Regulation of Phosphodiesterases by profibrotic regulators

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IV. List of abbreviations

AA	Amino acid
AKAP	A kinase anchoring protein
APS	Ammonium persulfate
BSA	Bovine serum albumin
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxiribonucleic acid
cGMP	Cyclic guanosine monophosphate
COPD	Chronic obstructive pulmonary disease
Cyt	Cytokeratin
DAPI	4',6-diamidino-2-phenylindole
DHE	Dihydroergotamine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E-Cad	E-Cadherin
EDTA	Ethylendinitrilo-N,N,N',N' tetra acetate
EGTA	Ethylene glycol-bis (2-amino-ethyleter)- N,N,N',N'-tetraacetic acid
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinases
FITC	Fluorescein-5-isothiocyanate
FCS	Fetal Calf Serum
FN	Fibronectin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IB	Immunoblotting
IBMX	Isobutylmethylxanthine

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ICCH	Immunocytochemistry
IL	Interleukin
NAC	*N*-acetyl-l-cysteine
NO	Nitric oxide
PAH	Pulmonary arterial hypertension
PBGD	Porphobilinogen deaminase
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline + 0,1% Tween 20
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PKA	Protein kinase A
Rho	Ras homolog gene family
ROCK	Rho kinase
ROS	Reactive Oxygen Species
RT-PCR	Reverse transcription PCR
ROCK	Rho-associated, coiled-coil containing protein kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SMA	Smooth muscle actin
TAE	Tris-acetate EDTA
TBS	Tris Buffered Saline buffer
TEMED	*N,N,N′,N′*-tetramethyl-ethane-1,2-diamine
TGF	Transforming growth factor
TGF-βRII	TGF-β receptor type II

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V. Summary

Epithelial-mesenchymal transition (EMT) is a series of events in which fully differentiated epithelial cells undergo transition to a mesenchymal phenotype giving rise to fibroblasts and myofibroblasts. EMT is increasingly recognized as important process not only in development, but also in adult life: in wound healing, fibrosis, and the invasion and metastasis of tumor cells. The phenotypic conversion involves loss of epithelial polarity, loss of epithelial markers, loss of adherens and gap junctions, cytoskeletal reorganization, and transition to a spindle-shaped morphology concomitant with acquisition of mesenchymal markers and an invasive phenotype.

Cyclic nucleotide phosphodiesterases (PDE) comprise a family of related proteins, which can be subdivided into 11 families (PDE1-PDE11) based on their amino acid sequences, sensitivity to different activators and inhibitors and their ability to preferentially hydrolyze either cAMP (PDE4, 7, 8) or cGMP (PDE5, 6, 9) or both (PDE1, 2, 3, 10, 11). cAMP and cGMP are ubiquitous second messengers and consequently PDEs propagate many signaling pathways, including proliferation, migration, and differentiation.

The present study was undertaken to evaluate the potential role of phosphodiesterases (PDE) in TGF-β1 induced EMT in the human alveolar epithelial type-II cell line-A549. TGF-β1 induced EMT was characterized by morphological alterations and by expression changes of EMT specific markers. TGF-β1 treatment decreased expression of epithelial phenotype markers such as E-cadherin, cytokeratin-18, zona occludens-1 and increased expression of mesenchymal phenotype markers such as collagen I, fibronectin-EDA and α-smooth muscle actin.

Interestingly, a 2-fold increase in total cAMP-PDE activity following TGF-β1 stimulation was found and attributed to increased PDE3 and PDE4 activities. Further mRNA and protein expression demonstrated upregulation of PDE4A and PDE4D isoforms in TGF-β1 stimulated cells.

Most importantly, treatment of TGF- β1 stimulated epithelial cells with the PDE4 selective inhibitor Rolipram potently inhibited EMT changes in a Smad-independent

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manner by decreasing reactive oxygen species (ROS). Rolipram pre-treatment resulted in E-Cad restoration as well as in Fibronectin abolishement.

In addition, Rho kinase signaling activated by TGF- β1 during EMT demonstrated as a positive regulator of PDE4. Our findings suggest that TGF-β mediated up-regulation of PDE4 promotes EMT in alveolar epithelial cells.

Thus, targeting PDE4 isoforms may be a novel approach to attenuating EMT-associated lung diseases, such as pulmonary fibrosis and lung cancer.

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VI. Zusammenfassung

Die Epithelial-Mesenchymale Transition (EMT) voll differenzierter Epithelzellen in Zellen mesenchymalen Phänotyps, wie (Myo) Fibroblasten, spielt nicht nur beim Wachstum und in der Entwicklung eine Rolle, sondern nimmt auch bei Prozessen wie der Wundheilung, der Entstehung von Fibrose und der Invasion und Metastasierung von Tumorzellen einen wichtigen Stellenwert ein. Während der phänotypischen Umwandlung verliert die Zelle, neben ihrer für Epithelzellen typischen Polarität, ihre epithelialen Marker und durchläuft eine Neustrukturierung ihres Zellskeletts. Dies hat eine spindelförmige Morphologie zur Folge und wird von der Aneignung mesenchymaler Marker begleitet.

Zyklische Nukleotid Phosphodiesterasen (PDEs) bilden eine Familie verwandter Proteine, die, basierend auf ihren Aminosäuresequenzen, ihrer Sensitivität gegenüber verschiedenen Aktivatoren und Inhibitoren und aufgrund ihrer Fähigkeit entweder cAMP oder cGMP oder beide zyklischen Nukleotide zu hydrolisieren, in 11 Familienmitglieder eingeteilt werden. Sie beeinflussen mehrere zelluläre Signalwege, sowohl durch den Abbau der ubiquitären *second messenger* cAMP und cGMP, als auch durch direkte Interaktion mit anderen Proteinen ,was Auswirkungen auf Proliferation, Migration und Differenzierung von Zellen hat.

In der vorliegenden Arbeit sollte untersucht werden, welche Rolle Phosphodiesterasen in der TGF-β1 induzierten EMT von humanen, alveolären Typ II-Zellen (A549-Zellen) spielen. Die durch die Stimulation mit TGF-β1 induzierte EMT wurde anhand morphologischer Veränderungen und durch Genexpressionsanalysen der epithelialen Marker E-Cadherin, Zytokeratin-18, Zona Occludens-1 und der mesenchymalen Marker Kollagen I, Fibronektin-EDA und α-Smooth Muscle Aktin überprüft. Interessanterweise zeigte sich nach der TGF-β1-Stimulation eine 2fach gesteigerte Aktivität der cAMP-hydrolysierenden PDEs, welche auf eine erhöhte Aktivität der PDEs 3 und 4 zurückgeführt werden konnte. Des Weiteren konnte auf Gen – und Proteinexpressionsebene gezeigt werden, dass sowohl die PDE4A, als auch die PDE4D in TGF-β1 stimulierten Zellen erhöht exprimiert werden. Zudem ist von Bedeutung, dass die Behandlung TGF-β1 stimulierter Zellen mit dem PDE4 spezifischen

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Inhibitor Rolipram zu einer geringeren Produktion reaktiver Sauerstoffspezies (ROS) führte und es so in Smad – unabhängiger Weise zur Epithelial-Mesenchymalen Transition kommen konnte. Zusätzlich zeigte sich, dass der Rho-Kinase-Signalweg, welcher während der EMT durch TGF-β1 aktiviert wird, einen positiv regulatorischen Effekt auf die PDE4 hat. Zusammenfassend weisen unsere Ergebnisse darauf hin, dass die durch TGF-β1 verursachte erhöhte Expression der PDE4 die EMT in alveolären Epithelzellen begünstigt. Daher wäre eine speziell auf die PDE4 abzielende Behandlung ein neuer Ansatz um EMT-assozierte Lungenkrankheiten wie Lungenfibrose und Lungenkrebs zu verbessern.

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1 Introduction

1.1 Epithelial to mesenchymal transition

1.1.1 Characteristic of epithelial to mesenchymal transition

Epithelial cells cover internal organs and other internal surfaces of the body. Major feature of epithelial cells is cell-to-cell contact and monolayers formation. It is important during embryonic life for development as well as during adult life for proper homeostasis and architecture of epithelium.

Epithelial cells are polarized and closely adhered by membrane associated specialized junctions such as tight junctions, gap junctions, adherens junctions (containing E-Cadherin in complexes with β-catenin and α-catenin, linked to the cytoskeleton) and desmosomes. In cell culture, epithelial cells grow as clusters that maintain complete cell-to-cell adhesion. Specific markers used to describe epithelial characteristic include E-Cadherin (E-Cad), zonula ocludens (ZO-1), cytokeratins (cyt), apical actin-binding transmembrane protein-1 (MUC-1)[1-3].

E-Cadherin (E-Cad), one of the major epithelial marker and one of the main target during EMT, is a calcium dependent adhesion molecule, responsible for morphogenetic rearragements and integrity of cells through the formation of adherens junctions. Cells are kept close to each other and adhere through the interaction between extracellular domains of cadherins. Moreover, also cytoplasmatic tail is required for strong, stable adhesions. Cytoplasmic domains have 90-100% similarity, what suggest that that sequences bind common adaptors and effectors[4].

E-Cad is able to form complexes with β-catenin and α-catenin. Seronine/threonine phosphorylation stabilizes, wheras dephosphorylation disrupts this complex. Interestingly, changes in expression and activity of kinases responsible for phosphorylation, correlate with changes in cell adhesion and migration[4]. During EMT, E-Cad is one of the main targets. When E-Cad level decrease, they can not form complexes with β-catenin. As a results, β-catenin localizes from membrane to the nucleus and contributes to the Wnt signaling pathway[2].

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Mesenchymal cells do not form layers and are not polarized. They contact each other only focally. The only junction they form are gap junctions when passing other mesenchymal cells[5]. In the cell culture they have spindle shape and fibroblast-like morphology. Specific markers used to define mesenchymal phenotype are alpha-smooth muscle actin (α-SMA), fibronectin (Fn), vimentin, desmin, collagen (coll), the transcription factors Snail and Slug and expression of matrix proteinases (MMPs)[2] (see Table 1).

Fibronectin is one of the major mesenchymal markers. Fibronectin is an extracellular matrix glycoprotein that binds to membrane receptor proteins called integrins. In addition to integrins, they also bind extracellular matrix (ECM) components such as collagen or fibrin. Fibronectin contributes to the cross-linked network within the ECM, due to its binding sites for other ECM components.

It is already known, that fibronectin is one of the markers gained during TGF-β induced epithelial-to-mesenchymal transition[6-8].

Epithelial-to-mesenchymal transition (EMT) is well known mechanism known for dispersing cells in verbrate embryos[9], forming fibroblasts in injured tissues[10] or intiating metastases in epithelial cancer[11,12] (see Figure 1.1).

During EMT epithelial cells are disaggregated and reshaped for movement. Cells lose polarity, adherens and tight junctions, desmosomes and cytokeratin intermediate filaments. They became highly motile and migrate what is the effect of rearrangement of the cytoskeleton and formation of new cell-subtratum contacts, and allow them to pass through the underlying basement membrane.

Table 1.   The EMT proteome[13]

Proteins gained or maintained	Proteins attenuated
Snail, Slug, Scratch, SIP1, E47, Ets, FTS binding protein, RhoB, FSP1, TGF- β, FGF-1,2,8, MMP-2,9, vimentin, α-SMA, Fibronectin, Collagen type I, III, Thrombospondin, PAI-1	E-Cadherin, ZO-1, β-catenin, Despoplakin, Muc-1, Syndecan-1, Cytokeratin-18

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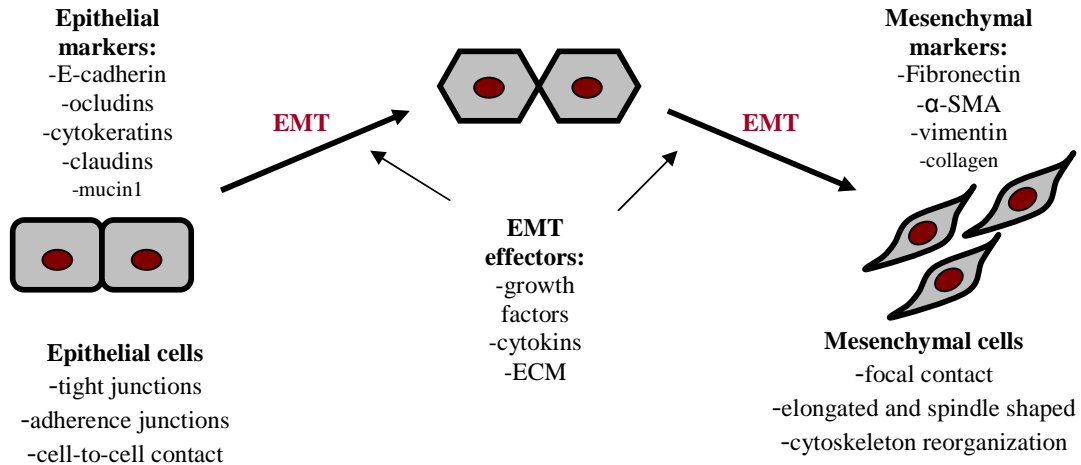
Figure 1.1. Basic mechanisms of epithelial-to-mesenchymal transition (EMT).

1.1.2 Induction of EMT

EMT can be triggered either by oncogenic activation either by specific growth factors (such as transforming growth factor β, TGF-β; fibroblast growth factor, FGF; epidermal growth factor, EGF; insulin-like growth factor, IGF; hepatocyte growth factor, HGF; connective tissue growth factor, CTGF; interleukin-1, IL-1) or by extracellular matrix components (for example collagen or hyaluronic acid)[2,3].

1.1.3 TGF-β and its role during EMT

It is already known that TGF-β is a major inducer of EMT in development, carcinogenesis and fibrosis[5]. Disturbance of TGF-β signaling pathway has been implicated in several developmental disorders and in various human diseases[14].

The first notes about TGF-β as a inducer of EMT, came from experiments with normal mammary epithelial cells[15]. Since that time, its effect was shown in a number of different epithelial cells.

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TGF-β is multifunctional cytokine that regulates tissue morphogenesis and differentiation through effects on cell proliferation, differentiation, apoptosis and extracellular matrix production[1].

TGF-β1 exerts its effects by binding to the TGF-β type II receptor (TβRII) and subsequently recruiting the TGF-β type I receptor (TβRI). Smad 2/3 and Smad 4 are known intracellular mediators of TGF-β1. Once phosphorylated by the activated TGF-β1 receptor, Smad 2 and/or Smad 3 complex with Smad 4 and translocate to the nucleus where they regulate TGF-β1 target genes[16,17,18].

On the other hand, TGF-β1 can also regulate its target genes via Non-Smad-dependent pathways that include the RhoA, Ras, MAPK, PI3 kinase, Notch, and Wnt signaling pathways[19,20,21].
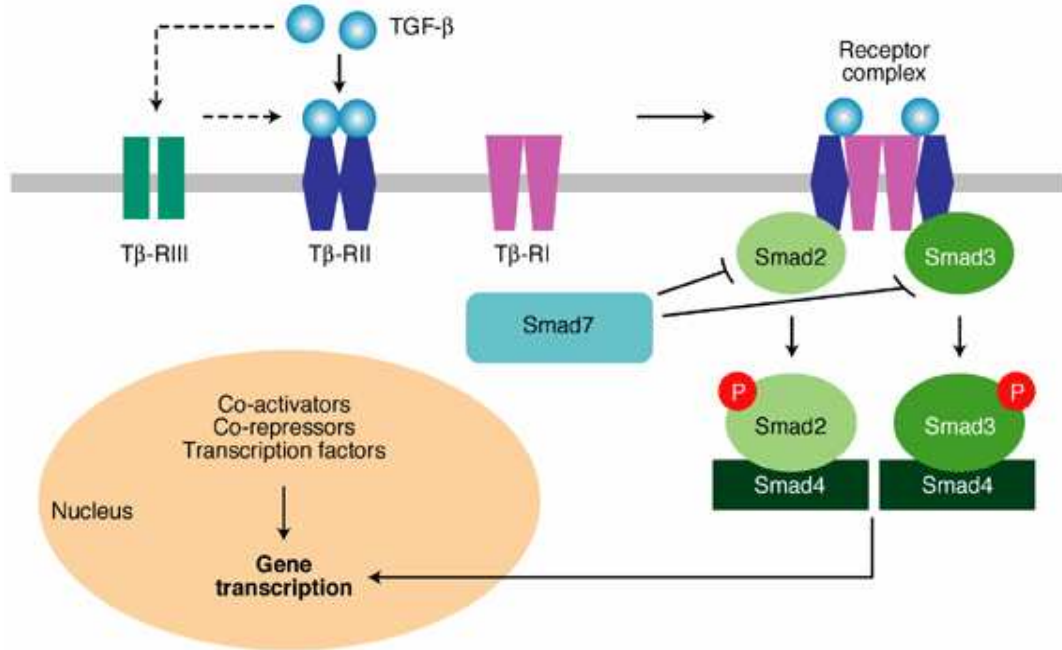
Figure 1.2. Transforming growth factor β1 (TGF- β1) signaling pathway. Adapted from Cambridge University Press 2003.

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1.1.4 EMT in embryo and adults

1.1.4.1   EMT in development

During embryogenesis, a number of extracellular signals can trigger the conversion of epithelial cells into mesenchymal cells by triggering EMT. Mesenchymal cells do not derive exclusively from the mesoderm primary germ layer, but they can be produced by epithelial endodermal cells[2].

Mesenchymal cells allow the shaping of the embryo during gastrulation. They are also able to move and settle at sites of critical epithelial–mesenchymal interactions or they can differentiate into new structures[2].

1.1.4.2   EMT in wound healing

Epithelial cells and fibroblasts are major regulatory elements of epithelialization and wound contraction. Fibroblasts contribute to contraction directly by producing contractile forces and indirectly via differentiating into mesenchymal cells such as myofibroblasts. The epithelium, once considered to be terminally differentiated, has the ability to differentiate into mesenchymal cells and the occurrence of EMT following epithelial stress such as inflammation or wound has been documented[10,22].

1.1.4.3   EMT in cancer

In order to develop cancer, tumour cells must be invasive and migrate from primary tumour sites into the surrounding tissue. To be able to do so, tumour cells undergo some phenotypic changes and it is believed that this happen during EMT. Recent studies indicated that EMT is indeed involved in tumour progression.

Tumour cells that gained mesenchymal characteristics such as enhance motility or activation of proteolysis, are able to metastasize and establish secondary tumours at distant sites[2]. It was also demonstrated that manipulation in E-Cad expression causes the effects on phenotype and invasive properties. It was shown that loss of E-Cad expression and function can be observed in most of carcinomas, and was proposed that this gene can be a invasion suppressor gene during EMT[23].

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1.1.4.4 EMT in fibrosis

The molecular basis of EMT with respect to fibrosis is starting to emerge as it is believed that inflammatory mediators that are produced in response to injury cause EMT, which can lead to fibrosis[2].

TGFβ levels are increased in the lungs of patients with fibrotic pulmonary diseases such as idiopathic pulmonary fibrosis. TGFβ induces EMT of alveolar epithelial cells and fibroblastic cells that express both epithelial and mesenchymal markers can be detected in human biopsies. Furthermore, a significant body of evidence points to TGFβ-induced EMT of lens epithelial cells as an important event during cataract formation and injury-induced lens-capsule fibrosis[2].

EMT has also been demonstrated in the fibrosis of various other organs including the lung, liver, anterior sub capsular cataracts in humans[1,10,24].

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1.2   Phosphodiesterases

1.2.1 Nomenclature of Phosphodiesterases

Cyclic nucleotide phosphodiesterases are the enzymes which effectively catalyze the hydrolysis of cAMP and/or cGMP.

PDEs are classified into I, II or III class. Mammalian PDEs belong to class I.

There have been identified twenty-one genes encoding PDEs in human genome and more then 50 different isoforms of PDEs, and they have been classified into 11 families (PDE1 – 11) according to the genes of which they are products, their biochemical properties, regulation and sensivity to pharmacological agents [25].

According to the nomenclature used for each PDE isozyme (eg. HSPDE4D), the first two letters indicate the animal species, the first Arabic number after PDE indicates PDE gene family, following a single capital letter indicates a distinct subfamily gene and the last Arabic number indicates a specific splice variant or a specific transcript generated from a unique transcription initiation site (http://depts.washington.edu/pde/pde.html).

1.2.2 Classification of Phosphodiesterases

Among the multiple isoforms of phosphodiesterases, the enzymes belonging to three families (PDE4, 7, 8) are belived to be only cyclic AMP specific, three families (PDE5, 6, 9) are cyclic GMP specific and five families (PDE1, 2, 3, 10, 11) display dual substrate specifity.

1.2.2.1   cGMP

Cyclic guanosine monophosphate (cGMP) is a cyclic nucleotide synthesized by soluble or particulate guanylate cyclases (GC) from guanosine triphosphate (GTP), wheras hydrolyzed to guanosine monophosphate (GMP) by PDEs (Figure 1.3.).

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cGMP acts as a second messenger mostly by activating intracellular protein kinases, for example PKG. cGMP is known to be a common regulator of ion channel conductance, glycogenolysis, and cellular apoptosis. It also plays a role in smooth muscle relaxation and migration[26,27].
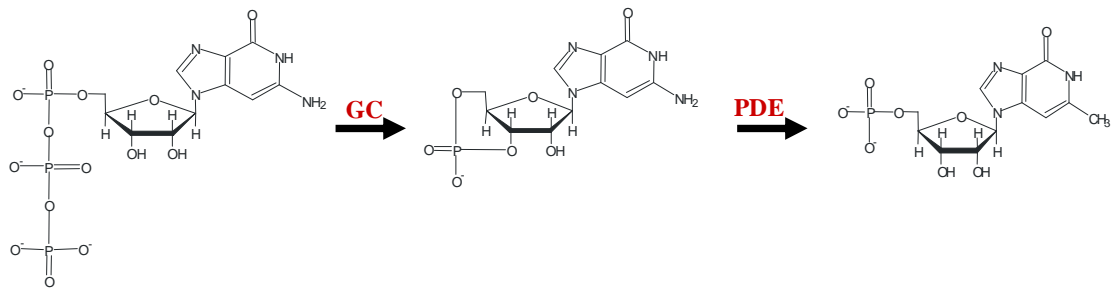
Figure 1.3. Synthesis and hydrolysis of cGMP. GC – guanylate cyclase, PDE – phosphodiesterase.

1.2.2.2   cAMP

Cyclic adenosine monophosphate (cAMP) is a cyclic nucleotide synthesized by adenylyl cyclases (AC) from adenosine triphosphate (ATP), wheras hydrolyzed to adenosine monophosphate (AMP) by PDEs (Figure 1.4)

Cyclic AMP mainly activates protein kinase A (PKA). PKA is a holoenzyme composed of two genetically distinct regulatory (R) and two catalytic (C) subunits that form a tetrameric holoenzyme (R2C2). In the absence of cAMP, PKA exists as a stable inactive tetramer; the catalytic activity of cAMP is suppressed when the C subunits form a complex with the R subunits. After an increase in intracellular cAMP, the regulatory PKA subunits bind to cAMP, which results in the disassociation of the holoenzyme into an R2(cAMP)4 dimer and two monomers of catalytically active C kinase. The R subunits remain in the cytoplasm, and the free catalytic subunits either translocate into the nucleus or remain in the cytosol. In both locations, PKA C subunits phosphorylate serine and threonine residues of specific substrates. In the nucleus, regulation of transcription by PKA is mediated by cAMP-responsive nuclear factors, which bind to and regulate the expression of genes containing a cAMP-response element binding element (CRE)

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consensus in their promoter region. Phosphorylation of the CRE binding protein (CREB) modulates its activity[28].
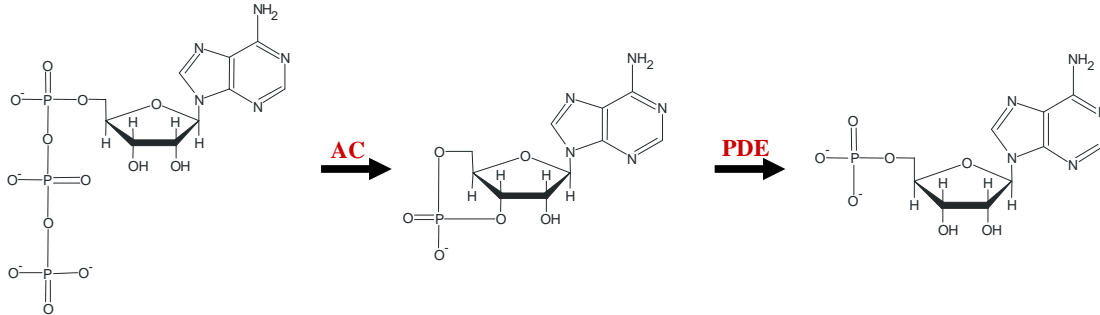
Figure 1.4. Synthesis and hydrolysis of cGMP. AC – adenylate cyclase, PDE – phosphodiesterase
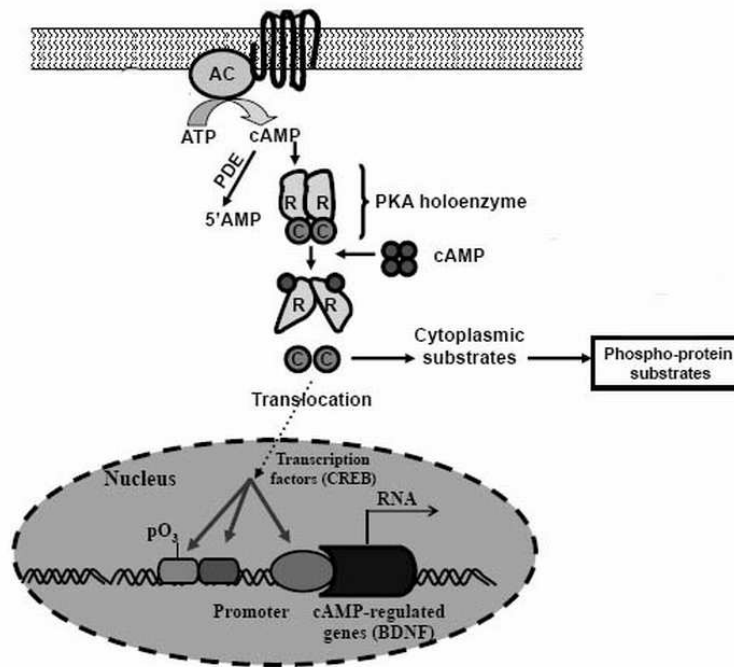
Figure 1.5. AC-cAMP-PKA signaling pathway. Adapted from Dwivedi et al. 2008

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1.2.3 Structure of Phosphodiesterases

Phosphodiesterases contain three functional domains: conserved catalytic domain, a regulatory N-terminus domain and C-terminus domain.

Catalytic domain (approximately 270 amino acids) constitutes the core of PDE and is a higly conserved region, with a 20 – 45% identity. It contains consensus metal binding domains: 2 Zn^{2+} binding motifs and a Mg^{2+} binding motif related to metal-ion phosphohydrolases [29] . The putative role of this ions include stabilization of the structure and activation of hydroxide to mediate catalysis.

Regulatory domains are placed between the amino terminus and the catalytic domain on which are located calmodulin binding sites for PDE1, allosteric cGMP binding sites (GAF domains for PDE2, 5, 6, 10, 11), phosphorylation sites, phosphatidic binding site for PDE4, PAS domain for PDE8, autoinhibitory sequences for PDE1 and PDE4, membrane association domain for PDE2 - 4, dimerization motifs[30,31] .

1.2.4 Cyclic nucleotide recognition and hydrolysis

It is known from structural studies that the cyclic nucleotide specifity of the catalytic site depends largely on one particular invariant glutamine. The idea is that this glutamine stabilizes the banding of the purine ring in the binding pocket through hydrogen bonds that form with cAMP or cGMP or both, depending on the orientation of the glutamine. Freely rotation of this glutamine is required for efficient binding of cyclic nucleotides. If the rotation is blocked by some neighboring residues, the preferred substrate will be either cAMP or cGMP, but not both. For the PDEs that show very high selectivity, the rotation hindered[32].

1.2.5 Biochemical characteristics and structure of Human PDE Families

A complete overview of the PDE isoforms, substrate specificity, relevance and localization is given in Table 2[33,34].

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Table 2. Biochemical characteristics of Human PDE families

Isoforms	Substrate specificity	Relevance	Tissue/Cellular localization	Intracellular localization
PDE1A	cAMP<cGMP	Calcium/ calmodulin regulated	SMC,heart,lung,brain,sperm	Mostly cytosolic
PDE1B	cAMP<cGMP		Neurons,lymphocytes, macrophages, SMC	Cytosolic
PDE1C	cAMP=cGMP		Brain,SMC,sperm, epithelium	Cytosolic
PDE2A	cAMP=cGMP	cGMP stimulated	Brain,naurons,heart,platelet, macrophage, endothelium	PDE2A1 cytosolic, PDE2A2 and PDE2A3 membrane
PDE3A	cAMP>cGMP	cGMP inhibited	Heart,SMC,platelet,oocyte, kidney, cardiovascular tissues	Membrane/ Cytosolic
PDE3B	cAMP>cGMP		SMC,adipocytes, kidney hepatocytes,β cells,sperm,T lymphocytes, macrophages	Membrane
PDE4A	cAMP	cAMP specific, Rolipram sensitive	Immune cells,testis,brain, olfactory system	Membrane/interaction with Src kinases or β-arrestin
PDE4B	cAMP		Immune cells,brain	Interaction with β-arrestin
PDE4C	cAMP		Lung,testis,neuronal origin cell lines	Cytosolic
PDE4D	cAMP		Variety of tissues, commonly used cell lines and inflammatory cells	Cytosolic/particulate
PDE5A	cGMP	cGMP binding, cGMP specific	SMC,brain,lung,heart, kidney,skeletal muscle	Cytosolic
PDE6A	cGMP	Photoreceptor	Rod cells,pineal gland	Association with δ subunit
PDE6B	cGMP		Rod cells,pineal gland	
PDE6C	cGMP		Cone cells,pineal gland	
PDE7A	cAMP	Rolipram insensitive	Immune cells, heart, skeletal muscle, endothelium	Cytosolic
PDE7B	cAMP		Brain,heart,liver, testis, skeletal muscle, pancreas,	Cytosolic
PDE8A	cAMP	cAMP specific	Testis,spleen,ovary, colon, kidney	Cytosolic/particulate
PDE8B	cAMP		Brain,thyroid	
PDE9A	cGMP	cGMP specific, IBMX insensitive	Nearly every tissue, mostly kidney,brain, spleen, prostate	PDE9A5 cytosolic; PDE9A1 nucleus
PDE10A	cAMP>cGMP	cAMP inhibited	Brain,testis,heart,thyroid,cardiac muscle,pituitary gland	PDE10A1 and 10A3 cytosolic; PDE10A2 particulate
PDE11A	cAMP>cGMP	Dual substrate	Skeletal muscle,prostate, testis, salivary gland,thyroid gland,liver	Cytosolic

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1.2.6 Phospodiesterase 4 family

1.2.6.1   General structure

      The PDE4 family are a cAMP-specific, rolipram-sensitive phosphodiesterases, encoded by four genes (PDE4A, 4B, 4C, 4D). These four genes are able to generate more then twenty different variants by means of alternative start sites and alternative splicing[33]. These splicing variants are divided according to N-terminal variant into three goups: so called "long-form" (presence of N-terminal "upstream conserved regions", UCR), "short-form" and "super short-form" (absence of N-terminal "upstream conserved regions"). All of the "long-forms" contains UCR1, linker region (LR) 1, UCR2, LR2 and the catalytic domain. The "short-form" lack UCR1 and "super short-form" lack UCR1 and contain truncated ULR2.

UCR1 (formed from ~60 aminoacids) comprises one PKA phosphorylation site, and UCR2 (forms from some ~80 aminoacids) a hydrophilic N-terminal region which interact with the hydrophobic C-terminal part of UCR1. Moreover, UCR1 and UCR2 are involved in enzymatic regulation, particulary in integrating the effect of phosphorylation[32].The catalytic domain is rather conserved and it shows around 75% sequence identity among all PDE4 members.
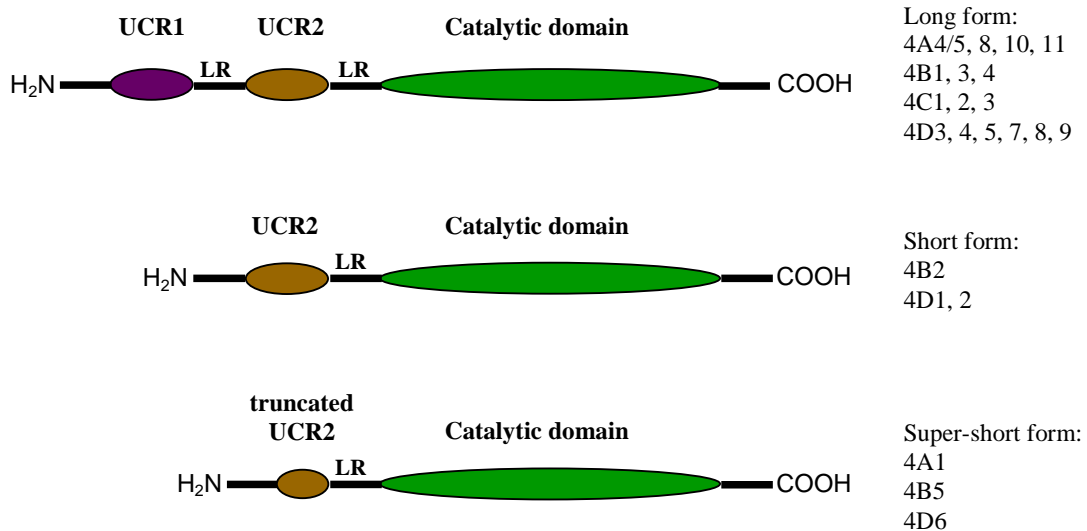
Figure 1.6.   PDE4 family structure.

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1.2.6.2   PDE4 interactions

PDE4 family can actively control and regulate level of cAMP through interactions with other proteins, such like A kinase anchoring protein (AKAP), arrestins, tyrosine kinases and receptor for activated protein C (RACK1).

1. A kinase-anchoring proteins (AKAPs)

Most interactions with kinases and phosphatases are mediated by proteins called A kinase-anchoring proteins (AKAPs). AKAPs are a family of more then 50 anchoring proteins which have ability to create complexes with target signal-termination enzymes such as phosphatases, kinases (PKA, PKC) and phospohodiesterases, and then anchor this complexes in specific subcellular domains. Through this they are able to regulate specific signaling pathways[32,35].

It has been shown that PDE4D forms complex with PKA subunit and mAKAP in rat heart extracts[36].Moreover, it has been shown that PDE4D3 directly interacts with mAKAP and that is co-localizes with mAKAP at the nuclear membrane of cardiomiocytes[36]. Though, there is a possibility that if PDE4D3 and PKA are associated with mAKAP, and regulate local cAMP level as well as PKA activity.

Additionally, the experiments with PDE4D null mice were performed. Absence of PDE4D from complex involving PKA, mAKAP and ryanodine receptor, causes constitutive phosphorylation of this channel[37].

It was shown that PDE4D3 together with mAKAP and PKA, can form complex with Epac (exchange protein activated by cAMP) and MAPK5. Through this complex, PDE4D3 is regulated by MAPK and is thought to play a role in cardiac hypertrophy[38].

2. Arrestins

PKA phosphorylates β_2-adrenoreceptor (β_2AR), what switches its predominant coupling from stimulatory guanine nucleotide regulatory protein (Gs) to inhibitory guanine nucleotide regulatory protein (Gi). Catalytic site of PDE4s binds to mediatory β-arrestin1/2 and this complex is recruited to the β_2AR, can degrade cAMP and through this PKA activity at the membrane can be controlled[39].

It has been also proposed that PDE4/arrestin complex plays a role in regulation of signaling from the T cell receptor during lymphocyte activation[40].

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3. Tyrosine kinases (Src, Lyn, Fyn)

N-terminal regions of PDE4A4/5 and PDE4D4 comprise proline-containing motifs, which are able to interact with SH3 domains of protein-tyrosine kinases, such as Src, Lyn, Fyn.

This interaction can have influence on the specificity as well as localization of PDE4A/D. For example, human PDE4A5 contain additional site for reaction with SH3 domains. This site is located in LR2 (see Rys.1 PDE structure) which is encoded by single exon. Moreover, this exon encodes an insertion of a ten aminoacids sequence (rich in proline and arginine). When Lyn and Src bind to this site, sensitivity of PDE4A to inhibition by rolipram is significantly enhanced[41].

Additionaly, PDE4A5 is associated with cell membrane and is localized at the cell periphery and is bound to ruffles. It was shown that when site for interaction with SH3 domain is deleted or disrupted, prevents this interaction and allows it to be distributed equally through the cell margin[42].

4. Receptor for activated C kinase 1 (RACK1)

PDE4D5 contain on N-terminal region domain called RAID1 (RACK1 interaction domain) through which can bind to RACK1. RACK1 is a signaling protein formed from seven tryptophan-aspartate repeats that binds to PKC isoforms. RAID1 contain hydrophobic amino acids, and reacts with a site on RACK1 that is formed primarily from tryptophan-aspartate. However, the functional role of this interaction is not known, because it does not change PDE4D5 activity, but it does change a little sensivity to inhibition by rolipram. The probable role may be that recruitment of PDE4D5 by RACK1 to signaling complex, modulates and controls cAMP levels and through this regulates PKA phosphorylation of RACK1-associated proteins[43,44].

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1.2.6.3   PDE4 regulation

Recent studies show that phosphorylation plays an important role for PDE4 activation.

PKA phosphorylates serine residue in UCR1 at the N-terminal end of PDE4, and though activates long PDE4 isoforms[45,46]. It is believed that PKA phosphorylation in UCR1 causing disruption of interaction between UCR1 and UCR2 through conformational changes[47].

It has been shown that PKA activation can be mimicked when serine residues was replaced by aspartate or glutamate residue. Moreover, it was also observed that this target serine is next to glutamate residue residue that is conserved in all PDE4 subfamilies and which attenuates phosphorylation by PKA. Mutation of this glutamate residues to a neutral amino acid also mimics activation by PKA phosphorylation [44,48].

Moreover, mutation of Ser54 in UCR1 to aspartic acid which also mimics PKA-dependent activation, reduced the interaction between UCR1 and UCR2[47]. Concerning this idea, it was also shown that PKA phopshorylation additionally increases the sensitivity of PDE4D3 and PDE4A4 to stimulation by Mg^{2+} that is found in catalytic domain and is essential for PDE activity[46].

On the other side, ERK (Extracellular Signal-regulated Kinase) regulates PDE4 activity via phosphorylation, causing inhibition as well as activation of PDE4.

All PDE4 subfamilies, except PDE4A, contain in a catalytic domain a single ERK consensus motif (Pro-Xaa-Ser-Pro). It is already known *in vitro* and *in vivo* that serine from this motif can be phosphorylated by ERK[49,50].

Moreover, phosphorylation of Ser579 by ERK2, inhibits PDE4D3 and through this probably modulate cAMP signaling. This effect could be mimicked by the Ser579 → Asp mutant form of PDE4D3[49-51]. In comparison, mutation of Ser579 → Ala ablated the ability of ERK2 to be co-immunoprecipitated with PDE4D3 and abolished the ERK2-mediated inhibition of PDE4D3[50]. In comparison, PDE4D1 has been shown to be activated by ERK2.

It was also demonstrated that PDE4B and PDE4C share an identical motif to that found in members of the PDE4D family[51]. Long form of PDE4B and PDE4C can be phosphorylated by ERK2 and this inhibits its activity, wheras activates PDE4B2[49].

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It is believed that UCR1 and UCR2 regions interact with each other and form a segment which can regulate the activity of catalytic unit[47]. Proposed model of this interaction shows that between carboxyl-terminal group of UCR1 and amino-terminal group of UCR2 communication occurs. Moreover, mutation of Ser54 → Asp or Ser54 → Ala in the UCR1, abolished UCR1–UCR2 interaction, wheras mutation of Ser54 → Thr or Glu53 → Ala did not block this interaction. Therefore, it is possible that Ser54 is essential for UCR1-UCR2 interaction. It was also observed that this interaction is PKA-depentent since this enzyme can phosphorylate Ser54. Such a phosphorylation prevents UCR1 region from reacting with UCR2[47].

1.2.6.4 PDE4 inhibitors

Rolipram (4-(3-cyclopentyloxy-4-methoxy-phenyl)pyrrolidin-2-one) is the first and prototype PDE4 inhibitor. It was developed as a anti-depressant drug by Schering AG.

According to accepted idea, catalytic domain of PDE4 can exist in at least two conformational states. This states differ between each other depending on the affinity to rolipram. "LARBS" is a "low-affinity-rolipram-binding-site" with IC_{50} in the range of 0,1-1.0 µM and "HARBS" is a "high-affinity-rolipram-binding-site" with with IC_{50} in the range 1-50nM. Rolipram has been found to bind to HARBS, distinct from the catalytic site.

High- and low-affinity rolipram binding is up to single site which can be in two conformational different forms. Switching between this states depends on modifications, such as interaction with other proteins and phosphorylation.

It was shown that the concentration of Mg^{2+} has ability to modify this states of enzyme. PDE4 conformations are the consequence of PDE4 binding to Mg^{2+}- its metal cofactor. Mg^{2+}-deficient enzyme is inactive and has low affinity for rolipram[52].

It was also demonstrated that Mg^{2+} binding is upregulated by phosphorylation of serine. A partially phosphorylated PDE4D3 shown much lower rolipram affinity state in compare to fully phosphorylated Ser54 and in consequence increased catalytic potency for rolipram[52].

It was also proposed that inhibitor selectivity is determined by a combination of different amino acids and subtle conformational changes at the active site of each PDE family[53].

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PDE inhibitors are currently known in therapeutical use and particular PDE4 inhibitors have proven as potential drugs, for example in asthma, COPD, rhinitis, arthritis, psoriais, depression. In recent years, new generation of PDE4 inhibitors have been developed as an attempt to improve the therapeutic index of the first generation of PDE4 inhibitors such as rolipram. New generation of such a inhibitors include roflumilast (Daxas) and cilomilast that are currently in phase III trials for treatment of COPD.

Moreover, in animal models treatment with rolipram seems to enhance several models of learning and memory[33].
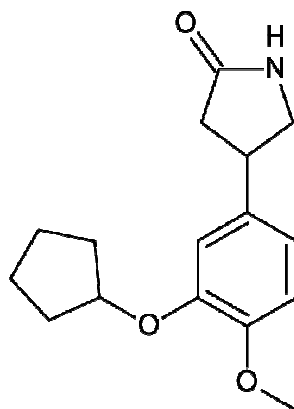
Figure 1.7.   Structure of Rolipram

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2   Aim of the study

It is well known that dysregulation of PDE isoforms may contribute to the development of several pulmonary diseases, such as pulmonary hypertension (PAH), chronic obstructive pulmonary disease (COPD) or asthma.

On the other hand, EMT is a process whereby fully differentiated epithelial cells undergo transition to a mesenchymal phenotype giving rise to fibroblasts and myofibroblasts, and is recognized as an important process not only in development, but also in wound healing, fibrosis, as well as in invasion and metastasis of tumor cells.

However, until now the direct involvement of PDE family members and therapeutic value of PDE specific inhibitors in the process of EMT has not been reported in detail.

In this context, the research focus was:

1. to ascertain the expression pattern of PDE isoforms upon TGF-β1 induced EMT of A549 cells;

2. to investigate whether altered PDE expression has a functional effect on cAMP accumulation and differentiation of A549 cells using PDE isoform specific inhibitors;

3. to elucidate the signaling pathways in the PDE-mediated regulation of EMT.

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3.　Materials and methods

3.1.　Materials

3.1.1. Equipment

Name	Company
Cell Culture Incubator, Hera Cell	Heraeus, Germany
Chromatography Columns	Bio-Rad, USA
Electrophoresis Chambers	Biometra, Germany
Falcon Tubes	Greiner Bio-One, Germany
Film Casette	Kodak, USA
Filter Tips: 10; 100; 1000μl	Eppendorf, Germany
Fluorescence Microscope, LEICA	Leica, Germany
Frezer -80°C	Bosh, Germany
Freezer -20°C	Bosch, Germany
Freezer +4°C	Bosch, Germany
Gel Blotting Paper	Bio-Rad, USA
Glass Bottles: 0,1; 0,2; 1l	Schott Duran, Germany
Glass Pipetes	Greiner Bio-One, Germany
Light Microscope	Hund, Germany
Multifuge Centrifuge	Heraeus, Germany
NanoDrop	PeqLab, Germany
PCR-thermocycler	Biometria, Germany
Petri Dish	Greiner Bio-One, Germany
Pipetboy	Eppendorf, Germany
Pipets	Eppendorf, Germany
Power Supply	Biometria, Germany
Radiographic Film Hypersensitive	Amersham Bioscences, UK
Radiographic Film Kodak	Kodak, USA
Serological pipette: 5, 10, 25, 50 ml	BD Falcon, USA
Tissue Culture Chamber Slides	BD Falcon, USA

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Tissue Culture Dish 100mm	Greiner Bio-One, Germany
Tissue Culture Flask 250mm	Greiner Bio-One, Germany
Tissue Culture 6well Plate	Greiner Bio-One, Germany
Transilluminator BioDocAnalyzer	Biometria, Germany
Water Bath for Cell Culture	Medingen, Germany
Western Blot Chambers	Biometra, Germany
Vortex Machine	VWR, Germany

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3.1.2. Reagents

Product Name	Company
Ammonium persulfate	Sigma-Aldrich, Germany
1-Butanol (n-butyl alcohol)	Merck, Germany
2-Mercapto-ethanol	Sigma-Aldrich, Germany
2-Propanol	Fluka, Germany
Acetic Acid, Glacial 99+%	Sigma-Aldrich, Germany
Acetone 99,5+% A.C.S. reagent	Sigma-Aldrich, Germany
Acrylamide solution, Rotiphorese gel 30	Roth, Germany
Agarose	Fluka, Germany
Albumine, Bovine serum	Sigma-Aldrich, Germany
Ammonium Acetate	Sigma-Aldrich, Germany
Ammonium formate	Sigma-Aldrich, Germany
Brillant Blue G	Sigma-Aldrich, Germany
Calcium Chloride	Sigma-Aldrich, Germany
DMEM-F12 medium	Gibco BRL, Germany
Dimethyl Sulfoxide	Sigma-Aldrich, Germany
DNA Ladder (100bp, 1kb)	Promega, USA
Dulbecco's phosphate buffered saline 1x	PAN, Germany
Ethanol absolut	Riedel-de Haen, Germany
Ethidium Bromide	Sigma-Aldrich, Germany
Ethylene glycol-bis(ß-amino-ethylether)-N,N,N',N'tetraacetic acid (EGTA)	Sigma-Aldrich, Germany
Ethylenediamine-Tetraacetic acid (EDTA)	Sigma-Aldrich, Germany
ECL Plus Western Blotting Detection System	Amersham Biosciences
Fetal Calf Serum (FCS)	Biowest, USA
Formaldehyde	Sigma-Aldrich, Germany
Glycerol	Sigma-Aldrich, Germany
Glycine, minimum 99% TLC	Sigma-Aldrich, Germany
GoTaq ® Flexi DNA polymerase	Promega, USA

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2-(-4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate (HEPES)	Sigma-Aldrich, Germany
Hydrochloric Acid	Sigma-Aldrich, Germany
ImProm-II^{TM} Reverse Transcriptase	Promega, USA
Magnesium chloride (anhydrous)	Sigma-Aldrich, Germany
Methanol	Fluka, Germany
Milk Powder	Roth, Germany
N,N,N',N'-Tetramethyl-1-,2-diaminomethane (TEMED)	Sigma-Aldrich, Germany
N,N'-Methylene-bis-Acrylamide	Sigma-Aldrich, Germany
Oligo(dT) Primer	Promega, USA
Penicillin-Streptomycin	PAA Laboratories, Austria
PCR Nucleotide Mix	Promega, USA
Ponceau S Solution	Sigma-Aldrich, Germany
Potassium hydroxide, Sigma Ultra	Sigma-Aldrich, Germany
Potassium Phosphate monobasic	Sigma-Aldrich, Germany
QAE Sephadex	Amersham Biosciences
RNase Away	Molecular Bioproducts
RNAsin inhibitor	Promega, USA
RIPA buffer	Santa Cruz, Germany
SDS Solution, 10% w/v	Promega, USA
Sodium Chloride	Riedel-de Haen, Germany
Sodium citrate tribasic dihydrate	Sigma-Aldrich, Germany
Sodium hydroxide	Sigma-Aldrich, Germany
Sodium Phosphate (monobasic, anhydrous)	Sigma-Aldrich, Germany
Tris-HCl 1M	Sigma-Aldrich, Germany
Trizma base, minimum 99.9% titration	Sigma-Aldrich, Germany
Trypsin/EDTA	PAA Laboratories, Austria

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3.2. Methods

3.2.1. RNA isolation

Total RNA was isolated from the cells using TRIzol™ reagent. Cells were resuspended in 1,0ml of TRIzol™ reagent and incubated for 10min in room temperature. After adding 200μl of chloroform, vortexing for 15sec and incubating in room temperature for 10min, samples were centrifuged for 25min (4°C, 13000xg). After centrifugation aqueous phase was transferred to new tube and 0,5ml isopropanol per 1,0ml of TRIzol reagent used was added for precipitation of RNA. Probes were incubated in room temperature for 10min and then centrifuged for 10min (4°C, 13000xg). Pellet was washed with 0,5ml of 75% ethanol per 1,0ml of TRIzol reagent used and centrifuged for 10min (4°C, 13000xg). The RNA pellet was redissolved in DEPC treated water, warmed for 10min in 55°C and stored in -80°C.

RNA purity and concentration were measured spectroscopically by its absorbance in 260nm and 280nm in NanoDrop.

3.2.2. Reverse transcriptase reaction

Reverse transcriptase reaction (RT) is an enzymatic technique for amplifying a defined piece of a RNA into complementary DNA (cDNA) by an enzyme reverse transcriptase.

cDNA was synthesized from total RNA using ImpromII Reverse Transcriptase Kit. 3μg of RNA was used per single reaction. cDNA synthesis was performed in 2 steps. In first step RNA was mixed with 1μl oligo(dT) (100μg/ml) and the nuclease free water till the final volume of 5μl. The reaction mixture was heated to 70°C for 5min.

In the second step RT mixture was added as follows:

RT reaction component	Volume	Final concentration
5xRT buffer	4μl	1x
25mM MgCl$_2$	4,8μl	6mM
10mM dNTP mix	1μl	0,5mM
RNAsin inhibitor (1U/μl)	1μl	1U
Reverse transcriptase (1U/μl)	1μl	1U
H$_2$O (RNase free)	3,2μl	-

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The reaction mixture was vortexed, spined down and kept at 25°C for 5min and then at 42°C for 1h. Synthesized cRNA was used for PCR or stored in -20°C.

3.2.3. Polymerase chain reaction

Polymerase chain reaction (PCR) is an enzymatic technique used to amplify cDNA by an enzyme DNA polymerase. Each PCR cycle contain 3 steps: denaturation (separation of double stranded cDNA into single stranded); annealing (primers binding to specific sites in single stranded cDNA) and elongation (synthesis of new DNA by DNA polymerase).

PCR was performed on cDNA prepared as described in (3.2.2) using GoTaq® Flex DNA Polymerase Kit (Promega).

PCR reaction Mix was added as follows:

PCR reaction component	Volume	Final concentration
5xPCR buffer	10µl	1x
25mM MgCl$_2$	5µl	2,5mM
10mM dNTP mix	1µl	0,2mM
10µM of forward primer*	1µl	0,2µM
10µM of reverse primer*	1µl	0,2µM
Taq DNA polymerase (5U/ml)	0,25µl	1,25U
cDNA	1µl	-
H$_2$O (nuclease free)	5,75µl	-

PCR components were mixed on ice, vortexed, spined down and placed in the Thermal Cycler. Number of cycles depend on the amplified sequence.

Programmed steps were as follows:

Step	Time	Temperature
Denaturation	30s	94°C
Annealing	30s	Variable*
Elongation	1min	72°C
Final elongation	5min	72°C

*Primers seguences and annealing temperature are listed in Appendix in **Table 3.**

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3.2.4. Agarose gel electrophoresis

DNA agarose gel electrophoresis was performed to visualize and analyze PCR products. The preparation of 1% gel was as follows: agarose was mixed with 1xTAE buffer and ethidium bromide (1μg/ml). PCR products and GeneRuler DNA ladder were loaded and the gel was run in 1xTAE buffer at 80V.

DNA bands were visualized under UV-transilluminator of Biometra system.

<u>1xTAE Buffer</u>

40mM Tris-acetate, pH=8,0

1mM EDTA, pH=8,0

3.2.5. Protein isolation

Harvested cells were lysed in RIPA buffer containing DMSO, protease inhibitor and PMSF. Lysates were incubated on ice for 30min and then subjected to a low-speed centrifugation (13000xg for 30 min). Supernatants were placed in new tubes and stored in -80˚C.

3.2.6. Protein quantification

Protein quantification was performed using Quick Start™ Bradford Dye Reagent (Biorad). The Bradford assay is determination method that involved the binding of Coomassie Brilliant Blue G-250 dye to proteins. When the dye binds to protein it is converted to a stable blue form. This form is detected at 595nm using a spectrophotometer reader. BSA in different concentration was used as a standard.

3.2.7. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed to separate proteins according to its size. Before loading, proteins were mixed with 5x SDS-loading buffer and denatured by heating in 100˚C for 10min. This prepared mix was loaded into the SDS acrylamide gel (consists of 10% resolving gel and 5% stacking gel) and run in SDS-running buffer at 130V.

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SDS-running buffer

25mM Tris

250mM Glycine

0,1% (w/v) SDS

5xSDS-loading buffer

0,25mol/l Tris-HCl, pH=6,8

10% (w/v) SDS

50% Glycerol

0,5mol/l DTT

0,5% Bromophenol Blue

Stacking gel (5%)

5% acrylamide:bisacrylamide

125mM Tris-HCl, pH=6,8

0,1% (w/v) SDS

0,1% (w/v) APS

0,1% (v/v) TEMED

Resolving gel (10%)

10% acrylamide:bisacrylamide

375mM Tris-HCl, pH=8,8

0,1% (w/v) SDS

0,1% (w/v) APS

0,1% (v/v) TEMED

3.2.8. Immunoblotting

Immunoblotting was performed to visualize proteins separated according to its size during SDS acrylamide gel electrophoresis.

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3.2.9. Protein blotting

Separeted on SDS acrylamide gel proteins were transferred into the nitrocellulose blotting membrane (0,45μM pores) using wet transfer technique. Transfer was performed in transfer buffer for 1h at 100V.

Transfer buffer

25mM Tris

192mM Glycine

20% (v/v)Methanol

3.2.10.   Protein detection

Membranes with transferred proteins were blocked in 5% milk for 1h at room temperature. After blocking, membranes were incubated with appropriate primary antibody diluted in 5% milk overnight at 4˚C*. The next day, membrane were washed 3x for 10min with TBST and incubated for 1h with HRP-conjugated secondary antibody diluted in 5% milk* and washed 3x for 10min with TBST. Proteins on the membrane were detected using ECL Western Blotting Reagent (Amersham Bioscencies). In order to re-probe membranes with another antibody, membranes were stripped for 30min in 60˚C and used again for protein detection.

*Primary and secondary antibody are listed in Appendix in **Table 4.** and **Table 5.**.

20x TBS

25mM Tris

0,15M NaCl

1xTBST

1xTBS

0,1% (v/v) Tween 20

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Blocking milk solution

5% non-fat dry milk

1xTBST

Stripping buffer

62,5 mM Tris-HCl, pH=6,8

2% (w/v) SDS

100mM β-mercaptoethanol

3.2.11. Densitometry

cDNA and protein densitometry were performed using Biometra Illuminator and BioDoc software. cDNA and protein expression were normalized to GAPDH.

3.2.12. PDE activity assay

PDE assays were done by a modification of the two-step method by Thompson and Appleman. Harvested cells were lysed in RIPA buffer (Santa Cruz) containing DMSO, protease inhibitor and PMSF. Lysates were incubated on ice for 30min and then subjected to a low-speed centrifugation (13000xg for 30 min) and aliquots of the resulting supernatant were used. 30μg of proteins were mixed with reaction buffer, appriopriate inhibitor and spiked with [^{3}H]cAMP (Amersham Bioscencies), as a substrate. Assays were carried out at 37°C for 15min and then terminated by boiling for 3min in 100°C. Samples were cooled down on ice and *Crotalus Atrox* venom at the final concentration of 80μg/ml was added to prevent resynthesis of cAMP. Next, products of cAMP were separated from unhydrolysed substrate on chromatography columns filled with Sephadex-Q25 beads. Scintillation liquid was added to each probe and radioactivity was measured using X software.

Total PDE activity in cell lysates was determined and expressed as pmol of cAMP or cGMP hydrolysed per minute per mg of lysate protein. PDE activities were determined using specific inhibitors diluted in DMSO: IBMX as a non-specific PDE inhibitor, rolipram for PDE4, motapizone for PDE3, calcium/calmodulin as a stimulator of PDE1.

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Reaction buffer was as follows:

Reagent	Volume	Final concentration
1% BSA	10μl	0,01%
40mM MgCl$_2$	12,5μl	0,4mM
1M HEPES, pH=7,6	10μl	0,2mM
100μM cAMP	0,5μl	1μM
Distilled H$_2$O	22,5μl	-

Inhibitors used:

Inhibitor	Volume	Final concentration
4mM IBMX	5μl	40μM
0,4mM Rolipram	2,5μl	1μM
0,25 mM Motapizone	2μl	5μM
10μM Cilomilast	6μl/ml	60 nM

3.2.13.　　Immunocytochemistry

Immunocytochemistry was performed for protein localization in the cells. Cells were seeded in 8-well chamber slides. After stimulation, cells were washed with 1xPBS. Cells were fixed in 1:1 methanol:acetone for 5min in room temperature. After washing 3x for 10min with PBS, cells were blocked in 5% PBS for 1h at room temperature and then incubated with appriopriate primary antibody* overnight at 4˚C. After washing 3x for 10min with PBS and 1h incubation with FITC-conjugated secondary antibody*, cells were incubated with DAPI for 5min at room temperature and covered with mounting medium. Visualization of protein localization was performed using fluorescent microscopy and Leica software.

*Primary and secondary antibody are listed in Appendix in **Table 4.** and **Table 5.**.

Blocking solution
5% BSA
1x PBS

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3.2.14.   Reactive oxygen species measurements

Superoxide release from A549 was measured using the superoxide sensitive dye DHE. The cells were grown on chamber slides and cultured in medium alone, medium of 0.1% FCS supplemented with TGF-β1 alone (2 ng/ml), TGF-β1 (2 ng/ml) and rolipram (1μM), TGF-β1 (2 ng/ml) and NAC (5mM), or H_2O_2 alone (1mM,or 10mM) for 24h. H_2O_2 treatment served as the positive control, whereas NAC treatment served as the negative control. Subsequently the cells were incubated with of DHE (5 μM) for 30min. The cells were washed with PBS, fixed in 1:1 methanol:acetone for 10min and stained with the nuclear stain DAPI. Cells were visualized under fluorescent microscopy (excitation: 514 nm; emission: 560 nm). A total of 10 images were captured from each group and in each image the total fluorescence-integrated density was analysed from all groups, respectively, in a blinded fashion using Image J software.

3.2.15.   Statistical analysis

Data are expressed as the mean ± SEM. Statistical comparisons between two populations were performed using paired and unpaired Student t-tests where appropriate or by one-way ANOVA in combination with a Student-Newman-Keuls post-hoc test, which was used to compare differences between multiple groups, with a probability value of $P<0.05$ considered to be significant.

3.2.16.   Cell culture condition

The A549 cell line was obtained from ATCC, Manassas, USA.

A549 cells were grown on $10cm^2$ dishes in DMEM-F12 supplemented with 10% FCS, 5% streptomycin/penicillin, 5% vitamins, and 5% non-essentials amino acids, at 37˚C, 5% CO_2 and 95-100% humidity. After achieving 80-90% of confluence cells were passaged as follows: first cells were washed 1x with PBS and then incubated with 3ml Trypsin/EDTA for 2-3min to detach the cells. Next, 10% FCS culture medium was added to neutralize effect of Trypsin, the cells were counted and cell suspension was seeded on new plate culture dishes.

Cells were cultured from the time of plating in medium alone, and medium 0.1% FCS supplemented with TGF-β1 (2ng/ml) for 24h. For experiments with rolipram, cells were

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pretreated with different concentrations of Rolipram (100nM or 1µM) for 12h followed by TGF-β1 (TGF-β1) stimulation. For experiments with Y27632, cells were pretreated with Y27632 (10µM) for 12h followed by TGF- β1 (2ng/ml) stimulation.

10% FCS cell culture medium

F-12 DMEM Medium

1% vitamins

1% non-essentials amino acids

1% Penicillin/Streptamycin

1% Glutamine

10% FCS

0,1% FCS cell culture medium

F-12 DMEM Medium

1% vitamins

1% non-essentials amino acids

1% Penicillin/Streptomycin

1% Glutamine

10% FCS

1x Trypsin/EDTA

0,025% Trypsin/EDTA

1xPBS

Cells stimulation/treatment was as follows:

Reagent	Volume (per 1ml of medium)	Final concentration
2ng/µl TGF-β1	1µl	2ng/ml
1mM Rolipram	1µl	1µM
2mM NAC	5µl	10mM
1µM Y27632	10µl	10µM
30 % H_2O_2	1:1000000	10µM

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4.   Results

4.1. Cell morphology and phenotype during EMT in A549 cells

4.1.1. Morphological changes

To investigate potential changes in cell morphology, A549 cells were stimulated with TGF-β1 (2ng/ml) for 24 h. Cell morphology was examined using phase contrast microscopy. A549 cells in the absence of TGF-β1 exhibited a cubic, epithelioid shape and cluster formation, the typical features of epithelial cells (Figure 4.1.). Upon TGF-β1 stimulation for 24 h, phase contrast microscopy revealed that cells underwent a morphological change, from cobblestone-like cell morphology to an elongated and spindle-like morphology, and reduced their cell-cell contact.

Control	TGF-β1 [2ng/ml]
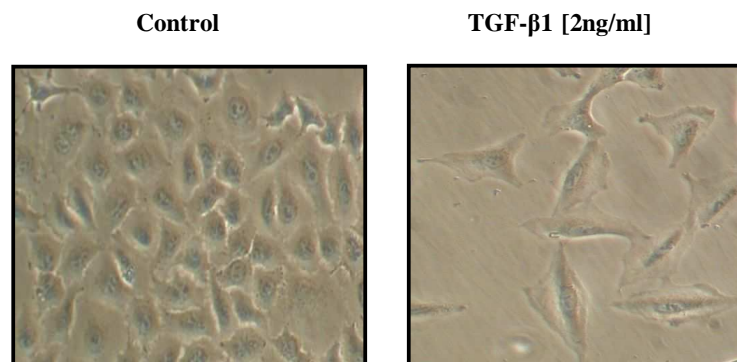
Figure 4.1.   Effects of TGF-β1 on cell morphology.
A549 cells were stimulated with TGF- β1 (2ng/ml) for 24 h. Cell morphology was examined using phase contrast microscopy.

4.1.2. Expression analysis of epithelial markers

To further investigate changes caused by TGF-β1, mRNA and protein expression was analyzed. Both mRNA and proteins were isolated from control A549 cells and cells stimulated with TGF-β1 (1ng/ml and 2ng/ml) for 24 h.

The analysis of expression on the mRNA level was performed by means of semi-quantitative RT-PCR. The mRNA expression of epithelial phenotype markers, E-cadherin (E-Cad), cytokeratin-18 (Cyt 18), and zona occludins-1 (ZO-1) were significantly altered in TGF-β1 stimulated cells compared to control cells. TGF-β1 stimulation significantly

Results

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decreased E-cadherin expression in a concentration-dependent manner. Concentrations as low as 1 ng/ml of TGF-β1 induced at least a 2 fold downregulation of E-Cad, Cyt-18 and ZO-1 expression (Figure 4.2. A, B).

Similarly, immunoblotting confirmed decreased protein expression of both E-cadherin and cytokeratin-18 in TGF-β1 stimulated cells (Figure 4.2. C, D).
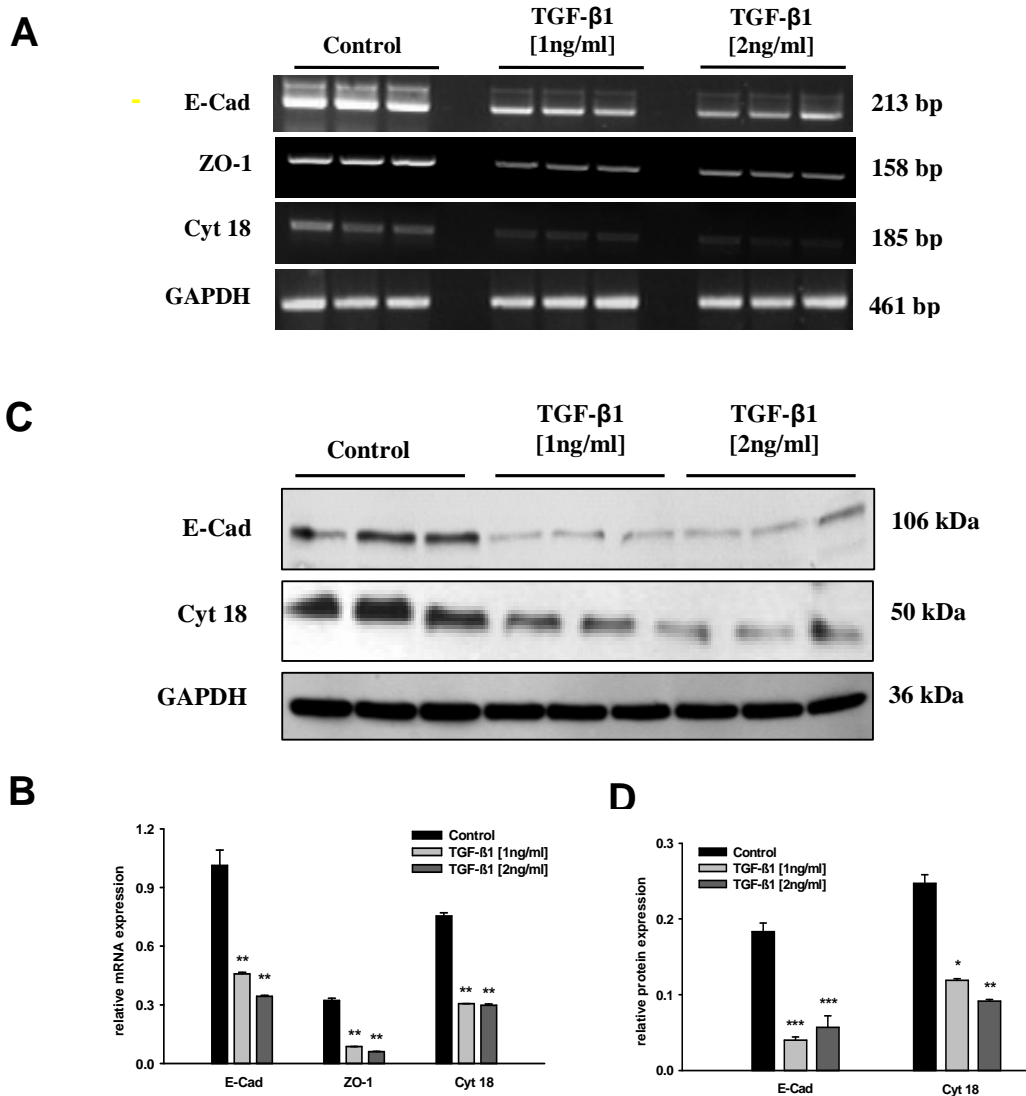
Figure 4.2. **Effect of TGF-β1 stimulation on epithelial phenotype marker expression.**
Expression levels of mRNA from control and TGF-β1 (1ng/ml and 2ng/ml) treated A549 cells for 24h assessed by semi-quantitative PCR. GAPDH served as a control gene. **A.** mRNA expression of epithelial markers, **B.** Densitometric analysis of mRNA expression of epithelial markers, **C.** Protein expression of epithelial markers, **D.** Densitometric analysis of protein expression of epithelial markers.
All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * p<0.05; ** p<0.01; *** p<0.001 versus control.

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4.1.3. Expression analysis of mesenchymal markers

In order to further study changes caused by TGF-β1, mesenchymal markers expression was analyzed on mRNA and protein level. Both mRNA and proteins were isolated from control A549 cells and cells stimulated with TGF-β1 (1ng/ml and 2ng/ml) for 24 h.

RT-PCR was performed to examine the mRNA levels of mesenchymal markers as shown on Figure 4.3. A and B. We observed that TGF-β1 significantly increased mRNA expression of the mesenchymal markers collagen I (Coll I), fibronectin-EDA (Fn-EDA) and α-smooth muscle actin (α-SMA) in a concentration-dependent manner. However, the extent of α SMA expression at low concentrations of TGF-β1 was not as profound as Fn-EDA and Coll I.

In line with the mRNA expression, protein levels of those markers revealed increase after TGF-β1 treatment compared to control cells (Figure 4.3. C, D).

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A

C

B

D
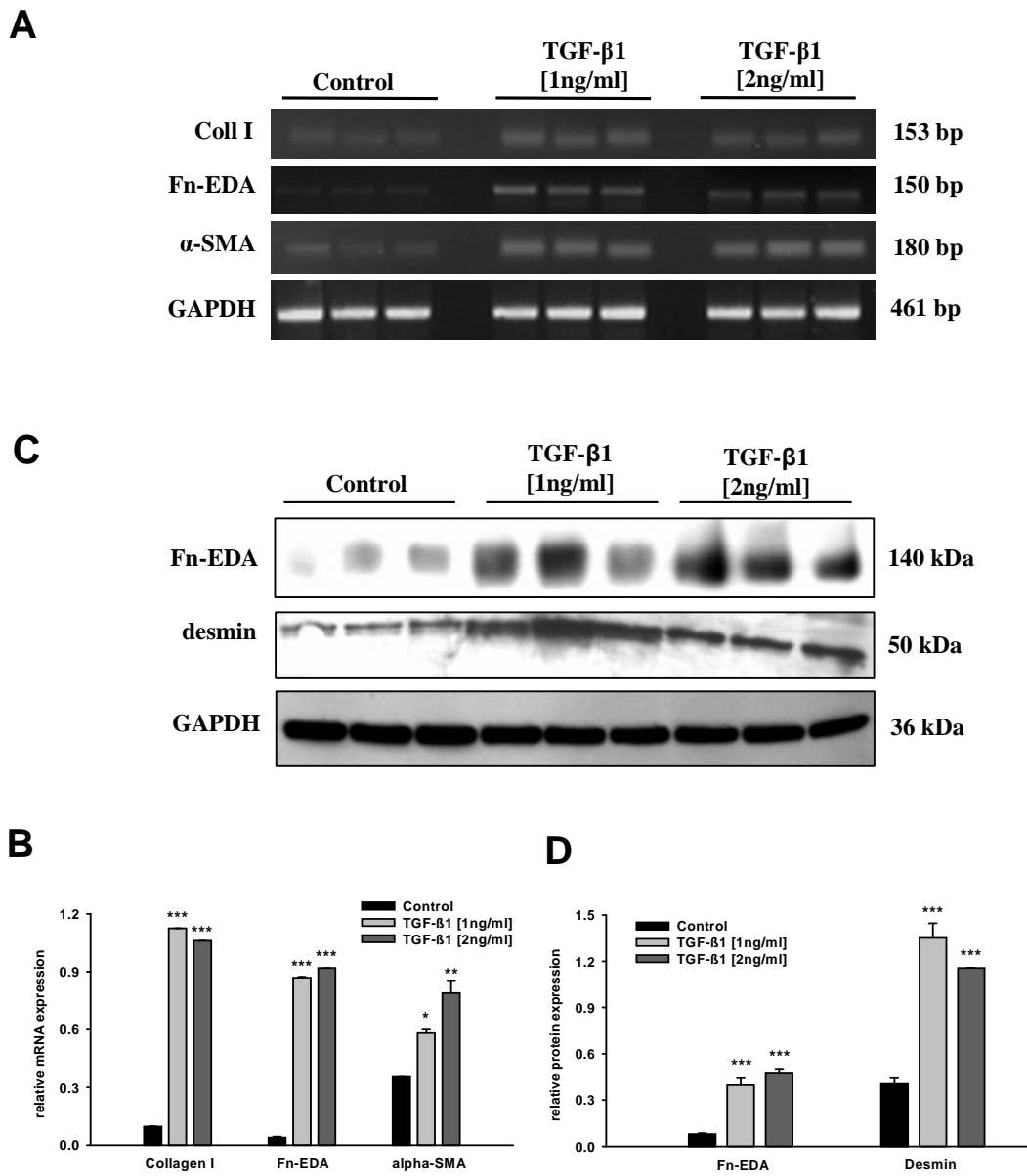
Figure 4.3. **Effect of TGF-β1 stimulation on mesenchymal phenotype marker expression.**
Expression levels of mRNA from control and TGF-β1 (1ng/ml and 2ng/ml) treated A549 cells for 24h assessed by semi-quantitative PCR. GAPDH served as a control gene. **A.** mRNA expression of mesenchymal markers, **B.** Densitometric analysis of mRNA expression of mesenchymal markers, **C.** Protein expression of mesenchymal markers, **D.** Densitometric analysis of protein expression of mesenchymal markers
All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * p<0.05; ** p<0.01; *** p<0.001 versus control**.**

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4.2. PDE expression during TGF-β1 induced EMT

To investigate potential influence of TGF-β1 treatment on PDE isoforms, mRNA expression of all 11 PDE families was analyzed. The mRNA was isolated from control A549 cells and cells stimulated with TGF-β1 (1ng/ml and 2ng/ml) for 24 h.

Semi-quantitative PCR demonstrated an increase in mRNA expression of four PDE isoforms in TGF-β1 stimulated cells compared with control: PDE4A, PDE4D, PDE7A, and PDE10A, with PDE4D showing the most prominent increase in mRNA. In contrast, PDE1A and PDE3A expressions were decreased by TGF-β1 stimulation (Figure 4.4 A, B).

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A

B
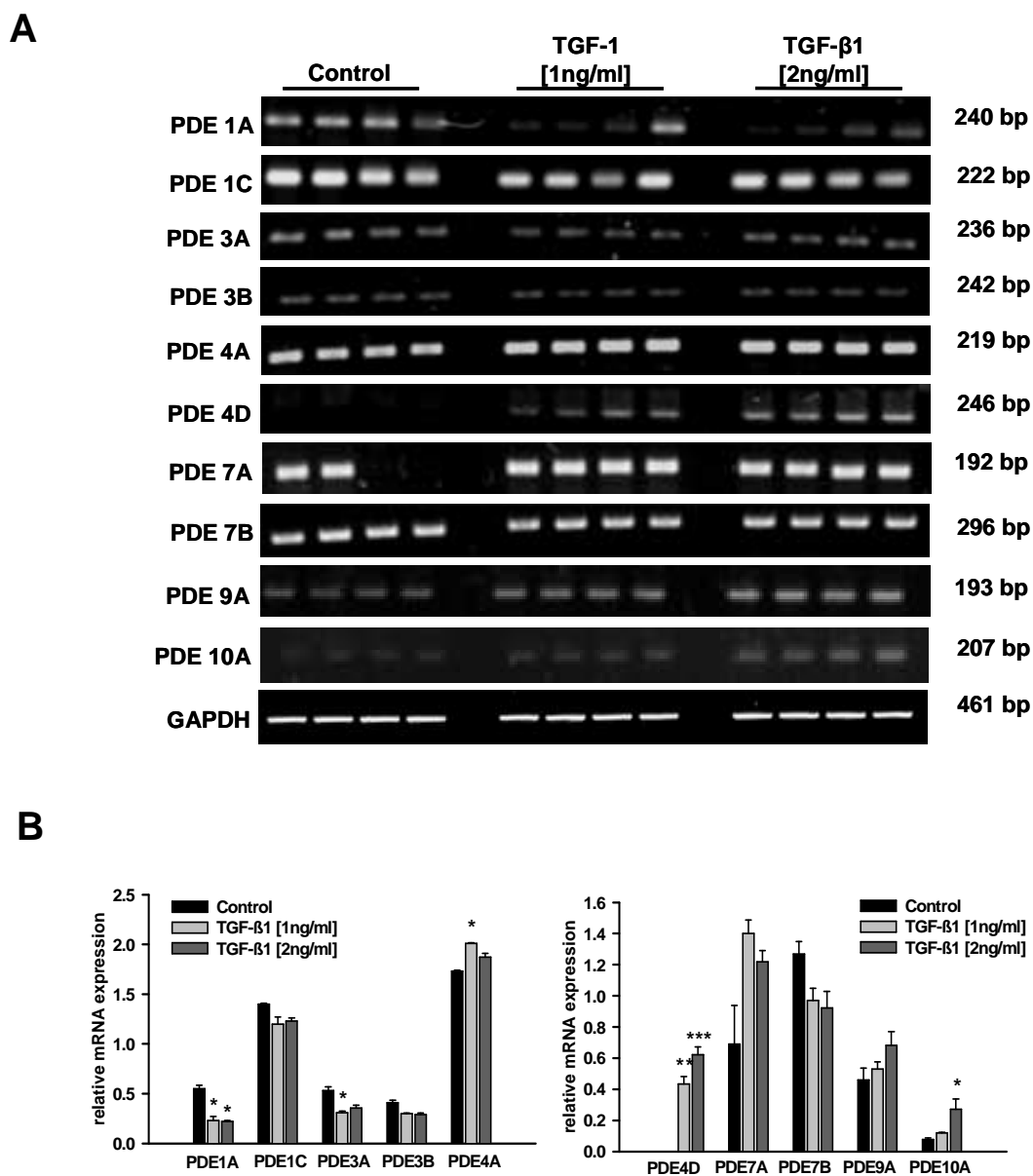
Figure 4.4. **mRNA expression of multiple phosphodiesterase (PDE) isoforms during TGF-β1 induced epithelial mesenchymal transition (EMT).**
Expression levels of mRNA from control and TGF-β1 (1ng/ml and 2ng/ml) treated A549 cells for 24h assessed by semi-quantitative PCR. GAPDH served as a control gene. **A**. mRNA expression of PDE isoforms, **B**. Densitometric analysis of mRNA expression of PDE isoforms. All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control.

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4.3. PDE activity during TGF-β1 induced EMT

In order to investigate whether the dramatic changes in mRNA expression of PDE1, PDE3, PDE4, PDE7 and PDE10 resulted in changes in activity of this enzymes, PDE activity assay was performed (Figure 4.5).

A549 cells were treated with TGF-β1 (2ng/ml) for 24 h. Using cAMP-PDE inhibitors [non-specific PDE inhibitor, 30 µM IBMX; PDE1, calmodulin in addition of excess calcium in the presence of EGTA; PDE3, 10 µM motapizone; PDE4, 10 µM rolipram], we calculated the relative contribution of each isoform to the total cAMP-PDE activity. Total PDE activity in cell lysates was determined and is expressed as pmol cAMP hydrolysed per minute per mg of lysate protein.

Interestingly, total cAMP-PDE activity was increased 2 fold in TGF-β1 stimulated cells compared with control (Figure 4.5). We calculated the relative contribution of each isoform to the total activity and we found PDE4 to be the major contributor to cAMP-PDE activity. In addition, PDE3 and PDE4 activities were also increased in TGF-β1 stimulated cells compared with control and other cAMP-PDE isoforms.
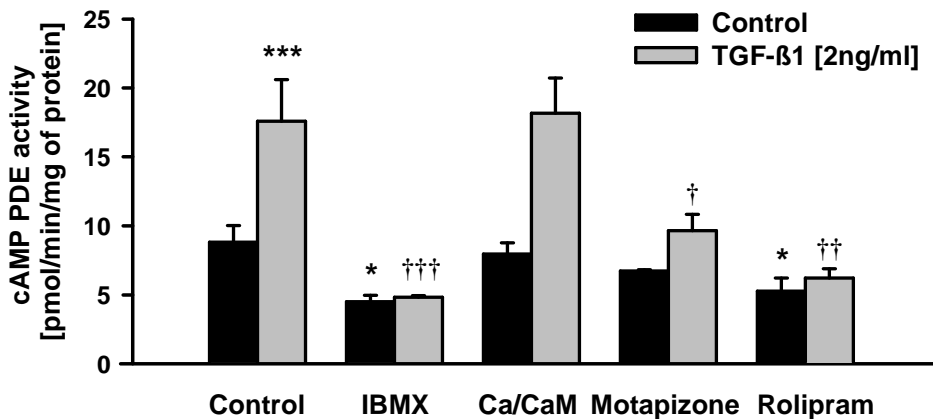
Figure 4.5.　　　**cAMP-PDE activities during TGF-β1 induced EMT.**
A549 cells were treated with TGF-β1 (2ng/ml) for 24 h. Using cAMP-PDE inhibitors [PDE1, addition of excess calcium in the presence of EGTA and 30 µM 8-MM-IBMX; PDE2, 10 µM EHNA in the presence of excess cGMP; PDE3, 10 µM milrinone; and PDE4, 10 µM rolipram]. Total PDE activity and is expressed as pmol cAMP hydrolysed per minute per mg of lysate protein. All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control

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ERK and PKA are known for activation PDE4 family by phosphorylation[25,29,32]. In order to check possible regulation of ERK and PKA by TGF-β1, immunoblotting analysis was performed (Figure 4.6). We couldn't detect either PKA nor P-PKA in control A549 cells, as well as in TGF-β1 treated cells. Results obtained from ERK immunoblotting demonstrated that after TGF-β1 stimulation ERK is strongly phosphorylated as compared to control, wheras no changes were observed in total ERK level (Figure 4.6.).
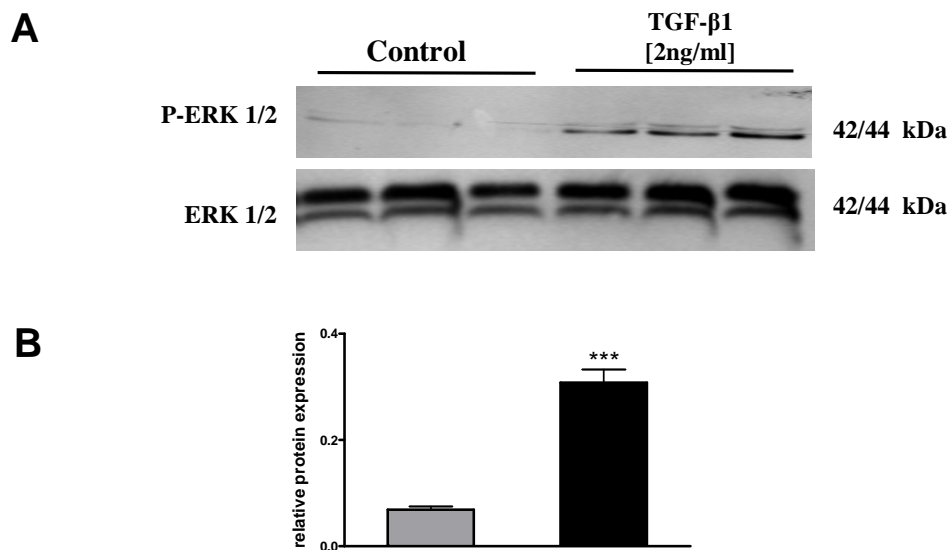
Figure 4.6.   Protein expression of ERK and P-ERK during TGF-β1 induced EMT.
Protein expression levels of total ERK and P-ERK from control and TGF-β1 (2ng/ml) treated A549 cells for 24h assessed by IB. Total ERK served as a protein loading control. **A.** Protein expression of ERK and P-ERK, **B.** Densitometric analysis of protein expression of P-ERK.
All values are given as the mean ± SEM (n=4) and are normalized to total ERK. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ versus control.

4.4. Expression of PDE4 isoforms in TGF-β1 induced EMT

To support our previous observations, protein expression and cell localization of PDE4 isoforms was analyzed.

Protein expression was detected by immunoblotting and subsequent densitometric quantification of PDE4 isoforms (PDE4A and PDE4D) in TGF-β1 (1ng/ml and 2ng/ml) stimulated and control cells for 24 h (Figure 4.7). Imunoblotting demonstrated upregulation of both PDE4Ax isoform and PDE4D isoforms compared to control.

A

B
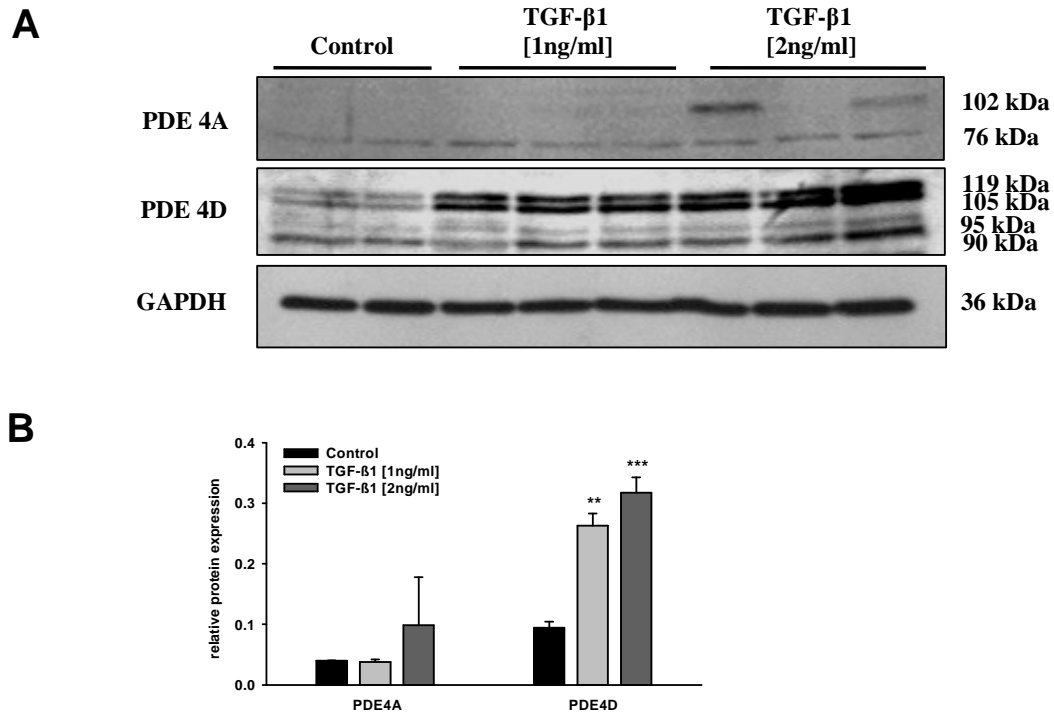
Figure 4.7.   Protein expression of PDE4 isoforms during TGF-β1 induced EMT.
Protein expression levels of control and TGF-β1 (1ng/ml and 2ng/ml) treated A549 cells for 24h assessed by IB. GAPDH served as a protein loading control. Gels are representative for three independent experiments. **A.** Protein expression of PDE4 isoforms, **B.** Densitometric analysis of protein expression of PDE4 isoforms.All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ versus control.

4.5. Localization of PDE4 isoforms in TGF-β1 induced EMT

Additionaly, localization of PDE4 isoforms (PDE4A and PDE4D) in TGF-β1 (1ng/ml and 2ng/ml) stimulated and control cells for 24 h, was analyzed by immunocytochemistry (Figure 4.8). Results displayed PDE4A and PDE4D-like immunoreactivity in the cytoplasm. PDE4A was sometimes found to be diffusely present in the membrane of the A549 cells.

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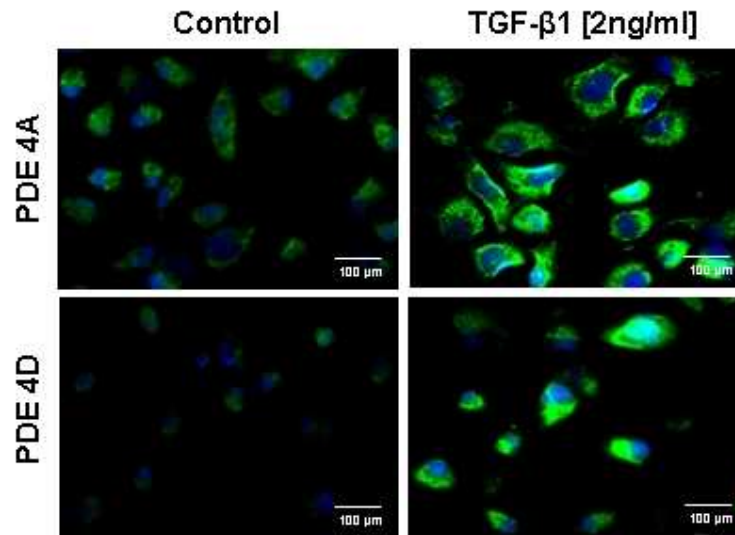
Figure 4.8. **Localization of PDE4A and PDE4D isoforms in TGF-β1 induced EMT**
PDE4A and PDE4D localization visualized with secondary FITC-labelled antibody (green). Cell nuclei were visualized with DAPI (blue). A549 cells were grown in serum-free medium or stimulated with TGF-β1 (2ng/ml) for 24 h.

4.6. Effect of PDE4 inhibition on TGF-β1 induced EMT

On the basis of the prominent increase in the mRNA and protein expression of PDE4A and PDE4D as well as PDE4 activity, during TGF-β1 induced EMT, we assessed the role of the PDE4 family by using the specific pharmacological inhibitor, Rolipram. A549 cells were pre-treated with the PDE4 inhibitor, Rolipram (100nM or 1μM) for 12 h followed by TGF-β1 (2ng/ml) stimulation for 24h or stimulated with TGF-β1 (2ng/ml) alone.

mRNA expression was analyzed by semi-quantitative PCR and subsequent densitometric quantification of epithelial and mesenchymal phenotype markers, (E-Cad, Cyt 18, α-SMA, Coll I and Fn-EDA) in the above treated cells.

Figure 4.9 shows changes in both epithelial and mesenchymal phenotype markers in TGF-β1 stimulated A549 cells after treatment with Rolipram. Rolipram (100nM and 1μM) significantly decreased the mRNA expression of mesenchymal markers Coll I and Fn-EDA in a concentration dependent manner (Figure 4.9 A, C). Incubation of cells with Rolipram (1μM) also helped to restore mRNA expression of the epithelial marker E-Cad, but not Cyt-18 (Figure 4.9. A, B).

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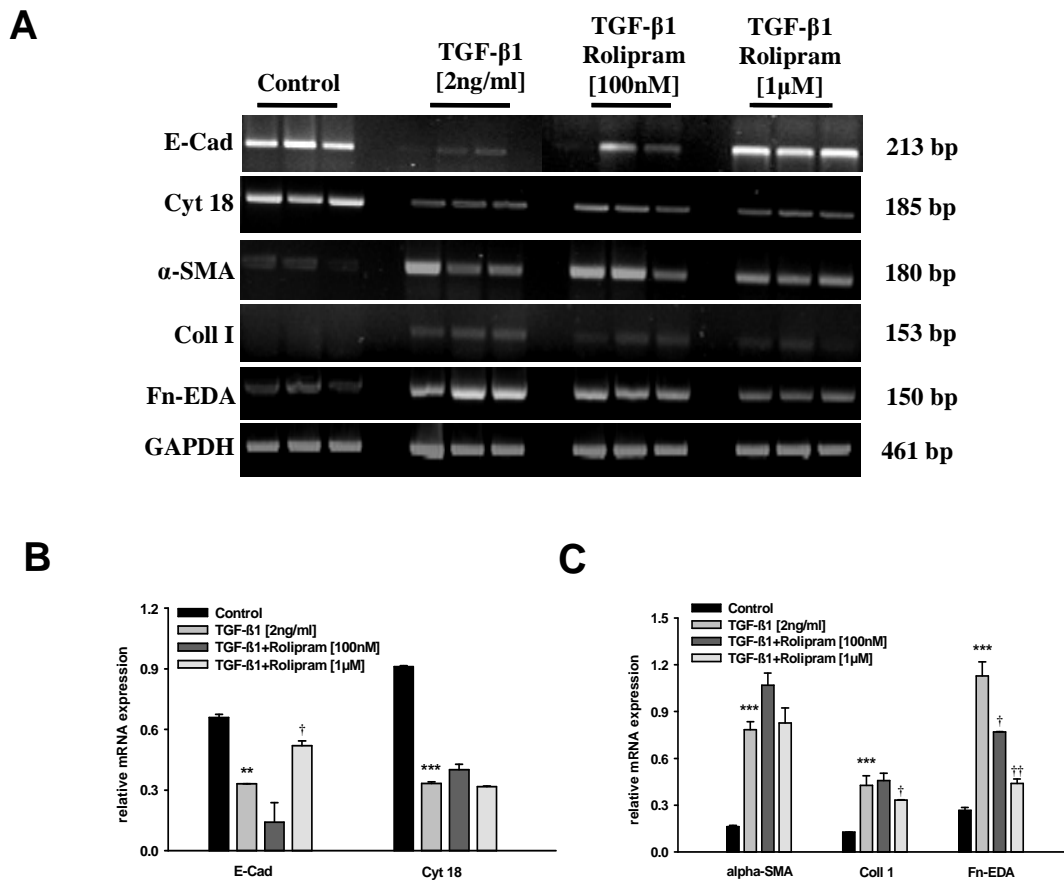
Figure 4.9. **Analysis of mRNA expression of epithelial and mesenchymal markers in PDE4 inhibited TGF-β1 induced EMT.**
Expression levels of mRNA from control, A549 cells pre-treated with Rolipram (1μM) for 12 h followed by TGF-β1 (2ng/ml) stimulation for 24h or A549 cells stimulated with TGF-β1 (2ng/ml) alone, were assessed by semi-quantitative PCR. GAPDH served as a control gene. **A.** mRNA expression of epithelial and mesenchymal markers**, B.** Densitometric analysis of mRNA expression of epithelial markers**, C.** Densitometric analysis of mRNA expression of mesenchymal markers.
All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ versus control; † $p<0.05$; †† $p<0.01$; ††† $p<0.001$ versus TGF-β1 stimulated cells

Similarly, immunoblotting suggested downregulation of E-Cad (1.5 fold) and upregulation of Fn-EDA (1.7 fold) with TGF-β1 stimulation. Treatment with Rolipram restored both E-Cad and Fn-EDA expression to nearly a normal level compared with TGF-β1 stimulated cells (Figure 4.10 A, B), thus demonstrating that PDE4 inhibition potentially abrogates TGF-β1 induced EMT.

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A

B
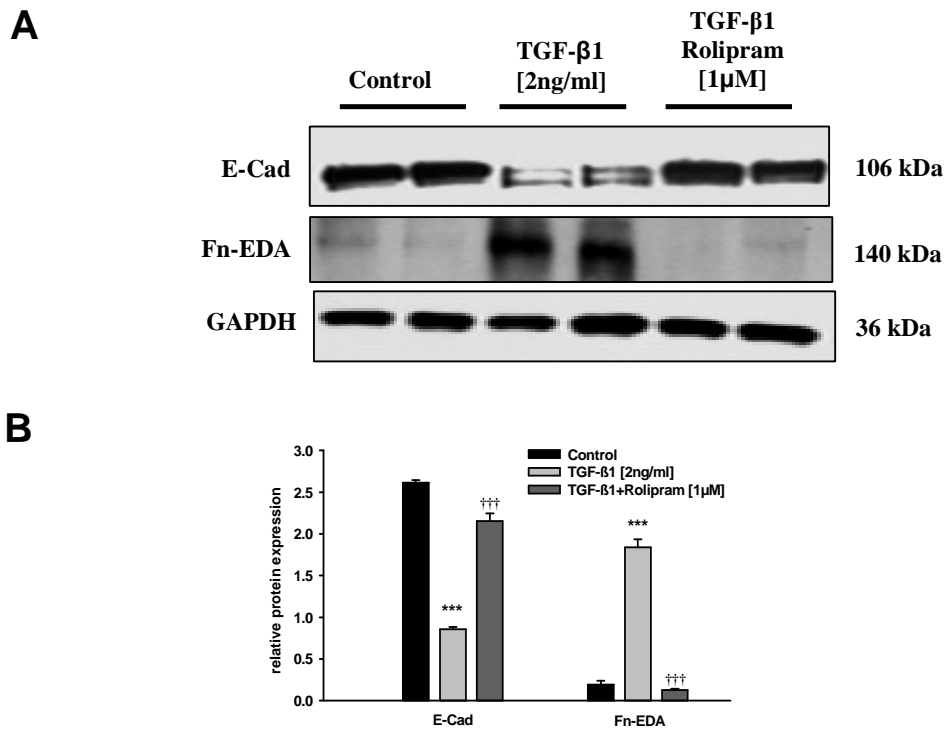
Figure 4.10. **Analysis of protein expression of epithelial and mesenchymal markers in PDE4 inhibited TGF-β1 induced EMT.**
Protein expression levels of mesenchymal markers from control, A549 cells pre-treated with Rolipram (1μM) for 12 h followed by TGF-β1 (2ng/ml) stimulation for 24h or A549 cells stimulated with TGF-β1 (2ng/ml) alone, were assessed IB. GAPDH served as a protein loading control. **A.** Protein expression of epithelial and mesenchymal markers, **B.** Densitometric analysis of mRNA expression of epithelial and mesenchymal markers.
All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ versus control; † $p<0.05$; †† $p<0.01$; ††† $p<0.001$ versus TGF-β1 stimulated cells

In order to localize E-Cad, cytokeratin, α-SMA and fibronectin in the cells, we performed immunocytochemistry analysis (Figure 4.11). Cells were treated as above. Results revealed that membrane localized E-Cad, completely disappeared after TGF- β1 stimulation, wheras pre-treatment with Rolipram partly restored its expression. Level of cytosolic fibronectin was strongly increased after TGF- β1 stimulation, whereas pre-treatment with Rolipram abolished its expression. Rolipram pre-treatment had no significant effect on cytokeratin and α-SMA expression and localization.

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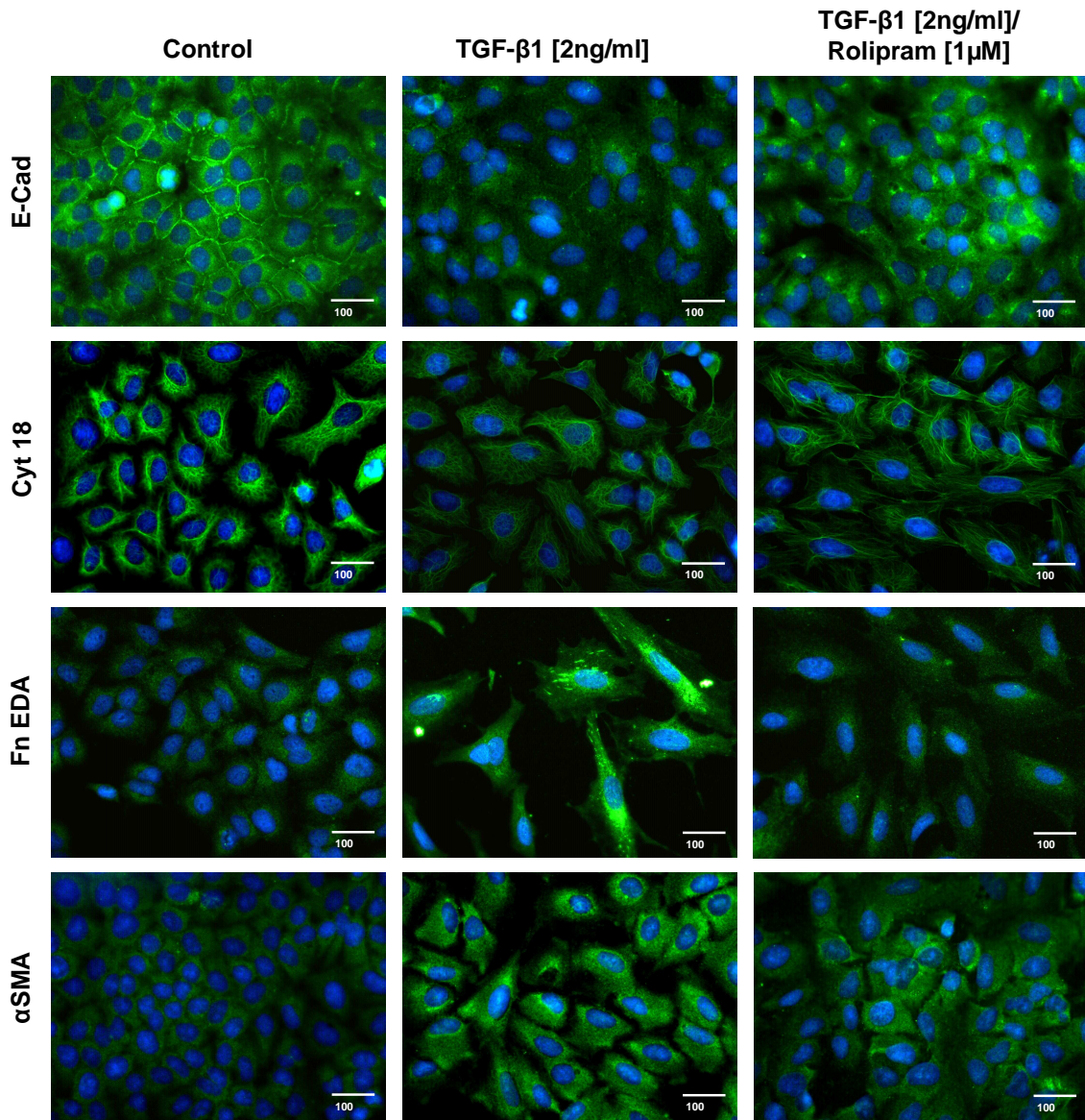
Figure 4.11. **Localization of epithelial and mesenchymal markers in PDE4 inhibited TGF-β1 induced EMT.**

E-Cad, Cytokeratin, Fibronectin and α-SMA localization visualized with secondary FITC-labelled antibody (green). Cell nuclei were visualized with DAPI (blue). A549 cells were grown in serum-free medium alone, or pre-treated with Rolipram (1μM) for 12 h followed by TGF-β1 (2ng/ml) stimulation for 24h or stimulated with TGF-β1 (2ng/ml) alone.

4.7. Effect of PDE4 inhibition on Smad signaling

TGF-β1 can regulate its target genes via Smad-dependent pathways that include recruitment TGF-β1 receptors and phosphorylation of Smad 2 and Smad 3.

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To investigate whether Rolipram is influencing Smad signaling pathway, A549 cells were pre-treated with the PDE4 inhibitor, Rolipram (100nM or 1μM) for 12 h followed by TGF-β1 (2ng/ml) stimulation for 24h or stimulated with TGF-β1 (2ng/ml) alone.

The effects of Rolipram on the activation of the Smad signaling pathway in A549 cells by TGF-β1 were studied by immunoblotting with Smad 4, phospho-Smad 2, phospho-Smad 3, and TGF-β1 type II receptor (TGF-β1 RII) antibodies. Phosphorylation of Smad 2 and Smad 3 protein increased in A549 cells upon TGF-β1 stimulation for 24 h; however, these increase was not blocked by treatment with Rolipram (Figure 4.12 A, B). Likewise, Smad 4 and TGF-β1 RII protein levels increased after TGF-β1 stimulation, and this increase was not blocked by Rolipram, indicating that PDE4 inhibitor did not inhibit TGF-β1 induced EMT through Smad-dependent pathway..
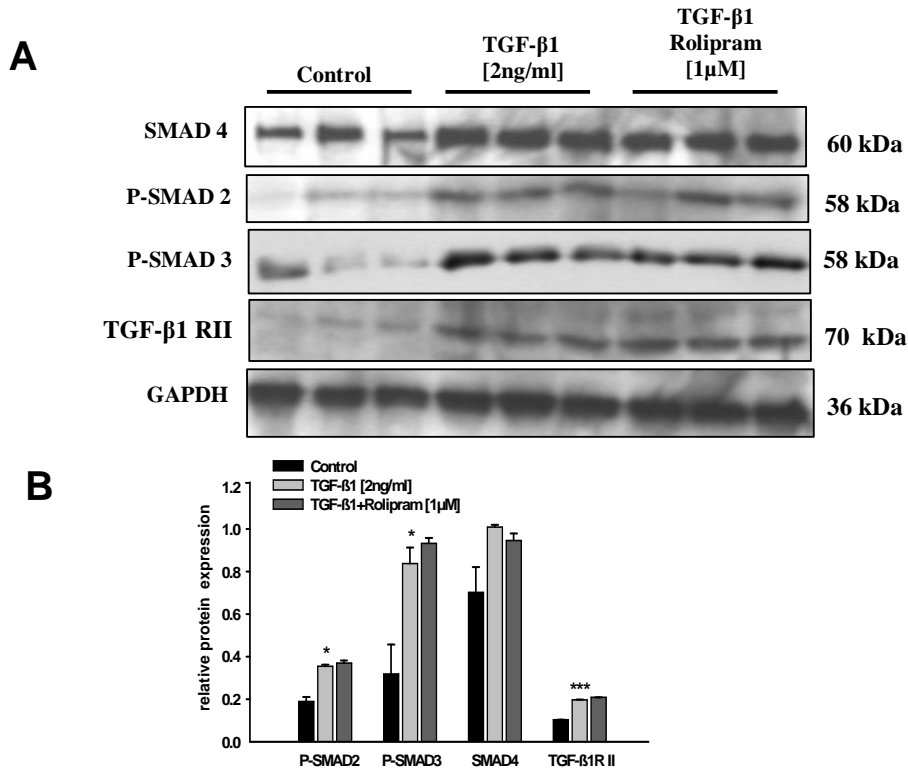
Figure 4.12.   Effect of PDE4 inhibition on TGF-β1 induced Smad signaling.
Protein expression levels of P-Smad 2, P-Smad 3, Smad 4 and TRβ II, from control, A549 cells pre-treated with Rolipram (1μM) for 12 h followed by TGF-β1 (2ng/ml) stimulation for 24h or A549 cells stimulated with TGF-β1 (2ng/ml) alone, were assessed by IB. GAPDH served as a protein loading control. **A.** Protein expression of Smad proteins and TRII**, B.** Densitometric analysis of protein expression of Smad proteins and TRII
All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * p<0.05; ** p<0.01; *** p<0.001 versus control.

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4.8. Effect of PDE4 inhibition on TGF-β1 induced ROS production

Since TGF-β1 was already shown to be able to induce ROS generation[54] and PDE4 involvement in this process was also suggested[55], we analyzed the effect of Rolipram in TGF-β1 induced EMT on ROS production.

To assess the role of ROS, DHE immunofluorescence was performed on cells treated with H_2O_2 (1mM and 10mM), TGF-β1 and/or Rolipram for 24 h. As shown in Figure 4.13, H_2O_2 used as a positive control increased ROS production in a concentration-dependent manner compared to control. Interestingly, TGF-β1 treatment induced ROS production, whereas co-treatment with Rolipram blocked the TGF-β1 mediated increase in ROS production.
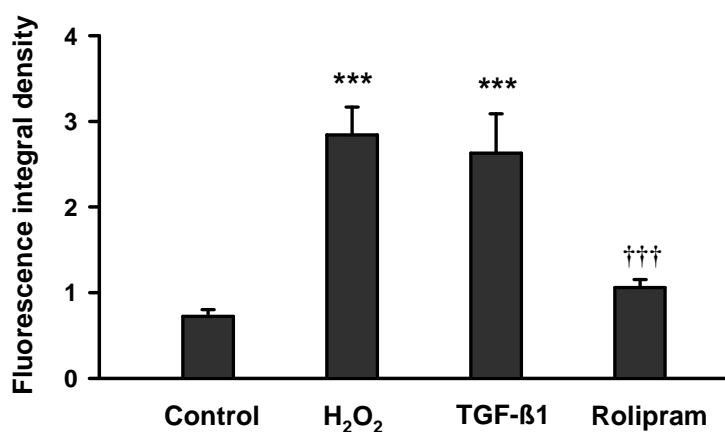
Figure 4.13.   Effect of PDE4 inhibition on ROS production.
Reactive oxygen species (ROS) production was measured by dihydroethidium (DHE) fluorescence in 10 randomly selected images from each group. A549 cells were treated with TGF-β1 (2ng/ml) for 24h, Rolipram (100nM and 1μM) pre-treatment for 12h and then TGF-β1 (2ng/ml) for 24h or H_2O_2 (1mM or 10mM) for 12 h as a positive control.
All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * p<0.05; ** p<0.01; *** p<0.001 versus control, † p<0.05; †† p<0.01; ††† p<0.001 versus TGF-β1 stimulated cells.

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4.9. Effect of ROCK inhibition

Since TGF-β1 stimulation increased the expression of ROCK1 in A549 cells, we assessed the involvement of Rho/ROCK signaling by using a specific pharmacological inhibitor of ROCK, Y27632. A549 cells were pre-treated with the Y27632 (100nM) for 12 h followed by TGF-β1 (2ng/ml) stimulation for 24h or stimulated with TGF-β1 (2ng/ml) alone.

Treatment with Y27632 partially abrogated TGF-β1 induced EMT and restored the expression of E-Cad. Most importantly, PDE4D protein level increased after TGF-β1 stimulation was decreased by Y27632 treatment (10μM) (Figure 4.14).
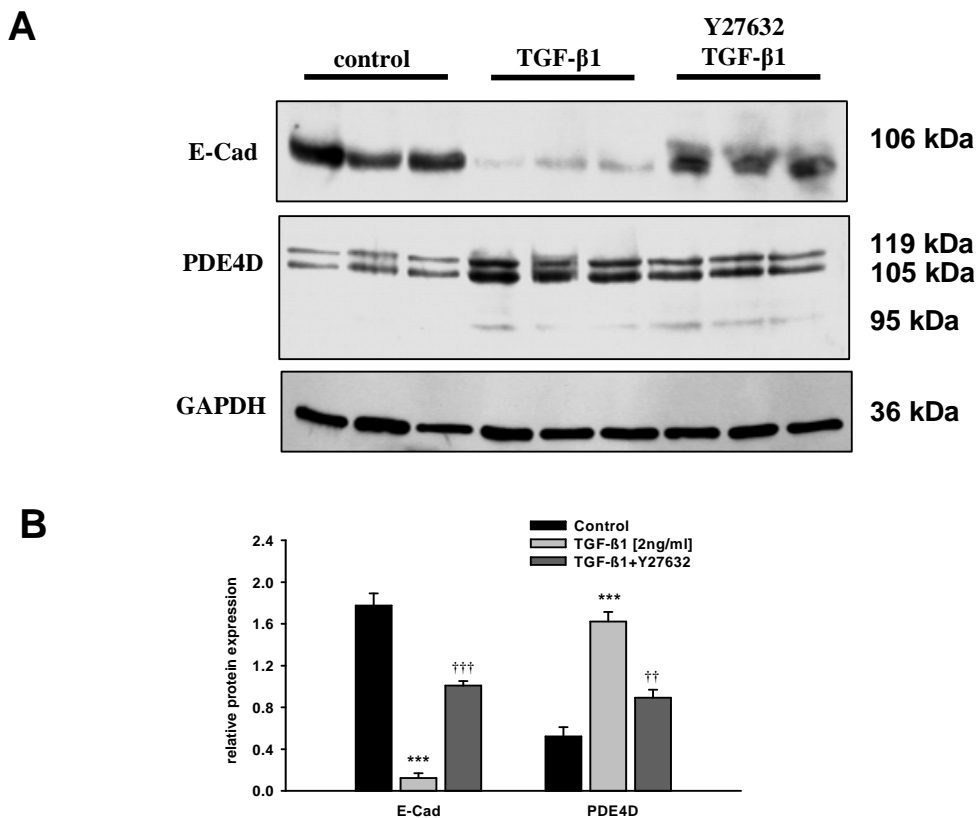
Figure 4.14.   Effect of ROCK inhibition on TGF-β1 induced EMT.
Protein expression levels of E-Cad and PDE4D from control, A549 cells pre-treated with Rolipram (1μM) for 12 h followed by TGF-β1 (2ng/ml) stimulation for 24h or A549 cells stimulated with TGF-β1 (2ng/ml) alone, were assessed by IB. GAPDH served as a protein loading control. **A.** Protein expression of E-Cad and PDE4D, **B.** Densitometric analysis of E-Cad and PDE4D.
All values are given as the mean ± SEM (n=4) and are normalized to GAPDH. * p<0.05; ** p<0.01; *** p<0.001 versus control; † p<0.05; †† p<0.01; ††† p<0.001 versus TGF-β1 stimulated cells.

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5. Discussion

5.1. TGF-β1 induced EMT in A549 cells

The epithelial mesenchymal transition has emerged as a critical event not only in development, but also in wound healing, fibrosis, and the invasion and metastasis of tumor cells[2,5,56]. Moreover, it has been recently established that EMT is critically involved in organ fibrosis, including the lung. It is increasingly being recognized that, following epithelial injury, epithelial cells can give rise to fibroblasts and thereby contribute to the pathogenesis of pulmonary fibrosis by undergoing EMT[57,58]. Similarly, EMT involvement in lung cancer has been recently reported to promote the migratory and invasive abilities of lung cancer cells, attributes essential for tumor metastasis[59].

Although a growing number of studies have shown the occurrence of EMT *in vivo* and *in vitro*, very little is known about mediators and molecular mechanisms involved in EMT. Better understanding and explanation of underlying mechanism and regulation of this process is being currently growing area of study.

TGF-β is considered as an important regulator of cell proliferation, differentiation, apoptosis, immune response, and extracellular matrix remodeling, depending on physiological context. Moreover, TGF-β1 has been shown as a major inducer of EMT in many organs such as lung[1,60-62], kidney[63], skin[64] and liver[24].

A549 cells retain important characteristics of alveolar type II epithelial cells and are among the best-characterized alveolar epithelial cells. They have been used in a number of studies to investigate promoter activity, apoptosis and alveolar epithelial cell DNA damage as well as various aspects of EMT[8,65,66]. The ability of TGF-β1 to promote EMT in A549 cells was reported in several studies[8,67,68].

In the present study we could observe that exposure of A549 cells to TGF-β1 for 24 h induced a complete conversion of the epithelial cells to myofibroblasts, as evidenced by the acquisition of a spindle-like morphology and loosing typical epithelial morphology – layers formation, cell to cell contact and cuboic shape (Figure 4.1). This is in line with the observed elevated expression of mesenchymal marker genes (fibronectin, desmin and

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α-smooth muscle actin) and at the same time loss of epithelial marker genes (E-cadherin, zona occludins-1, cytokeratin 18) (Figures 4.2 and 4.3).

We investigated the effect of two different concentrations of TGF-β1 - 1ng/ml and 2ng/ml, since previous studies show diversity in TGF-β1 concentrations used to induce EMT. We could observe that already as low concentration as 1ng/ml is sufficient to induce A549 to undergo EMT.

The time frame required for this transition was 24 h. Changes in epithelial and mesenchymal phenotypic markers on mRNA level as well as on protein level, illustrated that 24 h is sufficient time for TGF-β1 to induce EMT in A549 cells and our results were similar to previous reports[8].

Data from current study indicates that A549 cells undergo EMT and that TGF-β1 is the inducer of this process. This findings are in line with numerous studies[8,69,70].

5.2. PDEs in TGF-β1 induced EMT in A549 cells

Cyclic nucleotide phosphodiesterases through hydrolyzing cAMP and/or cGMP propagate many signaling pathways, including proliferation, migration, and differentiation[27,37,71,72].

Furthermore, epithelial and mesenchymal phenotype marker genes that are altered during EMT by TGF- β1 can be influenced by increased cAMP or cGMP levels. cAMP acts directly via reducing activation of collagen gene transcription, but also the expression of α-smooth muscle actin[73]. It was also demonstrated that cAMP is able to abolish the TGF-β1-induced reorganization of the actin cytoskeleton and inhibits the disruption of the E-Cadherin cell to cell interactions[74]. Stimulator of cGMP synthesis, nitric oxygen (NO), is able to conserve epithelial morphology, decrease collagen I expression, and keep the epithelial markers E-Cadherin and pro SP-B in the face of TGF-β1 exposure[75].

Concerning all of this reports, investigations of possible role of PDEs in EMT seems very promising.

Additionaly, PDEs have been already shown to be involved in several diseases, including lung.

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In lung cancer PDEs have been shown to be involved in apoptosis. PDE1 and PDE5 isoforms were found to be highly expressed in orthotopic tumors and their inhibition resulted in decreased proliferation and increased apoptosis within the tumor[76].

Concerning pulmonary fibrosis, it has been demonstrated that PDE5 is highly upregulated in transition of fibroblast to myofibroblast and that PDE4 inhibition can block the process of differentiation[77]. In cystic fibrosis, PDE4 is involved in signaling in apical membrane patches during cell-attached recordings in airway epithelium[78].

Also current findings in pulmonary arterial hypertension (PAH) demonstrate that PDEs play an important role in smooth muscle contraction and relaxation. PDE3 was shown to influence pulmonary vasodilatation in intact rabbits with elevated pulmonary artery pressure, PDE1 and PDE5 are strongly upregulated in the pulmonary artery media of human lungs with severe pulmonary hypertension, and PDE4 inhibition reversed pulmonary hypertension and right heart hypertrophy in rats [79-82].

5.2.1. PDE expression in TGF-β1 induced EMT

As PDEs proofed to be an important target on the single cell level as well as in several lung diseases, we found interesting to examine expression profile of those enzymes during TGF-β1 induced EMT in A549 cells.

To date, present study is the first one investigating the involvement of PDEs in TGF-β1 induced EMT process.

We investigated an effect of two concentrations of TGF-β1 on A549 cells. Our results indicated that TGF-β1 brought changes in PDE expression. Among 11 PDE families, TGF-β1 effects mostly PDE1A, PDE3A, PDE4A, PDE4D, PDE7A and PDE10A family, causing increased expression on mRNA level of PDE4A, PDE4D, PDE7A and PDE10A isoforms, wheras decreased expression of PDE1A and PDE3A isoforms. To our observations, PDE4D show the most prominent increase in mRNA (Figure 4.3).

There are no reports describing any correlation between PDE4 and EMT, but it was demonstrated that elevated levels of cAMP had influence on EMT[83,84]. Since PDE4 is cAMP specific it suggests that cAMP-PDEs may be involve in this process as well.

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5.2.2.PDE activation in TGF-β1 induced EMT

Above findings suggested that TGF-β1 is able to regulate PDEs expression. To further investigate the influence of TGF-β1 treatment on PDEs and in particular on their functional properties , activity of those enzymes was examined.

We found the most striking 2 fold increase in total cAMP-PDE activity after TGF-β1 stimulation. We observed that PDE4 family contributed the most to the total activity. Additionaly, PDE4 activity increased more then 2 fold (Fig. 4.5). These results were in line with mRNA expression, where we could observe the strongest increase in PDE4 family (PDE4A and PDE4D) and PDE3 family (PDE3A). Both of them are cAMP specific.

There are no reports so far exploring direct effect of TGF-β1 on PDE4 activity, but previous studies demonstrated that PDE4 activation and induction, occurs as an effect of regulation of phosphorylation/dephosphorylation of PKA and/or ERK. What is also interesting, there are reports showing that TGF-β1 can regulate ERK and PKA activation.

PKA phosphorylates serine residue in UCR1 at the N-terminal end of PDE4, and though activates long PDE4 isoforms[45,46]. It is believed that PKA phosphorylation in UCR1 causes disruption of interaction between UCR1 and UCR2 through conformational changes[47].

On the other hand, ERK regulates PDE4 activity via phosphorylation, causing inhibition as well as activation of PDE4. All PDE4 subfamilies, except PDE4A, contain in a catalytic domain a single ERK consensus motif (Pro-Xaa-Ser-Pro). It is already known *in vitro* and *in vivo* that serine from this motif can be phosphorylated by ERK[49,50].

Moreover, recent studies indicated that TGF-β1 can regulate ERK and PKA activation. It was demonstrated that in fetal lung fibroblasts, TGF-β is able to activate PKA. Moreover, TGF-β1 stimulates cell proliferation by activating the MEK-ERK pathway[85]. Elevated ERK activity can enhance Smad activity, and ERK inhibition reduces TGF-β1 stimulated Smad phosphorylation as well as collagen production and promoter activities. Additionally, cells of mesenchymal origin appear to show synergy in ERK-Smad interaction whereas epithelial cells generally appear to show inhibition[86]. But experiments on T-cells revealed that TGF-β1 can also decrease phosphorylation of ERK and thereby its activation[87].

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We investigated expression level of total PKA and its phosphorylated status in A549 cells after TGF-β1 treatment. Unfortunately, we were not able to detect either PKA nor phosphorylated form of PKA.

We also investigated total ERK and its phosphorylated status in A549 cells after TGF-β1 treatment. Our results indicated that upon TGF-β1 stimulation ERK is phosphorylated and though probably activated (Fig. 4.6). Thus, our observation that TGF-β1 treatment caused double increase in PDE activity, with the main contribution of PDE4, may be explained that TGF-β1 is able to regulate activation of PDE, and in particular PDE4, during EMT through ERK signaling pathway.

5.2.3. PDE4 family in TGF-β1 induced EMT in A549 cells

5.2.3.1. PDE4 expression

As observed above, after TGF-β1 treatment PDE4 family is the one with the most prominent changes concerning expression on mRNA level as well as activation. Since PDE activity assay is unable to distinguish which PDE4 isoforms are the main contributors to total PDE4 activity, we checked the protein expression of PDE4 family. Our results were in line with mRNA expression, and we observed increase in expression of PDE4A and PDE4D isoforms - PDE4A expression was slightly increased, wheras PDE4D expression level was more then 2 fold increased (Fig. 4.7).

5.2.3.2. PDE4 localization

PDE4 is specific for cAMP and it has been reported that cAMP is compartmentalized in the cell. cAMP is generated at the cytosol surface of the plasma membrane through the action of membrane bound adenylate cyclases. Increased levels of cAMP are translated into cellular response through the action of PKA[44]. cAMP forms a gradients in the cell and the basis of such gradients will depend upon the activity of both - AC and PDEs[32,44]. It is now well recognized that AC are not evenly distributed across the cellular membrane. Since PDE4 control and regulate cAMP, localization of those

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enzymes may differ according to the gradients of cAMP. PDE4 activity was found in cytosol, membrane, nucleus and cytoskeletal locations[32,44,88].

Moreover, distribution of PDE4 in the cell is changing according to targeting molecule PDE4 is reacting with. It has been shown that PDE4 form a complex with β-arrestins, thereby providing a means of delivering PDE4 to the active site of cAMP synthesis in the plasma membrane[44]. Findings in cardiac myocytes concerning PDE4 interactions with AKAP, indicate that hypertrophic conditions causes the dynamic redistribution of PDE4 from the cytosol into perinuclear compartment[44,71]. Other study show that PDE4 can interact with protein-tyrosine kinases (such as Src, Lyn, Fyn) and role of this interaction is intracellular targeting. Thus membrane associated PDE4A5 is found at the cell periphery where it is localized to ruffles and at a discreete perinuclear localization. But when interaction with kinases can not take place, PDE4A5 is distributed uniformly through the cell margin[44].

Concerning all of this reports, we performed an experiment to localize PDE4A and PDE4D in the cell before and after TGF-β1 treatment. Results displayed that PDE4A and PDE4D immunoreactivity were concentrated in the cytoplasm. Additionally, PDE4A was sometimes found to be diffusely present in the membrane of the A549 cells (Fig. 4.8). These results are in agreement with recently published data indicating that PDE4A isoform is mostly found on the cellular membrane, wheras PDE4D is found mostly in cytosol[32,44,88].

Cytosolic and membrane localization of PDE4A and PDE4D may suggest that TGF-β1 can directly regulate these PDEs via TGF-β1 receptors which are membrane-bound. Other possible explanation is that PDE4 is regulated by TGF-β1 indirectly via TGF-β1 signaling components such as Smad proteins which are localized in cytosol.

5.2.4. PDE4 inhibition in TGF-β1 induced EMT

Our findings indicated that mRNA, protein and activation level of PDE4 family is strongly elevated by TGF-β1. To asses a potential effect of PDE4 inhibition on TGF-β1 induced EMT, we investigated treatment with PDE4 selective inhibitor Rolipram.

Rolipram is a specific PDE4 inhibitor which proved to be significantly more potent in inhibiting PDE4D than the other subtypes, in particular PDE4C[89]. Rolipram was

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first used in mice and rats, and results from this investigation indicated that Rolipram show antidepressant activity[90,91]. Rolipram was also proofed to have anti-inflammatory activity. It has been shown to be a potent inhibitor of leukotriene and PGE_2 production[92]. Although so promising at the beginning, Rolipram demonstrated to have too many side effects *in vivo* and currently is being used *in vitro* mostly.

PDE4 inhibitors are known for suppressive activity on various *in vitro* and *in vivo* responses. *In vitro* they suppress production of cytokines, cell proliferation and chemotaxis, release of inflammatory mediators and NADPH activity. *In vivo,* attenuation of PDE has been shown to influence pathological processes by means of specific inhibitors which have demonstrated pre-clinical and clinical efficacy in the treatment of different lung diseases. PDE4 inhibitors are currently being developed in COPD, inflammatory diseases such as asthma, lung cancer, fibrosis and PAH[33,82,93,94].

In COPD, arthritis and asthma PDE4 inhibitors suppress multiple neutrophil responses, such as production of IL-8, leukotriene, and superoxide anions, degranulation, chemotaxis and adhesion. PDE4 inhibition reduces the transcriptional activity of the predominant genes involved in mucin secretion during inflammation[95].

In mice experimental model of autoimmune diabetes, PDE4 inhibition reduced severity of insulitis and prevented diabetes[96]

In allergic diseases PDE4 inhibitors suppress eosinophil activity such as superoxide production, adhesion and infiltration into airways. In addition T cell proliferation and production of TNF-α, IL-2, IL-5, IL-4 and PGE_2 are supported by PDE4 inhibition [95].

PDE4 and its inhibition also seems to play o role during cystic fibrosis. Two independent reports demonstrated that PDE4D is involved in controling the function of transmembrane conductance regulator in pulmonary epithelial cells[95].

PDE4 inhibition is also proposed to play a role in leukemias through its antitumor and anti-angiogenic effects. PDE4 inhibitors shown to have antiproliferative activity on T cells and B cells as well as on murine carcinoma cells[95].

Recently it was also shown that PDE3/4 inhibition can partially reverse MCT-induced PAH in rats[93].

Moreover, PDE4 knock-out experiments demonstrated that PDE4D$^{-/-}$ mice exhibit impaired airway contraction induced by cholinergic stimulation and abolished

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airway hyperreactivity what is caused by exposure to allergen. This indicates a role of PDE4D in cholinergic airway responsiveness and in development of hyperreactivity[34]

Silent finding of this study was that PDE4 inhibitor Rolipram exhibited a remarkable inhibitory effect on TGF-β1 induced EMT. Interestingly, these effects were observed with a PDE4 inhibitor at very low concentrations (100 to 1000 nM). This inhibition was partial, as evidenced by a restoration of epithelial morphology and E-cadherin expression and a complete abolishment of fibronectin stimulation (Fig. 4.9 and 4.10).

We observed that on mRNA as well as on protein level, E-Cad expression was restored like in the control cells. Additionally, we were able to localize E-Cad in control A549 cells in cytosol but mostly in cell membrane. After TGF- β1 stimulation E-Cad disappeared from cell membrane and was very slightly localized only in cytoplasm, wheras when Rolipram pre-treated we could observe that E-Cad expression level in cytoplasm dramatically increased. We could not detect membrane localization of E-Cad with Rolipram pre-treatment but tendency of gathering the cells in cluster-like origins was observed. Cytokeratin mRNA level was only slightly influenced by Rolipram. Cytokeratin fibers were very clearly visible in control A549 cells, wheras its disappearance was noticed after TGF- β1 treatment. Rolipram pre-treatment had no effect on TGF- β1 caused changes (Fig. 11). Our data demonstrating that E-cadherin expression is preserved in TGF-β1-exposed A549 cells, may have important implications in the prevention of EMT. E-Cad restoration may have serious implication during EMT – cell to cell contact via tight junction may be restored as well. As a result, migratory properties may be abolished. Therefore, inhibition of the TGF- β 1 induced downregulation of E-cadherin by Rolipram may be a critical first step in the prevention of EMT.

Concerning mesenchymal markers we could observe that Rolipram pre-treatment abolished fibronectin increase caused by TGF-β1 on mRNA as well as on protein level. Moreover, we were able to localize fibronectin in cytosol after TGF-β1 treatment, wheras no expression was observed in control cells. In the cells pre-treated with Rolipram fibronectin expression in cytosol was also not detectable anymore. Rolipram had no effect on α-SMA on mRNA level. When we localize α-SMA in the cell, it was expressed in cytoplasm after TGF-β1 treatment. No changes compare to TGF-β1 stimulation were observed with Rolipram pre-treatment. Fibronectin abolishment by Rolipram may be

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important for blocking EMT process since fibronectin plays crucial regulatory roles in migration and in interactions with extracellular matrix components (Fig. 4.11).

This is the first study showing direct effect of PDE4 inhibition on EMT. However, in general agreement with this results, similar observation were demonstrated in kidney epithelial cells where cAMP elevating agents, 8-Br-cAMP and forskolin exhibit an inhibitory effect on EMT[83]. Also cAMP directly acts reducing activation of collagen gene transcription, but also the expression of α-smooth muscle actin[73]. It was also demonstrated that cAMP is able to abolish the TGF- β1-induced reorganization of the actin cytoskeleton and also inhibits the disruption of the E-Cadherin cell to cell interactions[74].

5.2.5. PDE4 inhibition and Smad signaling

TGF-β1 network involves receptor serine/threonine kinases at the cell surface and their substrates, the Smad proteins. Each ligand of the TGF-β family binds to specific pairs of receptor serine/threonine kinases, belonging to groups known as the type I and type II receptors, respectively. Most mammalian cells express different members of this receptor family, some of which may be shared by different TGF-β ligands.When the ligand binds, it acts as a dimeric assembly factor, bringing together two type I and two type II receptors. In this complex, receptor II exerts its only known function, which is to activate receptor I by phosphorylation. The type I receptor then phosphorylates Smad proteins that propagate the signal. Once phosphorylated by the activated TGF-β1 receptor, Smad2 and/or Smad3 complex with Smad4 and move into the nucleus, where they activate target gene transcription in association with DNA-binding partners. One mechanism for switching off the TGF-β signal involves Smad ubiquitylation in the nucleus, followed by proteasome-mediated degradation of the Smad protein. A separate ubiquitylation mechanism controls the basal level of Smad through the ubiquitin ligase Smad ubiquitylation regulatory factor (SMURF1). A more common mechanism for returning a phosphorylated mediator to its basal state, protein dephosphorylation, has not been described for the deactivation of Smads[16,97].

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As mentioned already above, it was shown that TGF-β1 can activate ERK via interaction of Smad pathway on ERK directly[86]. ERK phosphorylates and thus activates PDE4, but there are no reports demonstrating correlation between PDE4 and its inhibition, and Smad pathway.

It was also reported that TGF-β1 induced fibroblast to myofibroblast differention was blocked by PGE$_2$ in Smad independent manner. Stimulation with PGE$_2$ limited collagen I and α-SMA expression, whereas had no effect on translocation of Smad 2/Smad 3 complex to the nucleus. Therefore it suggest that cAMP plays a role in TGF-β1 non-Smad signaling pathways. Moreover; PGE$_2$ was reported to block any of the well known signal transduction events that occur as a result of TGF-β1 stimulation in fibroblasts[98,99].

What was also interesting, is the report that cells of mesenchymal origin appear to show synergy in ERK-Smad interaction whereas epithelial cells generally appear to show inhibition[86].

In our study we show that TGF-β1 caused increase in PDE4 expression and activity. Moreover, PDE4 inhibition during TGF-β1 induced EMT can partially reverse this process. But since direct correlation between PDE4 pathway and Smad signaling were not demonstrated so far, we investigated Smad expression during TGF-β1 induced EMT as well as during PDE4 inhibited TGF-β1 induced EMT. We found that TGF-β1 induces Smad 2 and Smad 3 phosphorylation in A549 cells, as has been shown by several other groups[16,17,85]. Interestingly, these phosphorylation events are not altered by PDE4 inhibition, suggesting that PDE4 inhibitors may use alternative Smad-independent pathways to regulate EMT marker genes (Fig. 4.12).

These observation are in agreement with the data demonstrated that in fibroblasts cAMP elevation through PGE$_2$ does not block translocation of Smad 2/Smad 3 complex to the nucleus. Moreover, elevation of cAMP levels by PGE$_2$ was accompanied by changes in cell shape, cell adhesion to the matrix and cytoskeletal organization and it occur also in Smad independent way[100-102].

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5.3. PDE4 family and ROS generation

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radical are generated as byproducts of the metabolism of oxygen. There is a growing evidence that ROS play important roles as a second messenger in signal transduction in processes including wound repair, host defense, blood vessel relaxation, and neurotransmission[103].

Increased generation of ROS can occur when inflammatory cells such as neutrophils, eosinophils and macrophages are recruited to the alveolar spaces, either in response to toxins or antigens or as a consequence of infection. Data obtained from cell culture studies indicates that after exposure to prooxidants, lung epithelial and endothelial cells are able to generate ROS. Various experimental models strongly support the hypothesis that oxidative stress generated by these agents and inflammatory cells can lead to the development of many acute and chronic airway diseases, including fibrosis, asthma, emphysema, acute respiratory distress syndrome (ARDS), and bronchial carcinogenesis[104].

Considering the established role of both, PDE4 and TGF-β1, in ROS generation, we postulated ROS signaling to be a candidate mediator of the PDE4 inhibitory effects on EMT.

PDE4 inhibition triggered a cAMP-independent inhibition of ROS[55]. It was also reported that elevation of cAMP produces the inhibition of different inflammatory processes, such as cellular trafficking, cytokine release or reactive oxygen species production[105]. What is also interesting, PDE4 inhibition has been linked already with inhibition of ROS generation. Data obtain from experiments performed in macrophages and A549 cells, suggest that ROS-mediated lung inflammation could be mediated at least in part by elevated PDE activity associated with decreased cAMP in those cells[106].

Also TGF-β1 influence on ROS production was already reported. ROS have been shown to mediate TGF-β1 induced cellular responses in various cells[54,107-109]. Moreover, it has been demonstrated that antioxidants effectively reversed TGF-β1 induced Smad 2 phosphorylation as well as changes in E-Cad and α-SMA expression[54].

Consistent with our hypothesis, we found that TGF-β1 induced a threefold increase in DCF-sensitive cellular ROS, what confirmed previous reports. But to our

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surprise, 1μM of PDE4 inhibitor Rolipram completely blocked TGF-β1 induced ROS production, thus establishing ROS as a target of PDE4 during EMT (Fig. 4.13). Our data strongly suggest that PDE4 inhibition has ability to reverse of EMT. Though PDE4 inhibition was not directly correlated with TGF-β1 Smad dependent pathway, it seems that it is triggered via ROS.

This results are similar to finding that inhibiting ROS production by antioxidants prevented changes in E-Cad and α-SMA[54].

5.4. PDE4 family and Rho kinases

Members of the Rho family of small GTPases have emerged as key regulators of the actin cytoskeleton, and furthermore, through their interaction with multiple target proteins, they ensure coordinated control of cellular activities such as gene transcription and adhesion[110]

Proper epithelial cell-to-cell require cadherin-based adherens junctions, which found to be associated with the actin cytoskeleton, and a recent study demonstrated that Rho is required for their assembly in keratinocytes[110]. It was in agreement with report where downregulation of Rho activity caused in fibroblasts nonmigratory and epithelioid phenotype. Restoration of Rho activity resulted in a migratory behavior and fibroblastoid phenotype[111]

But what is interesting to our project, recent studies have implicated the small GTPase Rho and its downstream effector Rho kinase (ROK) in TGF-β1 induced remodeling of cell contacts in mammary epithelial cells, and as necessary components for the acquisition of stress fibers and a fibroblastic morphology in NMuMG and primary mouse keratinocytes[20].

In this study, we examined the possibility that PDE4 may influence EMT via Rho signaling. To our surprise, we found that TGF-β1 induced E-cadherin expression is potently suppressed by the Rho kinase inhibitor Y-27632. Interestingly, Y-27632 also decreased PDE4D expression, and subsequently, PDE4 activity (Fig. 4.14). Hence, to our knowledge, this is the first report of an alternative signaling pathway for TGF-β1 via RhoA activation in the positive regulation of EMT and negative regulation of PDE4 in alveolar epithelial cells.

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5.5. Conclusions and future directions

Phosphodiesterases are known propagators in many signaling pathways, including proliferation, migration, and differentiation[32,112] thanks to their ability to hydrolyze cAMP and/or cGMP. PDE4 family of enzymes have proofed already their potential role in diseases in animal models and in clinical trials[93].
There is a growing interest in epithelial to mesenchymal transition, and especially its role in lung fibrosis and lung cancer[1,5,10-13,23,24,76,113].

In this investigation, we hypothesized that PDEs are altered during TGF-β1 induced EMT. In order to explore this idea, an initial characterization of enzymes expression was performed. The upregulation of PDE4 family was demonstrated, which correlated with increased protein levels and elevation in PDE4 activity. The main PDE4 family members influenced by TGF-β1 were PDE4A and PDE4D. Both PDE4A and PDE4D isoforms were localized in cytosol and membrane of the cells. Since PDE4 family is cAMP specific and is the main one which contributed to total PDE activity in TGF-β1 induced EMT, this localization can be probably correlated with compartmelization of cAMP in the cell. In order to further investigations, cAMP level in the cells before and after TGF-β1 stimulation should be measured.

Inhibition of PDE4 activity with specific inhibitor Rolipram, significantly attenuated TGF-β1 induced EMT – E-Cad which is believed to be the main epithelial phenotypic marker, was restored with Rolipram pre-treatment almost to control level. In pararel, fibronectin which is considering as one of the mesenchymal phenotypic marker, was abolished with Rolipram pre-treatment. These results strongly suggest that Rolipram has ability to reverse partially EMT in A549 cells. We couldn't detect any effect of Rolipram on α-SMA. α-SMA is reported to be the main phenotypic marker of myofibroblast which according to hypothesis, are formed during idiopathic pulmonary fibrosis.

To investigate specific PDE4 isoforms involvement in EMT process, siRNA experiments for PDE4A and PDD4D would be a good tool.

Additionally, we observed that PDE4 inhibition had no influence on TGF-β1 Smad-dependent pathway. This may suggest possibility that PDE4 inhibition resulted in reverse of EMT, influences alternative Smad-independent pathway. To make story complete it

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would be interesting to investigate the RhoA, Ras, MAPK, PI3 kinase, Notch, and Wnt signaling pathways.

We found that ROS is possible one of the main targets of PDE4 during TGF-β1 induced EMT. TGF-β1 increase ROS production, wheras Rolipram pre-treatment abolished this elevation. As one of the future directions, it would be a benefit to investigate in detail ROS signaling and the way how PDE4 is triggering ROS generation. It would be interesting to investigate whether there is any correlation between PKA and PKC, since PKA is strictly connected with PDE4 wheras PKC seems to be related to p47 pbox and NADPH oxidases.

Moreover, we found out that Rho kinases and in particular its inhibition during TGF-β1 induced EMT, are able to regulate PDE4. Additionally, Rho inhibition regulate EMT related epithelial marker E-Cad. To our knowledge, this is first report of an alternative signaling pathway for TGF-β1. Exploring in details how this correlation occurs would be interesting.

Since all of this experiments were performed in A549 cells which is a cell line, it would be a benefit to perform similar set of experiments on freshly isolated primary cells according to pulmonary fibrosis, as well as on tumor cells according to lung cancer. It could be more relevant to human diseases and possible treatment.

In conclusion, the current study provides new evidence for a biochemical and physiologically important role for PDE isoforms in the epithelial mesenchymal transition in alveolar epithelial cells. Based on its high level of expression and activity, PDE4 appears to be a particularly useful marker of the phenotypic switch mediated by TGF-β1. The use of specific inhibitors targeted to cAMP hydrolyzing PDEs whose expression is increased during TGF-β1 induced EMT has the potential to provide a means to regulate the magnitude and duration of cAMP levels and response, and thereby to attenuate EMT. PDE4 selective inhibitors appear to be particularly attractive as novel therapeutics to attenuate EMT-associated lung diseases, such as pulmonary fibrosis and lung cancer.

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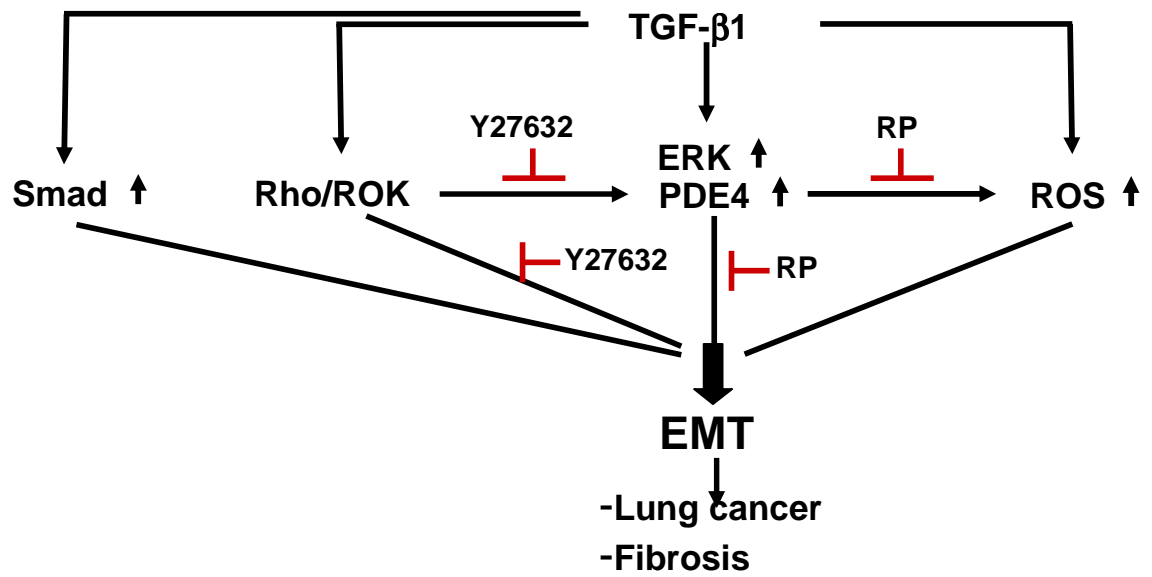
Figure 5.1.   Possible signaling pathway during PDE4 and/or Rho inhibition in TGF-β1 induced EMT.

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6.   Appendix

6.1. List of primers used for PCR amplification

Gene Bank Acession Number	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing Temperature (˙C)	Amplicon size (bp)
alpha SMA human NM_001613.1	gagaagagttacgagttgcctga	tgttagcatagaggtccttcctg	60	180
cyt 19 human NM_002276.3	acttccgaaccaagtttgag	cagcgtactgatttcctcct	56	185
coll-1 human NM_000088	tctgactggaagagtggagagta	gttcttgctgatgtaccagttct	58	153
Desmin human NM_001927.3	gaggagattcagttgaaggaagaag	gatctcctcttcatgcactttctta	60	156
E-Cad human NM_004360	agaggtgggtgactacaaaatca	agcaagagcagcagaatcagaat	60	213
Fn-EDA human NM_212478.1	gagtacacagtcagtgtggttgc	cgatatccagtgagctgaacatt	60	150
GAPDH human M33197	caccgtcaaggctgagaac	cagtagaggcagggatgatgtt	56	461
ZO-1 human NM_003257	aagagaaaggtgaaacactgctg	gtttgctccaacgagataatttg	60	158
PDE1A human NM_001003683	ctggttggcttctacctttacac	ctatgctctccacttgcttcatt	59	240
PDE1C human NM_005020	gtcacttccaacaaatcaaagc	ggtcacacagaggagaaaaagg	59	222
PDE2A human NM_002599	atgctctgtgtccctgtcatc	cttgagtttctgttccttctgga	59	183
PDE3A human NM_000921	cctcacctctccaagggact	acttctctccaggggctcat	59	236
PDE3B human NM_000922	acagatgattctttgggattgg	caggaaatggtgtgagaggatta	59	242
PDE4A human NM_006202	ataactactccgaccgcatcc	caataaaacccacctgagacttc	59	219
PDE4D human NM_006203	tacacggagatgacttgattgtg	tctgttatggtggctttgttga	59	246
PDE5A human AY264918	ctattccctgttccttgtctgtg	tgctttgtgtcttgtagcctgta	59	245
PDE7A human L12052	cagtgggcttattgagagaatca	caaatgtctgtgtctggtgtctt	59	192
PDE7B human NM_018945	gttgagaggtgtggcgaaat	tgtccaaggtagtcttcatccag	59	296
PDE8A human NM_002605	gggtggatagagaagagttgtcc	gactgatagcccattgttgtttc	59	265
PDE8B human NM_003719	agagattttacggaccacagaac	attgagagatacaaggggaaca	59	212
PDE9A human AF067226	acctgatgggttcaagcagat	tggacggacctcgttagagata	59	193
PDE10A human NM_006661	gagatgaacgatttccaagagg	gcacctgatgtattgctactgaa	59	207
PDE11A human NM_016953	actgtgtaggtggcttttgaca	cattgttggttcccctgtg	59	203

Table 3.   List of primers used for PCR amplification

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6.2. List of primary antibodies

Primary Antibody	Source	Dilution IB	Dilution ICCH	Company
Anti - αSMA	Mouse		1:200	Sigma Aldrich
Anti - cyt	Mouse	1:1000	1:500	DakoCytomation
Anti - desmin	Rabbit	1:1000		Sigma
Anti - E-cad	Rabbit	1:1000	1:400	Upstate
Anti - GAPDH	Mouse	1:5000		Novus
Anti - ERK 1/2	Rabbit	1:1000		Santa Cruz
Anti - P-ERK 1/2	Rabbit	1:1000		Santa Cruz
Anti - Fibronectin	Rabbit	1:1000	1:200	Abcam
Anti - PDE4A	Rabbit	1:1000		Abcam
Anti - PDE4D	Rabbit	1:1000		Abcam
Anti - SMAD4	Mouse	1:500		Santa Cruz
Anti - SMAD2/3	Mouse	1:1000		BD Bioscences
Anti - P-SMAD2	Rabbit	1:500		Cell Signaling
Anti - P-SMAD3	Rabbit	1:500		Cell Signaling
Anti - ROCK1	Rabbit	1:1000		Cell Signaling
Anti - TGFβRII	Rabbit	1:1000		Santa Cruz

Table 4.   List of primary antibodies

6.3. List of secondary antibodies

Secondary Antibody	Dilution IB	Dilution ICCH	Company
Alexa Fluor® 488 goat anti–mouse IgG		1:1000	Molecular Probes
Alexa Fluor® 488 goat anti–rabbit IgG		1:1000	Molecular Probes
HRP-conjugated anti-mouse IgG	1:30000		Sigma
HRP-conjugated anti-rabbit IgG	1:30000		Sigma

Table 5.   List of secondary antibodies

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8. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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1. Ciuman M, Siednienko J, Czyzyk R, Witwicka H, **Kolosionek E**, Kobiałka M, Gorczyca WA. "Cyclic GMP-dependent protein kinase and soluble guanylyl cyclase disappear in elicited rat neutrophils." *Biochim Biophys Acta*. 2006 Nov;1760(11):1618-23

2. Schermuly RT, Stasch JP, Pullamsetti SS, Middendorff R, Müller D, Schlüter KD, Dingendorf A, Hackemack S, **Kolosionek E**, Kaulen C, Dumitrascu R, Weissmann N, Mittendorf J, Klepetko W, Seeger W, Ghofrani HA, Grimminger F. "Expression and Function of Soluble Guanylate Cyclase in Pulmonary Arterial Hypertension." *Eur Respir J*. 2008 Jun 11

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POSTER PRESENTATIONS:

Ewa Kolosionek, Soni Savai Pullamsetti, Werner Seeger, Friedrich Grimminger and Ralph Theo Schermuly "Activation of Phosphodiesterases is required for TGF-beta-induced Epithelial-to-Mesenchymal Transition in Lung Fibrosis". Internistenkongress der Deutschen Gesellschaft für Innere Medizin (DGIM), 26 April, 2006, Wiesbaden, Germany

Ewa Kolosionek, Soni Savai Pullamsetti, Werner Seeger, Friedrich Grimminger and Ralph Theo Schermuly„Activation of Phosphodiesterases is required for TGF-beta-induced Epithelial-to-Mesenchymal Transition in Lung Fibrosis" American Thoracic Society, 18-23 May 2006 San Francisco, California, USA

Ewa Kolosionek, Soni Savai Pullamsetti, Werner Seeger, Friedrich Grimminger and Ralph Theo Schermuly„Activation of Phosphodiesterases is required for TGF-beta-induced Epithelial-to-Mesenchymal Transition in Lung Fibrosis" 6th *ERS* Lung Science Conference 14-16 March, Estoril, Portugal

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