INVESTIGATIONS ON mRNA MATURATION DISTURBANCIES IN THE PATHOGENESIS OF PSORIASIS

Theses of doctoral dissertation

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1. AIMS

1. Characterization of luc-7 like protein 3 (*LUC7L3*), peptidyl-prolyl cis-trans isomerase G (*PPIG*) and arginine/serine-rich 18 (*SFRS18*) splicing factor gene expression differences in psoriasis and during the proliferation /differentiation states of keratinocytes.

2. Examination of *LUC7L3, PPIG* and *SFSR18* influence on the mRNA maturation process of the extracellular matrix protein fibronectin.

3. To study additional gene expression and mRNA maturation alterations upon the modified expression of splicing factors in human keratinocytes

2. INTRODUCTION

Despite the advances in the field of psoriasis research, several questions remain that need to be answered considering the exact molecular pathogenesis of the disorder, including the description of molecular triggers and the mechanism of disorder initiation. However, the altered response of keratinocytes to the T-cell signals is undoubtful an essential factor of psoriasis development [1-3].

To date, relatively few studies have been conducted on mRNA maturation disturbances in psoriasis. In the study of Ting *et al.* was

shown that the EDA+ domain containing fibronectin isoform (EDA+ fibronectin) is overexpressed in psoriatic non-involved epidermis [4]. We found that proliferating keratinocytes (both normal cultured keratinocytes and HaCaT cells) are able to produce this isoform [5]. EDA+ fibronectin expression was modest in healthy keratinocytes whereas keratinocytes from psoriatic non-involved epidermis proved to be effective producers of this splice variant.

In our cDNA microarray study we examinated the T-lymphokine induced gene expression changes [6]. In the applied experimental setup, organotypic cultures were generated from both healthy and psoriatic epidermis samples, and half of them were treated with a lymphokine mixture containing GM-CSF, IFN-γ and IL-3. It was previously proven that IFN-γ in the presence of GM-CSF and IL-3 is able to promote proliferation of the keratinocyte precursors originating from the non-involved epidermis, therefore, these mediators could play a crucial role in the early steps of disorder development [7]. In the following analysis, a comparison has been made between the autologuous pairs of untreated and treated samples, and the differentially regulated genes among healthy and psoriatic non-involved epidermis samples were selected for further analyses [6].

An important outcome of the study was that due to T-lymphokine treatment, several identified genes showed upregulation in healthy epidermis, while in the non-involved epidermis, downregulation or unchanged gene expression was experienced. Moreover, we have also proved that certain SR-rich splicing regulators showed altered responsiveness to T-lymphokine stimuli. In this experiment, we identified splicing factor, luc-7 like protein 3 (*LUC7L3*) [8-12], peptidyl-prolyl cis-trans isomerase G (*PPIG*) [13-15] and arginine/serine-rich 18 (*SFRS18*) [15], differentially regulated among healthy and psoriatic non-involved epidermis, therefore they could contribute to the responsiveness changes of keratinocytes.

3. MATERIALS AND METHODS

Organotypic skin cultures

Organotypic skin cultures were generated from half cuts of shave biopsies, obtained from 4 healthy volunteers and 4 psoriasis patients [6]. Half of the organotypic cultures were treated with a lymphokine mixture containing of 1 ng/ml IFNγ, 1 ng/ml GM-CSF and 0.3 ng/ml IL-3. Standard culturing conditions were applied (at 37°C in a 5% CO_2 atmosphere), at the air/liquid interface for 72 h. The epidermis/dermis

separation was executed by overnight incubation in Dispase solution at 4°C, finally, the samples were placed in TRIreagent.

Real-time RT-PCR

One microgram of total RNA was reverse transcribed by the iScript TM cDNA Synthesis kit, and custom primer sets, Universal Probe Library and iQ Supermix were used. Relative gene expression was calculated using the ΔΔCt method, normalization process was done with the expression data of the 18S ribosomal RNA.

Immunofluorescent staining

Healthy, psoriatic non-involved and involved skin biopsies were cut into 6-μm sections. Primary antibodies: anti-LUC7L3 (1:300), anti-PPIG (1:300) and anti-SFRS18 (1:250). Secondary antibodies: Anti-mouse IgG-Alexa Fluor 647 and anti-rabbit Alexa Fluor 546 at 1:500 dilution as secondary antibodies. Negative staining controls: normal rabbit IgG or incubation without the primary antibody (in case of PPIG). Nuclear staining: 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI). Fluorescent intensity quantification of microscopic images was determinated by ImageJ software.

Culturing of HPV-KER cells

HPV-KER cells were cultured in 75 cm^2 flasks (standard culturing conditions were used) and maintained in 1% antibiotic/antimycotic solution and 1% L-glutamine supplemented keratinocyte serum-free medium. HPV-KER cells were synchronized via contact inhibition and withdrawal of growth factors.

Western blot analysis

Equal amounts of proteins were separated on a 10% SDS-PAGE gel and were transferred to Pure Nitrocellulose Membrane. Primary antibodies: anti-LUC7L3 (1:300), anti-PPIG (1:300), anti-SFRS18 (1:300). Secondary antibodies: anti-mouse and anti-rabbit IgG alkaline phosphate conjugate. Signal visualization: Sigma Fast TM BCIP/NBT. Loading control: α-actin-specific antibody.

Gene specific silencing

HPV-KER immortalized keratinocytes were transiently transfected by scrambled or gene-specific siRNA products at approximately 70% confluency. The most suitable effectiveness was achieved in serum-free culture medium, not containing additives and supplements.

Fibronectin polymerase chain reaction

Human fibronectin specific primers and PCR reactions were carried out as described previously [50]. The results were photographed and evaluated by Bio-Rad Gel Doc XR densitometer.

Flow cytometry

siRNA silenced HPV-KER keratinocytes were collected with careful trypsinization, fixed in Fixation/Permeabilization Concentrate and Diluent and resuspended in phosphate-buffered saline (PBS). Primary antibodies: anti-EDA^{+}-fibronectin (1:500) and anti-fibronectin (1:1000). Secondary antibody: anti-mouse IgG-Alexa Fluor 647 (1:500). Fluorescent detection: FACSCalibur flow cytometer.

Sequencing

Sequencing was carried out on the Illumina HiScan SQ platform. Illumina-compatible ScriptSeq RNA-Seq Library Preparation Kit was used for 2x100 bp paired-end library construction, by sequencing two technical replicates per condition (2 control and 2 siRNA-silenced samples derived from immortalized HPV-KERs).

Statistical analysis and bioinformatics

For the statistical analysis of PCR, densitometry and flow-cytometry mesurements, GraphPad Prism 5.0 Software was used. Predictive interaction analyses were performed in the STRING database. Raw

sequencing reads were submitted to quality control using FASTQC, followed by quality and adapter trimming. Mapping was carried out using the STAR aligner, ambiguously mapping reads were discarded from further analysis. TopHat2 was used for potential fusion transcript discovery using the fusion-search algorithm. Transcript assembly and annotation were carried out using Cufflinks. Count data were then processed using the DESeq and DEXSeq packages to quantify differential gene expression and differential exon usage, respectively. Functional annotation of differentially expressed genes was carried out using Gene Ontology (GO) enrichment, data visualization was carried out in R version 3.0.1, and Cytoscape.

4. RESULTS

SR-rich splicing factor genes exert decreased response to T-lymphokine stimuli in psoriatic non-involved epidermis as compared to healthy samples

Real-time RT-PCR verified the results in the case of both *LUC7L3* and *PPIG*: while their expression is upregulated in healthy epidermis, no changes or even downregulation were observable in psoriatic non-involved epidermis. Although we were unable to validate the microarray results for *SFRS18*, we included this gene in the further

studies, because it is involved in similar biological processes as *LUC7L3* and *PPIG*.

In addition, we decided to compare the mRNA expression levels of the splicing regulators in untreated healthy and psoriatic non-involved skin specimens of the cDNA microarray experiment: in this manner of comparison, the expression of LUC7L3 and SFRS18 was found to be slightly higher in psoriatic non-involved samples than in healthy epidermis, while PPIG did not exhibit any alterations.

LUC7L3, PPIG and SFRS18 show altered expression in psoriasis.

LUC7L3 could be detected in significantly higher amounts in psoriatic non-involved epidermis, whereas SFRS18 presents only minimal, non-significant upregulation in non-involved epidermis. Both LUC7L3 and SFRS18 exhibit the highest levels of expression in psoriatic involved epidermis. Pattern of expression for PPIG was distinct from that of the other splicing regulators, because PPIG exerts a significant decrease in psoriatic non-involved epidermis. However, the highest amount of LUC7L3, PPIG and SFRS18 was detected in psoriatic involved epidermis.

LUC7L3, PPIG and SFRS18 exhibit similar pattern of expression in synchronized, immortalized keratinocytes

Both mRNA and protein expression patterns were characterized in synchronized, immortalized cell lines. During comparison of splicing

factor expression in different states of the proliferation and differentiation, we observed a very similar mRNA and protein expression pattern for LUC7L3, PPIG and SFRS18. As they localized on different chromosomes, genetic linkage is not liable for the experienced similarities. The most plausible explanation is that these splicing factors might share similar upstream regulatory elements.

siRNA silencing of *PPIG*, *SFRS18* and *LUC7L3* gene expression alter the ratio of EDA+/total fibronectin

As former results of the research group suggested that certain fibronectin splicing abnormalities might possess pathogenic role in psoriasis [5], first, we aimed to assess the relevance of LUC7L3, PPIG and SFRS18 in the mRNA maturation processes of this protein.

As other types of undifferentiated cells, HPV-KERs contain higher amount of the EDA+ fibronectin isoform than the EDA- variant. In single silencing experiments, siRNA transfection of *LUC7L3* diminished the relative amount of the EDA+ isoform, and a slight decline in this ratio was also also demonstrated with *PPIG* and *SFRS18* silencing.

Bioinformatics prediction using the STRING database suggests that LUC7L3 and SFRS18 are presumably interactors. In accordance with the predictions, the most robust alteration in the fibronectin EDA+/total ratio was achieved by the combined silencing of *LUC7L3* and *SFRS18*.

Using flow cytometry, we also demonstrated that the double silencing of *LUC7L3* and *SFRS18* significantly decreased the quantity of the EDA+ isoform without changing the amount of total fibronectin. This result refers to an altered mRNA maturation mechanism in the background.

Global transcriptome analysis of immortalized keratinocytes

In our further experiments, we intended to analyze other additional targets of LUC7L3, PPIG and SFRS18 in order to identify biological patways affected by these regulators. For the subsequent experiments, we have chosen the dual silencing of *LUC7L3/SFRS18*, which proved to be most robust in the generation of splicing pattern alterations of fibronectin. This experimental design enables a powerful methodological approach, therefore we carried out RNA-Seq profiling of immortalized HPV-KER cells, which is also capable of the detection of distinct splice variants and non-coding transcripts, as well. We detected suitable silencing efficacies (between ~70-80%.) and excellent RNA quality (RIN: 10)

Paired-end RNA-Sequencing reveals differential expression and exon usage patterns upon the silencing of the LUC7L3 and SFRS18 splicing factors

Sequencing reads of 2x100 bp were mapped to the Hg19 human reference genome, followed by de novo transcript assembly, in order

to identify transcript isoforms expressed in the HPV-KER cell line, and to provide accurate exon-level annotations for downstream analyses. Differential exon usage and differential expression metrics were generated separately, to assess the alterations caused by the dual silencing of splicing factors. Differential exon usage was analysed instead of differential isoform expression in order to circumvent the complexity, and possible errors introduced by isoform reconstruction methods.

Differential gene expression

The combined silencing of the *LUC7L3* and *SFRS18* resulted in moderate changes in gene expression, however extensive disturbances in exon usage. This finding was in accordance with expectations based on the experimental model. Thirty-five protein-coding genes were identified as differentially expressed ones (logFC > 0.5, FDR < 0.05), with IFI6 and MX1, ISG15 and KRT6A mRNAs showing the most robust fold-changes. Regarding the functional enrichment analysis of significantly differentially expressed genes, the majority of *LUC7L3* and *SFRS18* regulated genes share functions related to Type I interferon signaling. The most significant enriched GO terms were cellular response to type I interferon, cellular response to cytokine stimulus, regulation of viral genome replication.

The Real-Time RT-PCR validation of *IFI6*, the most highly expressed gene already known to implicated in psoriasis pathogenesis [16, 17],

displayed up to 4-fold upregulation, in agreement with the high-throughput results, altogether three biological replicates were used for validation.

Differential exon usage

Following *de novo* transcript assembly, the extent of differential exon usage was measured, and indicated changes in 224 exons of 217 genes at a significance cutoff level of logFC >0.5, FDR <0.1. The differential usage of multiple exons per gene is also shown in a considerable amount of genes, possibly indicating fine-tuned transcriptional changes induced by silencing. Differential exon usage was detected in several non-coding RNAs, including NEAT1 and TINCR, a long non-coding RNA implicated in terminal differentiation of keratinocytes [18].

In contrast with the observations on the gene expression level, transcripts regulated with altered splicing pattern did not exhibit extensively shared molecular functions. On the other hand, coordinated transcriptional regulation could be identified by mining co-expression-level databases. The identification of several functional relations between the regulated genes indicate their coordinated transcriptional regulation, and possibly shared post-transcriptional processing. The resulting functional network of transcripts displaying differential exon usage contains 172 genes, represented by nodes, with an average connectivity degree of 13.4, indicating high connectivity

of the network. The majority of connecting edges between genes correspond to co-expression relations, followed by physical and genetic interactions.

Among differential exon usage detections, we confirmed the decreased inclusion of the EDA-domain, which agrees well with prior results. In addition, splicing alterations of the transcription element *CREB1*, along with *HERC6* and *CUL1*, which are implicated in ubiquitination [19, 20], were also identified.

CONCLUSION

In recent years, we gained a deeper insight into psoriasis pathogenesis. Much of our current knowledge based on wide-spread application of high-throughput methods, including cDNA microarrays and RNA-Seq, however, the initiation phase of the disorder is still only partially uncovered. In our recent microarray study, three splicing regulators - luc-7 like protein 3 (*LUC7L3*), peptidyl-prolyl cis-trans isomerase G (*PPIG*) and arginine/serine-rich 18 (*SFRS18*) - have been identified, which may be accountable for the pathogenic response of keratinocytes to T-lymphokine stimuli. Subsequent characterization of expression revealed the definite presence of splicing regulators in immature keratinocytes of cell lines and psoriatic epidermis, suggesting their multiple involvement in disorder pathogenesis.

Furthermore, *LUC7L3, PPIG* and *SFRS18* might share common upstream regulatory elements.

It was also proved that SR-rich splicing factors function as regulators of the fibronectin mRNA maturation, and facilitate inclusion of the disease-associated EDA domain. In this process, a synergistic interaction has been confirmed for LUC7L3 and SFRS18. Thus, the subsequent RNA-Seq experiment on *LUC7L3/SFRS18* double silenced cells showed the influence of splicing factors on several well-known psoriasis-associated pathways, especially IFN signaling, antiviral immunity, and ubiquitination. These could be responsible for the balancing between pro- and antiapoptotic events in keratinocytes, determine keratinocyte differentiation as in case of lncRNA TINCR, and could be part from multiple feed-back loops, responsible for the maintenance of molecular discrepancies seen in the disease. These findings are of special interest considering that interferon-related molecular abnormalities are not clarified yet in detail, however, their role in disease pathogenesis besides TNF-α and the IL17/IL23/IL22 axis is undoubted. LUC7L3, PPIG and SFRS18 might be contributors of disorder initiation, but presumably also participate in the establishment phases.

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LIST OF PUBLICATIONS

Publications directly related to the subject of the thesis:

I. Szlavicz E, Szabo K, Groma G, Bata-Csorgo Z, Pagani F, Kemeny L, Szell M (2017) Analysis of psoriasis-relevant gene expression and exon usage alterations after silencing of SR-rich splicing regulators. Article submitted to Experimental Dermatology, under review. **IF: 2,68**

II. Szlavicz E, Szabo K, Groma G, Bata-Csorgo Z, Pagani F, Kemeny L, Szell M (2017) Splicing factors differentially expressed in psoriasis alter mRNA maturation of disease-associated EDA+ fibronectin Molecular and Cellular Biochemistry, doi: 10.1007/s11010-017-3090-1 **IF: 2,67**

III. Szlavicz E, Szabo K, Bata-Csorgo Z, Kemeny L, Szell M (2014) What have we learned about non-involved psoriatic skin from large-scale expression studies? World Journal of Dermatology, 3(3):50-57. doi: 10.5314/wjd.v3.i3.50

Publications not related to the subject of the thesis:

I. Szlavicz E, Perera PS, Tomboly C, Helyes Z, Zador F, Benyhe S, Borsodi A, Bojnik E (2015) Further Characterization of Hemopressin Peptide Fragments in the Opioid and Cannabinoid Systems. Anesthesia and Analgesia, 121(6):1488-94. doi: 10.1213/ANE.0000000000000964. **IF: 3,83**

II. Zador F, Samavati R, Szlavicz E, Tuka B, Bojnik E, Fulop F, Toldi J, Vecsei L, Borsodi A (2014) Inhibition of opioid receptor mediated G-protein activity after chronic administration of kynurenic acid and its derivative without direct binding to opioid receptors. CNS Neurol Disord Drug Targets. 13(9):1520-9. doi: 10.2174/1871527314666141205164114 **IF: 2,6**