins are located on the cytoplasmic surface of the TJs (8, 11, 12). Tight junction proteins are also used as markers of the epithelial cell phenotype in lung studies (13, 14). Epithelial markers such as E-cadherin, occludin, and ZO-2 as one of the main adaptor proteins were chosen to determine the epithelial phenotype of cells.

A mesenchymal cell phenotype can be assessed by the expression of the alpha-smooth muscle actin (α-SMA), which gives a cell the ability to move; collagen I and III expression, which are typical mesenchymal extracellular proteins; and vimentin expression, which is specific for cytoskeleton of mesenchymal cells (3, 4, 15-17). It was previously shown that alveolar epithelial type II cells are capable of producing cytokines, particularly transforming growth factor beta (TGF-β) (18, 19), which affects the expression of epithelial and mesenchymal markers (20-24).

Alveolar epithelial cell lines are commonly used as *in vitro* models to study physiological and pathophysiological processes occurring in tissues (15, 23, 25-28). Stimuli and molecular cascades inducing EMT lead to up-regulating of the mesenchymal-specific genes in the alveolar epithelial cells and to down-regulation of the genes coding for epithelial markers (25, 29, 30). Therefore, assessment of transcriptional levels of epithelial and mesenchymal markers can be used to track EMT in cells.

In this study we aimed to provide a uniform quantitative assessment and comparison of the transcriptional levels of the epithelial (*E-cadherin, occludin, and ZO-2*) and mesenchymal (*α-SMA, collagen III, and vimentin*) markers in three rat alveolar epithelial cell lines: L2, R3/1, and RLE-6TN.

Materials and Methods

Rat lung epithelial cell lines L2 (CCL-149, ATCC, USA) and RLE-6TN (CRL-2300, ATCC, USA) were obtained from ATCC (Manassas, VA, USA). The R3/1 cell line (31) was a kind gift from Prof. M. Kasper (Institute of Anatomy, University of Technology, Dresden, Germany). The L2 cell line originating from the lung tissue of the adult female Lewis strain rat is characterized by the formation of lamellar bodies specific for type II alveolar epithelial cells (32). The R3/1 cell line isolated by Knebel and colleagues (33) from the bronchopulmonary tissue of the 18-day-old fetal Han-Wistar rat is thought to have predominantly the phenotype of alveolar epithelial cells type I, but with a simultaneous low level of expression of several proteins characteristic only of alveolar epithelial cells type II. So, the R3/1 cell line derived from fetal lung tissue is presumably characterized by the phenotypic features of low-differentiated lung cells (31, 33). The RLE-6TN cell line was derived from alveolar epithelial cells type II isolated from 56-day-old male Fischer 344 rat (34).

Cell lines culture

L2 cell line was cultured in DMEM medium (Gibco, Invitrogen, USA) (35). R3/1 and RLE-6TN cell lines were maintained in DMEM/F-12 medium (Gibco, Invitrogen, USA) (36-38). All cell culture mediums were

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supplemented with 10% FBS and 100 U/ml penicillin / 100 µg/ml streptomycin (Gibco, Invitrogen, USA). All cells were grown at 37 °C in a humidified atmosphere with 6.5% CO_2 (35). The cells were routinely passaged 1-2 times per week; the cell growth media was changed every 2–3 days.

mRNA extraction and qPCR

Total mRNA was extracted from cells monolayers with peqGOLD Total RNA Kit (PeqLab, VWR, Germany) followed by purification with DNase I digestion kit (PeqLab, VWR, Germany). Reverse transcription and qPCR were performed with qScript cDNA SuperMix (qScript, Quanta BioSciences, USA) and GoTaq qPCR MasterMix (Promega, Madison, USA) respectively. The primers were designed with Primer- BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) tool assuring that the PCR products will contain parts of at least two exons and synthesized by biomers.net. Efficiency and specificity of the primers was verified before the experiments. Nucleotide sequences of the primer pairs used are listed in Table 1. qPCR was performed using an iCycler iQ Real-Time PCR Detection System (Bio-Rad, USA) with the following conditions: 5 min 95 °C, followed by forty 20 sec cycles at 95 °C, 60 °C 30 sec, 72 °C 30 sec, 10 min of final elongation at 72 °C. All qPCR tests were done in duplicates or triplicates. After the amplification process was completed, the SYBR Green luminescence threshold cycles for each reaction were determined in the cycler software. Primary data in the form of threshold cycle numbers were obtained as Excel spreadsheets. β-actin was used as a reference gene.

Table 1. Nucleotide sequences of the primers used in the study.

Gene	Sequence (5'→3')		Product length, bp
E-cadherin	Forward	TTGAGAATGAGGTCGGTGCC	155
	Reverse	CAGAATGCCCTCGTTGTTCT	
Occludin	Forward	TCTCAGCCGGCATACTCTTT	162
	Reverse	ATAGGCTCTGTCCCAAGCAA	
ZO-2	Forward	GGCCTGGACCATGAAGACTA	232
	Reverse	GTTCATAGCGGGTCTCTGGA	
Alpha-smooth muscle actin (α-SMA)	Forward	ATGCATCATGCGTCTGGATCT	148
	Reverse	TCCAGGGCGACATAACACAGTT	
Collagen III	Forward	AGTGGCCATAATGGGGAACG	331
	Reverse	CACCTTTGTCACCTCGTGGA	
Vimentin	Forward	GCACGTCTTGACCTTGAACG	151
	Reverse	TGAGGTCAGGCTTGGAAACG	
TGF-β	Forward	TGCTGCCTTCGCCCTCTTTACATT	149
	Reverse	AGGCTGAGGACTTTGGTGTGTTGT	
β-actin	Forward	AACCCTAAGGCCAACCGTGAAA	133
	Reverse	AGTCCATCACAATGCCAGTGGT	

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Statistical analysis

The data analysis was performed using Microsoft Excel professional version 2016 and Prism 8 for Windows Version 8.2.1 (441), GraphPad Software, Inc. All differences of genes transcriptional levels were determined by one-way ANOVA with Tukey correction. All genes transcriptional levels (normalized to β-actin) are expressed as means ± standard error of mean. The probability of type I error was set as 5%.

Results

All three cell lines expressed both epithelial and mesenchymal markers (Table 2). The mRNA levels of all studied epithelial markers (E-cadherin, occludin and ZO-2) were higher in RLE- 6TN cells compared to L2 and R3/1 cell lines. Whereas there were no differences between L2 and R3/1 cell lines in the expression levels of E-cadherin, occludin, and ZO-2.

Table 2. Comparison of the mRNA levels (normalized to β-actin) of epithelial (E-cadherin, occludin, and ZO-2) and mesenchymal (α-SMA, collagen III, and vimentin) markers in the L2, R3/1, and RLE-6TN cell lines detected in the current study and previously published ones.

Marker		L2	R3/1	RLE-6TN	References
E-cadherin	*mRNA*	**7.0±1.8x10^{-7}**	**2.6±0.7x10^{-7}**	**1.6±0.4x10^{-5}**	
	mRNA	+	+ -	+	(25, 29, 30, 36, 39)
	protein	ND	+ IF staining	+ WB	(25, 26, 30, 31, 40-44)
Occludin	*mRNA*	**2.8±0.9x10^{-8}**	**1.1±0.5x10^{-7}**	**2.4±0.4x10^{-4}**	
	mRNA	ND	-	ND	(39)
	protein	+ WB	ND	ND	(14)
ZO-2	*mRNA*	**5.7±1.1x10^{-4}**	**5.9±1.6x10^{-4}**	**5.0±0.4x10^{-3}**	
	mRNA	ND	+	ND	(39)
	protein	ND	ND	ND	
α-SMA	*mRNA*	**1.3±0.3x10^{-5}**	**5.5±1.5x10^{-5}**	**9.4±3.7x10^{-5}**	
	mRNA	+	+	+	(30, 36)
	protein	+	+ IP staining	+ WB - WB	(23, 26, 30, 36, 40, 41, 43, 44)
Collagen III	*mRNA*	**0.5±0.1**	**0.2± 0.04**	**0.3±0.1**	
	mRNA	+	+	ND	(36)
	protein	ND	ND	+ WB	(42)
Vimentin	*mRNA*	**1.1±0.2**	**0.5±0.1**	**1.3±0.4**	
	mRNA	+	+	+	(25, 29, 30, 36)
	protein	ND	ND	+ WB + IF staining	(25, 26, 30, 40, 42, 43)

+: presence of mRNA or protein expression; **-**: absence of mRNA or protein expression; ND: no data was found; WB: Western Blot; IF: immunofluorescent staining; IP: immunoperoxidase staining. Bold font: our original data.

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We were able to detect mRNA for α-SMA, collagen III, and vimentin in all three cell lines investigated (Figs. 1 and 2). It was found that α-SMA transcriptional levels in L2 cells were lower than in RLE-6TN cells. We did not detect any significant difference in the collagen III and vimentin mRNA levels among the three cell lines. We also found no statistically significant difference in the transcriptional levels of TGF-β in all cell lines studied (not shown). Taken together, the data show that the RLE-6TN cell line had the highest expression levels of the investigated epithelial markers, and no differences in mesenchymal marker mRNA levels were found in the studied cell lines.

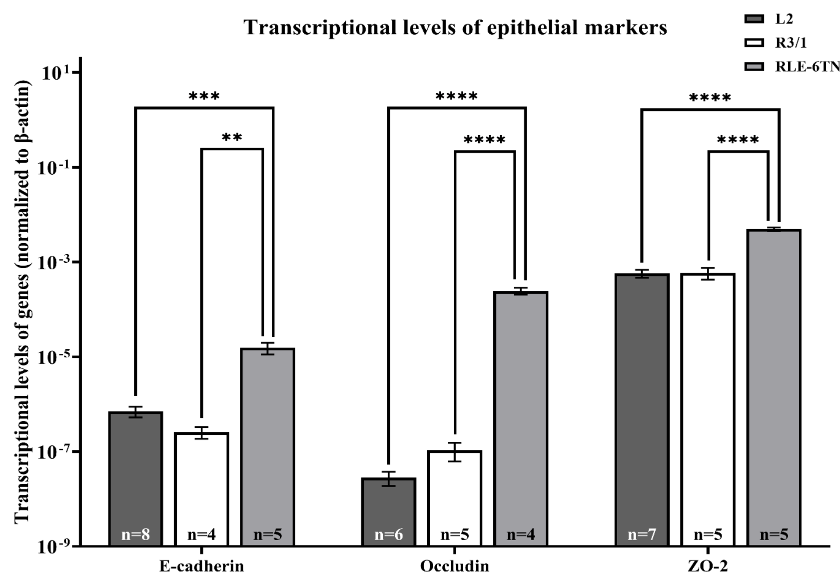

Fig. 1. Transcriptional levels (normalized to β-actin) of epithelial markers (*E-cadherin, occludin, and ZO-2*) in L2, R3/1, and RLE-6TN cell lines. Data are presented as mean ± standard error of the mean and the number of replicates is depicted on the figure. One-way ANOVA; ** $p<0.01$, *** and **** $p<0.001$.

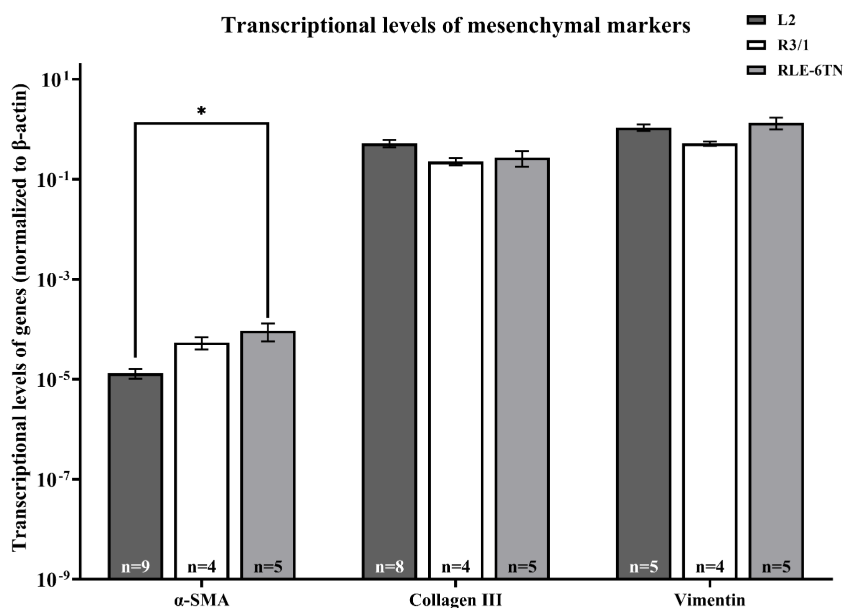

Fig. 2. Transcriptional levels (normalized to β-actin) of mesenchymal markers (α-SMA, collagen III, and vimentin) in L2, R3/1, and RLE-6TN cell lines. Data are presented as mean ± standard error of the mean and the number of replicates is depicted on the figure. One-way ANOVA; *$p<0.05$.

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Discussion

The results showed that, firstly, the genes of epithelial and mesenchymal markers are transcribed simultaneously in L2, R3/1 and RLE-6TN cell lines. These data led us to conclude that the cell lines studied are at one of the intermediate stages of EMT at the level of gene expression. Secondly, higher mRNA levels of epithelial markers in RLE-6TN cells indicate that this cell line retained a more pronounced epithelial gene expression profile compared to L2 and R3/1 cell lines.

Our data shows that *E-cadherin* is present at mRNA level in all examined alveolar cell lines. This protein was also found by other authors in L2 (36), R3/1 (36), and RLE-6TN (25, 29, 30) cell lines. However, the group of Horalkova and colleagues was not able to detect *E-cadherin* mRNA expression in the R3/1 cell line (39). We believe that the low level of *E-cadherin* mRNA expression in the R3/1 cell line and methods differences led to discrepancies in our results. According to the best of our knowledge, the tight junction proteins have not been investigated in the L2 and RLE-6TN cell lines so far, while the group of Horalkova was able to detect mRNA for *ZO-2* in the R3/1 cell line but not for occludin (39). In our study we performed simultaneous determination of the transcriptional levels of tight junction proteins (*occludin* and *ZO-2*) in the L2, R3/1, and RLE-6TN cell lines for the first time. Moreover, we detected a significantly higher level of these transcripts in the RLE-6TN cell line compared to L2 and R3/1 cells, which suggests that the phenotype of the RLE-6TN cell line is more epithelial-like than the phenotype of the L2 and R3/1 cell lines.

Transcription of a number of mesenchymal markers in L2, R3/1, and RLE-6TN cell lines has been mentioned in several earlier studies. Specifically, the presence of *α-SMA, collagen III*, and *vimentin* mRNA has been shown in L2 and R3/1 cell lines (36), and transcriptional levels of *α-SMA* and *vimentin* have been identified in the RLE-6TN cells (25, 29, 30). In our work, we for the first time quantified the levels of mRNA expression of cell motility protein (*α-SMA*), extracellular matrix protein (*collagen III*), and cytoskeleton protein (*vimentin*) in L2, R3/1, and RLE-6TN cell lines. The presence of *α-SMA, collagen III*, and *vimentin* is consistent with cells at advanced steps in the epithelial-mesenchymal transition (4, 5, 45, 46). This is particularly relevant to *α-SMA* expression, which is crucial for myofibroblasts motility (47). Interestingly, while the RLE-6TN cell line had the highest transcriptional levels of epithelial markers, we did not detect and significant differences in the mesenchymal markers' expression levels in all the three investigated cell lines.

We compared our results on the transcriptional levels of epithelial and mesenchymal markers in L2, R3/1, and RLE-6TN cell lines with previously published data on the protein expression levels of these markers (Table 2). It should be noted that protein expression of epithelial and mesenchymal markers has not been studied thoroughly. Most of the studies on epithelial and mesenchymal markers expression in the rat cell lines were performed on the RLE-6TN cell line. As for epithelial markers, earlier works mainly evaluated the E-cadherin expression. In all studies performed on the RLE-6TN cell line, E-cadherin was detected on a protein level with the use of the Western blotting technique (25, 26, 30, 40-44). Occludin has also been shown to be present in the L2 cell line (14). Regarding mesenchymal markers, the closest association between mRNA expression and protein presence was shown for vimentin in the RLE-6TN cell line (25, 26, 30, 40, 42, 43). Likewise, Western blot revealed the presence of collagen III in RLE-6TN cells (42). However, the data on α-SMA protein expression is contradictory. In some of the studies the protein was detected in the RLE-6TN cell line by Western blot (40, 41, 43, 44), while other groups could not reproduce this finding (23, 26, 30, 43).

In summary, our data indicate that the immortalized alveolar epithelial cell lines investigated in this study are at one of the intermediate steps of EMT, as shown by simultaneous expression of epithelial and mesenchymal markers in the cells. Potentially,

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this finding opens up new possibilities for the study of epithelial-mesenchymal transition in cell lines. Determination of the direction of changes in epithelial and mesenchymal markers will make it possible to establish the factors responsible for both epithelial-mesenchymal transition and reverse mesenchymal-epithelial transition.

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Compliance with ethical standards

There are no studies in the article that have used animals or biological material derived from humans. The study was approved by the Bioethics Committee of St. Petersburg State University no. 131-03-3 (issued 03-12-2020)

Conflict of interest

The authors declare that they have no conflict of interest. All authors have read and agreed to the published version of the manuscript.

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