Journal of Clinical & Experimental Dermatology Research

Magnucki et al., J Clin Exp Dermatol Res 2013, 4:1
DOI: 10.4172/2155-9554.1000171

Research Article Open Access

Up-Regulation of 14-3-3σ in Hacat upon Co-Cultivation with HEPM

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Abstract

Background: To generate an *in vitro* system of the dermal epithelial-mesenchymal interaction we co-cultured HaCaT (keratinocytes) and HEPM (embryonic mesenchyme). This model allowed a local disjunction with preserved cell-cell communication.

Methods: For the analysis of different protein expression patterns between co- and pure-cultured HaCaTs we performed 2D-electrophoresis and mass spectrometry. Afterwards the mass spectromertically identified protein 14-3-3σ was silenced by siRNA in HaCaT cells.

Results: We analyzed 28 spots and found 17 different expressed mainly metabolic and cytoskeletal proteins. Interestingly, stratifin (14-3-3σ), maspin and Profilin-1 (PFN1) were up-regulated, whereas Peroxiredoxin-5 (PRDX5), PDZ-and-LIM-domain-protein-1 (CLIM1) and Annexin A1 (ANXA1) were decreased in co-cultured HaCaTs. The specific *knock-down* of 14-3-3σ resulted in a down-regulation of RhoA, Rac1/2/3, LIMK1 and phosphorylated cofilin, which are involved in cytoskeleton dynamics. Furthermore, reduction of 14-3-3σ coincided with significantly decreased proliferation rates and levels of Ki67.

Conclusions: We concluded that the interaction with mesenchymal cells may initiate the alternation of epidermal precursor cells to differentiated keratinocytes, what was confirmed by the up-regulation of different keratins. Furthermore, 14-3-3σ may influence the proliferation-rate of keratinocytes via Rho GTPases.

Keywords: 14-3-3σ; HaCaT; Keratinocytes differentiation; Small GTPases; Proliferation

Introduction

Many studies showed that 14-3-3σ, also called stratifin, plays an important role in the interaction between keratinocytes and fibroblasts [1]. The highly-conserved family of 14-3-3 proteins was named after their migration in a 2D-diethylaminoethyl-cellulose chromatography and in starch-gel electrophoresis [2]. Moreover, nowadays seven ubiquitously expressed isoforms have been described. They are engaged in many intracellular functions, like the regulation of the cell cycle and apoptosis, cellular trafficking, proliferation and differentiation. The σ-isoform was shown to be deeply involved in the DNA interactions, the control of the cell cycle and the apoptosis, the cellular differentiation and the ubiquitin metabolism [1,3,4].

Interestingly, 14-3-3σ was not detected in the *stratum basale*, but the expression increased in all other epidermal layers [1,5,6]. These investigations were supported by 14-3-3σ *knock-outs*, in which keratinocyte precursor cells maintained their immortal stem cell character [6]. Therefore, the σ-isoform was considered as a marker for differentiated keratinocytes [1,6,7].

Also numerous invasive carcinomas like prostate, ovarian and breast cancer, which are characterized by increased proliferation, demonstrated a silenced 14-3-3σ gene, caused by methylation of CpG-island [8-10]. Due to the deficit of 14-3-3σ expression this protein seemed to be involved in early stages of the cancer development and progression [2,6,9]. On the other hand, many hyperproliferative cutaneous conditions like actinic keratosis, psoriasis and condylomata showed an over expression of 14-3-3σ [10].

An *in vitro* co-culture-model mimicking the human skin barrier was established to reconstruct the wound healing mechanisms [11,12]. It was demonstrated that co-cultured fibroblasts over express matrix metalloproteinases (MMP) like MMP-1, MMP-3, MMP-8, MMP-10 and MMP-24 in comparison to pure culture. Authors showed that 14-3-3σ was secreted by keratinocytes and influenced the expression of collagenase in fibroblasts via surface receptor CD13 or aminopeptidase-N and the intracellular p38-MAP-kinase activation. Elimination of 14-3-3σ with a 30 kDa filter from the co-culture medium suppressed the expression of collagenase in fibroblasts. Conversely, the stimulation of dermal fibroblasts with recombinant 14-3-3σ induced also MMP1 up-regulation. These interactions were suggested to be crucial for the wound healing retardation in the non-healing disorders [11-15].

The aim of our study was to evaluate different protein-expression patterns of pure- and with HEPM co-cultured HaCaT cells. Possible protein markers could be identified that contribute to the dermal epithelial-mesenchymal interaction. Furthermore, we focused on the influence of the identified protein 14-3-3σ and on its effects on the cytoskeleton and the proliferation of keratinocytes.

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Received January 07, 2012; **Accepted** April 12, 2013; **Published** April 18, 2013

Citation: Magnucki G, Białek J, Hammje K, Prenzel H, Klohs G, et al. (2013) Up-Regulation of 14-3-3σ in Hacat upon Co-Cultivation with HEPM. J Clin Exp Dermatol Res 4: 171. doi:10.4172/2155-9554.1000171

Material and Methods

Cell culture

In order to investigate the effects of epithelial-mesenchymal interactions we used an *in vitro* model established by Karimi-Busheri and Ghahary and co-cultured HaCaT in upper and HEPM (CRL-1486, ATCC®, LGC Standars Gmbh, Wesel, Germany) in lower chamber of the system [11,12]. Pure cultures of HaCaT cells served as control. HaCaT cells were kindly provided by Dr. Fusenig (DKFZ Heidelberg, Germany). The cells were cultivated with DMEM (Invitrogen, Karlsruhe, Germany) containing 10% FCS (Biowest, Nuaille, France) at 37°C and 5% CO_2.

Protein extraction and purification for 2D gel electrophoresis

For the protein extraction, the 2D gel electrophoresis and the mass spectrometry recently published protocols were used [16]. After the PBS-washing the proteins were extracted with the 2D lysis buffer (8µM Urea (Sigma-Aldrich®), 4% CHAPS, 1% dithiothreitol (DTT), 0,8% pharmalyte (all; Amersham Biosciences)) used for silver staining. The co- and pure-cultured cells were incubated with the lysis buffers for 30 min at room temperature. Afterwards, the isolated proteins were purified with the 2D Clean Up Kit (Amersham Biosciences). Purified proteins were dissolved in rehydration solution for use in silver stained gels (8 M Urea (Sigma-Aldrich®), 2% CHAPS, 0,5% pharmalyte, 40 mM DTT (all; Amersham Biosciences)) and stored at −80°C until use.

Two-dimensional gel electrophoresis

The first dimension (the isoelectric focusing) was carried out with IPGphor (Amersham Biosciences). Total protein (30 or 150 µg) was loaded onto nonlinear, 18 cm (pH 3-10) immobilized pH gradient (IPG) strips and rehydrated under low voltage conditions (30 V) for 12 h. The isoelectric focusing was performed at 8000 V for 9 h. IPG strips were equilibrated first in 10 ml equilibration solution containing 6 M urea, 2% SDS, 50 mM Tris-HCl (pH 8,8) and 30% glycerol with 100 mg DTT (Roth) for 15 min and in 10 ml equilibration solution with 250 mg iodoacetamide (IAA, Sigma) for another 15 min. Then the IPG strips were arrested on a 12,5% polyacrylamide gel (37,5:1 Rothiphorese Gel 30, 10% SDS, 1,5 M Tris–HCl (pH 8,8), 10% APS, TEMED) using 0,5% agarose.

The second dimension was performed in an Ettan Dalt Unit (Amersham Biosciences) with SDS electrophoresis buffer (25 mM Tris-base, 192 mM glycine, 0,1% SDS) at 2,5 W/gel for 30 min and at 5 W/gel for the next 5-6 h. Afterwards the gels were fixed for 20 min with 0,25% silver nitrate, 0,00925% formaldehyde. The gels were washed thrice for 20 s in distilled water, developed in 3% sodium carbonate, 0,0185% formaldehyde and the silver staining was stopped after 10 min in 5% acetic acid and 3×10s washing in distilled water. For semi-quantitative protein spot evaluation, silver stained gels were scanned using a visual light scanner Hewlet Packard scanjet 7400C and analyzed with Phoretix 2D software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

The different expression of protein spots between HaCaT and coHaCaT was analyzed by the Kodak Image System 440 cf (Eastman Kodak, New York, USA). GAPDH was used as normalization and the pure-cultured HaCaTs were set 100%. The results were classified in ↑ (over-expression of 25-50%), ↑↑ (over-expression of >50%), ↑ (down-regulation of 25-50%) and ↑↑ (down-regulation of >50%).

Protein preparation for mass spectrometry

The spots of interest were cut out of the gel as ~1 mm^3 large cubes and dried in a vacuum concentrator. Afterwards, the spots were destained with 100 mM potassium ferricyanide/30 mM sodium thiosulfate and washed with HPLC grade water (Roth). The samples were shrunk with acetonitrile followed by another drying step in the vacuum concentrator. The gel pieces were rehydrated with cold trypsin (15µg/ml) and digested for 16-24 h at 37°C. The peptides were extracted with acetonitrile and 5% trifluoroacetic acid. Next the dried proteins were reduced by a solution of 100 mM DTT in 100 mM NH_4HCO_3, followed by the alkylation with 55 mM iodoacetamide in 100 mM NH_4HCO_3, succeeded in a digestion 12,5 ng/µl trypsin dissolved in 5 mM $CaCl_2$/50 mM NH_4HCO_3. Finally, the peptides were extracted with acetonitrile and 5% formic acid, dried, desalted using the ZipTip Kit (Millipore Corporation, Billerica, USA) and dissolved in 50% ACN/0,1% trifluoroacetic acid and for Q-ToF MS/MS in 70% methanol with 1% formic acid.

Mass spectrometric analysis

MALDI-ToF MS identification of peptide mixtures was performed on a VoyagerDE Pro mass spectrometer (Applied Biosystems, Forester City, USA). The dissolved peptides were combined with a α-cyano-4-hydroxy-trans-cinnamic-acid-matrix in a 1:1 ratio. The calibration of the mass spectra was externally performed with the Sequazyme Protein Digest Standards Kit (Applied Biosystems). The proteins were identified by using the Mascot DataBase, where peptide mass tolerance was set to 100 ppm. Spectra were reconstructed with Data Explorer and identified with Mascot Data Bank.

RT-PCR

Total-RNA was extracted from HaCaT and coHaCaT using the TriZol reagent according to the manufacturer's instructions (Invitrogen). The cDNA was synthesized employing the reverse transcriptase kit (Superscript II, Gibco BRL, Invitrogen, Karlsruhe, Germany). RT-PCR was performed with specific primer pairs for keratin 6a (sense 5'-CAACAACCGCAACCTGGACC-3'; antisense 5'-AACGCCTTC-GCCATTCAGC-3') (Sequence ID: NM_005554.3), keratin 10 (sense 5'-AATGAAAAAGTAACCATGCAGAATCTG-3'; antisense 5'-CAC-GAGGCTCCCCCTGAT -3') (Sequence ID: NM_000421.3), keratin 16 (sense 5'-GCCAGTTCGTGCTCATAC-3'; antisense 5' GTCT-GTCTCCTCTCGCTTC-3') (Sequence ID: NM_005557.3), keratin 17 (sense 5'-TCTGGCTGCTGATGACTTC-3'; antisense 5'-CTT-GCGGTTCTTCTCTGC-3') (Sequence ID: NM_000422.2) and 18S (sense 5'-GTTGGTGGAGCGATTTGTCTGG-3'; antisense 5' AGGGCAGGGACTTAATCAACGC-3') (Sequence ID: NR_003286.2) as normalizing markers. The PCR-products were resolved on 1% agarose gel containing 0.05% Ethidium Bromide, scanned (Kodak Digital Science Image station® 440CF). The intensity of the PCR amplicons was semi-quantitatively calculated in comparison to positive control defined as 100%. Kodak Digital Science 1D® V.3.0.2.software was used for all calculations. For the investigation of mRNA-expression of REC8 transcription variant (TV) 1 (sense 5'- GCGTCTCAGTTATCCTGGT-GT-3'; antisense 5'-TCCCTCTGGTCTTTCACCCT-3') (Sequence ID: NM_005132.2), REC8 TV2 (sense 5'-CCACCTTGCCACCAGAGA-AG-3'; antisense 5'- ATCCCCGTTCGGATCTGAGT-3') (Sequence ID: NM_001048205.1), 14-3-3σ (sense 5'-TGTCACTAAAGTGGCT-GCGT-3'; antisense 5'-ACATAACACTCAGGGTGGCG-3') (Sequence ID: NM_020992.3), 14-3-3ε (sense 5'-CATTTTTGCTGCCC-GGACG-3'; antisense 5'-ACCATTTCGTCGTATCGCTCA-3') (Sequence ID: NM_006761.4) 14-3-3ζ (sense 5'-CGTCCCTCAAACCTT-GCTTCT-3'; antisense 5'-GCTCCTTGCTCAGTTACAGACT-3') (Sequence ID: NM_003406.3) and the reference gene GAPDH (sense 5'-ACCCAGAAGACTGTGGATGG-3'; antisense 5'-TTCTAGACG-

GCAGGTCAGGT-3') (Sequence ID: NM_002046.4) a qPCR was performed with the 2xRotor-Gene SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) and the Rotor-Gene Q 2 Plex (Qiagen, Hilden, Germany). Rotor-Gene Q Series Software (Qiagen, Hilden, Germany) was used for the calculation.

Western blot

Extracted total protein lysates (20 µg) of co-, pure-cultured and 14-3-3σ *knock-down* HaCaT cells and their respective controls (nonsilencing siRNA and Lipofectamine treated cells) were separated under reducing conditions on 10% SDS-polyacrylamide gels and blotted (1 mA for 120 min) on a prepared PVDF-membrane (Amersham Biosciences). Non-specific bindings were blocked by 5% non-fat milk powder in TBS-T 0,05% (Tris buffered saline (Amersham Biosciences); 0,05% Tween20 (Serva)). After washing with TBS-T 0,05%, the membranes were incubated with primary antibodies against 14-3-3σ, Rho A, Rac1/2/3, LIMK1, Cdc42, phosphorylated cofilin, cofilin (all; Cell Signaling, 1:500 in TBS-T 0,05%), maspin, cyclin A, cyclin D, cyclin E, α-tubulin, acetylated tubulin (all; Santa Cruz, 1:1000 in TBS-T 0,05%) and β-actin (Sigma, 1:10000 in TBS-T 0,05%) at 4˚C for 8 h. Secondary anti-goat (1:5000 in TBS-T 0,05%) and anti-mouse (1:5000 in TBS-T 0,05%) antibodies were used for 1 h at 20˚C (both Santa Cruz). Immunoreactive protein bands were visualized using the ECL Detection Kit (Amersham Biosciences) and Kodak Image System 440cf (Eastman Kodak, Rochester, USA).

siRNA *knock-down* experiments

For the silencing of 14-3-3σ in HaCaT cells transient transfection with siRNA against 14-3-3σ (Qiagen, SI02653679) was performed. Not treated and non-silencing/no-target siRNAs as well as Lipofectamine 2000 (Invitrogen) treated cells were used as negative controls. Transfection was performed in OptiMem medium (Invitrogen) at 60% confluence, in six-well plates. Proteins were isolated with 2D lysis buffer (7 µM Urea (Sigma-Aldrich®)), 2 µM Thiourea, 4% CHAPS, 2% Pharmalyte (all; Amersham Biosciences), 2% DTT (Invitrogen)) after transfection and analyzed by western blotting and qPCR.

Proliferation assay

To analyze the influence of 14-3-3σ on the cells proliferation and metabolic activity, siRNA *knock-down* HaCaT cells and their respective controls (nonsilencing siRNA and Lipofectamine treated cells) were cultured in 96-well plates in 100 µl culture medium DMEM (Invitrogen) containing 10% FCS (Biowest). After 24 h MTT assay was performed according to the manufacturer's instructions. Results of colorimetric reaction were measured by using Tecan Elisa Reader (Tecan, Grodig, Austria).

Immunohistochemistry

The siRNA *knock-down* HaCaT cells and their respective controls were seeded on a microscope slide for 24 h. Before the immunostaining the cells were washed with PBS and fixed for 20 min in 97% ice-cold methanol and 3% H_2O_2. Afterwards the cells were incubated overnight at 4°C with monoclonal antibodies against Ki67 (Santa Cruz) at dilutions of 1:100. After washing in PBS, the samples were incubated for 30 min with a 1:1000 dilution of biotinylated goat anti-mouse secondary antibody (DAKO, Hamburg, Germany). Detection of immunoreaction was accomplished by the chromogen 3,3V-diaminobenzidine (DAKO), followed by a counterstaining with haematoxylin. Finally, the results were visualised by optical microscopy (Zeiss, Jena, Germany).

Statistical analysis

Statistical analysis was carried out with SPSS software. All experimental parameters were calculated for statistical significance and p-values were defined as *($p< 0.05$) and **($p<0.005$).

Results

Comparison of proteomic pattern of co- and pure-cultured HaCaT by 2D-electrophoresis and MALDI-TOF MS analysis

Analysis of 2D-PAGE revealed 28 influenced protein spots, which were further investigated by mass spectrometry (Figure 1A). The peptide mass fingerprinting of all spots was obtained by Mascot software using the NCBI Prot-no- and Swiss-Prot-database. Based on the molecular weight and isoelectric point shown in the 2D all 28 proteins were identified. 17 peptides showed different expression patterns between HaCaT and coHaCaT. The results revealed that the metabolic proteins AHCY, PGK1 and LDH-A were up-regulated under co-culture-condition of HaCaT cells with HEPM, whereas the expression of PPIA, PRDX2, PRDX5, PGAM1, PSMB2 and TPI1 were decreased. The untreated HaCaTs characterized the high level of ANXA1, CLIM1 and PCBP1 in comparison to the co-cultured cells. Furthermore, the co-HaCaT cells demonstrated a stronger expression of 14-3-3ε, 14-3-3σ, PFN and maspin (Table 1) (Figures 1a and 1b).

Afterwards, we confirmed the results of the 2D-electrophoresis by western blotting. We investigated the expression rate of the previously found proteins 14-3-3σ, maspin, LDHA and GAPDH (Figure 2A) and detected correlating protein levels in compare to the 2D-PAGE (Figure 1). Moreover, the further investigations revealed an up-regulation of 14-3-3σ in fibroblasts-keratinocytes co-culture when compared to the purely cultivated HaCaTs (Figures 2a and 2b).

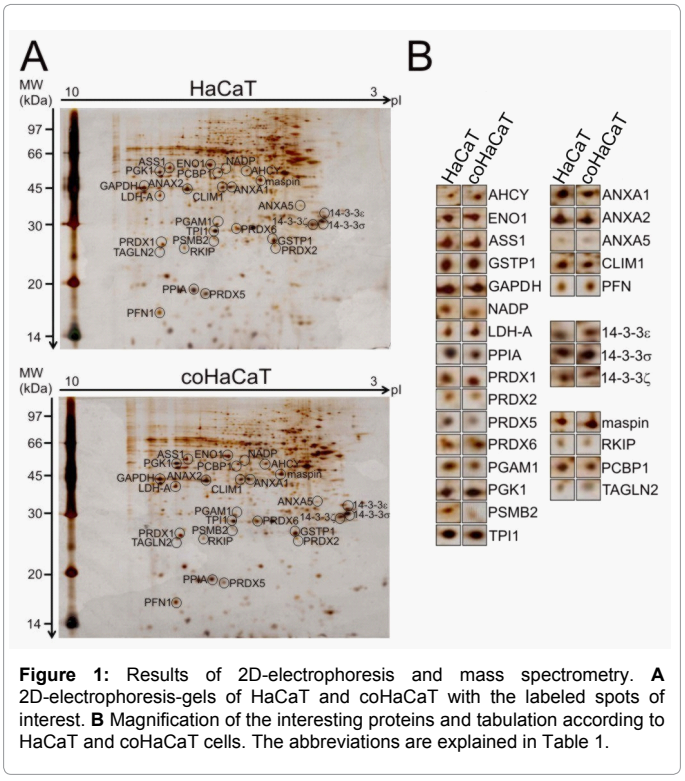

Figure 1: Results of 2D-electrophoresis and mass spectrometry. **A** 2D-electrophoresis-gels of HaCaT and coHaCaT with the labeled spots of interest. **B** Magnification of the interesting proteins and tabulation according to HaCaT and coHaCaT cells. The abbreviations are explained in Table 1.

Protein Abbreviation	Molecular Weigth [Da]	IP [pH]	NCBI Prot-no	UniProtKB / Swiss-Prot.	Protein name	coHaCaT
Metabolism						
AHCY	47,716	5.82	GI:20141702	P23526	Adenosylhomocysteinase	↑
ENO1	47,169	7.01	GI:119339	P06733	Alpha-Enolase	-
ASS1	46,530	7.15	GI:20141195	P00966.2	Argininosuccinate synthase	-
GSTP1	23,356	5.43	GI:121746	P09211.2	Gluthadion S-transferase P	-
GAPDH	36,053	8.57	GI:120649	P04406	Glyceraldehyde-3-phosphate dehydrogenase	NM
NADP	46,659	6.53	GI:21903432	O75874	Isocitratedehydrogenase	-
LDH-A	36,689	8.44	GI:126047	P00338	L-Lactate dehydrogenase A chain	↑
PPIA	18,012	7.68	GI:51702775	P62937	Peptidyl-prolylcis-trans isomearase A	↑
PRDX1	22,110	8.27	GI:548453	Q06830	Peroxiredoxin-1	↑
PRDX2	21,892	5.66	GI:2507169	P32119	Peroxiredoxin-2	↓
PRDX5	22,086	8.93	GI:317373539	P30044	Peroxiredoxin-5	↓↓
PRDX6	25.035	6.0	GI:1718024	P30041	Peroxiredoxin-6	-
PGAM1	28,804	6.67	GI:130348	P18669	Phosphoglyceratemutase 1	↓
PGK1	44,615	8.3	GI:52788229	P00558	Phosphoglycerate kinase 1	↑
PSMB2	22,836	6.49	GI:28559000	P49721	Proteasome subunit beta type 2	
TPI1	30,791	5.65	GI:353526311	P60174	Triosephophateisomerase 1	↓
Cytoskeleton and cell membrane compartments						
ANXA1	38,714	6.57	GI:113944	P04083	Annexin A1	↓
ANXA2	38,604	7.57	GI:113950	P07355	Annexin A2	-
ANXA5	35,937	4.94	GI:113960	P08758	Annexin A5	-
CLIM1	36,072	6.56	GI:20178312	O00151	PDZ and LIM domain protein 1	↓
PFN1	15,054	8.44	GI:130979	P07737	Profilin-1	↑
14-3-3 proteins						
14-3-3ε	29,174	4,63	GI:51702210	P62258	14-3-3 protein epsilon	↑
14-3-3σ	27,774	4,68	GI:398953	P31947	14-3-3 protein sigma	↑↑
14-3-3ζ	27,745	4,73	GI:52000887	P63104	14-3-3 protein zeta/delta	-
Others						
maspin	42,100	5.72	GI:229462757	P36952	Maspin	↑
RKIP	21,057	7.01	GI:1352726	P30086	Phosphatidylethanolamine-binding protein 1	-
PCBP1	37,498	6.66	GI:42560548	Q15365	Poly (rc)-bindingprotein 1	↓
TAGLN2	22,391	8.41	GI:586000	P37802	Transgelin-2	-

Table 1: Identification of proteins with consistent expression in HaCaT and coHaCaT cells. GAPDH was used as normalization marker (NM) and the pure cultured HaCaTs were set 100%. The results for coHaCaTs were classified in ↑ (over-expression of 25-50%), ↑↑ (over-expression of >50%), ↓ (down-regulation of 25-50%) and ↓↓ (down-regulation of >50%).

mRNA-expression of keratins in HaCaT and coHaCaT

For the further investigation of the keratinocyte maturation implied by the up-regulation of differentiation-associated proteins like 14-3-3σ, ANXA1, PDX1 or maspin we analyzed the expression of different keratins. We focused on keratin 6a, 10, 16 and 17 because of their expression-profile in the suprabasal layers and their interaction with the epidermal development [17-19]. Interestingly, the keratins 6a, 10 and 16 were significantly up-regulated in co-cultured HaCaTs in comparison to pure-cultured once. Keratin 17 was not significantly increased in coHaCaT (Figure 3).

siRNA *knock-down* in HaCaT

Our further investigation focused on 14-3-3σ, because of its important role explored in the interaction of keratinocytes and fibroblasts. Furthermore, this protein is associated with fibro-proliferative disorders of the skin and chronic non-healing wounds [1]. To further investigate the role of 14-3-3σ we *knocked-down* this protein in HaCaT cells. Immuno-blot and mRNA analysis confirmed reduced levels of 14-3-3σ after siRNA treatment when compared to lipofectamine or non-treated control cells (Figure 4). The RNA analysis excluded influence of 14-3-3 si-RNA-treatment on expression of related genes 14-3-3ε and 14-3-3ζ. Furthermore, the 14-3-3σ closely related genes REC8 TV1 and 2 were unaffected by the *knock-down* (Table 2).

Proteins received from 14-3-3σ *knock-down* cells revealed significant down-regulation of tested Rho-family members; Rho A, Rac1/2/3 as well as the Rho-dependent LIMK1. Total cofilin was increased, whereas the phosphorylated form of this protein was down-regulated. On the other hand the small Rho-GTPase Cdc42 was unaffected by the siRNA-treatment. Expression of σ-tubulin, acetylated tubulin and maspin were not reduced by the 14-3-3σ *knock-down*. Furthermore, we investigated the expression of mitosis-controlling cyclins. Cyclin A and E were decreased significantly, whereas the level of cyclin D was slightly reduced (Figure 4). Afterwards, the influence of 14-3-3σ on the proliferation rate was evaluated via an MTT-assay.

Silencing of 14-3-3σ significantly decreased metabolic/mitotic activity of HaCaT cells in the MTT-assay (Figure 5 A). To verify the data we stained the *knock-down* and control cells with antibodies against the proliferation marker Ki67. The 14-3-3σ siRNA-treated HaCaT cells showed significant weaker immunoreactivity in comparison to the control (Figure 5b) confirming the MTT assay results.

Discussion

The comparative analysis of protein expression of HaCaT and

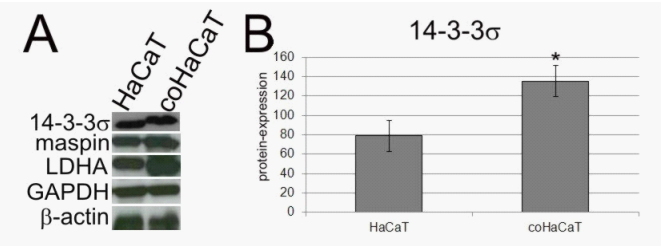

Figure 2: 14-3-3σ–expression upon co-cultivation. **A** Western blot results of 14-3-3σ–expression showed the detection of protein-bands in HaCaT and coHaCaT cells. **B** Graphical analysis of the protein-expression showed significant up-regulated 14-3-3σ in coHaCaT. * (p< 0.05).

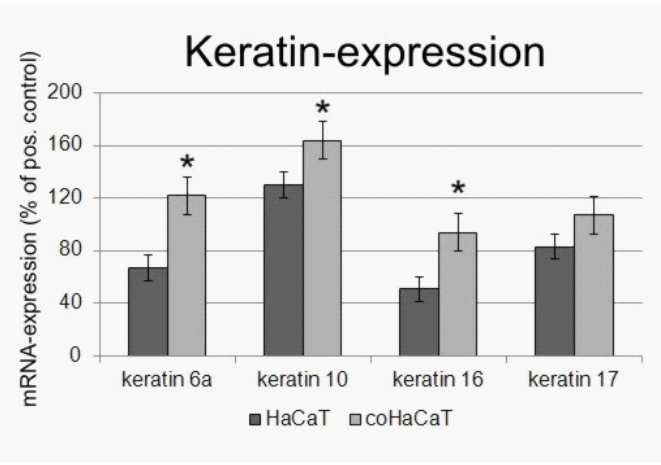

Figure 3: mRNA-expression of keratins in HaCaT and coHaCaT. Upon the co-cultivation of HaCaT and HEPM the keratinocytes significantly up-regulate keratin 6a, 10 and 16. Furthermore, keratin 17 was increased insignificantly. * (p< 0.05).

co-cultured HaCaT demonstrated that the differentially expressed proteins were involved in cellular metabolism, differentiation, changes in the cytoskeleton and the cell membrane. These expression alterations depend on the interaction of HaCaT cells with mesenchymal HEPM. Examination of these proteins may support the identification of protein markers for the epithelial-mesenchymal interaction and contribute to the understanding of this molecular mechanism. The following discussion highlights the function of the differentially expressed proteins identified in the present study and their relation to the cutaneous homeostasis.

ANXA, PFN and CLIM1

Our study documented that the interaction of keratinocytes and mesenchyme decreased the synthesis of ANXA1 in epithelial cells. Annexins are a family of calcium-dependent phospholipid-binding-proteins with numerous cellular functions including exo- and endocytosis. Interestingly, ANXA1 was only detected in the basal and suprabasal layer of human skin [20-22]. These results support the concept that the appearance of ANXA1 is linked to a certain level of keratinocyte´s differentiation [20,21]. Therefore, we hypothesized that the co-cultured HaCaT differentiated to mature keratinocytes. Furthermore, annexins are known to interact with a complex molecular mechanism that regulates the actin cytoskeleton [23]. Interestingly, other proteins involved in the actin metabolism were found in our investigation like PFN1 and CLIM1. We observed an up-regulation of PFN1 in coHaCaT cells induced by mesenchymal cells. The main function of PFN1, an actin-binding protein, is the turnover and restructuring of the actin cytoskeleton. PFN1 controls the growth of actin microfilaments, which is crucially for the cellular morphology and trafficking [24]. On the other hand CLIM1 was

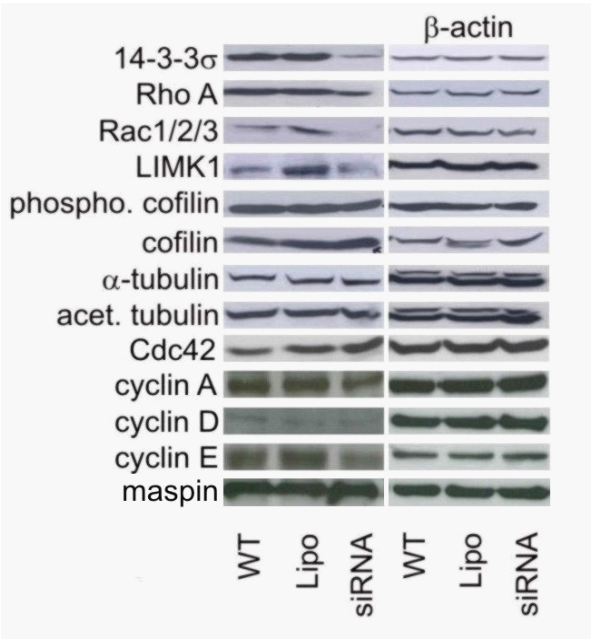

Figure 4: Western blot results of the 14-3-3σ *knock-down*. WT-untreated control; Lipo-Lipofectamine control; siRNA-siRNA-*knock-down*. Western blot demonstrated the *knock-down* of 14-3-3σ in the HaCaT cells. Parallel the expression of Rho A, Rac1/2/3, LIMK1 and phosphorylated cofilin decreased in siRNA transfected HaCaT cells, whereas cofilin was up-regulated. Furthermore, the expression of cyclin A and E were reduced significantly, but cyclin D was weakly decreased by the *knock-down*. Acetylated, α-tubulin, Cdc42 and maspin were unaffected by the siRNA treatment.

mRNA		**protein**	
gene name	**expression after siRNA**	**gene name**	**expression after siRNA**
14-3-3 and related genes		*Small GTPases and related proteins*	
14-3-3σ	↓	Rac1/2/3	↓
14-3-3ε	→	RhoA	↓
14-3-3ζ	→	LIMK1	↓
REC8 TV1	→	Cdc42	→
REC8 TV2	→	*Cytoskeleton*	
		phospho. cofilin	↓
		cofilin	↑
		acet. Tubulin	→
		Tubulin	→
		Cyclines	
		cyclin A	↓
		cyclin D	→
		cyclin E	↓
		Others	
		maspin	→
		14-3-3σ	↓

Table 2: Tabulation of investigated proteins and there regulation upon 14-3-3σ *knock-down* in HaCaT cells. The results were classified in ↑ (over-expression), ↓ (down-regulation) and (no regulation).

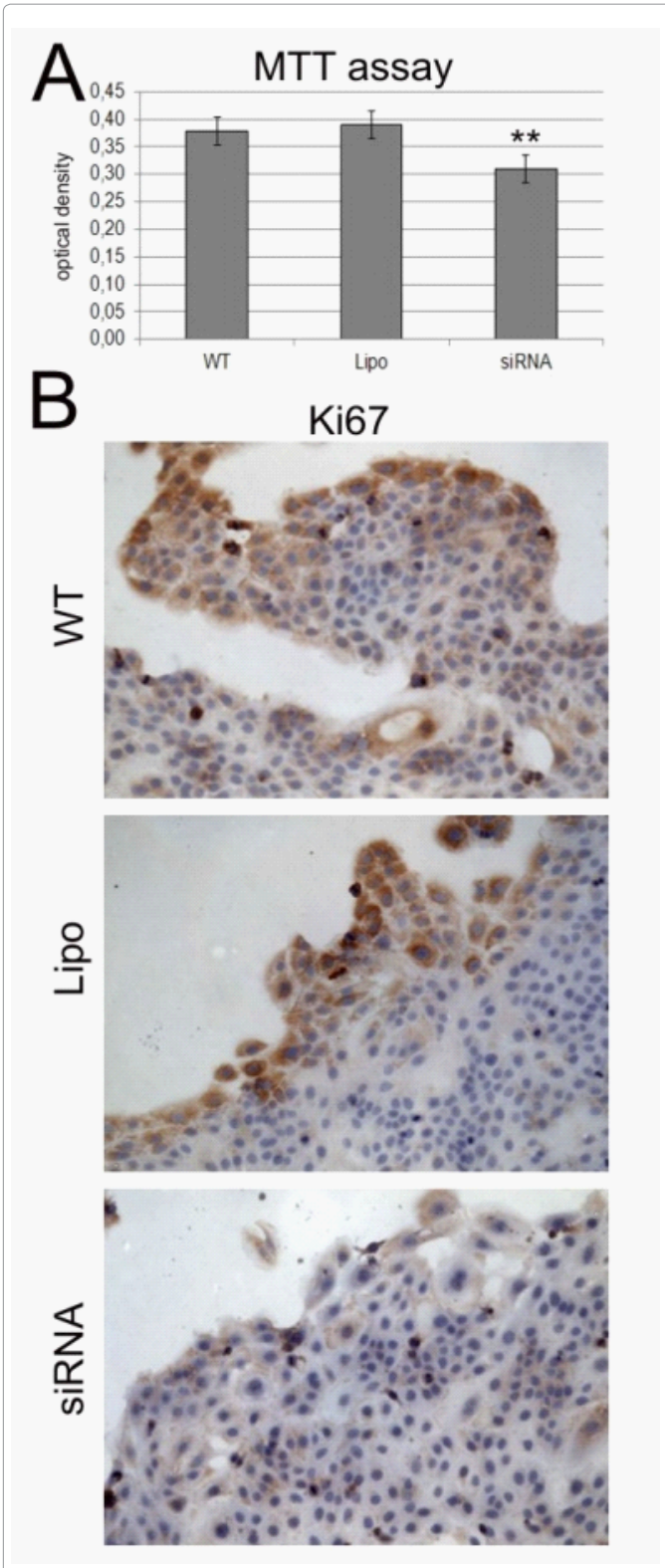

Figure 5: Results of the MTT-Assay and the Ki67-staining. WT-untreated control; Lipo-Lipofectamine control; siRNA-siRNA-*knock-down* **A** siRNA *knock-down* HaCaT cells showed a significantly decreased proliferation rate compared to the control cells. **B** The immunohistochemical staining with the proliferation marker Ki67 demonstrated weaker immunoreactivity in 14-3-3σ *knock-down* cells in comparison to the WT- and Lipo-control. * ($p< 0.05$) and ** ($p<0.005$).

decreased in keratinocytes through the influence of mesenchymal cells. The cytoskeletal adapter CLIM1 binds through the PDZ domain to α-actinin and through the LIM domain to other proteins like signaling molecules [25]. In mouse epithelial cells CLIM1 was localized in actin stress fibers [26,27]. We concluded that the communication of keratinocytes and mesenchyme induced a change of cytoskeleton, because the regulator of the actin-filament-synthesis PFN1 was increased. Simultaneously, the stress fiber associated CLIM1 was decreased. These cytoskeletal alterations may lead to a promotion of cell motility and/or a morphologic differentiation.

PRDX

PRDX is a peroxidase family protecting cells or tissues from oxidative damage by eliminating hydrogen peroxide. In human skin it was also shown that PRDX1 and PRDX2 were induced by keratinocyte differentiation. Furthermore, PRDX1 was mainly expressed in the suprabasal layer, whereas PRDX2 was detected in the later stages of the *stratum granulosum* and *spinosum* [28]. The up-regulation of PRDX1 in keratinocytes interacting with mesenchymal cells supported our hypothesis of an epidermal differentiation induced by HEPM. The decreased expression of PRDX2 implied unfinished terminal maturation to cells of the spinal or granular layer.

Maspin

Maspin belongs to the serine protease inhibitor superfamily [29] and is expressed in the suprabasal and granular layer of the human skin. Association of this peptide with specific lines of differentiation in the human epidermis was previously investigated [30]. Furthermore, maspin is known to be secreted by epidermal keratinocytes [31]. Our results demonstrated positive influence of mesenchymal cells on the expression of the protein in HaCaTs, however, an interaction of maspin and 14-3-3σ was not identified. Further studies should focus on the effects of maspin on the keratinocyte differentiation, its regulation in skin cancer progression and possible interactions with other identified proteins.

Effects of 14-3-3σ on HaCaT cells

As a result of the cooperation of mesenchyme and keratinocytes we demonstrated drastic changes in the expression of 14-3-3σ. 14-3-3σ is a specific epithelial protein, strongly expressed in differentiated keratinocytes [5,32]. Our studies demonstrated that the expression of this protein in keratinocytes depends on mesenchymal activity (mesenchyme-derived soluble factors). In presence of mesenchymal cells keratinocytes expressed higher levels of 14-3-3σ as a result of premature differentiation and reduced number of progenitor stem cells *in vivo* [33]. That concluded our hypothesis of mesenchymal induction on kerationcyte´s maturation. Furthermore, the investigated keratins 6, 10 and 16 were found in suprabasal layers and can therefore be concerned as additional differentiation markers [17-19]. Therefore, the up-regulation of these keratins under co-culture conditions supported our thesis of an HEPM-induced maturation of keratinocytes.

However, the exact role of 14-3-3σ in keratinocytes is still not known. To investigate the issue of this protein we silenced 14-3-3σ in HaCaT cells. 14-3-3σ-down-regulation induced a reduction of Rho GTPases synthesis as well as alternation of cytoskeleton-related protein cofilin-expression, suggesting its role in cytoskeleton dynamics. It was previously reported that Rho GTPases affect cells through p21-activated kinase-1 and Rho kinase, which stimulates kinase activity of LIMK-1 [34,35]. Thereafter, LIMK1 induces phosphorylation and therewith the inactivation of cofilin, probably leading to the decrease

of actin-depolymerization [36] (Figure 6). The detected cytoskeletal changes could further indicate a cellular differentiation. However, 14-3-3σ accelerated the mitotic activity of keratinocytes directly or via Rho GTPases, whereas an overexpression of this marker for differentiated keratinocytes suppressed the number of proliferating cells [33].

The cellular communication of the mesenchymal stroma and the basal layer can induce the expression of 14-3-3σ and differentiate these cells to mature keratinocytes. Furthermore, the proliferation rate of keratinocytes is increased upon the interaction with mesenchymal cells, probably caused by an up-regulation of 14-3-3σ and its activation of the Rho GTPases (Figure 6). The mechanisms of keratinocytes maturation and mitosis were normally localized in cells delaminating the basement membrane, where high rates of 14-3-3σ were detectable [1,5,6]. Therefore, the mesenchyme could mediate the homeostasis of the skin by activating 14-3-3σ.

This study also detected other proteins related to epithelial-mesenchymal interaction, such as AHCY, LDH-A, PRDX5, PPIA, PGAM1, PGK1, PSMB2, TPI1 and PCBP1. Although their role and function in the connection of mesenchyme and epidermis is not entirely clear, they are expected to become candidate markers for future study of the epithelial-mesenchymal communication.

In summary, our results identified previously unknown proteins involved in the epithelial-mesenchymal interaction and suggest that co-cultivation with mesenchymal cells initiate a differentiation of HaCaT cells. Furthermore, we suggest the role of 14-3-3σ during modulation of cytoskeleton in keratinocytes by activating Rho A family GTPases and accelerating the proliferation. Finally, 14-3-3σ seems to be a promising therapeutic tool in dermatology to conduct the proliferation and differentiation of keratinocytes.

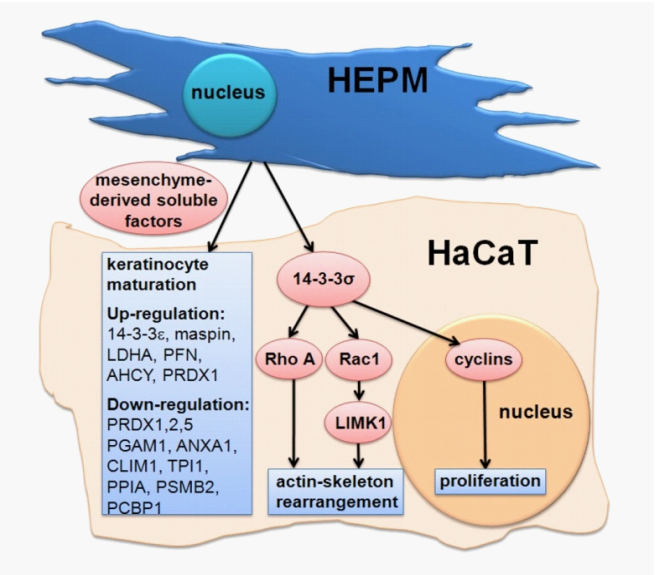

Figure 6: Schematic drawing of raised conclusions of this study. Upon the interaction with HEPM the HaCaT cells differentiate into mature keratinocytes. Furthermore, there were several up- and down-regulated proteins found in HaCaTs including the majorly focused protein 14-3-3σ, which were induced by mesenchyme-derived soluble factors. It was concluded that 14-3-3σ increased the proliferation by activating the cyclins and on the other hand it leads via Rho A and Rac1 to a cytoskeletal rearrangement.

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