INVESTIGATIONS ON mRNA MATURATION DISTURBANCIES IN THE PATHOGENESIS OF PSORIASIS

Ph.D. Thesis

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Szeged
2017

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: 2,63**

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LIST OF ABBREVIATIONS

IIICS: type III connecting segment

CCL: chemokine ligand

CI: confidence interval

CRE: cAMP responsive element

CUL1: cullin-1

DC: dendritic cell

DEG: differentially expressed genes

DMEM: Dulbecco's modified Eagle's medium

EDA: extra domain A

EDB: extra domain B

FACS: fluorescence-activated cell sorting

FC: fold-change

FN1: fibronectin

GO: gene ontology

GM-CSF: granulocyte-macrophage colony-stimulating factor

GWAS: genome wide association studies

HERC: HECT and RLD domain containing

HLA: human leukocyte antigene

HPV: human papilloma virus

IFI: interferon-induced

IFN: interferon

IL: interleukin

ISGs: interferon-stimulated genes

KER: keratinocyte

KGF: keratinocyte growth factor

lncRNAs: long non-coding RNAs

LUC7L3: luc-7 like protein 3

MHC: major histocompability complex

NBEAL1: neurobeachin like 1

NEAT1: nuclear paraspeckle assembly transcript 1

OAS2 2'-5'-oligoadenylate synthetase 2

PBS: phosphate-buffered saline

PPIG: peptidyl-prolyl cis-trans isomerase G

PASI: psoriasis area and severity index

PNISR: PNN interacting serine and arginine rich protein

PNN: pinin

RIN: RNA integrity number

RNA-Seq: RNA-Sequencing

RT-PCR: reverse transcription polymerase chain reaction

SDC4: syndecan-4

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE: standard error

SFM: serum-free medium

SFRS18: splicing factor, arginine/serine-rich 18

siRNA: small interfering RNA

SRPK: serine/arginine-rich splicing factor kinase

STAT1: signal transducer and activator of transcription 1

TGF-β: transforming growth factor beta

Th: T-helper

TINCR: tissue differentiation-inducing non-protein coding RNA

TLR: toll-like receptor

TNC: tenascin-c

TNF: tumor-necrosis factor

WGCNA: weighted correlation network analysis

1. INTRODUCTION

1.1 Etiology and clinical characteristics of psoriasis

Psoriasis is one of the most common chronic inflammatory skin disorders, affecting appr. 2% of the population. In Europe, its prevalence is between 1,6% and 4,8% [1, 2]. Considering disorder onset, a dual peak can be observed: approximetely 75% of patients are under the age of 40 years (type I), and usually exhibit a more serious prognosis in contrast to late onset cases (type II, between ages 55-60) [3]. Psoriasis vulgaris is the most frequent manifestation, which accounts for appr. 80-95% of all cases; the characteristic lesions of the disorder are the erythematosus, scaly, demarcated and squamousus plaques, which could cover predominantly the skin of knees, elbows and the scalp (Fig. 1., a-c). However, multiple clinical forms exist besides the well-known plaque-type disorder, with various morphology and localization of the symptoms: the different phenotypes consist of guttate, pustular (acrodermatitis continua of Hallopeau, palmoplantar and general pustulosis), inverse and erythrodermic psoriasis [1, 4, 5]. Cutaneous histopathological features of the symptoms are also typical (Fig. 1. d-e), containing hyper- and parakeratosis, acanthosis, dilatation of blood vessels and collection of neutrophil granulocytes (Munro's microabscesses) [5, 6].

The negative impact of psoriasis on life quality is self-explanatory, and there is still a discussion whether it should be considered a skin or a systematic disorder. Joint involvement is present in a significant number of cases (approximatly one fifth of patients, between 10-34%), moreover, cardiovascular comorbidities are also frequent in psoriasis, and several psoriatic patients suffer from the consequences of metabolic syndrome [2, 4, 7]. Assocation with immune-mediated, autoimmune disorders, including inflammatory bowel disease, Hashimoto's thyreoditis and multiple sclerosis has been also reported [7]. In addition, psoriasis is associated with serious psychosomatic stress, and concomitant psychiatric diseases, such as anxiety and depression, the latter one affecting appr. 60% psoriatic cases [4, 8, 9].

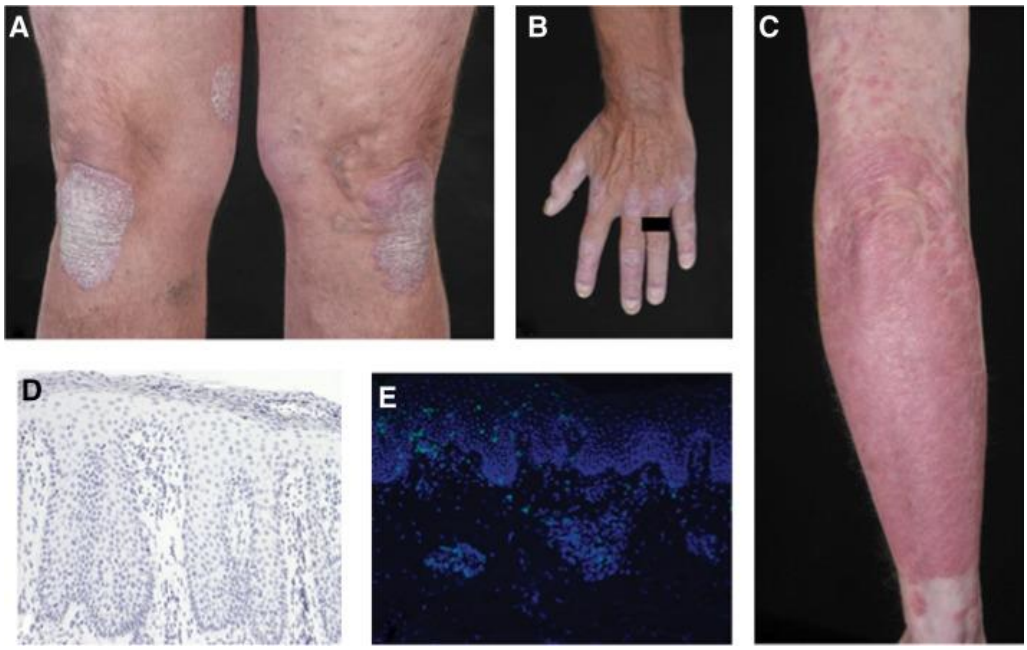


Fig. 1. Characteristic clinical presentations of psoriasis, including psoriatic plaques and nail involvement (a-c). Histopathologic features include acanthosis, papillomatosis and Munro's microabscesses (d) and the infiltrates of CD3+ T-lymphocytes (e) (Di Meglio et al., 2013)

Regarding disorder development, we should emphasize a multifactorial origin, in which genetic and environmental factors are also determinant. Twin studies shed light on the importance of genetic susceptibility of psoriasis and reported 2-3,5 fold higher risk for monozygotic twins as compared to dizygotic ones [1, 5]. Classic genome-wide linkage analysis and Genome Wide Association Studies (GWAS) provide valuable data about the genetic loci predisposing to psoriasis development. According to our current understanding, a major portion (approximately 35-50%) of heritability is related to the *PSORS1* loci, which includes genes of the major histocompability complex (MHC), encoding the human leukocyte antigens (HLAs) [1]. It is well documented, that within the MHC, the HLA-C*06:02 allele presents the greatest risk for psoriasis, accompanied by more serious manifestation and early disease onset [1, 4, 10, 11]. Besides *PSORS1*, numerous other genetic susceptibility factors have been described, also containing keratinocyte-derived and both adaptive and innate immunity-related components, however the majority of publications suggest that the contribution of these genomic regions to psoriasis formation are relatively modest [4, 5, 11]. On the other hand, rare mutations of the

IL36RN and *CARD14* genes could result in considerable effects [6]. Among environmental triggers, the involvement of mechanical trauma, infections, certain medications, UV-exposure, smoking, stress and some alimentary factors are the most notable ones. The Köbner phenomenon demontrates the importance of mechanical distress as a promoter of skin symptoms [1, 4, 12, 13, 14, 15].

1.2 Molecular background of psoriasis pathogenesis

To date, conceptions of psoriasis underwent serious changes: the current reports emphasize the critical role of the IL23/IL17/IL22 axis and TNF-α, as opposed to the previous idea that psoriasis is a Th1 and IFN-γ dominated disorder [4, 16, 17, 18, 19]. Their importance was also supported by studies specifically targeting these cytokines [6, 19, 20, 21, 22, 23]. However, the molecular background of psoriasis is quite complex, positive and negative feedback mechanisms and amplification signals are substantial in the disorder pathogenesis. Thus, psoriasis cannot be characterized by a linear pathway, as interactions of cytokines constitute a rather extensive, self-sustaining network, where either innate or adaptive immune system is involved, and the reciprocal actions of keratinocytes, different types of dendritic cells and T-lymphocytes, as well as other inflammatory cellular elements account for layers of molecular complexity [5, 6]. Moreover, cytokine products showing a characteristic distribution during the disease course, while the disorder initiation is dominated by a Th1 profile, in the maintained chronic inflammatory phase, Th17 cytokines gain an emerging importance [4, 5].

Despite the advances in the field of psoriasis research, molecular triggers and the mechanism of disorder initiation are still only partially understood, and many questions remain that need to be answered [6]. Literature data support that the activation of a circulatory dendritic cell (DC) subset, the plasmacytoid DCs could be a determinant event in the early pathogenic steps. In healthy skin, pDCs are almost absent, and they are stimulated via their TLR7/9 receptors, which interact with the complexes of the self DNA/RNA derived from stressed keratinocytes and cathelicidin (LL37), one of the so-called antimicrobial peptides (AMPs) [4, 24, 25, 26]. AMPs are cationic antimicrobial peptides, their release from keratinocytes are critical components of

the innate immune response in psoriasis. Besides LL37, S100 proteins are also important in psoriasis pathogenesis, since they amplify inflammatory processes due to activation of TLR receptors and the chemotactic feature [6, 27, 28].

In the further pathogenic steps, the stimulated pDCs release type I interferons [4, 26, 29]. Type I interferons along with the keratinocyte-derived mediators such as IL-1β, IL-6 and TNFα facilitate maturation and activation of the myeloid dermal dendritic cells (DDCs) [4, 19, 20, 26]. Furthermore, this step provides the connection of innate and adaptive immune processes, since DDCs are the producers of IL12, which stimulates the Th1 subset of keratinocytes and leads to the release of IFN-γ. On the other hand, another important DDC and macrophage-derived cytokine, IL23 triggers the Th17 and Th22 T-cell lineages [1, 4, 19]. It is well-documented, that in the interaction of DDCs and T-cells, presence of TNF-α is essential [6, 20]. The Th17 products promote T-cell (CCL20) and neutrophil (CXCL1, CXCL2, CXCL5, CXCL8) recruiting factors; LL37 and S100 protein production is also facilitated, perpetuating the inflammatory circuit [4, 30]. In addition, IL22 induces the psoriasis-specific accumulation of the innate keratinocytes [6, 31].

A schematic of the pathogenic events is demonstrated in Fig. 2. Besides the listed pathogenic events, autocrin loops, recruitement of tissue-resident memory T cells and release of angiogenic/vasoactive mediators (leading to punctual bleedings after removement of scales, called the Auspitz-sign) are also important components of the disorder development [4, 5, 19, 32, 33, 34]. In summary, as a consequence of the above detailed molecular processes, the turnover time of keratinocytes shows considerable accelaration compared the normal approximately 40 days, and the immature psoriatic keratinocytes reach the skin surface altogether in 6-8 days [10, 35]. The altered response of keratinocytes to the T-cell signals is presumably an essential factor of psoriasis pathogenesis, however, the exact mechanism of disorder initiation is still unclear.

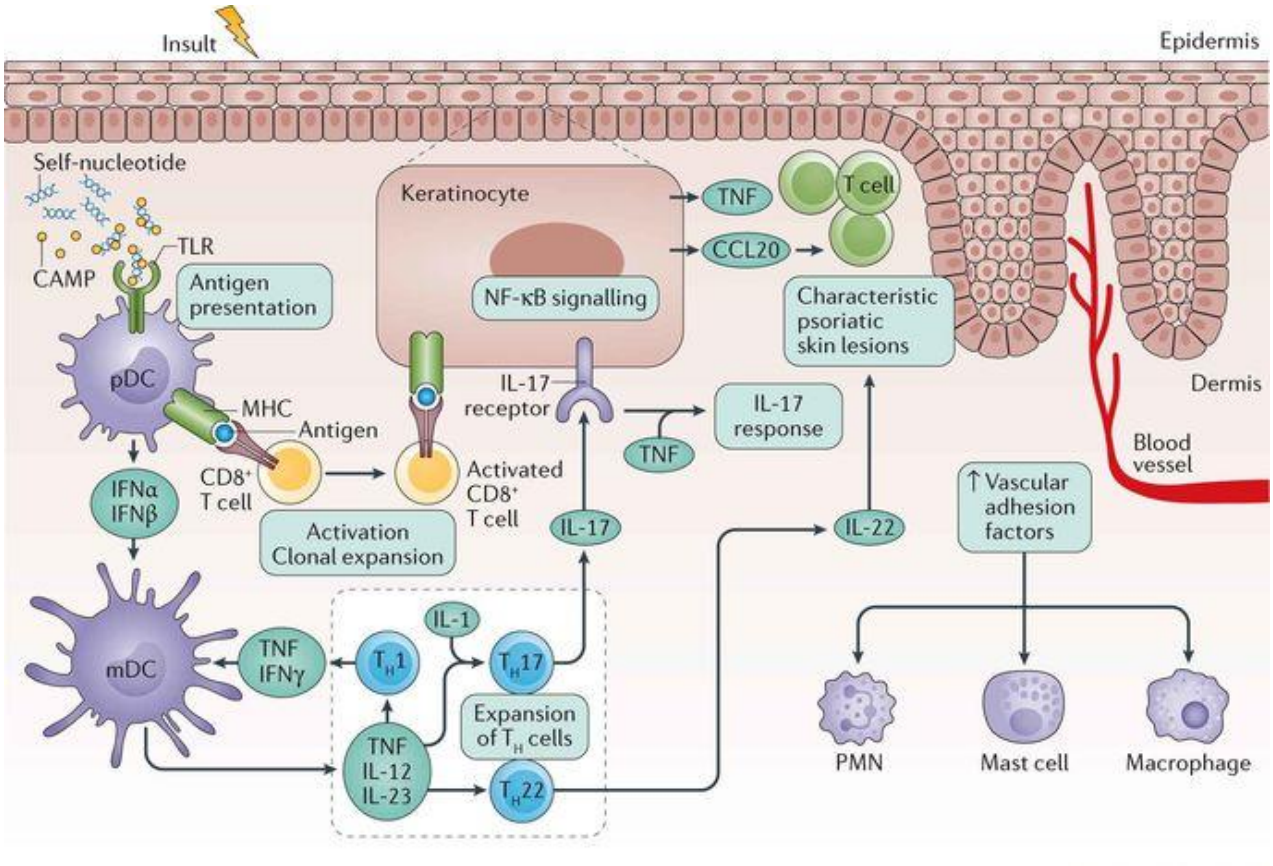


Fig. 2. Summary of initiatory pathogenic steps and the molecular background of the established psoriasis disorder (Greb et al., 2017)

1.3 EDA+ fibronectin and its potential impact in psoriasis pathogenesis

To date, relatively few studies have been conducted on mRNA maturation disturbances in psoriasis. One of the first reports established that the extracellular matrix protein tenascin-C showed differences in the inclusion of fibronectin type III fibronectin modules. The study found that the larger tenascin-C variant is more abundant in both epidermal keratinocytes and fibroblasts, altough the relative amount of the smaller transcript is higher in epidermal keratinocytes as compared to the fibroblasts. IFN-γ, TNF-α and IL4 have been described as regulators of the splicing process, and protein-level distribution of tenascin-C splice variants was also examined in healthy skin, wounds, psoriatic lesions and epidermal tumours [36]. Moreover, in the study of Ting *et al.* altered splicing patterns for another type III fibronectin

module were demonstrated: it was shown that EDA+ domain of the extracellular matrix protein fibronectin is overexpressed in psoriatic non-involved epidermis [37]. The study suggested that EDA+fibronectin might be a putative factor which makes epidermal keratinocytes prone to the proliferative stimuli of the professional immune cells.

Although fibronectin is a suitable model for analyzing mRNA maturation processes, the complete mechanism of splicing regulation as well as the EDA+ domain inclusion are not fully revealed. The production of splice variants has been shown to be an important point of regulation for several genes, including fibronectin, which produces at least 20 mRNA variants as a result of alternative splicing [38, 39, 40]. As we mentioned previously, the EDA domain belongs to the Type III repetitive modules (Fig. 3.) of fibronectin, together with EDB and Type III connecting segment (IIICS) [38, 39, 41]. The EDA module is excluded from plasma fibronectin, whereas cellular fibronectin could include variants with the EDA domain [41, 42]. α5β1 integrin is the classic receptor for fibronectin, but splicing events could impact the fibronectin related signalling: in case of the EDA+ variant, TLR4 binding is well-established [41, 42, 43, 44, 45, 46].

EDA+ fibronectin is typically present during embryonic development, nevertheless, there is a body of evidence indicating that this splice variant plays an important role in wound healing, inflammatory, angiogenic and malignant processes in adults [38, 47, 48, 49]. Besides the results of Ting *et al.*, our research group has also demonstrated that EDA+ fibronectin could play a crucial role in psoriasis pathogenesis: we found that proliferating keratinocytes (both normal cultured keratinocytes and HaCaT cells) are able to produce this isoform [37, 50]. In addition, it has also been established that EDA+ fibronectin expression was modest in healthy keratinocytes whereas keratinocytes from psoriatic non-involved epidermis proved to be effective producers of the splice variant. The ratio of the normal/oncofetal fibronectin isoforms depends on the proliferation and differentiation states, and a peak of total and EDA+ fibronectin expression was observable in highly proliferating cells. These data supported the theory that EDA+ fibronectin promotes the sensitization of keratinocytes to the mitogenic stimuli of professional immune cells [50].

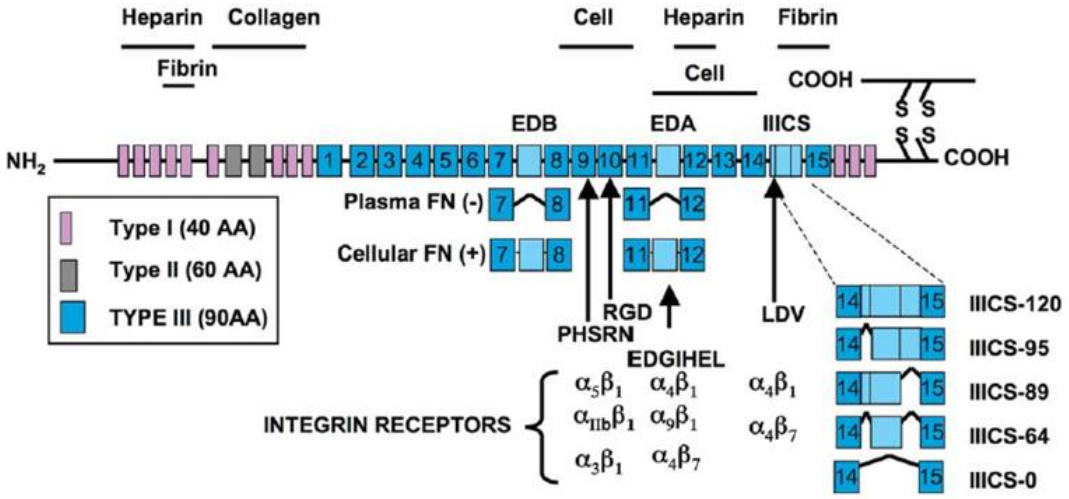


Fig. 3. Structure of fibronectin gene. The EDA+ domain belongs to the type III repetative moduls, such as EDB and Type III connecting segment (IIICS) (White et al., 2008).

1.4 High-througput methods in the identification of psoriasis-related signalling mechanisms

In recent years, several powerful methods have been developed for large-scale gene expression profiling. Among these tools, the most widespread were the cDNA microarrays and via the evolution of next-generation sequencing, pair-end RNA-Sequencing gained an emerging importance in experimental dermatology as well [51, 52, 53, 54].

The recent cDNA microarrays provided remarkably valuable information about the gene expression profiles of healthy, psoriatic non-involved and involved epidermis and they were also able to verify discrepancies related to the innate and adaptive immune responses. Besides altered immune functions, these studies emphasized the pathogenic role of epidermal keratinocyte proliferation, apoptosis and lipid metabolism related genes. Considering immunological processes, the importance of the proinflammatory T-cell lymphokine products such as INF-γ and IL17, or genes encoding chemokines and their receptors was also demonstrated in psoriasis development by the recent cDNA microarray experiments [55, 56,

57, 58, 59, 60]. Wnt pathway was found to be significantly elevated in the involved epidermis compared to non-involved samples, verifying the effects on cellular proliferation and differentiation [61]. In addition, other data supported the involvement of mTOR, NFκB, BCL-2 and BAX [62, 63]. PPARδ, the inflammatory and metabolic process-related transcription factor, is also implicated in the induction of keratinocyte proliferation, and exhibits proangiogenic effects as well [64]. Other research groups have found down-regulation of lipid metabolism-related pathways in non-involved samples and further downregulation was observed in the involved skin [58]. Considering that the results of former cDNA microarrays show distinct variance across the experiments, meta-analyses of the available datasets were also carried out. These studies identified among the most significant molecular pathways cellular proliferation/differentiation and immunomodulation, respectively. Moreover, meta-analyses confirmed the importance of metabolic disturbances, including atherosclerosis-related molecular abnormalities and transcripts associated with impaired glucose tolerance [65, 66].

Deep RNA-Sequencing harbors numerous advances as compared to microarrays, and eventually were put to general use in psoriasis research as well. In contrast to limitations of microarrays, RNA-Seq generates relatively low background noise and has a wide dynamic range, in addition, it does not require known template sequences to transcript abundance identification [54, 67]. One of the first reports was published by Jabbari and co-workers, where three pairs of psoriatic non-involved and involved biopsies were compared, and at the same time, the samples underwent microarray hybridization, as well. Their results indicated that RNA-Seq is capable of identifying low abundance transcripts, thus, a larger set of differentially expressed transcripts (DEGs) could be detected as compared to microarrays [67]. Li et al. worked with a larger sample size as they sequenced polyadenylated RNA of 92 psoriatic patients and 82 healthy volunteers. Consistent with previous microarray data, they could display up-regulation of inflammatory response-related genes, including Th17 associated genes and IFN-γ; and the upregulation of cell proliferation, and keratinization was also supported by this study [68].

Due to advances of RNA-Seq technology, splicing pattern detection and measurement of non-coding transcript abundance are also available. Li et al. has already reported differential usage of 343 exons among healthy and psoriatic non-involved samples [68]. In a recent report, a different approach was used and compared the abundance of distinct RNA isoforms, identifying

more than 9000 differential RNA isoforms, which are present in different amount between healthy, psoriatic non-involved and involved epidermis [69]. Considering non-coding transcripts, Antonini et al. introduced the most important long non-coding RNAs (lncRNAs) identified in the course of dermatological research using high-throughput RNA-Sequencing and they also discussed the advantages and limitations of the examinations of lncRNAs [70].

1.5 Identification of SR-rich splicing regulators

The vast majority of former cDNA microarray experiments analyzed the steady-state gene expression pattern of two or three disctinct states, which includes healthy, psoriatic non-involved and involved epidermis samples. Paralelled with other works published in the same field, the approach of our research group was quite different. The aim of our cDNA microarray study was the examination of the T-lymphokine induced gene expression changes, considering that altered keratinocyte responsiveness of psoriatic non-involed epidermis is essential in the early pathogenesis of psoriasis [71].

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 [72]. In the following analysis, a comparison has been made between the autologuous pairs of untreated and treated samples. After that, differentially expressed transcripts which showed a fold change >2 have been selected for further characterization and a comparisons have been made to identify differentially regulated genes among healthy and psoriatic non-involved epidermis samples [71]. To facilitate better understanding, the experimental design and comparisons applied during evaluation of the cDNA microarray are illustrated in a flow-chart (Fig. 4.).

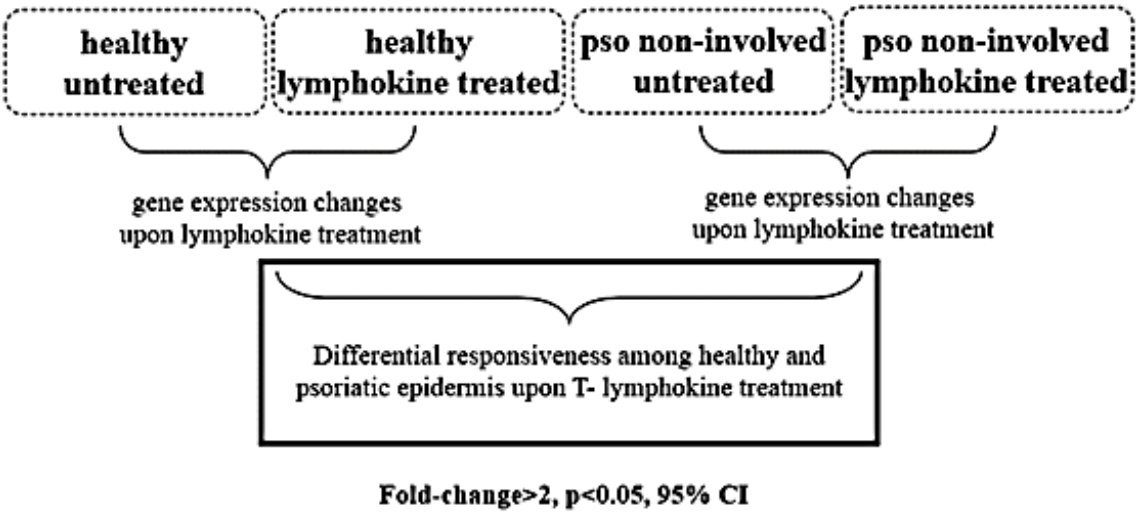


Fig. 4. Experimental set-up and the evaluation process of cDNA microarray results

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. In addition, we could demonstrate by *in silico* analysis using publicly available databases, that the most influenced cellular processes are apoptosis and the small-lipid metabolism [71]. 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*), peptidyl-prolyl cis-trans isomerase G (*PPIG*) and arginine/serine-rich 18 (*SFRS18*), differentially regulated among healthy and psoriatic non-involved epidermis, therefore they could contribute to the responsiveness changes of keratinocytes. Since mRNA maturation disturbances of psoriasis were a limitedly studied topic so far, and our previuos results regarding the EDA+ fibronectin indicated that certain splicing abnormalities might contribute to psoriasis pathogenesis, we decided to perform further experiments related to the identified splicing factors.

2. 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

3. MATERIALS AND METHODS

3.1 Skin biopsies

Previous cDNA microarray experiments were performed on organotypic skin cultures, which were established from tissue samples of 4 healthy volunteers and 4 psoriasis patients (age 18–60 years). Patients enrolled in the study suffered from moderate-to-severe chronic plaque-type psoriasis, in each case Psoriasis Area and Severity Index (PASI) score was calculated by dermatologists. Another criterion for enrollement was a medication-free period (≥ 2 weeks without local therapy and/or ≥ 4 weeks without systemic therapy). Tissue biopsies were obtained after the informed consent of participants. Tissue collection was carried out according to the guidelines of the Declaration of Helsinki and was approved by the Regional and Institutional Research Ethics Committee (2799, 3517).

3.2 Organotypic skin cultures

Organotypic skin cultures were generated from half cuts from shave biopsies [71]. First, epidermis samples were placed on 2.2 μm porosity cellulose acetate/cellulose nitrate filters (Millipore) and after that, they were transferred to a stainless steel grid platform, in a 6-well plate. Dulbecco's modified Eagle's medium (DMEM) was used as culture medium, which was supplemented with 12 mM glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), all of them were obtained from Life Technologies (Carlsbad, CA). In case of all donor skin specimens, a lymphokine mixture was added to one half of the organotypic cultures containing of 1 ng/ml IFNγ, 1 ng/ml GM-CSF and 0.3 ng/ml IL-3. The other half of the samples remained untreated. In order to the maintain organotypic skin cultures, standard culturing conditions were applied (at 37°C in a 5% CO_2 atmosphere), at the air/liquid interface for 72 h [71, 72]. Following these steps, separation of the epidermis from the dermis was executed by overnight incubation in Dispase solution (grade II, Roche Applied Science) at 4°C, finally, the samples were placed in TRIreagent (Molecular Research Center Inc., Cincinnati, OH).

3.3 Real-time RT-PCR

Total RNA was purified from the organotypic skin cultures, and immortalized cell lines, such as HPV-immortalized keratinocytes (HPV-KER), HaCaT cells, respectively. One microgram of total RNA was reverse transcribed by the iScript TM cDNA Synthesis kit (#1708891, Bio-Rad, Hercules, CA), based on the protocol of manufacturers. To determine the transcript abundance, Real-Time RT-PCR was performed using custom primer sets, Universal Probe Library (Roche, Basel, Switzerland) and iQ Supermix (#1708862, Bio-Rad, Hercules, CA). Relative gene expression was calculated using the ΔΔCt method, normalization process was done with the expression data of the 18S ribosomal RNA.

3.4 Immunofluorescent staining

Biopsies from healthy, psoriatic non-involved and involved skin were frozen, embedded, and stored at –80 °C, before cutting into 6-μm sections. Primary antibodies used during immunfluorescent stainings were as follows: anti-LUC7L3 (1:300, Abcam, Cambridge, UK), anti-PPIG (1:300; Abcam) and anti-SFRS18 (1:250, Novus Biologicals, Littleton, USA). In addition, we used Anti-mouse IgG-Alexa Fluor 647 and anti-rabbit Alexa Fluor 546 at 1:500 dilution as secondary antibodies (Life Technologies, Carlsbad, CA). For negative staining controls, sections were incubated with normal rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA) or without the primary antibody (in case of PPIG). Nuclear staining was carried out with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI; Sigma-Aldrich, St Louis, MO, USA). Fluorescent intensity quantification of microscopic images was performed using ImageJ software: during evaluation, corrected-total-cell-fluorescence values were calculated based on integrated density of stained cell areas.

3.5 Culturing of HPV-KER and HaCaT cells

The HPV-KER cell line immortalization was achieved by the HPV E6 oncogene, description was provided by Polyanka et al., 2011 [73]. HPV-KER cells were cultured in 75 cm^2 flasks (culturing conditions: 37 °C; humidified atmosphere containing 5% CO2) and maintained in

1% antibiotic/antimycotic solution (PAA, Pasching, Austria) and 1% L-glutamine (PAA) supplemented keratinocyte serum-free medium (Gibco® Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark). The medium of cells was changed every two days. The synchronization process was as follows: after confluence was obtained, the HPV-KER cells were maintained for one week in glutamine- and growth factor-free keratinocyte SFM. Cells were harvested with brief trypsinization (0.25% trypsin solution Sigma Aldrich, St. Louis, MO, USA). HaCaT cells were kindly provided by Dr. Fusenig, N.E. (Heidelberg, Germany). Cells were cultured and synchronized as described previously [74].

3.6 Western blot analysis

Equal amounts of proteins were separated on a 10% SDS-PAGE gel and were transferred to Pure Nitrocellulose Membrane (Bio-Rad Laboratories) in the course of western blot analysis. Primary antibodies used in our experiments and their dilutions: anti-LUC7L3 (1:300, Abcam, Cambridge, UK), anti-PPIG (1:300, Abcam), anti-SFRS18 (1:300, Novus Biologicals, Littleton, US). As secondary antibodys, anti-mouse and anti-rabbit IgG alkaline phosphate conjugate (Sigma Aldrich, St. Louis, MO, USA) antibodies were applied, and signal visualization was done with Sigma Fast TM BCIP/NBT (Sigma Aldrich, St. Louis, MO, USA). α-actin-specific antibody was provided as a loading control (1:2000, Sigma Aldrich, St. Louis, MO, USA).

3.7 Gene specific silencing

siRNA mediated transfection method was used to achieve gene-specific silencing. HPV-KER immortalized keratinocytes were transiently transfected by siRNA products at approximately 70% confluency. Reagents for silencing (medium and transfection reagent) were ordered from Santa Cruz Biotechnology (Dallas, TX, USA). In the experiments, scrambled and gene-specific LUC7L3, PPIG, SFRS18 siRNA duplexes were also applied. The most suitable effectiveness was achieved in serum-free culture medium, which does not contain additives and supplements. The silencing efficacy was controlled by real-time RT-PCR.

3.8 Polymerase chain reaction

Total RNA collected from siRNA transfected HPV-KERs was isolated from the keratinocyte cultures by TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA), following the instructions provided in the manual. cDNA synthesis was accomplished from 1 μg total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Human fibronectin specific primers were applied for the amplification of an 847 bp product from the EDA-, and a 1221 bp product from the EDA+ fibronectin template. PCR reactions were carried out as described previously, and semiquantitative detection of the differentially spliced fibronectin isoforms are also detailed in this report [50]. To the analysis, 10μl of the PCR products were run on 1% agarose gel at 90 V, and after that, the results were photographed and evaluated by Bio-Rad Gel Doc XR densitometer.

3.9 Flow cytometry

siRNA silenced HPV-KER keratinocytes were collected with careful trypsinization (0.25% trypsin solution Sigma Aldrich, St. Louis, MO, USA), fixed in Fixation/Permeabilization Concentrate and Diluent (eBioscience, San Diego, USA) and resuspended in phosphate-buffered saline (PBS). The anti-EDA^+-fibronectin and anti-fibronectin primary antibodies were used for 45 min at dilution 1:500 and 1:1000, respectively (both purchased from Sigma Aldrich, St. Louis, MO, USA). The PBS-washed keratinocytes were then incubated with the secondary antibody (anti-mouse IgG-Alexa Fluor 647, dilution of 1:500; Life Technologies, Carlsbad, CA). Fluorescent detection was executed on a FACSCalibur flow cytometer equipped with 488 and 633 nm lasers (Becton–Dickinson, Franklin Lakes, NJ, USA)

3.10 Sequencing

Sequencing was carried out on the Illumina HiScan SQ platform in the Center for Clinical Genomics and Personalized Medicine, University of Debrecen. Illumina-compatible ScriptSeq RNA-Seq Library Preparation Kit (Epicenter) 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). The average sequencing depth was ~35 million raw reads per sample.

3.11 Statistical analysis and bioinformatics

For the statistical analysis of the results from polymerase chain reactions, densitometry and flow-cytometry mesurements, GraphPad Prism 5.0 Software (GraphPad Prism, San Diego, CA) was used, using one-sample *t*- and unpaired *t*-tests as necessary, with $p<0.05$ as the threshold of statistical significance. Predictive interaction analyses were performed in the publicly available STRING database (http://string-db.org/). FASTQ format raw reads were submitted to quality control using FASTQC, followed by quality and adapter trimming using the fastx toolkit. The Hg19 human genome reference was used for mapping filtered reads with the STAR aligner, using parameters optimized for splice-site discovery [75]. Subsequently, alignment to potential contaminant sequences and the HPV genome were carried out, and ambiguously mapping reads were discarded from further analysis. TopHat2 was used for potential fusion transcript discovery using the fusion-search algorithm [76]. Transcript assembly and annotation were carried out using Cufflinks (Trapnell et al., 2010) after mapping, and read counts were summarized at the gene and exon levels by using htseq-count [78]. Count data were then processed using the DESeq and DEXSeq packages to quantify differential gene expression and differential exon usage, respectively [79]. Exons with a coverage <5 reads were discarded from the analysis. Functional annotation of differentially expressed genes was carried out using Gene Ontology (GO) enrichment, and extensive comparisons with previous literature-based gene sets and data visualization were carried out in R version 3.0.1, and Cytoscape.

4. RESULTS

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

In the course of our previous cDNA microarray experiment, 61 previously annotated genes and additional 11 transcipts were found to be differentially regulated in psoriasis upon T-lymphokine treatment [71]. The subsequent Gene Ontology Analysis revealed three splicing regulators among the identified genes: luc-7 like protein 3 (*LUC7L3*), peptidyl-prolyl cis-trans isomerase G (*PPIG*) and arginine/serine-rich 18 (*SFRS18*). Real-time RT-PCR was performed to control whether the identified genes showed an altered responsiveness due to T-lymphokine induction. The lymphokine-stimulated changes were verified 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 (Fig. 5.) 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*.

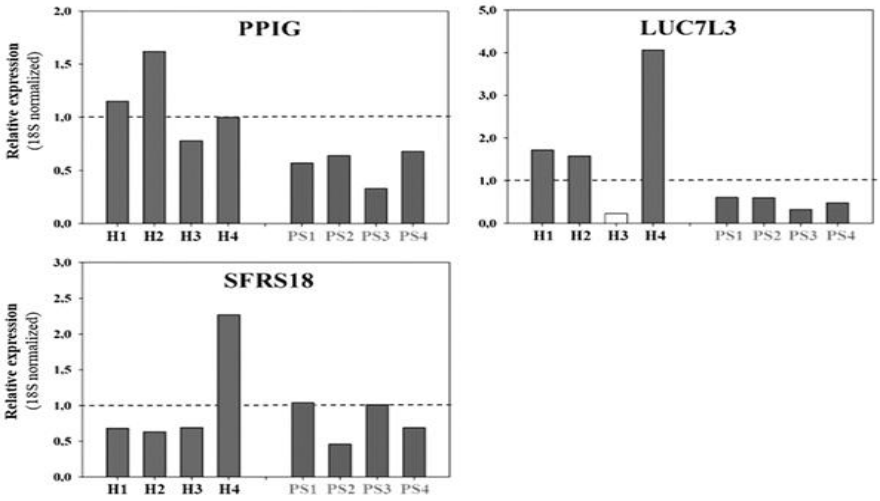


Fig. 5. T-lymphokine induced altered gene expression of splicing factors was compared in healthy (H, n=4) and psoriatic non-involved (PS, n=4) epidermis. As baseline, the average expression of untreated samples was considered (relative unit of 1 indicated by dashed line).

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 (Fig. 6.).

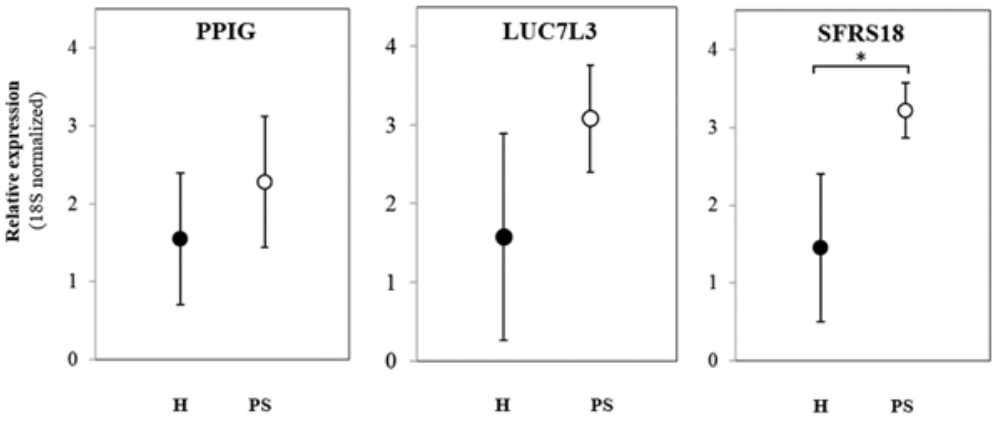
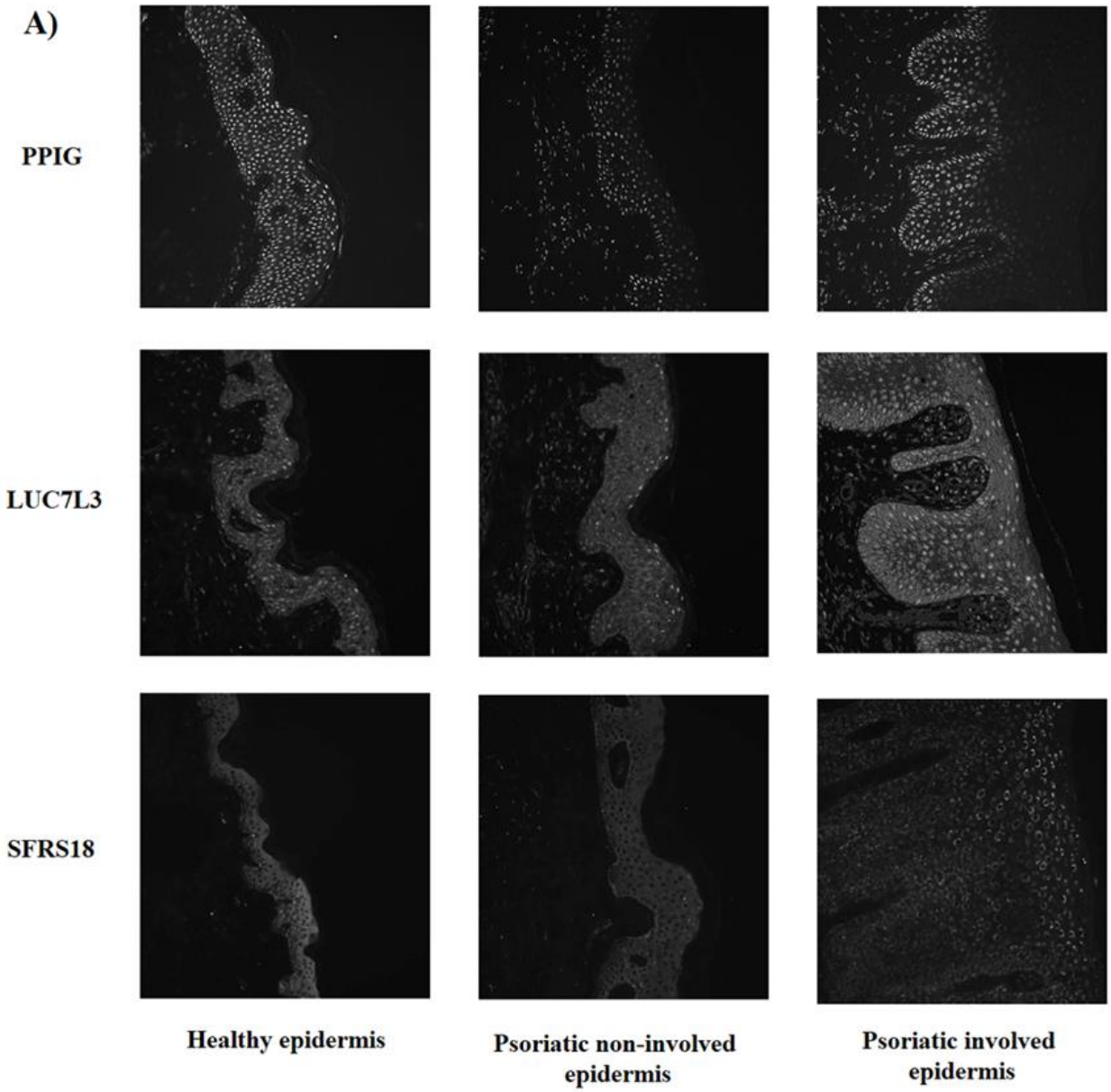


Fig. 6. LUC7L3, PPIG and SFRS18 basal mRNA expression in untreated healthy (H, n=4) and psoriatic non-involved (PS, n=4) samples. P<0.05 was considered as significance treshold

4.2 LUC7L3, PPIG and SFRS18 show altered expression in psoriasis.

Since LUC7L3, PPIG and SFRS are poorly characterized, we investigated their protein expression pattern among healthy, psoriatic non-involved and involved skin samples. LUC7L3 and PPIG exerted a nuclear localization while staining for SFRS18 was rather perinuclear (Fig. 7. a). 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 (Fig. 7. b.).


A)
PPIG
LUC7L3
SFRS18
Healthy epidermis
Psoriatic non-involved epidermis
Psoriatic involved epidermis

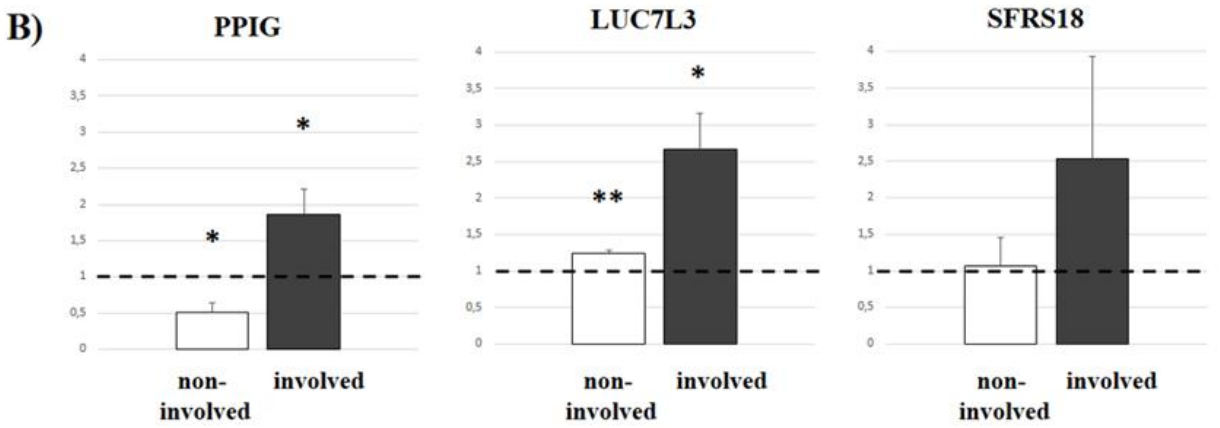

B)
PPIG
LUC7L3
SFRS18
non-involved
involved
*
**

Fig. 7. Immunofluorescent staining of PPIG, LUC7L3 and SFRS18 proteins (a) were performed on healthy, psoriatic non-involved and involved epidermis (n=3, in all cases). For each protein, a representative staining is introduced. Magnification: 40x. Fluorescent intensity detection of tissue stainings were also performed (b) and non-involved and involved samples were compared to healthy specimens: dashed lines indicates the unaltered condition, where the ratio of non-involved/healthy and involved/healthy is 1; significance levels are represented by asterisks ($p<0.001$ ***, $p<0.01$ **, $p<0.05$ *)

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

As the further step of the study, we aimed to determine the expression features of LUC7L3, PPIG and SFRS18 during keratinocyte proliferation and differentiation. Both mRNA and protein expression patterns were characterized in synchronized, immortalized cell lines. To this end, HPV-KER cells were synchronized via contact inhibition and withdrawal of growth factors. After release from cell quiescence, HPV-KER cells were harvested at various time points, and we applied real-time RT-PCR and western blot analysis to assess RNA and protein levels of splicing factors.

During comparison of splicing factor expression in different states of the proliferation and differentiation, we observed a very similar mRNA expression pattern for LUC7L3, PPIG and SFRS18 (Fig. 8.a). After a transient mRNA abundance decrease around 12 h, a peak of mRNA expression was found at 24 h, accompanied with a steady elevation, which started from 48 h.

Similarly to the mRNA level changes, the protein level expression changes were very similar during the proliferation-differentiation cycle of HPV-KER cells. However, protein levels were proven to be more even compared to mRNA level changes. The highest amount of splicing regulator proteins was displayed at 48 h after release from cell quiescence (Fig. 8.b).

Moreover, the synchronization experiment was repeated in another type of immortalized keratinocyte: the synchronized HaCaT cells - similarly to the results obtained in HPV-KERs - express splicing factors with an identical pattern (Fig. 9.).

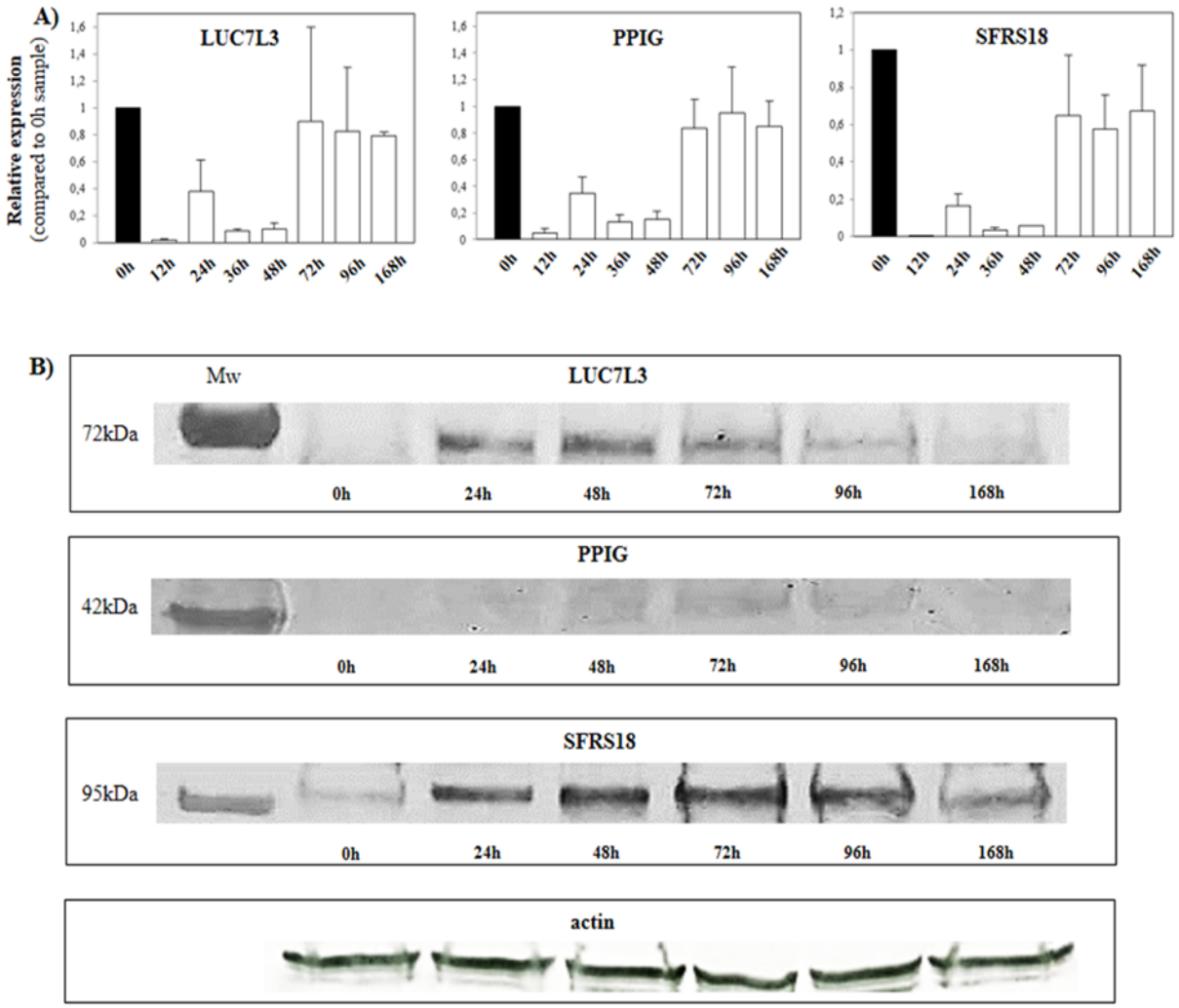


Fig. 8. Synchronization of HPV-KER cells was achieved via contact inhibition and supplement withdrawal, and sample collection from the re-passaged (0 h) cells was done at the indicated time points. Alterations in mRNA abundance **(a)** were measured by real-time RT-PCR, regarding protein levels **(b)** western blot analysis was used for determination. The experiments were performed with three independent series of cell cultures, and the mRNA amounts were calculated as the average of the parallels. For each splicing factor, one representative picture of western blot is provided. α-actin served as a loading control.

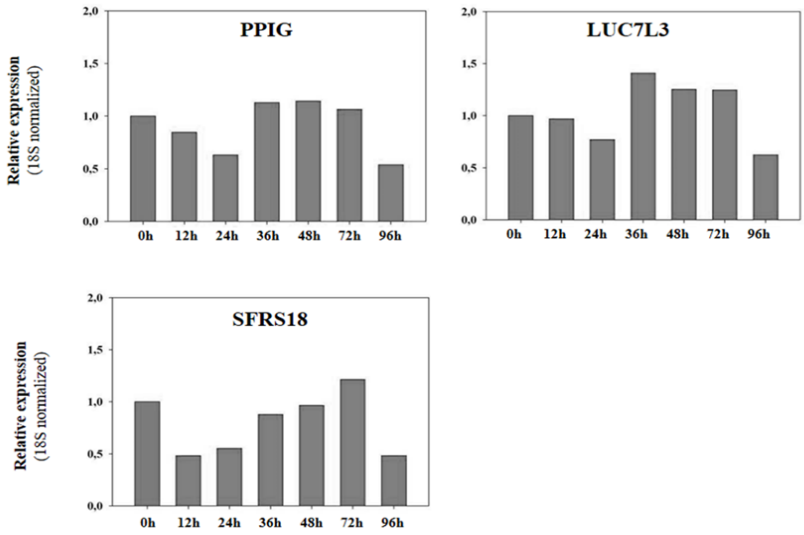


Fig. 9. Detection of LUC7L3, PPIG and SFRS18 mRNA changes in synchronized, immortalized HaCaT cells (n=1). Synchronization was executed according to Pivarcsi *et al.*, and samples were taken at the indicated time points following the re-passage (0 h) (Pivarcsi et al., 2001). Considering the mRNA expression, the three splicing regulators exert identical expression patterns after the release from cell quiescence.

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

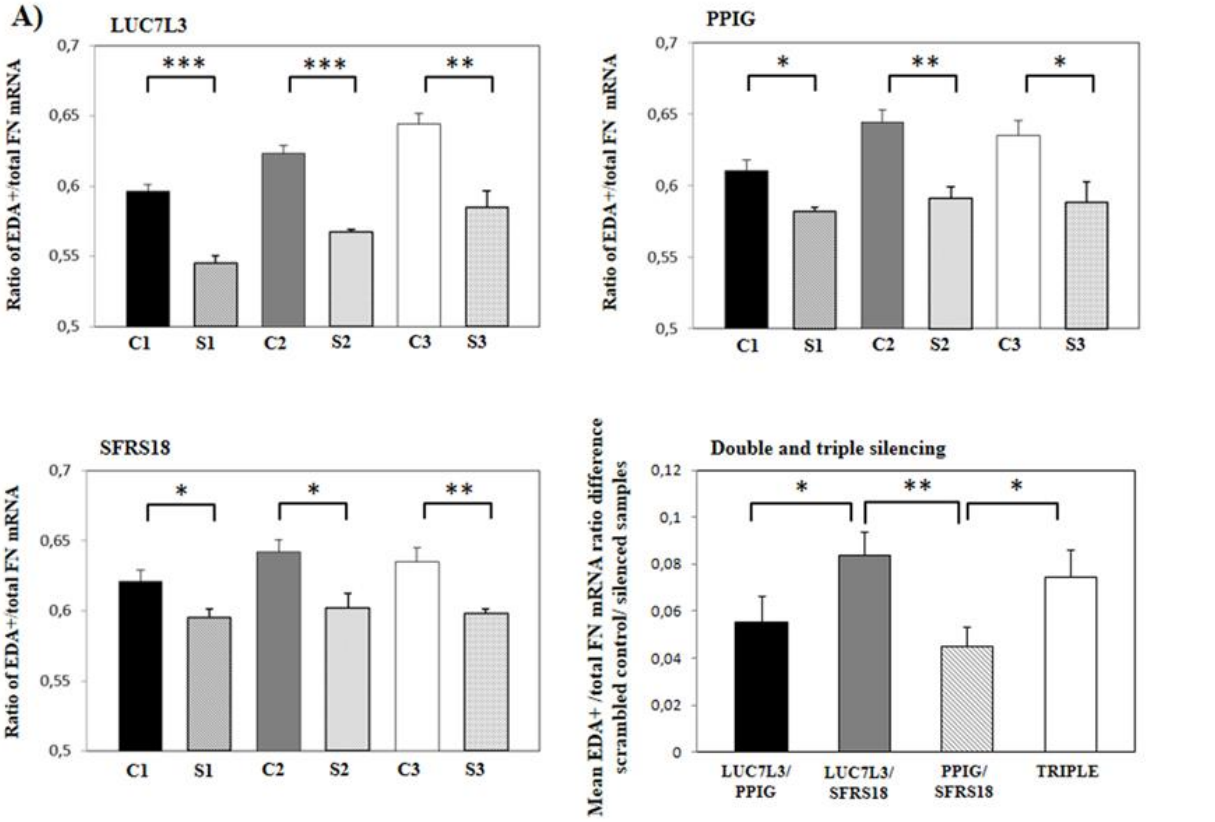
After the analysis of *LUC7L3, PPIG* and *SFRS18* gene expression characteristics, we decided to explore molecular mechanisms influenced by these splicing regulators. As former results of the research group suggested that certain fibronectin splicing abnormalities might possess pathogenic role in psoriasis, first, we aimed to assess the relevance of LUC7L3, PPIG and SFRS18 in the mRNA maturation processes of this protein. Therefore, HPV-KER cells were submitted to siRNA-mediated silencing of the splicing factor genes. The EDA+/total fibronectin ratios were compared in scrambled and specifically transfected cells, the effect of the silencing was high, with an average of approximately 80%. Alterations in the fibronectin splicing pattern were detected by RT-PCR, and densitometry was employed as a semiquantitive technique to investigate the EDA+/total fibronectin ratio changes upon silencing of the three

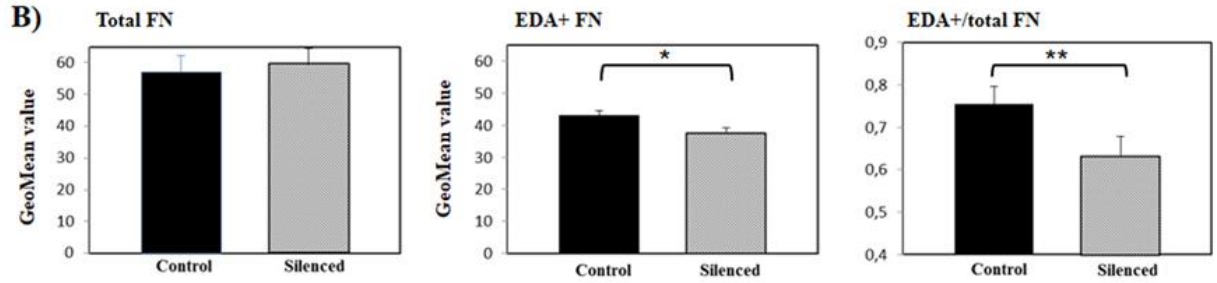
splicing factors. During the course of the experiments either single or combined silencing was performed, in order to observe possible interactions among the LUC7L3, PPIG and SFRS18 splicing factors.

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 (Fig. 10.a).

Bioinformatics prediction using the STRING database suggests that LUC7L3 and SFRS18 are presumably interactors (Fig. 10.c). As mentioned above, to examine the interference of splicing factors, simultaneous silencing of two or three genes was executed. 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* (Fig 10.a). Furthermore, triple silencing does not exert additional effect on the ratio compared to the combined silencing of *LUC7L3* and *SFRS18*.

The consequences of silencing were also evaluated at the protein level. The EDA+ and total fibronectin levels were analyzed by flow cytometry using the most robust silencing combination determined in the course of the previous experiments. In agreement with the gene expression experiments, the EDA+ fibronectin isoform was present in higher amounts in the immortalized keratinocytes as compared to the EDA- variant. The double silencing of *LUC7L3* and *SFRS18* significantly decreased the quantity of the EDA+ isoform without changing the amount of total fibronectin (Fig. 10.b). In conclusion, these data indicate that the decresed levels of the splicing regulators result in diminished EDA+/total fibronectin ratio both at mRNA and protein levels.


A)
LUC7L3
Ratio of EDA+/total FN mRNA
0,7
0,65
0,6
0,55
0,5
***
***
**
C1
S1
C2
S2
C3
S3
PPIG
Ratio of EDA+/total FN mRNA
*
**
*
SFRS18
Ratio of EDA+/total FN mRNA
*
*
**
Double and triple silencing
Mean EDA+ /total FN mRNA ratio difference scrambled control/ silenced samples
0,12
0,1
0,08
0,06
0,04
0,02
0
LUC7L3/ PPIG
LUC7L3/ SFRS18
PPIG/ SFRS18
TRIPLE


B)
Total FN
GeoMean value
60
50
40
30
20
10
0
Control
Silenced
EDA+ FN
GeoMean value
*
EDA+/total FN
0,9
0,8
0,7
0,6
0,5
0,4
**

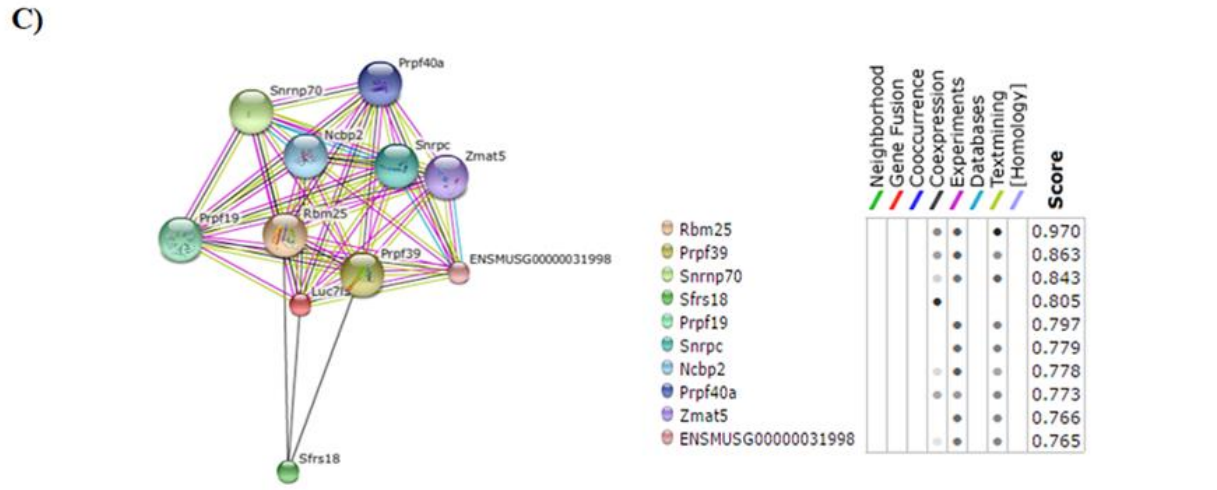

C)
Prpf40a
Snrnp70
Ncbp2
Snrpc
Zmat5
Prpf19
Rbm25
Prpf39
ENSMUSG00000031998
Luc7l3
Sfrs18
Neighborhood
Gene Fusion
Cooccurrence
Coexpression
Experiments
Databases
Textmining
[Homology]
Score
Rbm25 0.970
Prpf39 0.863
Snrnp70 0.843
Sfrs18 0.805
Prpf19 0.797
Snrpc 0.779
Ncbp2 0.778
Prpf40a 0.773
Zmat5 0.766
ENSMUSG00000031998 0.765

Fig. 10. HPV-KER cells were transfected with scrambled and specific siRNA constructs targeting *LUC7L3, PPIG* and *SFRS18* (a) and the EDA+/total fibronectin ratio was detected in each series (S1, S2, S3). The changes in this ratio were compared in each experiment to that of the scrambled RNA transfected controls (C1, C2, C3). Effect of double and triple silencing was also demonstrated. Columns represent means (+S.E.) of three independent experiments, which are all performed in triplicate and significance levels are marked by asterisks ($p<0.001$ ***, $p<0.01$ **, $p<0.05$ *)

Total fibronectin and EDA+ fibronectin were determinated by flow cytometry in *LUC7L3/SFRS18* double silenced HPV-KERs (n=5) (b). Silencing had not any affect on the quantity of total fibronectin, while a significant ($p<0.05$) EDA+ decrease was detected upon silencing, also resulted a significant ($p<0.01$) changes in the EDA+/total fibronectin ratio. Bioinformatics prediction suggested a possible interplay between the LUC7L3 and SFRS18 regulators (c)

4.5. Global transcriptome analysis of immortalized keratinocytes

4.5.1 Determination of silencing efficacies and quality control

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. Silencing efficiencies were screened via Real-Time RT-PCR prior to sequencing, which suggested a suitable silencing efficacy, between ~70-80% (Fig. 11.). Quality control of silenced sample isolates was performed by Bioanalyzer and indicated excellent quality (RIN: 10) **(Fig. 12.)**.

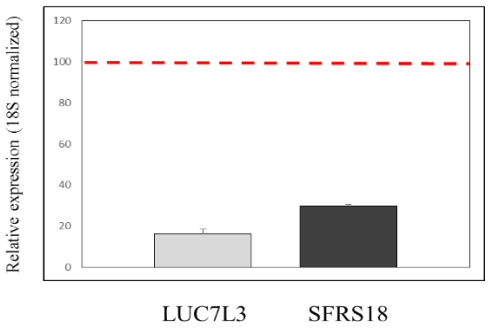


Fig. 11. Double silencing of *LUC7L3/SFRS18* was found to be the most effective in modifying fibronectin mRNA maturation. The silencing efficacy was found to be sufficient 83.6%±4,24 for *LUCL73* and 70,1%±1,56 for *SFRS18,* in two replicates

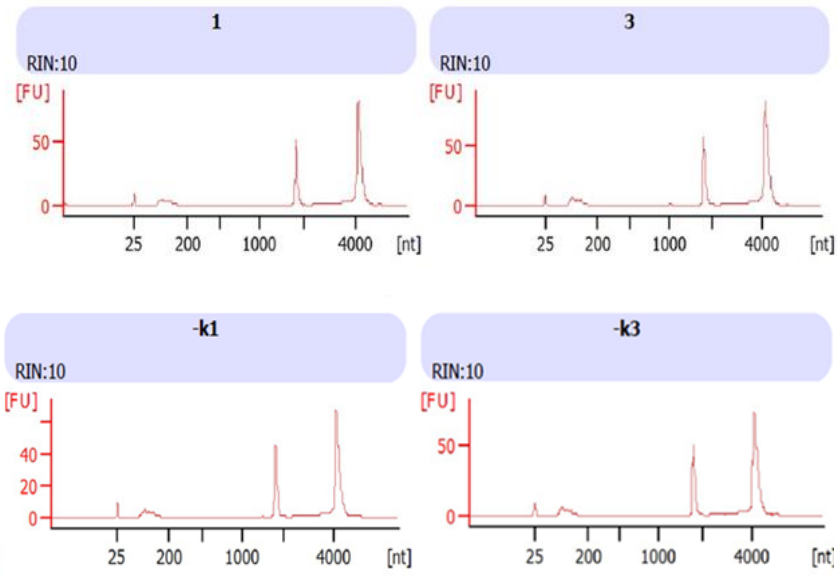


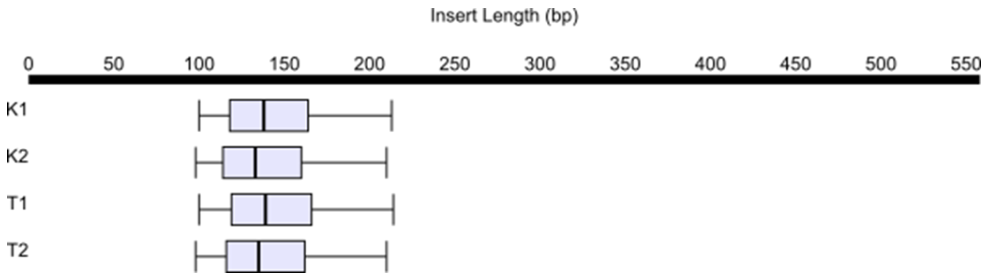
Fig. 12. Quality control of *LUC7L3/SFRS18* siRNA transfected samples (1, 3) and scrambled controls (-k1, -k3).

4.5.2 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 [80, 81].

A



B

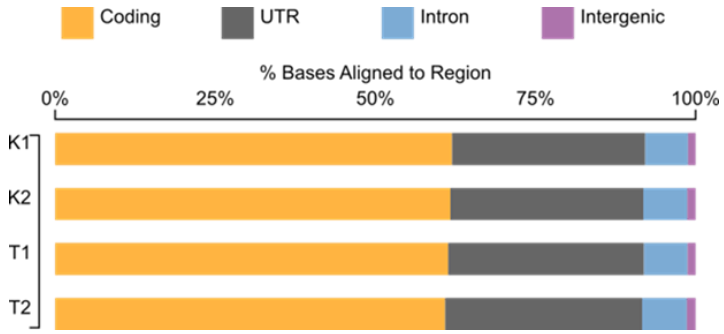


Fig. 13. Box-plot of insert length distributions in the 2x100 bp RNA-Seq library (a). Relative percentages of read coverage per transcript type in the alignments (b).

4.5.3 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 signalling. The most significant enriched GO terms were cellular response to type I interferon (GO:0071357), cellular response to cytokine stimulus (GO:0071345), regulation of viral genome replication (GO:0045069); the functional relationships of significantly differentially expressed genes are illustrated in a functional interaction network based on the Reactome and GeneMania databases (Fig. 14.).

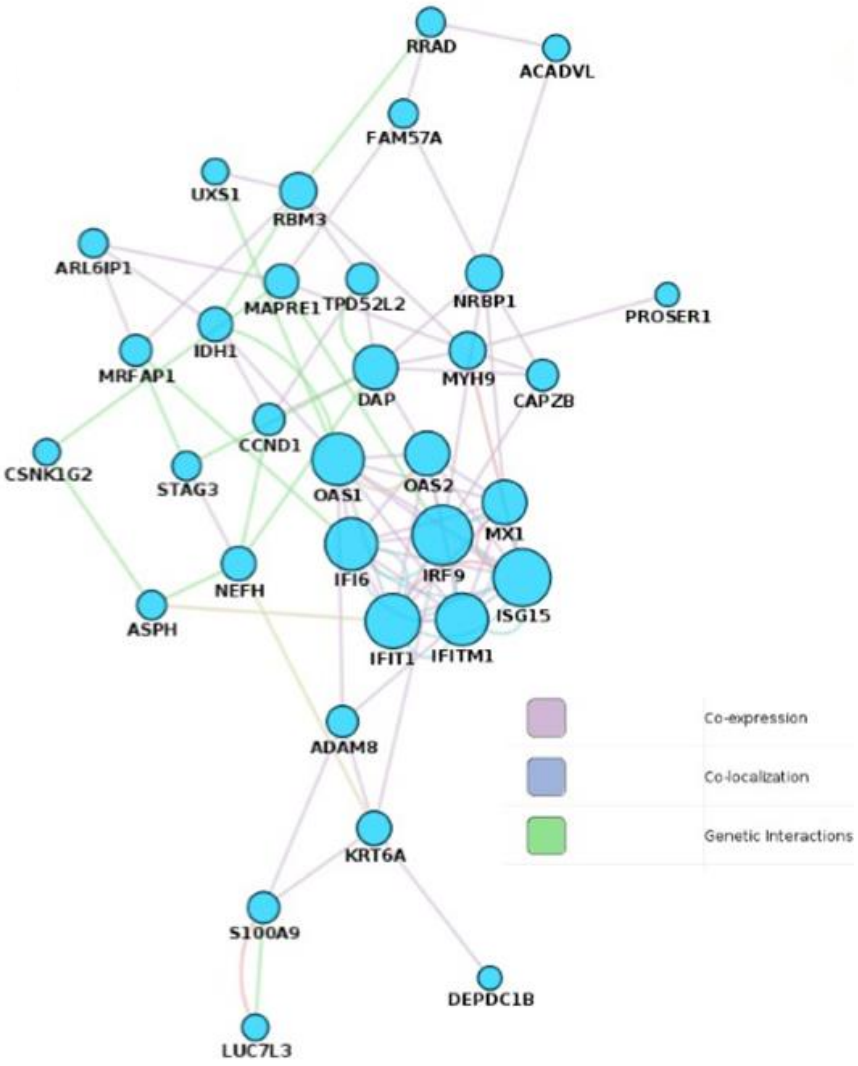


Fig. 14. Functional network of significantly differentially expressed genes (log2FC>0.5, FDR<0.05) in response to dual silencing of *LUC7L3* and *SFRS18*, with edges corresponding to GeneMania and Reactome annotation categories, and nodes corresponding to significantly regulated genes.

According to the Real-Time RT-PCR validation, IFI6 displayed up to 4-fold upregulation, in agreement with the high-throughput results. Altogether three biological replicates (with double technical replicates of the RNA-seq experiment and double technical replicates of two prior *LUC7L3/SFRS18* silencing experiments) were used for validation. An integrated figure of the three independent measurements is presented in Fig. 15.

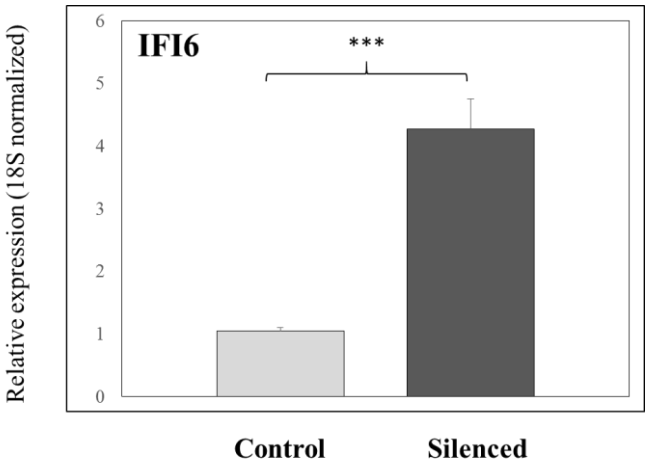
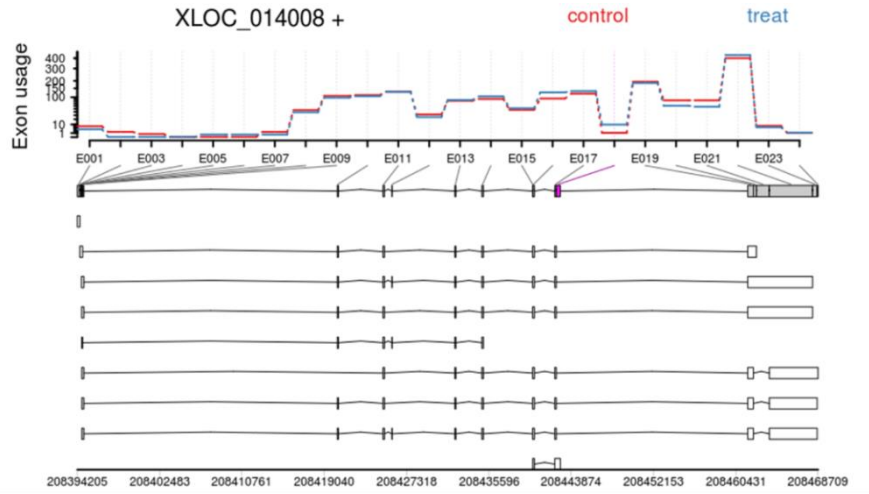


Fig. 15. Real-time RT-PCR validation of IFI6 mRNA expression changes. Columns indicate the mean of three independent experiments, with a significance threshold of $p<0.05$.

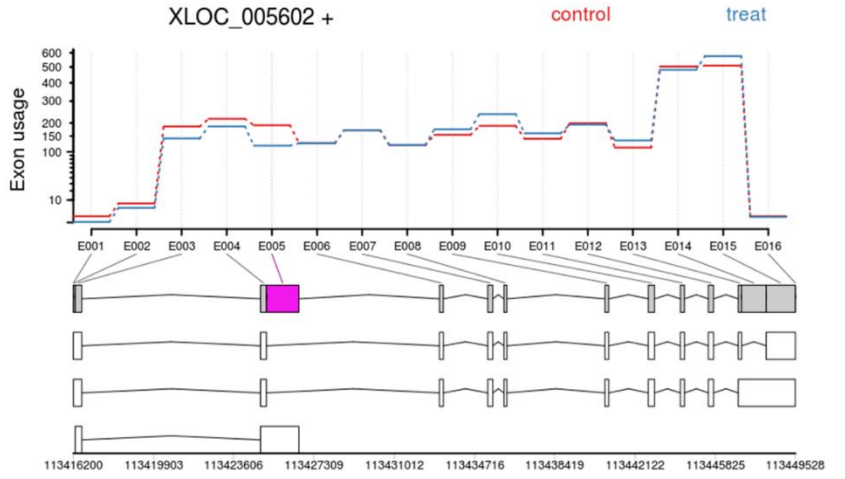
4.5.4 Differential exon usage

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 cut-off 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 (Fig. 16.). Differential exon usage was detected in several non-coding RNAs. Coordinated transcriptional regulation could be identified by mining co-expression-level databases. Functional enrichment analysis of the most significant genes shows an extensive co-expression, suggesting the possibility of overlapping regulation at transcriptional and/or post-transcriptional levels, and less pronounced enrichment of molecular functions (Fig. 17.).

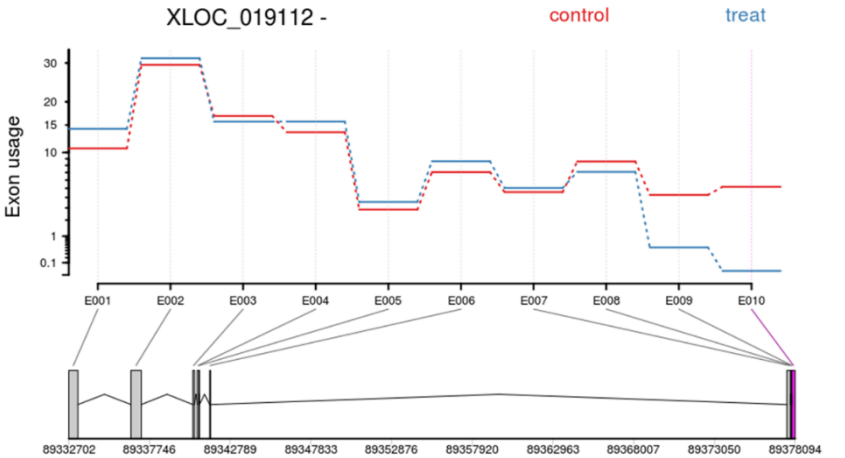
CREB1



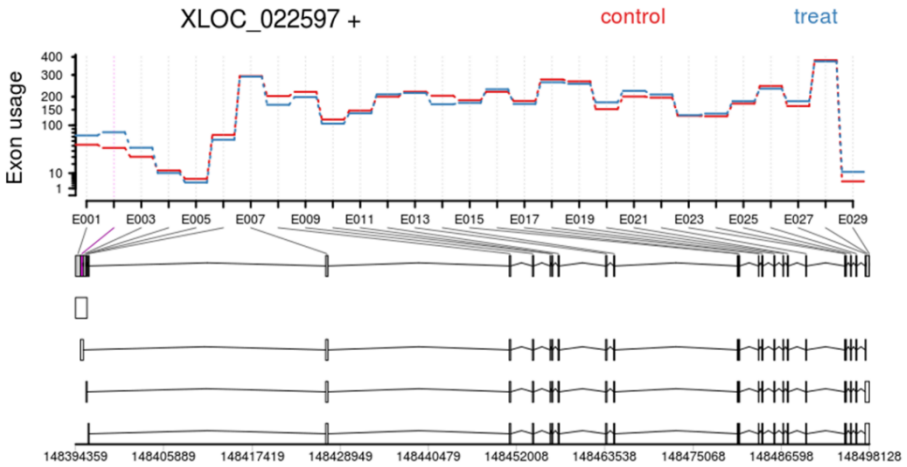
OAS2



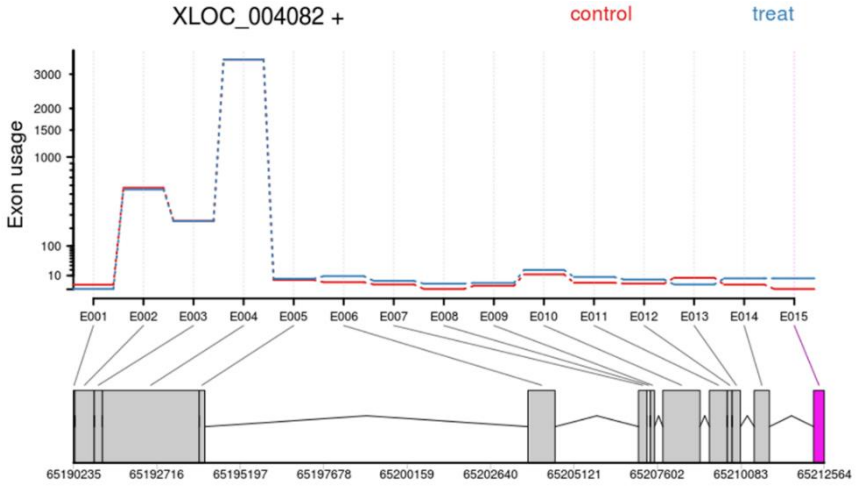
HERC6



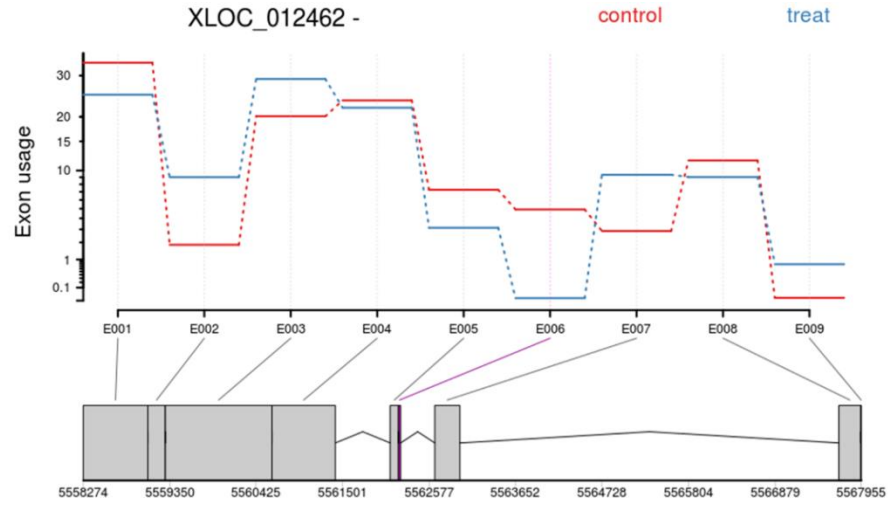
CUL1



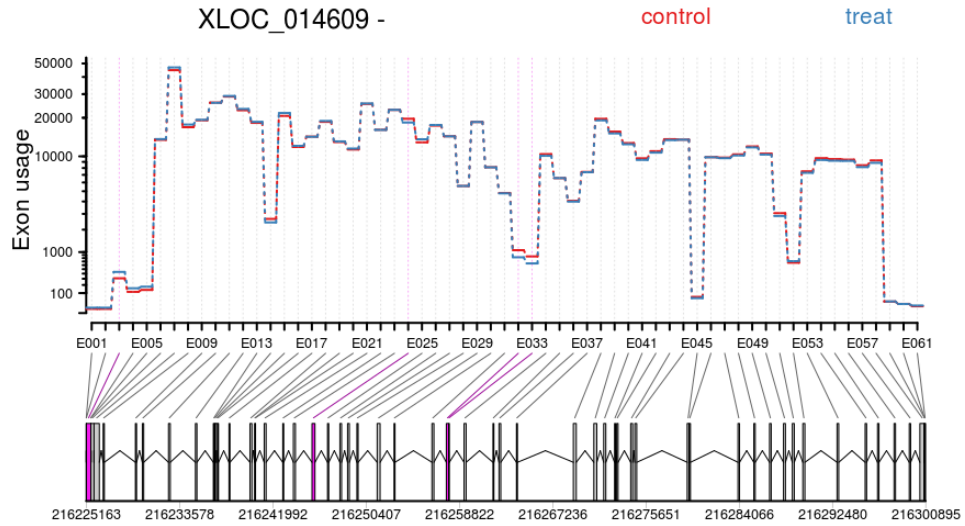
NEAT1



TINCR



FN1



NBEAL1

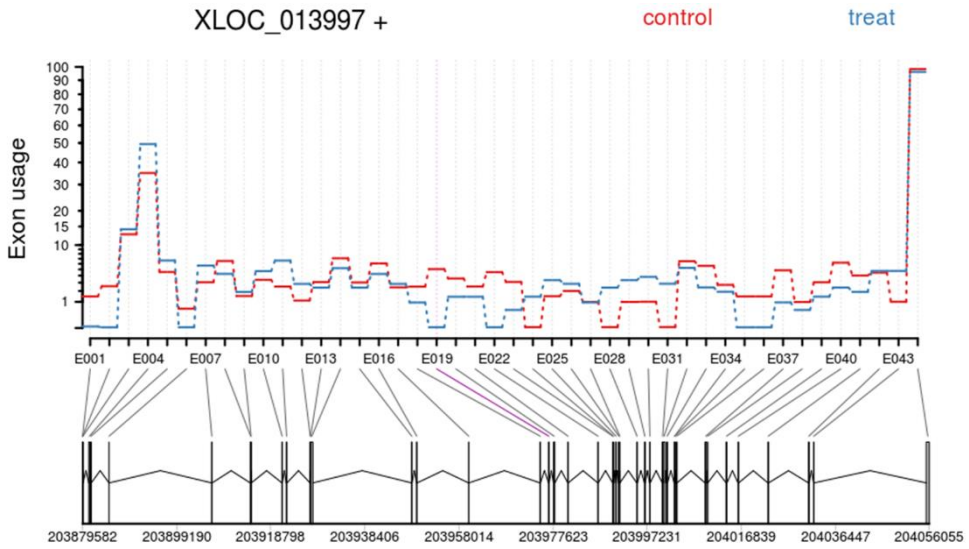


Fig. 16. DEXSeq differential-exon-usage plots of selected transcripts show significant alterations (logFC>0.5, FDR<0.1). Red: average exon usage frequency in control samples; blue: average exon usage frequency in treated samples; pink: significantly differentially expressed exons. *De novo* assembled transcript diagrams are depicted in bottom rows.

Based on these results, a functional network of genes showing significant exon usage alterations was generated. 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. As the complete network is difficult to visualize, a smaller sub-network showing the most highly connected genes is illustrated in Fig. 17.

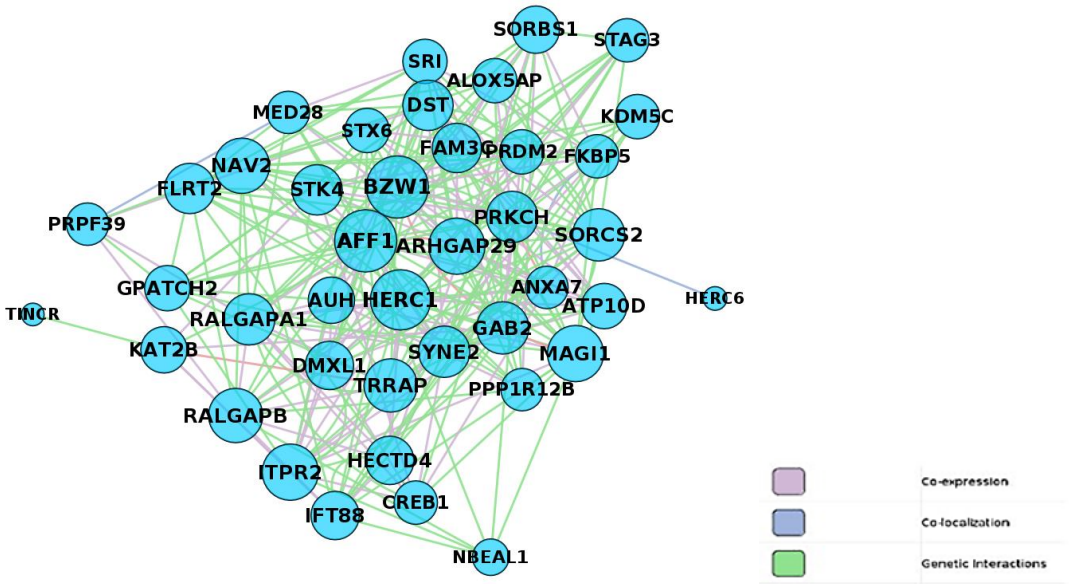


Fig. 17. Sub-network representation of a functional network constructed from genes exhibiting significant exon usage changes, with edge color corresponding to GeneMania and Reactome annotation categories.

Independent validations using RT-PCR were carried out for the exon inclusion/exclusion of fibronectin (FN1). The use of exon-specific, junction spanning primers for FN1 transcripts, followed by agarose gel electrophoresis, resulted in the detection of altered exon inclusion, and the decreased relative abundance of the EDA+ isoform, respectively (Fig. 18).

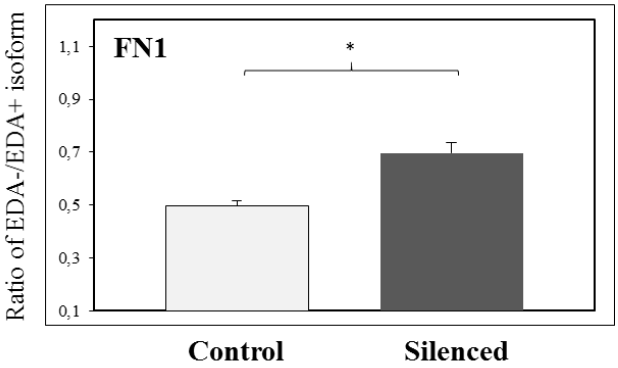


Fig. 18. Independent RT-PCR-based validation of differential exon usage, indicating abundance of alternative splicing variants in control (grey) and treated (black) samples in two replicates.

5. DISCUSSION

Widespread application of large-scale gene expression studies proved to be fruitful in psoriasis research: the methodological developments provided a closer insight into disorder pathogenesis and facilitated the discovery of novel therapeutical targets [52, 53, 54]. In our recently conducted cDNA microarray experiment, we have focused on the early steps of psoriasis pathogenesis, which remain still only partially understood. In a cDNA microarray analysis, we used a unique approach, where the T-lymphokine stimulated altered responsiveness of keratinocytes was investigated [71]. The applied lymphokine mixture consisted INF-γ, an inflammatory mediator, which facilitates the proliferation of immature keratinocytes, and its effect was potentiated by IL-3 and GM-CSF. These data suggest that the listed lymphokines might implicated in the early disorder pathogenesis, thus, our microarray experimental design was suitable to describe novel factors participating in the primary phase of psoriasis [71, 72]. Currently, the IL-17 /23/IL22 axis and TNF-α attracts more attention in psoriasis compared to INF-γ, but it is unquestionable that IFN-γ plays an essential role in disorder initiation and establishement. However, to reveal its exact mechanism of action in psoriasis, further studies are needed [6, 19].

Among differentially regulated genes between the healthy and psoriatic non-involved epidermis, we identifed three SR-rich splicing regulators, *LUC7L3*, *PPIG* and *SFRS18*. This finding is of special interest, as neither the splicing disturbances nor the biological function of these SR-rich splicing regulators have been characterized in depth in psoriasis. In the Results section, we demonstrated that splicing regulators are downregulated or do not show any changes in psoriatic non-involved epidermis upon T-lymphokine treatment, as compared to healthy tissue specimens. We described the same phenomenon in case of several transcripts identified in our cDNA microarray experiment. This seeming contradiction could be explained by the following: the gene products are already upregulated in the non-involved epidermis, hence, the lymphokine treatment does not result in further elevation of expression [71]. Furthermore, an upregulation tendency of splicing regulators was revealed for splicing regulators in psoriatic non-involved epidermis.

Besides the participation in mRNA maturation, LUC7L3 influences other molecular processes, for instance, it is involved in cysplatine resistence of tumor cells [82, 83, 84]. Novel data suggests that it might behave as an antiviral compound via inhibition of hepatitis-B virus

replication [85]. Moreover, LUC7L3 could be one of the protein interactors of cAMP responsive element (CRE), thus, it may play a role in cellular signal transduction processes [86].

PPIG is implicated in protein folding, and its cell-cycle dependent phosphorylation *via* SRPK1 and SRPK3 is also described [87, 88]. Literature and data mining indicates that PPIG together with SFRS18 (also known as PNISR) might be physical and functional interactors of PNN, a desmosome-associated particle, playing roles in splicing and cellular connections, repectively [88, 89].

The previous results of the research group already indicated that certain mRNA isoforms might promote psoriasis development. We and others have previously verified that the fibronectin splice variant containing the oncofetal EDA domain (EDA+ fibronectin) might be responsible for the altered responsiveness of the psoriatic non-involved epidermis. The amount of the EDA domain is elevated in non-involved epidermis compared to healthy skin, and we also demonstrated that keratinocytes are effective producers of this domain [37, 50]. Regulation of fibronectin splicing has been studied in detail, however, our knowledge regarding the formation of its isoform is still incomplete [40]. Other reports described, that TGF-β and another type of SR-rich protein (SF2/ASF) promote the EDA domain inclusion while snRNPs possess an opposite effect [39, 90, 91, 92, 93]. STAT1 promotes EDA inclusion in fibroblast supported by the current data of our research group [94].

In the first study presented in the dissertation, we aimed to clarify whether SR-rich splicing regulators identified to be differentially regulated in psoriasis are implicated in the production of the EDA+ fibronectin. In addition, investigation of expression characteristics of *LUC7L3, PPIG* and *SFRS18* was another aim of our study. We observed their gene expression differences among healthy, psoriatic non-involved and involved epidermis, and the expression of splicing regulators was also monitored during keratinocyte proliferation/differentiation phases of immortalized keratinocytes. By comparing splicing factor abundance in different types of epidermis samples, we concluded that all of the three splicing regulators are upregulated in the involved epidermis, exhibiting approximately 2-3 fold elevation. LUC7L3 showed a moderate, but significant increase in non-involved epidermis, which can suggest its association with EDA+ fibronectin. In contrast to LUC7L3, PPIG is diminished in the non-involved epidermis, therefore, its pattern of expression resembles that of STAT1, moreover, both STAT1 and PPIG

are the component of the molecular network including keratinocyte growth factor (KGF) and fibronectin signalling [94].

The congruent expression of the three splicing factors in synchronized keratinocytes is a remarkable outcome of our study. As *LUC7L3, PPIG* and *SFRS18* are localized on different chromosomes, genetic linkage is not liable for the experienced similarities, and leaves way to alternative explanation for the phenomenon. The most plausible answer is that these SR-rich splicing factors might share similar upstream regulatory elements, including transcription factors and their binding sites. If we consider the results of the Western blot and immunostaining experiments together, the expression of splicing regulators both in involved epidermis and immortalized keratinocytes is well-defined. This finding suggests that abundant LUC7L3, PPIG and SFRS18 expression might be involved in the shortened and disturbed maturation of keratinocytes that is typical of psoriatic skin and is also an attribute of the immortalized HPV-KER cells.

We constructed an *in vitro* experimental system to analyze whether altered expression of *LUC7L3, PPIG* and *SFRS18* influenced the ratio of the oncofetal and normal fibronectin isoforms. siRNA silencing of these splicing factors verified, that single silencing leads to significant decrease in the relative amount of the EDA+ fibronectin isoform, which is present in majority in the immature keratinocytes. Moreover, we also investigated the effects of combined silencing, to observe the existence of synergistic effects. The previous analysis of publicly available databases supported the relationship of LUC7L3 and SFRS18, and accordingly to the prediction, double silencing of *LUC7L3/SFRS18* enhanced the decrease of EDA domain inclusion. Triple silecing did not cause significant difference compared to the combined silencing of *LUC7L3* and *SFRS18*. Using the *LUC7L3/SFRS18* double-silenced combination, we tested whether the fibronectin related isoform alterations are also present at protein level. Flow cytometry validated the mRNA-level findings, since we were able to demonstrate the decrease of EDA+ fibronectin amount in HPV-KER keratinocytes, whithout changing the quantity of total fibronectin: this result refers to an altered mRNA maturation mechanism in the background. Based on prior reports, SR-rich proteins induces exon inclusion via interaction of enhancer splicing elements, therefore the finding possess special importance [95, 96, 97].

Findings introduced so far suggested, that the SR-rich proteins might be implicated in psoriasis in multiple manners, thus, tissue stainings and the results related to synchronized keratinocytes and mRNA maturation of fibronectin prompted us to examine other pathways influenced by the aforementioned splicing factors. To obtain a deeper insight into molecular functions associated with splicing regulators, we applied again a high-throughput gene expression tool, paired-end RNA-Sequencing. For further analysis, we have chosen for further analysis the double silencing of *LUC7L3/SFRS18*, which resulted in the most robust splicing pattern alterations regarding the EDA+ fibronectin formation.

In accordance with our primary hypothesis, siRNA mediated silencing of *LUC7L3/SFRS18* caused relatively modest changes in gene expression, and definite alterations in splicing patterns. However, several significant results were obtained concerning differential expression, the most important one is related to an interferon-inducible gene, the *IFI6* (*G1P3*). *IFI6* belongs to the family of interferon-stimulated genes (*ISG*s), together with *ISG12A, ISG12B, ISG12C*. *ISG12A* is also referred as *IFI27* [98]. In contrast to other members of ISGs, IFI6 was shown to be rather antiapoptotic: this feature was observed in cancer cells, and our research group presented its overexpression both in non-involved and involved epidermis [99, 100]. Via elevated expression IFI6 could be responsible for the survival of immature keratinocytes which is an important phenomenon in psoriasis. Moreover, we have also analyzed the interaction of G1P3 and PRINS, a non-coding RNA implicated in the cellular stress responses of keratinocytes [99, 101]. Our RNA-Seq results enrich our knowledge on the relation of IFI6: in response to *LUC7L3/SFRS18*, overexpression of IFI6 could be observed, moreover the result have been validated in independent samples as well. Real-Time RT-PCR indicated up to 4-fold upregulation in siRNA silenced samples as compared to the scrambled controls. An important coincidence is that we could also detect the upregulation of the apoptotic IFI27, which exerts its effect via Bax activation and causes mitochondrial cytocin-c release, and decrease the mitochondial membrane potential [98]. Function of IFI27 has been also assessed in psoriasis: some experimental data suggest high abundance in psoriatic skin samples [102]. Taken together, we can conclude that SR-rich proteins could be involved in the fine-tuning of pro- and antiapoptotic events in human keratinocytes. This is not the only proof for interferon-SR-rich splicing factor connection, since a previous report demonstrated that serine/arginine-rich

splicing factor 1 (SRSF1) facilitated the type-I IFN production, via the double-stranded RNA sensor RIG-1, which is also an abundant molecular component in psoriasis [103].

We also analysed the predicted interactors of IFI6 using the STRING database (Fig. 19.). During the review of the gene list and its comparison to the RNA-Seq results, we found that several of them exerted significant upregulation upon silencing. OAS1, OAS2, IFIT1, IFIT3, ISG15, MX1 were all identified to be upregulated in our RNA-Seq experiment, besides that, literature data supported their implication in antiviral immunity [104, 105]. ISG15, MX1 and OAS2 was previously decribed to be upregulated in psoriatic epidermis as compared to the healthy samples [104]. To date, Raposo and his co-workers demonstrated in their recent RNA-Seq study that 16 antiviral genes exhibit at least two-fold elevation in psoriatic epidermis compared to the healtly and non-involved epidermis samples [105]. This gene list also includes *ISG15*. The results related to the antiviral immunity might have a huge importance, considering that novel experimental data suggests that LUC7L3 inhibits hepatitis B virus replication [85]. Furthermore, there is several evidence that psoriatic skin is less prone to viral infections due to the upregulation of the type I interferon induced antiviral pathway [104, 105].

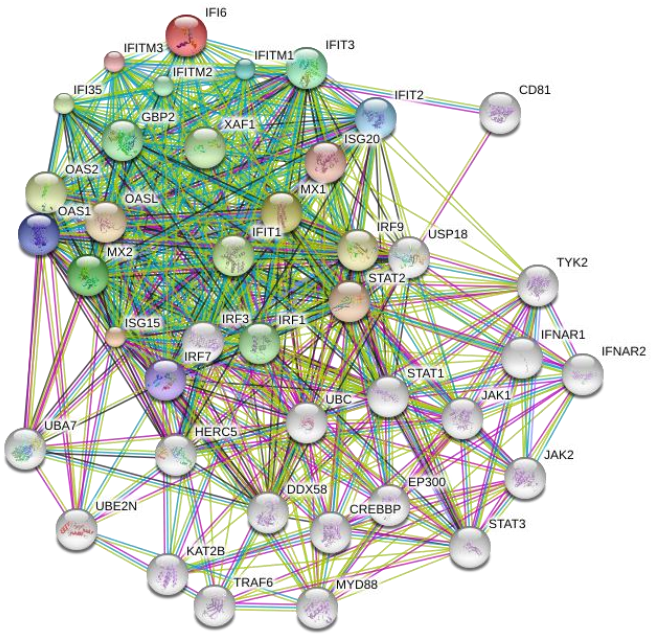


Fig. 19. Interaction network of IFI6 predicted by STRING database (https://string-db.org/cgi/input.pl?taskId=_notask&UserId=gz5TU6vnllRU&sessionId=FpUklt2OTNm7)

In our RNA-Seq experiments we also demonstrated, that innate immunity components, like KRT6A and the calcium binding protein S100A9 are regulated by LUC7L3/SFRS18. S100A9 forms a heterodimeric complex with S100A8, and together, they exert antimicrobial and chemoattractant functions [106, 107]. S100A9 and KRT6A belong to the same molecular network related to barrier function, innate immunity and danger signals. KRT6A is also involved in wound healing processes [108].

Splicing pattern alteration related to the EDA+ fibronectin isoform (the drop of inclusion tendency) was reproducible in the samples of RNA-Seq experiment. Besides that, we were also able to demonstrate the elevated level for integrin 5 (ITGA5), the known fibronectin receptor, also having the capacity to bind the EDA domain of fibronectin. As consequence of the diminished relative amount of the EDA+ fibronectin, ITGA5 abundance is elevated, which could be considered to be a cellular compensatory mechanism. This phenomenon is a novel evidence for the suggestion, that EDA domain inclusion alters the binding partners of fibronectin [39, 41, 42, 45]. Previous data indicated that the presence of the EDA domain increases the affinity towards α5β1 integrin receptor, which is the most relevant fibronectin interactor [42, 43, 45]. However, a contradicting report claims that the EDA+ fibronectin does not bind to this receptor, and the α4β1 and α9β1 integrins recognize the EDGIHEL sequence located in the EDA domain. Additional results support the attachment of EDA+ fibronectin to the innate immune system component TLR4 receptor [44, 45, 46]. The complexity of this regulatory network is indicated by the fact that the effect of S100A9 - upregulated also in response to *LUC7L3/SFR18* silencing - is also mediated by TLR4 [106, 107]. Moreover, other fibronectin signalling related components, as syndecan-4 (SDC4) and tenascin-C show altered expression upon silencing of splicing factors [109].

LUC7L3, which was formerly designated as CREAP-1, contains a binding domain for the cAMP regulatory element (CRE) [86]. However, based on silencing experiments, it may also have influence on the mRNA maturation of CREB1, besides the potential direct interplay with CRE. Therefore LUC7L3 potentially exerts a modulatory role on CREB1: altered splicing might regulate the protein binding of this fundamental element of transcription. In addition, various cellular processes are affected by differential exon usage, such as CUL1, HERC1 and HERC6 implicated in ubiquitination. CUL1 is part of the E3 ubiquitin ligase complex and known to be overexpressed in melanoma, mediating hyperproliferative signals [110]. The

HERC gene family contributes to multiple molecular pathways, including DNA repair, cell-cycle regulation, and ubiquitination. Köks and co-workers demonstrated the isoform ratio changes of HERC6 in psoriatic skin compared to healthy samples [69]. HERC6 is also referred in E3 conjugation of ISG15 [111]. The antiviral OAS2 exhibited not only upregulation but differential exon usage as well. Multiple genes with relatively sparse functional annotation available in the present literature exerted markedly altered exon usage. These comprises the *HERC2* paralog *RCC1, NBEAL1* and AUH, with RNA-binding and hydratase activities [112, 113, 114].

Using RNA-Seq, exon usage alterations of long non-coding RNAs (lncRNAs) are also demonstrable [70, 115]. In our study, we have demonstrated several non-coding RNAs with differential exon usage, such as NEAT1 and TINCR. The contribution of NEAT1 to tumorigenesis is well studied: its overexpression has been validated in esophagus, colorectal, hepatocellularand lung carcinoma [116]. Since hyperproliferation of keratinocytes is a rudimental step in the pathogenesis, NEAT1 could presumably act in psoriasis pathogenesis.

The widespread functionality of TINCR in keratinocyte maturation has been currently delieneated: in contrast with another non-coding transcript, ANCR, which is required for the maintenance of keratinocyte precursors, TINCR promotes terminal differentiation. TINCR is a low-abundance transcript in undifferentiated keratinocytes, and shows increase in mature keratinocytes [117, 118]. Moreover, TINCR and its modulated gene STAU1 exerted elevated expression in the psoriatic gene module, based on a WGCNA gene network analysis of a former RNA-Seq [68]. We have demonstrated that TINCR is present at low levels in HPV immortalized keratinocytes. The binding capacity of TINCR is determined by several „TINCR box" motifs, and inclusion or exclusion of these motifs by altered splicing might modulate binding features [118].

6. 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 signalling, 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.

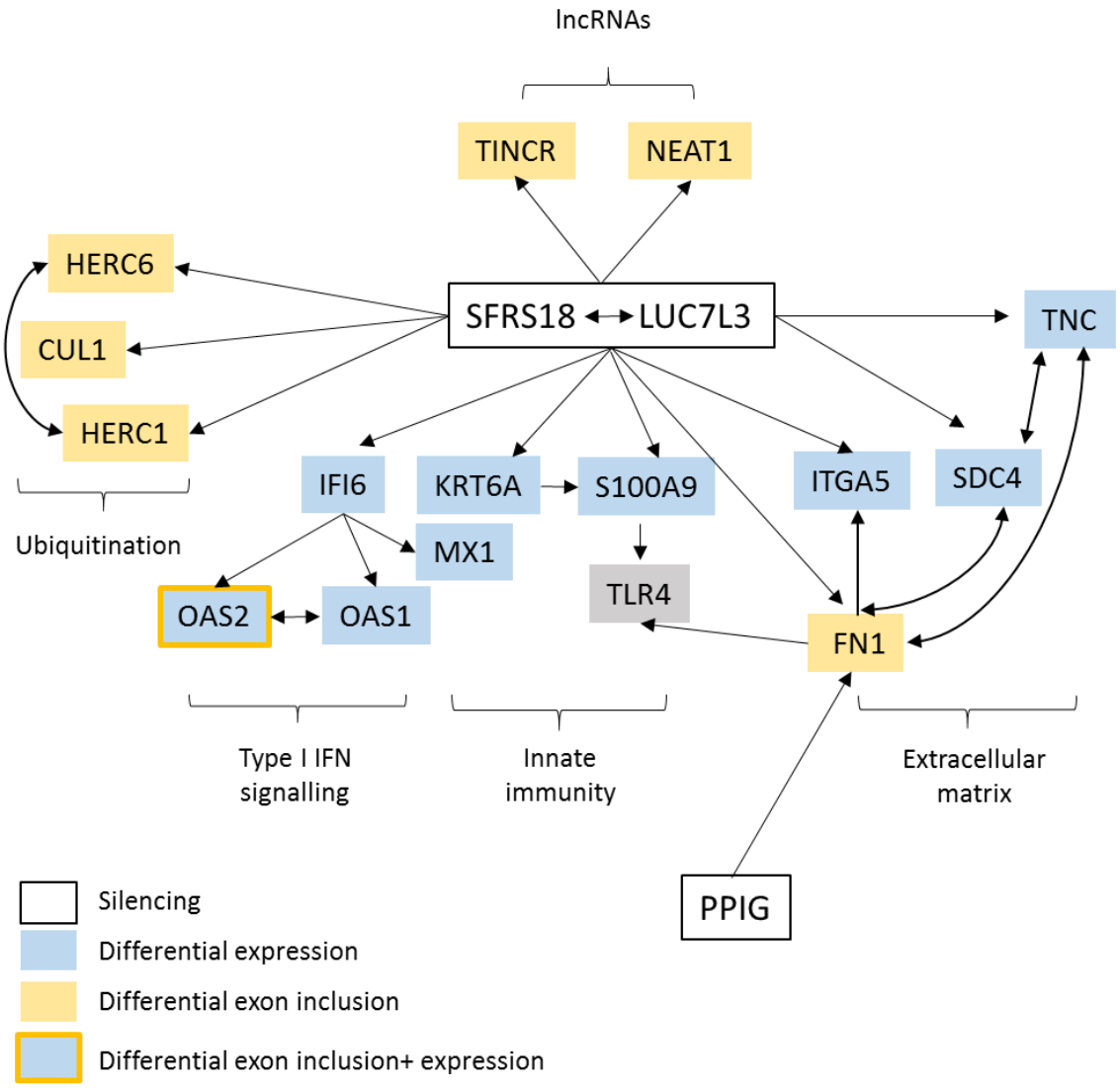


Fig. 20. Schematic model of molecular pathways, which might be influenced by LUC7L3, PPIG, and SFRS18 splicing regulators during psoriasis development

7. ACKNOWLEDGEMENT

Foremost I thank my supervisor, Prof. Dr. Márta Széll for her continuous motivation and guidance throughout the Ph.D. program, without her support any of the presented work would have been impossible.

I am grateful for the support of Prof. Dr. Lajos Kemény and Prof. Dr. Zsuzsanna Bata-Csörgő for providing the background for the current study and for the practical help of Dr. Kornélia Szabó and Dr. Gergely Groma during the experimental work. I would like thank Dr. Franco Pagani for the possibility to spend a studentship in ICGEB Trieste to practice research techniques facilitating the preparation of the Ph.D. thesis. I am also grateful to Prof. Dr. Rolland Gyulai for the encouragement of Ph.D. work during my resident years at University of Pécs.

I would like to thank all my colleagues and staff of the Department of Dermatology and Allergology for their kind support.

I would like to also express gratitude to Prof. Dr. Anna Borsodi for the help in the start of my scientific work and the continous kind support.

Last, I give my special thanks to my loving husband, Péter, who participated in the bioinformatics workflow in the present study and my supporting family, as well.

Illumina short-read library preparation, sequencing and primary bioinformatic analysis of the samples was performed at the Genomic Medicine and Bioinformatic Core Facility of the University of Debrecen, Faculty of Medicine.

The study was supported by OTKA K105985, OTKA K111885 and TÁMOP-4.2.2.A-11/1/KONV-2012-0035, GINOP-2.3.2-15-2016-00015 research grants.

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Analysis of psoriasis-relevant gene expression and exon usage alterations after silencing of SR-rich splicing regulators

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Word count: 3095

Number of Display items: 4 Figures, 2 Supplementary Figures

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ABSTRACT

In our recent cDNA microarray experiment, three SR-rich splicing factors — *SFRS18, PPIG* and *LUC7L3* — were shown to exert altered responsiveness upon T-lymphokine stimulation of psoriatic non-involved and healthy epidermis samples. We have also demonstrated that double silencing LUC7L3 and SFRS18 efficiently decreased production of the psoriasis-associated EDA+ fibronectin isoform. These findings prompted the further investigation of signaling pathways affected by LUC7L3 and SFRS18.

To detect gene expression and splicing pattern alterations upon double silencing of LUC7L3 and SFRS18 in an HPV-immortalized keratinocyte cell culture, paired-end RNA sequencing was carried out. Marked changes in exon usage were revealed, in contrast to the modest alterations detected in gene expression, providing a closer delineation of the potential targets of the examined splicing factors. The most prominent gene expression change was detected for IFI6, an interferon-inducible gene highly expressed in psoriasis. Interacting partners of IFI6 and certain psoriasis-associated transcripts also exhibited significantly increased expression upon silencing.

In addition to elevated abundance of the EDA+ fibronectin interactor ITGA5, we confirmed decreased EDA-domain inclusion, which agrees well with our prior experimental data. Furthermore, differential exon usage was established for the transcription element CREB1, along with HERC6 and CUL1, which are implicated in ubiquitination. Although immortalized keratinocytes express low levels of TINCR, a long non-coding RNA involved in terminal differentiation of keratinocytes, splicing alterations were successfully demonstrated for this RNA as well.

We believe that the targeted investigation of mRNA maturation disturbances may help us gain deeper insight into the molecular pathogenesis of psoriasis.

KEYWORDS:

-RNA Sequencing

-mRNA maturation

-inflammation

-non-coding RNAs

-antiviral immunity

INTRODUCTION

Psoriasis is one of the most common chronic inflammatory skin disorders, affecting 2–3% of the Caucasian population with unquestionable negative impacts on quality of life. Characterization is complicated by the multifactorial origin of the disease, as the interplay of genetic and environmental factors has great influence on the formation of psoriatic lesions. It is well established that innate and adaptive immunological processes both interact in disease pathogenesis [1-3]. Recent findings indicate that activation of plasmacytoid dendritic cells could be a determinant step in the initiation phase of psoriasis, which is triggered by the complexes of cathelicidin (LL37) and the self-DNA/RNA of stressed keratinocytes [3-6]. This step is followed by the release of type I interferons, as well as the consecutive production of other inflammatory mediators, such as IL-1β, IL-6 and TNFα, which are activators of myeloid dermal dendritic cells [3, 6, 7]. This cell type is an important source of IL-12 and IL-23, which are cytokine products that stimulate multiple T-cell lineages implicated in further pathogenic steps, including Th1, Th17 and Th22 lymphocytes [1, 3, 7, 8]. Different stages of the disorder are characterized by unique cytokine profiles: whereas the primary phase is dominated by Th1 cells, the propagation of the disorder entails increasing involvement of the Th17 pathway [3, 9]. Despite the wealth of data, our knowledge regarding the complexity of molecular mechanisms in psoriasis is still incomplete, and further experiments are needed for the detailed description of the molecular background of this inflammatory skin disorder.

In recent years, large-scale gene expression studies have become more accessible and several studies have been conducted to describe gene expression differences of healthy, psoriatic non-involved and involved epidermis [10-13]. The majority of these studies, however, investigated steady-state gene expression levels of various patient-derived samples. In contrast to previous works, we recently investigated T-lymphokine-induced gene expression changes between healthy and psoriatic non-involved epidermis in a cDNA microarray experiment [14]. In our experimental setup, organotypic skin cultures were generated, and half of the samples, both healthy and non-involved epidermis, were treated with a lymphokine mixture containing IFN-γ, IL3 and GM-CSF. This lymphokine combination has been shown to facilitate the proliferation of keratinocyte precursors in psoriatic non-involved epidermis; thus, it is likely that these cytokines are important participants of early disorder development [15].

When comparing T-lymphokine-induced gene expression between healthy and psoriatic epidermis, three SR-rich splicing factors — splicing factor, arginine/serine-rich 18 (SFRS18), peptidyl-prolyl cis-trans isomerase G (PPIG) and luc-7 like protein 3 (LUC7L3) — were found to be differentially regulated in healthy and non-involved psoriatic keratinocytes. The finding is notable, as only a few articles concerning psoriasis and mRNA maturation have been published: we and others have shown that, compared to the healthy samples, EDA+ fibronectin is overexpressed in psoriatic non-involved epidermis and modifies the response of keratinocytes to T-lymphokine stimuli [16-18]. In subsequent experiments, we successfully verified that the aforementioned splicing regulators are able to facilitate EDA domain inclusion. Data mining using publicly available interaction databases also supported the credibility of the interaction of LUC7L3 and SFRS18 splicing regulators [19]. These data suggest that these splicing factors indeed contribute to disease pathogenesis and their effect is – at least partially –mediated by the regulation of psoriasis-associated EDA+ fibronectin.

In the present study, we aim to determine additional molecular pathways influenced by the SR-rich splicing regulators, LUC7L3 and SFRS18, as the combined effects of these two genes proved to be the most potent in prior experiments [19]. Global transcriptome analysis was performed using pair-end RNA-Sequencing to define gene expression changes and differential exon usage in HPV-immortalized keratinocytes.

MATERIALS AND METHODS

Culturing of HPV-KER cells

As we described previously, immortalization of the HPV-KER cell line was achieved by the HPV E6 oncogene [20]. HPV-immortalized keratinocytes were grown in 75 cm^2 cell culture flasks at 37°C in a humidified atmosphere containing 5% CO_2. For the maintenance of the cultures, keratinocyte serum-free medium was used (Gibco® Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark), supplemented with 1% L-glutamine (PAA, Pasching, Austria) and 1% antibiotic/antimycotic solution (PAA). The medium was changed every two days.

Gene-specific silencing

HPV-KER cells were transiently transfected with gene-specific siRNAs at approximately 70% confluency. Transfection medium and reagents were ordered from Santa Cruz Biotechnology (Dallas, TX, USA). In our siRNA-mediated silencing experiments, scrambled and gene-specific LUC7L3 and SFRS18 siRNA duplexes were used. The most effective silencing results were obtained in serum-free culture medium without additive and supplements. Real-time RT-PCR was used to confirm the effectiveness of silencing. Quality control of samples submitted to RNA-Seq was carried out by Bioanalyzer (Agilent, Santa Clara, CA, USA), and the measurements indicated excellent RNA quality (RIN: 10).

Sequencing

Libraries were prepared with cDNA using the Illumina-compatible ScriptSeq RNA-Seq Library Preparation Kit (Epicenter) for 2x100 bp paired-end library construction, for two technical replicates per condition (silenced and control). Sequencing was carried out on the Illumina HiScan SQ platform, resulting in an average depth of ~35 million raw reads per sample. Library preparation and sequencing were carried out in the Center for Clinical Genomics and Personalized Medicine, University of Debrecen.

Statistical analysis and bioinformatics

Raw reads in FASTQ format were submitted to quality control using FASTQC, followed by quality and adapter trimming using the *fastx* toolkit. Filtered reads were mapped to the Hg19 human reference with the STAR aligner, using parameters optimized for splice-site discovery [21]. *In silico* contaminant screening was also carried out, and ambiguously mapping reads were discarded from further analysis. Potential fusion transcripts were predicted by TopHat2 using the fusion-search algorithm [22]. Following mapping, transcript assembly and annotation were carried out using Cufflinks, and read counts were summarized at the gene and exon levels by using htseq-count [23, 24]. Count data were then processed using the DESeq and DEXSeq packages to quantify differential gene expression and differential exon usage, respectively [25]. Exons with a coverage <5 reads were discarded from the analysis. Functional annotation of differentially expressed genes was carried out using Gene Ontology (GO) enrichment, and extensive comparisons with previous literature-based gene sets and data visualization were carried out in R version 3.0.1, and Cytoscape.

Real-time RT-PCR

Using the iScript TM cDNA Synthesis kit (#1708891, Bio-Rad, Hercules, CA), 1 mg total RNA purified from HPV-KER cells was reverse transcribed. Subsequently, real-time RT-PCR was carried out using custom primer sets and the Universal Probe Library (Roche, Basel, Switzerland) with an iQ Supermix (#1708862, Bio-Rad, Hercules, CA) to quantify transcript abundance. Relative gene expression data were calculated by the ΔΔCt method, normalizing the expression to the 18S ribosomal RNA.

PCR

Samples were collected from the siRNA silenced HPV-KER cell cultures, and total RNA was isolated using the TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA), according to the instructions of the manual. cDNA synthesis was performed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) from 1 μg total RNA. To amplify a 847 bp product from EDA– fibronectin and a 1221 bp product from EDA+ fibronectin, specific primers were used with PCR conditions summarized in Szell et al. (2004). Semiquantitative analysis of the differentially spliced fibronectin isoforms are also described in the referred paper. Amplification products (10μl) were size fractionated on a 1% agarose gel at 90 V. Image acquisition and analysis were carried out on a BioRad GelDoc XR densitometer.

RESULTS

Immortalized HPV-KER cells in which both the LUC7L3 and SFRS18 splicing regulators were silenced were subjected to RNA-Seq profiling. Silencing efficiencies, determined by qPCR prior to sequencing, were between ~70–80% (Suppl. Fig 1). Following read mapping to reference Hg19, de novo transcript assembly was performed to identify the potentially novel transcripts and isoforms in the HPV-KER cell line, as well as to provide accurate exon-level annotation for downstream analyses. To assess the alterations caused by the dual silencing of the splicing factors, differential expression and differential exon usage metrics were generated; allowing the complexity and possible errors introduced by isoform reconstruction methods to be circumvented [26, 27].

Differential gene expression

As expected from the experimental model, combined silencing of the LUC7L3 and SFRS18 splicing factors resulted in moderate changes in gene expression (Suppl. Fig. 2), accompanied by extensive disturbances in exon inclusion and exclusion rates. As a result, 35 protein-coding genes were identified as differentially expressed (logFC > 0.5, FDR < 0.05), with IFI6 and MX1, ISG15 and KRT6A mRNAs showing the most robust fold-changes. Notably, the majority of these regulated genes share functions related to type I interferon signaling based on enrichment analysis. The most significant enriched GO terms were cellular response to type I interferon, cellular response to cytokine stimulus (GO:0071345), regulation of viral genome replication (GO:0045069); these functions were also identified in the functional interaction network based on the Reactome and GeneMania databases (Fig. 1. a). Independent qPCR validation of IFI6 expression indicated up to four-fold changes, which agrees well with our high-throughput results (Fig. 1. b).

Differential exon usage

Differential exon usage was measured for the assembled transcript models, and detected in 224 exons of 217 genes at logFC >0.5, FDR <0.1, corresponding to p-value < $7x10^{-4}$. It is notable that differential usage of multiple exons per gene was detected for a considerable number of

genes throughout the dataset, possibly indicating fine-tuned changes induced by silencing. Differential exon usage was detected in several non-coding RNAs, whereas functional network analysis of the most significant genes indicted an extensive co-expression background, suggesting overlapping regulation at the transcriptional and post-transcriptional levels (Fig. 2). Transcripts regulated with regard to splicing pattern alterations did not exhibit extensively shared molecular functions, in contrast to regulation detected at the gene expression level. Notably, mining of co-expression-level databases resulted in the identification of abundant relations between regulated genes, indicating their coordinated transcriptional regulation, and, as supported by our current dataset, possibly shared post-transcriptional processing events. The resulting complete functional network contained 172 genes, with only 4 disconnected nodes, and an average node connectivity degree of 13.4. Most connecting edges corresponded to co-expression, followed by physical and genetic interactions. The core set of the most highly connected genes is presented in Fig 3.

Experimental validation of fibronectin exon usage was also carried out on the scrambled controls and siRNA silenced samples of the RNA-Seq experiment. RT-PCR using exon-specific, junction-spanning primers for fibronectin transcripts, followed by visualization with agarose gel electrophoresis, indicated significantly altered inclusion of exon 33 (encoding the EDA domain) and the decreased relative abundance of the EDA+ isoform (Fig. 4).

DISCUSSION

In recent years, NGS-based gene-expression profiling techniques, such as RNA-Seq, have revolutionized psoriasis research. RNA-Seq possesses a wider dynamic range compared to microarrays and permits the investigation of potentially novel transcripts, non-coding RNAs and different splicing isoforms [28-33]. Nonetheless, only a few psoriasis studies to date address differential exon usage or altered expression of RNA isoforms [30, 32, 33].

LUC7L3 and SFRS18 splicing factors have been selected based on the results of the aforementioned cDNA microarray experiment, where we identified genes showing T-lymphokine-induced differential expression among control and psoriatic non-involved epidermis [14, 19]. Products encoded by LUC7L3 and SFRS18 genes are both SR-rich splicing regulators. LUC7L3 — which has a strong sequence similarity with yeast U1 snRNP component Luc7p — has also been shown to participate in the cisplatin-induced resistance of tumor cells [34, 35]. Another report suggests LUC7L3 protein binding to the cAMP responsive element, and a recent article claimed antiviral property for this splicing regulator [36, 37]. Relatively few data are available for SFRS18. Zimowska and Lin et al. reported interaction of the cell-adhesion and splicing protein pinin (PNN) and SFRS18 [38, 39].

The main purpose of the present study was to determine differential gene expression and exon usage upon altered expression of LUC7L3 and SFRS18. In agreement with our primary hypothesis, the extent of gene expression changes was relatively moderate; in contrast, several significant alterations in differential gene expression were induced by the siRNA transfection. Among these data, the most pronounced change was observed for IFI6 (also known as G1P3), an interferon stimulated gene (ISG), which mediates anti-inflammatory, antiviral and antiproliferative effects of interferons. IFI6 is a member of the human ISG12 family, which also includes ISG12A (IFI27), ISG12B, ISG12C. In contrast to other ISG12 isoforms, IFI6 was demonstrated to possess an antiapoptotic feature [40]. In addition to its involvement in cancer, biological function of IFI6 in psoriasis pathogenesis has also been reported [40, 41]. Based on the previous results of our research group, IFI6 might contribute to keratinocyte survival due to its antiapoptotic effect. Experimental data suggest elevated IFI6 gene expression both in the psoriatic non-involved and involved epidermis as compared to the healthy samples [41]. Gene expression changes of IFI6 have been successfully validated, exhibiting up to ~fourfold

elevation in expression. In addition, we demonstrated increased levels of the ISG12 (IFI27) mRNA, also implicated in psoriasis [42]. Thus, LUC7L3/SFRS18 might contribute to balancing pro- and antiapoptotic events.

Furthermore, several predicted interactors for IFI6 (https://string-db.org/cgi/network.pl?taskId=Mq3bitC7x2Bp) exhibited significant upregulation in the silenced cells. Of these predicted genes, we detected OAS1, OAS2, IFIT1, IFIT3, ISG15, MX1, all of which are related to antiviral immunity [43, 44]. The elevated expression of MX1, ISG15 and OAS2 in psoriatic skin samples has been verified in a previous experiment [43]. In a recent RNA-Seq experiment, Raposo and his co-workers found that 16 antiviral genes — including ISG15 — showed at least twofold elevation in psoriatic epidermis [44]. We have also demonstrated LUC7L3/SFRS18-regulated expression of KRT6A and S100A9, which are known innate immunity-related molecules [45, 46].

In contrast to our results for differential gene expression, our investigation of differential exon usage indicated more robust alterations. The extracellular matrix protein fibronectin is one of the most suitable models for studying splicing events. Presence of the EDA domain is associated with intrauterine development, wound healing and cancer pathogenesis [47, 48]. In addition, EDA+ fibronectin was demonstrated to be a putative factor modifying keratinocyte response to mitogenic signals [17]. In our previous work, we verified that, similarly to other SR-rich proteins (such as SF2/ASF), LUC7L3, PPIG and SFRS18 also facilitate inclusion of the EDA domain [49-51]. We were able to validate the significant changes of EDA+/EDA– fibronectin abundances in our present large-scale experiment as well [19]. In addition to differential splicing of fibronectin, we also demonstrated the significant upregulation of ITGA5, a fibronectin receptor. Data in the literature demonstrate that presence of the EDA domain alters the binding features of fibronectin and increases its affinity for ITGA5 [49, 52, 53]. Elevated levels of ITGA5 might be a compensatory mechanism against the decreased relative amount of EDA+ fibronectin.

LUC7L3, formerly designated CREAP-1, is characterized by its binding domain for the cAMP regulatory element [36]. However, in addition to potential direct interaction with CRE, silencing of the splicing factors also alters the splicing pattern of CREB1. Thus LUC7L3 potentially plays a modulatory role in this fundamental element of transcription: altered splicing might influence the protein binding of CREB1. Components of various cellular processes, such as CUL1, HERC1 and HERC6 were are also affected. CUL1 is a component of the E3 ubiquitin ligase

complex and is overexpressed in melanoma, leading to hyperproliferative processes [54]. The HERC gene family is involved in multiple molecular processes, including cell-cycle regulation, DNA repair and ubiquitination, and is also differentially regulated in psoriatic skin. HERC6 is indicated in E3 conjugation of ISG15 [55]. OAS2, indicated in antiviral processes, exhibited marked gene expression upregulation as well as differential exon usage. Several genes with relatively sparse functional characterization available in the current literature presented markedly altered exon usage. These include NBEAL1, AUH, with RNA-binding and hydratase activities, and the HERC2 paralog RCC1 [56-58].

Differential exon usage for long non-coding RNAs has been detected using RNA-Seq [31]. We have also detected lncRNAs with differential exon usage, most notably TINCR and NEAT1. The role of NEAT1 is relatively well studied in tumorigenesis: its overexpression has been verified in lung, esophagus, colorectal and hepatocellular carcinoma [59]. NEAT1 might also be involved in psoriasis, as keratinocyte hyperproliferation is an elementary step in the pathogenesis. The widespread functionality of TINCR lncRNA in keratinocyte maturation has been recently described: whereas another non-coding transcript, ANCR, is needed for the maintenance of keratinocyte precursors, TINCR helps terminal differentiation. In undifferentiated keratinocytes, TINCR is regarded as a low abundance transcript, whereas in differentiated keratinocytes, TINCR exhibited elevated expression [60, 61]. Accordingly, we have also shown that TINCR is expressed at low levels in HPV-immortalized cells. Altered splicing patterns might affect TINCR binding capacity through the inclusion of one of its several "TINCR box" motifs.

In our present RNA-Seq experiment, we demonstrated that the LUC7L3 and SFRS18 splicing factors contribute to the regulation of several well known psoriasis-associated pathways, including the IFN signaling pathway, antiviral immunity and ubiquitination. Regulatory roles of these SR-rich splicing factors have also been verified for fibronectin mRNA maturation. These results, together with our results for long non-coding RNA expression and exon usage changes, might open new insights to molecular disturbances in early psoriasis development caused by altered mRNA maturation.

ACKNOWLEDGEMENT

The work was supported by OTKA grants 5K321 and K105985, and by GINOP-2.3.2-15-2016-00015 and GINOP-2.2.1-15-2016-00007 research grants.

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FIGURES AND LEGENDS

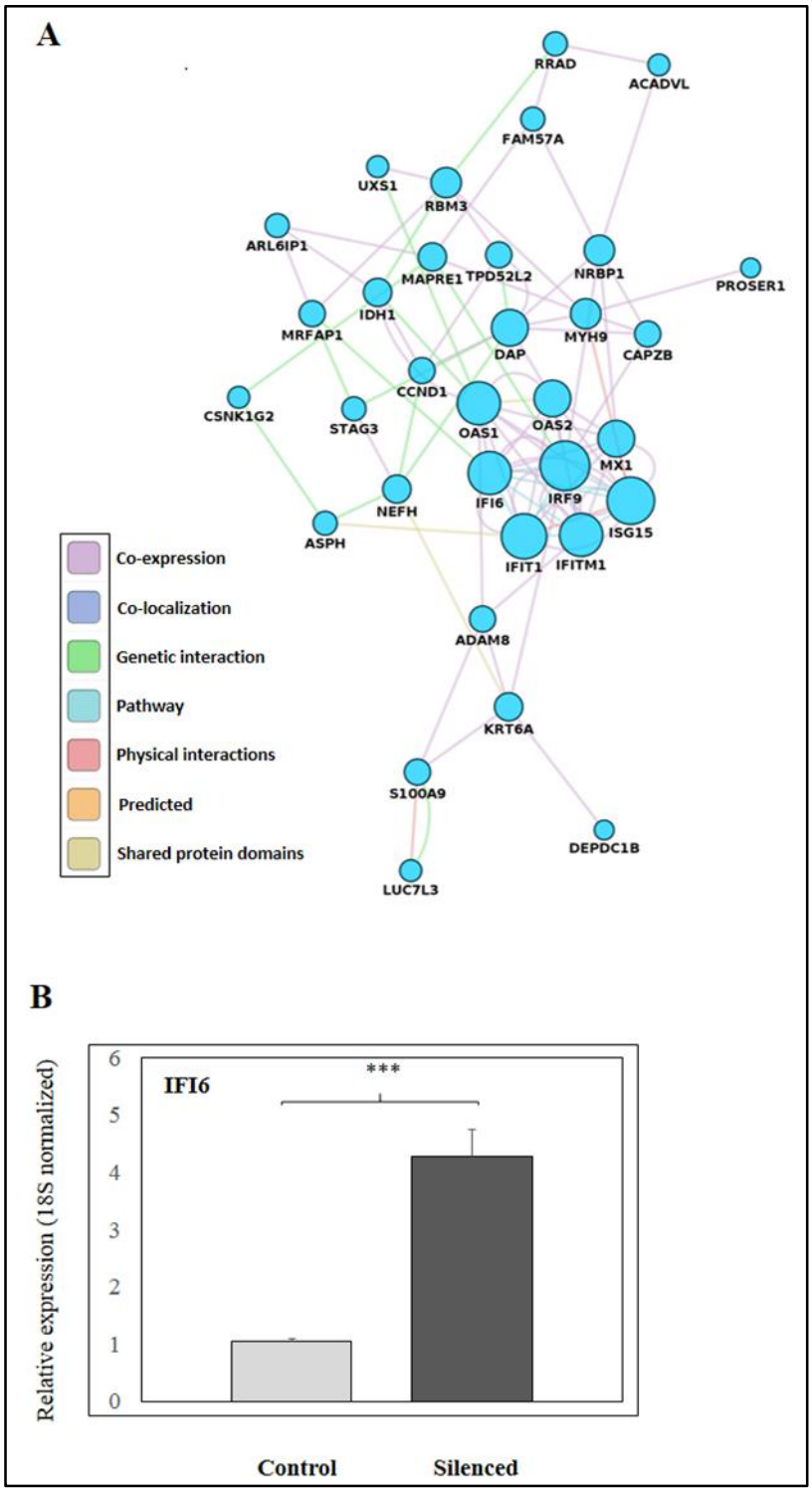


Figure 1: Functional network of significantly differentially expressed genes (log2FC>0.5, FDR<0.05) in response to dual silencing of LUC7L3 and SFRS18, with edges corresponding to GeneMania and Reactome annotation categories **(a)**. qRT-PCR validation of IFI6 expression

changes in siRNA-silenced samples; columns present the mean of three independent experiments. p<0.05 was considered as statistically significant **(b)**.

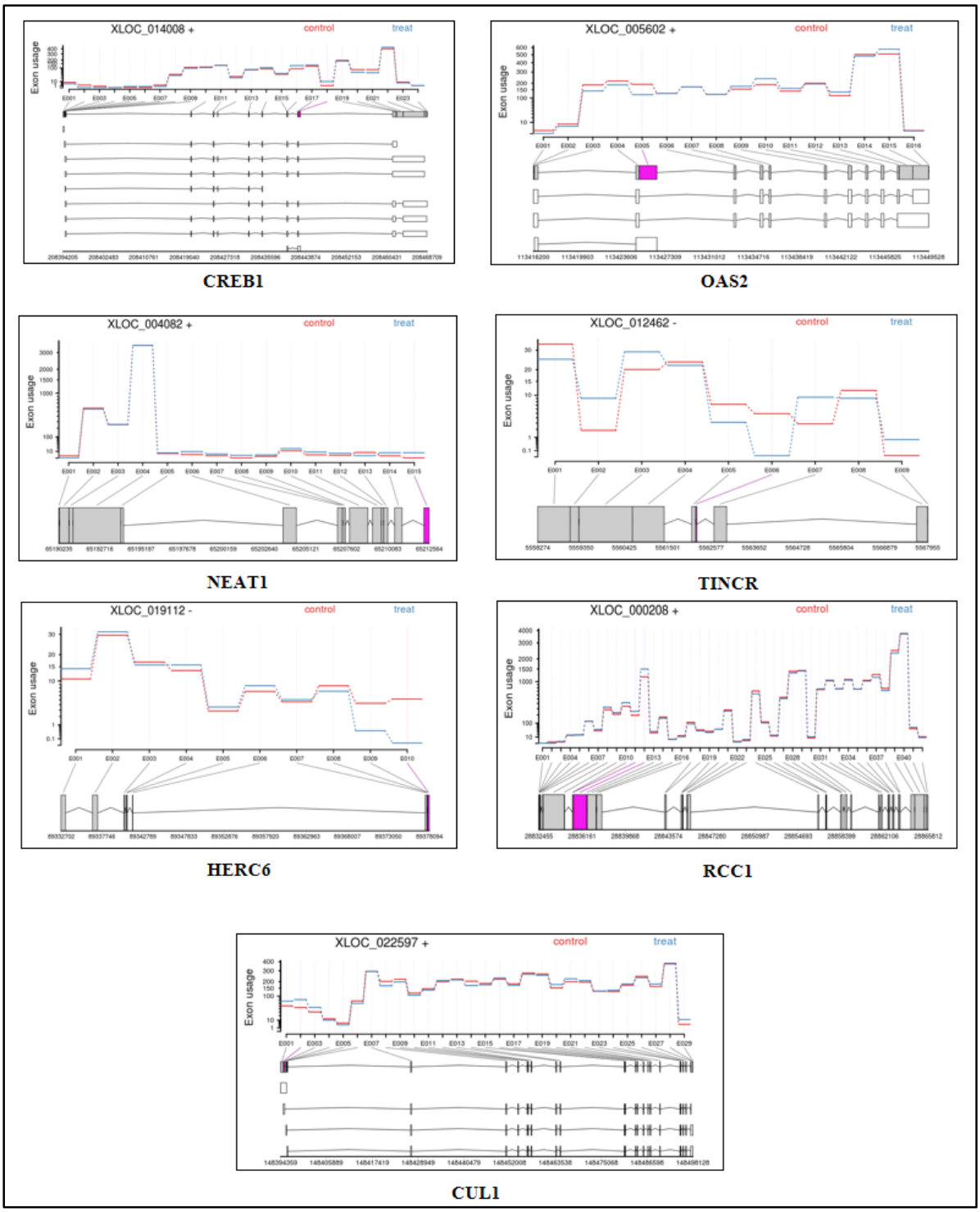


Figure 2: DEXSeq differential-exon-usage plots of selected transcripts showing significant alterations (logFC>0.5, FDR<0.1). Red: average exon usage frequency in control samples; blue: average exon usage frequency in treated samples; pink: significantly differentially expressed exons. De novo assembled transcript diagrams depicted in bottom rows.

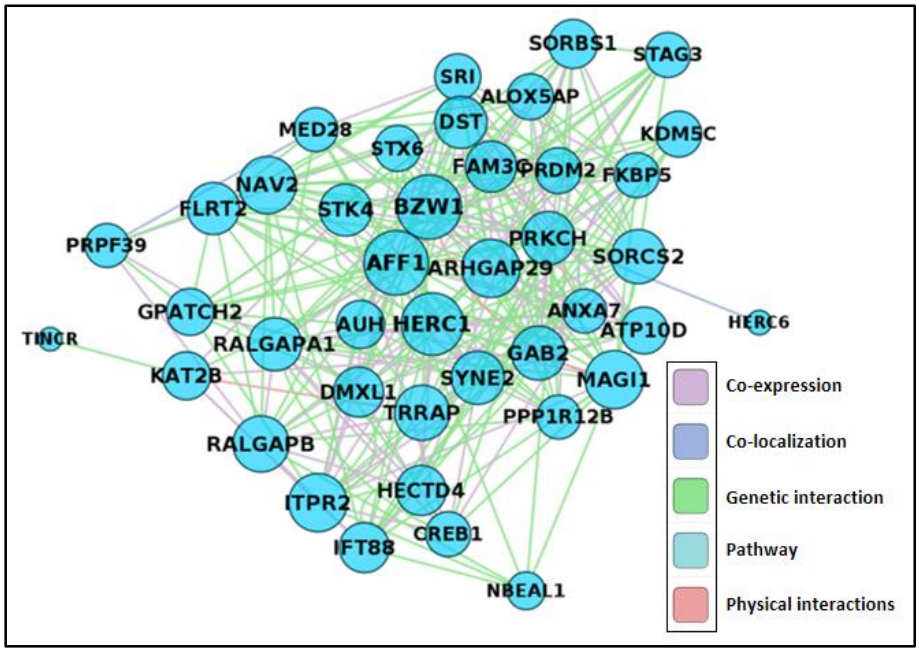


Figure 3: Sub-network representation of a functional network constructed from genes exhibiting significant exon usage changes, with edge color corresponding to GeneMania and Reactome annotation categories.

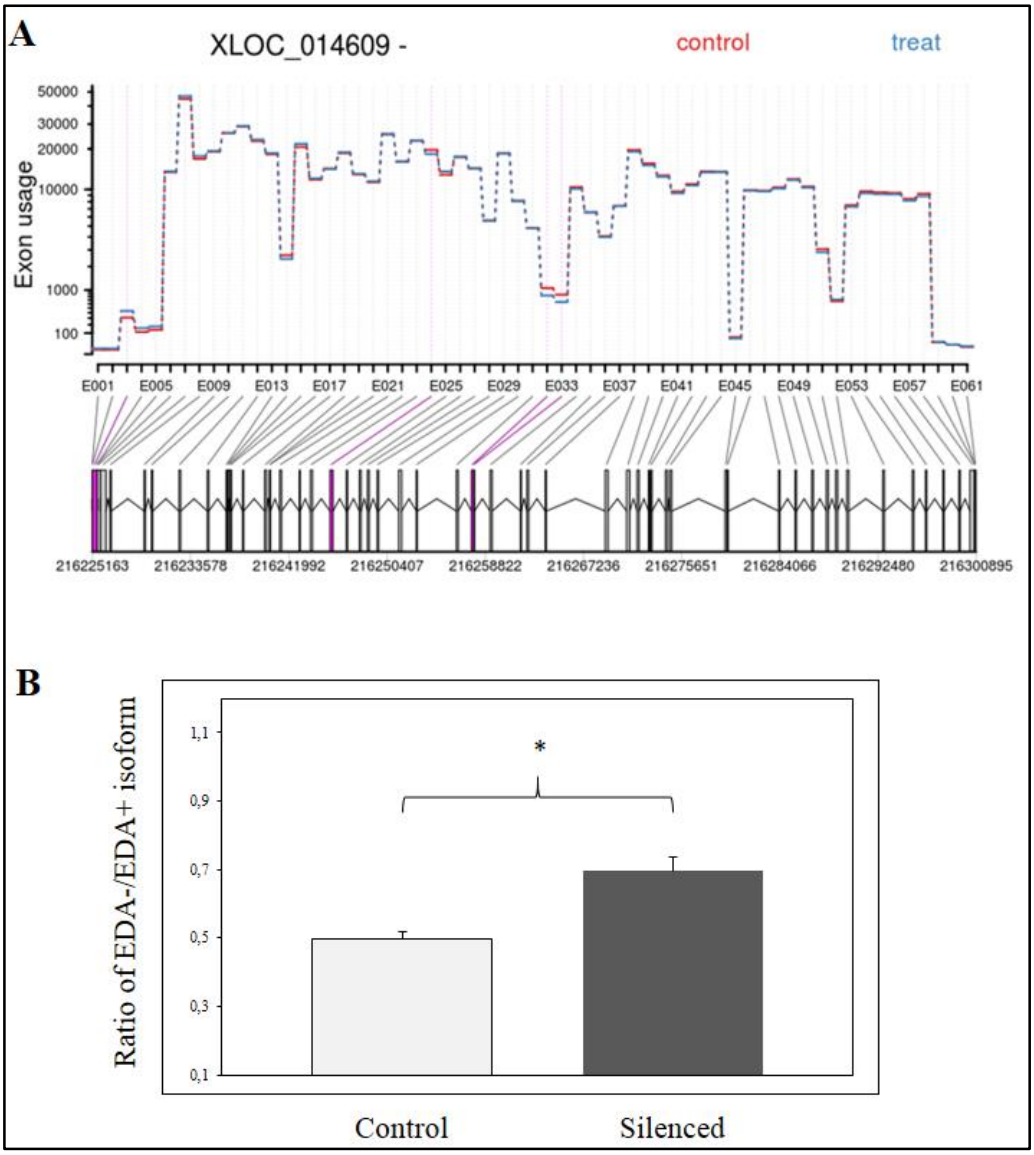
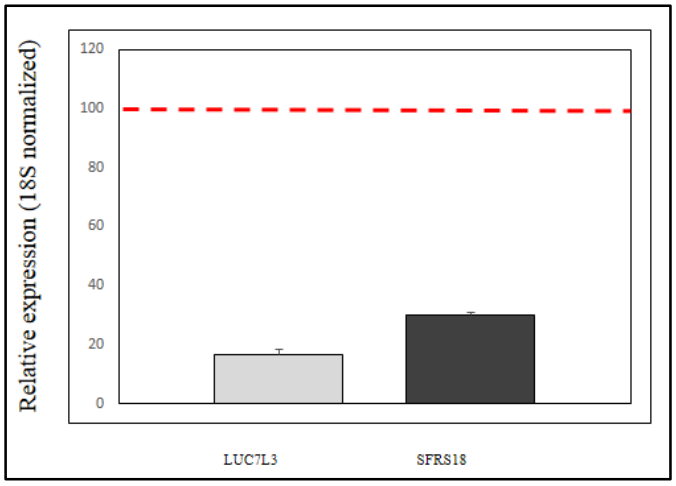
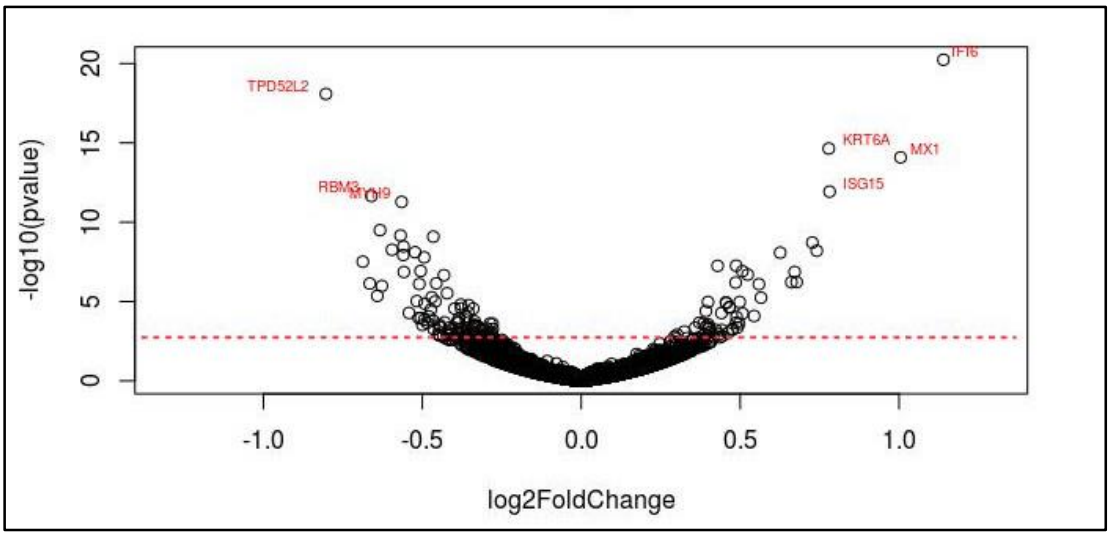


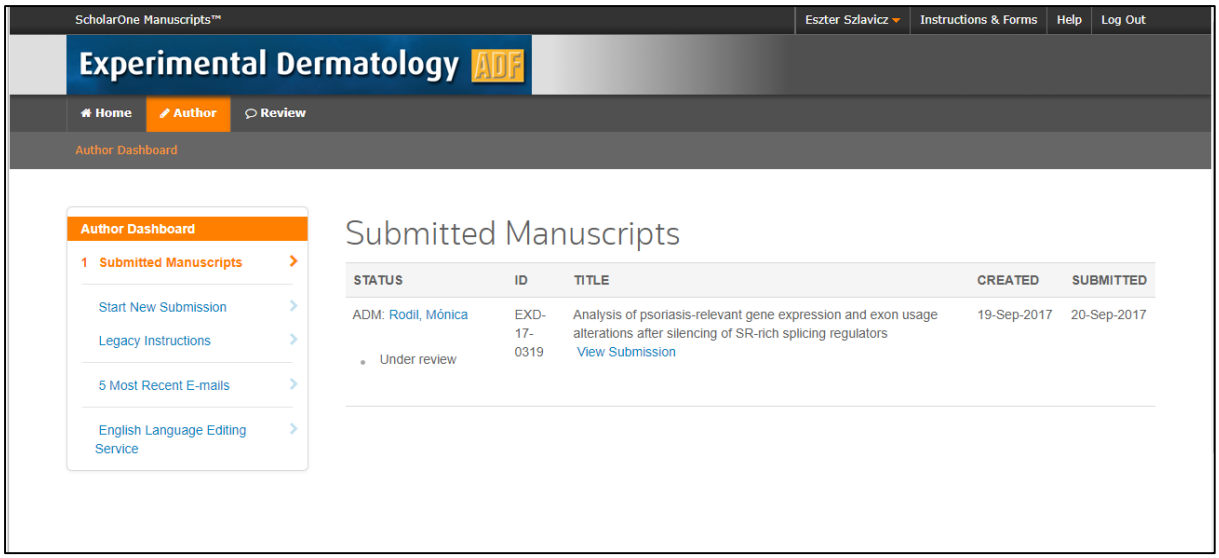
Figure 4: DEXSeq differential-exon-usage plot of fibronectin (FN1), showing the differential inclusion of exon 33, encoding the oncofetal EDA domain. Red indicates the average exon-usage frequency in control samples, and blue in silenced samples. Significantly differentially expressed exons are indicated in pink. **(a)**. Independent qRT-PCR-based validation of differential exon usage, indicating abundance of alternative splicing variants in control (grey) and treated (black) samples in double replicates. Significance threshold: $p<0.05$ **(b)**.



Supplementary Figure 1: Combined siRNA silencing of LUC7L3 and SFRS18 splicing factors was the most effective for the alteration of EDA domain inclusion of fibronectin during mRNA maturation. The efficacy of silencing was 83.6%±4.24 for LUCL73 and 70.1%±1,56 for SFRS18, in two replicates



Supplementary Figure 2: Volcano plot of differentially expressed genes upon LUC7L3/SFRS18 double silencing. Dashed line indicates p-value cut-off=0.001.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ADM: Rodil, Mónica
Under review
EXD-17-0319
Analysis of psoriasis-relevant gene expression and exon usage alterations after silencing of SR-rich splicing regulators
View Submission
19-Sep-2017
20-Sep-2017

II.

Mol Cell Biochem
DOI 10.1007/s11010-017-3090-1

Splicing factors differentially expressed in psoriasis alter mRNA maturation of disease-associated EDA+ fibronectin

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Received: 17 February 2017 / Accepted: 1 June 2017

Abstract The EDA+ fibronectin splicing variant is over-expressed in psoriatic non-lesional epidermis and sensitizes keratinocytes to mitogenic signals. However, regulation of its abundance is only partially understood. In our recent cDNA microarray experiment, we identified three SR-rich splicing factors—*splicing factor, arginine/serine-rich 18 (SFRS18), peptidyl-prolyl cis–trans isomerase G (PPIG),* and *luc-7 like protein 3 (LUC7L3)*—which might be implicated in the preactivated states of keratinocytes in psoriatic non-involved skin and could also contribute to the regulation of fibronectin mRNA maturation. In this study, we investigated the role of LUC7L3, PPIG, and SFRS18 in psoriasis and in the mRNA maturation process of fibronectin. Regarding tissue staining experiments, we were able to demonstrate a characteristic distribution of the splicing factors in healthy, psoriatic non-involved and involved epidermis. Moreover, the expression profiles of these SR-rich proteins were found to be very similar in synchronized keratinocytes. Contribution of splicing facwwtors to the EDA+ fibronectin formation was also confirmed: their siRNA silencing leads to altered fibronectin mRNA and protein expression patterns, suggesting the participation in the EDA domain inclusion. Our results indicate that LUC7L3, PPIG, and SFRS18 are not only implicated in EDA+ fibronectin formation, but also that they could possess multiple roles in psoriasis-associated molecular abnormalities.

Keywords Psoriasis · mRNA splicing · EDA+ fibronectin · SR-rich splicing factors

Introduction

During the past few years, huge advances have been made in the field of psoriasis research; however, the exact molecular background of this chronic inflammatory disorder has not been fully revealed and many questions remain to be answered. Development of psoriatic lesions is associated with keratinocyte hyperproliferation and abnormal T-lymphocyte function, although it is still controversial whether epithelial or immunological elements are determinant in the primary phase of the disease. The extent to which genetic and environmental factors lead to the disorder is also a subject of discussion [1–3].

Recently, a novel molecular factor has been identified that might be responsible for the elevated responsiveness of keratinocytes to the stimuli of professional immune cells. This putative factor is a splice variant of fibronectin that contains the oncofetal EDA domain (EDA+ fibronectin) [4–6]. Although fibronectin is an excellent model for studying splicing (having more than 20 mRNA variants), processes regulating mRNA maturation and the mechanisms affecting EDA+ fibronectin formation are not fully

Electronic supplementary material The online version of this article (doi:10.1007/s11010-017-3090-1) contains supplementary material, which is available to authorized users.

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Published online: 06 June 2017

understood [7, 8]. The EDA domain is a Type III repetitive module of fibronectin, typically present during embryonic development. Although the tendency is exclusion of the EDA domain in the adult organism, EDA+ fibronectin is elevated under some circumstances, including wound healing, inflammation, and malignant processes [8–13]. In psoriasis, elevated EDA+ fibronectin expression was described for psoriatic non-involved epidermis, and we showed that proliferating keratinocytes are able to produce this isoform [4, 5].

Several molecular factors in addition to EDA+ fibronectin have been implicated in psoriasis pathogenesis. Application of large-scale gene expression studies extended our knowledge regarding molecular abnormalities in involved and non-involved epidermis [14–19]. Recent RNA-Sequencing-based experiments have indicated that a large number of mRNAs are consistently differentially expressed in healthy and involved skin samples across independent studies, at both the transcript and gene levels [20, 21]. Specifically, a systematic comparison of the above-mentioned datasets indicated over 10,000 differentially expressed transcripts, suggesting the potential role of mRNA maturation processes in psoriasis development [22].

In a recently performed cDNA microarray experiment, we aimed to compare the responsiveness of healthy and psoriatic non-involved epidermis to treatment with a mixture of T-lymphokines (GM-CSF, IFN-γ, IL-3) previously shown to induce psoriatic plaque formation [23, 24]. Functional characterization of the identified genes exhibiting differential regulation upon T-lymphokine treatment showed that they play a role in several cellular procedures including the influence of cell morphology, development, and cell death. Moreover, a set of these genes participate in the metabolism of small lipids [23]. Among these genes were three that are implicated in mRNA maturation: *splicing factor, arginine/serine-rich 18 (SFRS18,* also known as *PNN-interacting serine/arginine-rich protein, PNISR), peptidyl-prolyl cis–trans isomerase G (PPIG),* and *luc-7 like protein 3 (LUC7L3)*. Products of these genes belong to the family of SR-rich proteins and function as trans-regulators of splicing events (UniProtKB-O95232 (LC7L3_HUMAN); UniProtKB-Q13427 (PPIG_HUMAN); UniProtKB-Q8TF01 (PNISR_HUMAN)). This finding is notable, as relatively few articles mention the relationship between psoriasis and disturbances of mRNA maturation.

In our study, characteristics of splicing-factor expression were examined in synchronized, immortalized cell cultures and skin samples. Since the regulation of fibronectin splicing is only partially elucidated, we aimed to determine whether these newly identified splicing factors participate in the production of the EDA+ fibronectin isoform. For this purpose, an in vitro functional assay was constructed to detect changes in fibronectin splicing patterns during decreased expression of the LUC7L3, PPIG, and SFRS18 splicing factors.

Materials and methods

Skin biopsies

To carry out our cDNA microarray experiment, 4 healthy volunteers and 4 patients (age 18–60 years) with moderate-to-severe chronic plaque-type psoriasis (Psoriasis Area and Severity Index score determined by dermatologists) were enrolled in the study after a medication-free period of ≥4 weeks without systemic therapy and/or ≥2 weeks without local therapy. Prior to surgical intervention, participant-informed consent was obtained. Tissue collection complied with the guidelines of the Declaration of Helsinki and was approved by the Regional and Institutional Research Ethics Committee (2799, 3517).

Organotypic skin cultures

Organotypic skin cultures were established from each half-cut from shave biopsies [23]. Briefly, epidermis samples placed on cellulose acetate/cellulose nitrate filters (2.2 μm porosity, Millipore) were transferred to a stainless steel grid platform in a 6-well plate. As culture medium, Dulbecco's modified Eagle's medium (DMEM) supplemented with 12 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Carlsbad, CA) was applied. For all donor skin specimens, one-half of the organotypic cultures was treated with the lymphokine mixture, containing 1 ng/ml IFNγ, 1 ng/ml GM-CSF, and 0.3 ng/ml IL-3, and the other half was not treated. Using standard culturing conditions (at 37 °C in a 5% CO_2 atmosphere), organotypic skin cultures were maintained at the air/liquid interface for 72 h. Subsequently, the epidermis was separated from the dermis by overnight incubation in Dispase solution (grade II, Roche Applied Science) at 4 °C, and then placed in TRI Reagent (Molecular Research Center Inc., Cincinnati, OH).

Real-time RT-PCR

One microgram of total RNA purified from organotypic skin culture samples, HPV-KER and HaCaT cells were reverse transcribed using the iScript TM cDNA Synthesis kit (#1708891, Bio-Rad, Hercules, CA). RT-PCR was performed with the cDNA to quantify transcript abundance using custom primer sets and the Universal Probe Library (Roche, Basel, Switzerland) with an iQ Supermix (#1708862, Bio-Rad, Hercules, CA). Relative gene expression data were calculated by normalizing the

expression data for the 18S ribosomal RNA and using the ΔΔCt method.

Immunofluorescent staining

Biopsies were frozen, embedded, stored at −80 °C, and then cut into 6-μm sections. The following primary antibodies were used: LUC7L3 (1:300, Abcam, Cambridge, UK), PPIG (1:300, Abcam), and SFRS18 (1:250, Novus Biologicals, Littleton, USA). Anti-mouse IgG-Alexa Fluor 647 and anti-rabbit Alexa Fluor 546 were used as secondary antibodies, both at 1:500 dilution (Life Technologies, Carlsbad, CA). For negative staining controls, sections were incubated without the primary antibody (in case of PPIG) or with normal rabbit IgG as isotype control antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Nuclei were stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI).

Fluorescent microscopic images were analyzed using ImageJ software, by calculating corrected-total-cell-fluorescence values based on integrated density of stained cell areas.

Culturing of HPV-KER and HaCaT cells

The HPV-KER cell line was immortalized by the HPV E6 oncogene as described by our research group [25]. HPV-KER cells were grown in 75 cm^2 cell culture flasks and maintained in keratinocyte serum-free medium (Gibco® Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark) supplemented with 1% antibiotic/antimycotic solution (PAA, Pasching, Austria) and 1% L-glutamine (PAA) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every two days.

During synchronization of the HPV-KER cells and after confluence was obtained, cells were maintained in glutamine-free and growth factor-free keratinocyte SFM for one week. Cells were harvested with brief trypsinization (0.25% trypsin solution Sigma Aldrich, St. Louis, MO, USA)

Immortalized HaCaT keratinocytes were kindly provided by Dr. Fusenig, N.E. (Heidelberg, Germany). Cells were synchronized as described previously [26].

Western blot analysis

For western blot analysis, equal amounts of proteins were separated on a 10% SDS-PAGE gel and transferred to Pure Nitrocellulose Membrane (Bio-Rad Laboratories). Primary antibodies were the following: LUC7L3 (1:300, Abcam, Cambridge, UK), PPIG (1:300, Abcam), and SFRS18 (1:300, Novus Biologicals, Littleton, US). Anti-mouse and anti-rabbit IgG alkaline phosphate conjugate (Sigma Aldrich, St. Louis, MO, USA) was used as a secondary antibody, and signals were visualized with Sigma Fast TM BCIP/NBT (Sigma Aldrich, St. Louis, MO, USA). An α-actin-specific antibody was used as a loading control (1:2000, Sigma Aldrich, St. Louis, MO, USA).

Gene-specific silencing

Gene-specific silencing was performed with an siRNA transfection method. HPV-KER cells were transiently transfected at approximately 70% confluency. siRNA silencing (transfection reagent and medium) was ordered from Santa Cruz Biotechnology (Dallas, TX, USA). In silencing experiments, scrambled and gene-specific *LUC7L3, PPIG, SFRS18* siRNA duplexes were applied. The most effective silencing was achieved in serum-free culture medium without additive and supplements. The effectiveness of silencing was confirmed by real-time RT-PCR.

Polymerase chain reaction

Samples were collected from the cultures of silenced HPV-KER cells. Total RNA was isolated from cell cultures by TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA), following the instructions provided in the manual. cDNA was synthesized from 1 μg total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Primers specific for human fibronectin (5′-AAGC-CAATTTCCATTAATTACCGAAC-3′ and 5′-TCTCA-TACT TGATGATGTAGCCGGTAA-3′) were used to amplify a 1221 bp product from the EDA+ template and an 847 bp product from the EDA− fibronectin template. PCR conditions are described in Szell et al., and detection and semiquantitative analysis of the differentially spliced fibronectin form are also demonstrated in this paper [4]. Ten microliters of the PCR products were run on 1% agarose gel, photographed and evaluated using Bio-Rad Gel Doc XR densitometer.

Flow cytometry

Cells were collected after a brief trypsinization (0.25% trypsin solution Sigma Aldrich, St. Louis, MO, USA), fixed in Fixation/Permeabilization Concentrate and Diluent (eBioscience, San Diego, USA), and resuspended in PBS. Primary antibodies (anti-EDA+-fibronectin, anti-fibronectin, 1:500 and 1:1000, respectively, Sigma Aldrich, St. Louis, MO, USA) were applied for 45 min. Cells were then washed in PBS and incubated with anti-mouse IgG-Alexa Fluor 647 (Life Technologies, Carlsbad, CA) as secondary antibody, at 1:500 dilution. Samples were analyzed on a FACSCalibur flow cytometer equipped with 488 and 633 nm lasers (Becton–Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis and bioinformatics

Statistical analysis was performed in GraphPad Prism 5.0 Software (GraphPad Prism, San Diego, CA), using one-sample *t*- and unpaired *t*-tests with a $p < 0.05$ significance threshold.

Predictive interaction analysis was carried out using the publicly available STRING database (http://string-db.org/).

Results

Differential expression of splicing-factor genes confirmed for psoriasis

LUC7L3, PPIG, and SFRS18 splicing regulators have been identified in our above-mentioned cDNA microarray experiment. An overview of our approach is presented in Fig. 1.a. Briefly, gene expression before and after lymphokine treatment was compared in healthy epidermis, and we applied the same approach to non-involved psoriatic skin. Genes exhibiting at least a two-fold change in relative expression have been chosen for further comparison. We then selected the genes showing significant differences in mean expression between the control and psoriatic non-involved samples [23]. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was carried out to verify whether the identified genes are differentially regulated in psoriasis. The T-lymphokine induced alterations were confirmed in the case of both *LUC7L3* and *PPIG* splicing factors (Fig. 1b) Although we were not able to validate the cDNA microarray results for *SFRS18* in the RT-PCR experiment, we decided to include this gene in further experiments as it participates in similar biological processes as *LUC7L3* and *PPIG*.

Expression of the splicing factors was also compared in untreated healthy and psoriatic non-involved epidermis samples of the microarray experiment: in this setup, the expression of *LUC7L3* and *SFRS18* was slightly higher in psoriatic non-involved epidermis than in healthy samples (Fig. 1c).

Differentially expressed splicing factors in psoriasis

As the splicing factors showing altered mRNA expression in psoriasis are poorly characterized, we examined the pattern of protein expression in healthy, psoriatic non-involved and involved skin samples. LUC7L3 and PPIG exhibited nuclear localization, whereas SFRS18 showed perinuclear staining (Fig. 2a). LUC7L3 is present in significantly higher amounts in psoriatic non-involved epidermis, whereas SFRS18 shows only minimal, non-significant elevation in non-involved epidermis. The highest levels of expression of LUC7L3 and SFRS18 were found in psoriatic involved epidermis samples. The expression pattern of PPIG was different from that of the other splicing factors: PPIG amounts were significantly lower in psoriatic non-involved epidermis than in healthy and psoriatic samples; however—similarly to LUC7L3 and SFRS18—the highest expression was also detected in psoriatic involved epidermis (Fig. 2b).

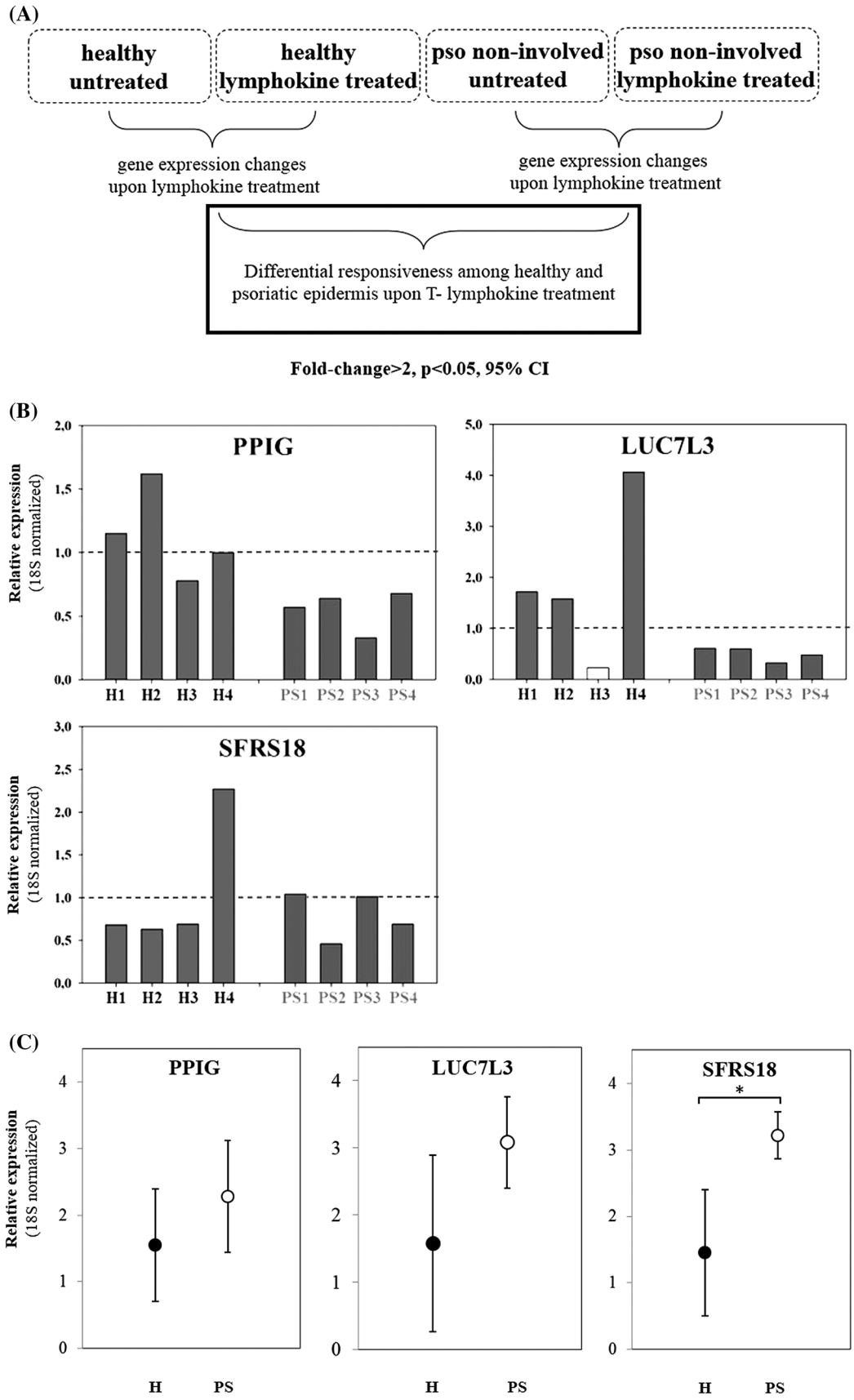
Fig. 1 Experimental design and comparisons used in our previous cDNA microarray study is summarized in (**a**). Gene expression changes of *LUC7L3*, *PPIG,* and *SFRS18* splicing factors induced by T-lymphokine treatment were compared in healthy (H, $n = 4$) and psoriatic non-involved (PS, $n = 4$) epidermis samples (**b**), where the average expression level of untreated samples was considered as baseline expression (relative unit of 1 indicated by *dashed line*). Comparison of *LUC7L3*, *PPIG,* and *SFRS18* basal mRNA expression levels in untreated healthy (H, $n = 4$) and psoriatic non-involved (PS, $n = 4$) epidermis. $p < 0.05$ was considered significant (**c**) ▶

LUC7L3, PPIG, and SFRS18 expression patterns in synchronized cells

To compare the expression levels in proliferating and differentiating keratinocytes, both mRNA and protein expression patterns of the selected splicing factors were analyzed in synchronized, immortalized cells. HPV-KER cells were synchronized by contact inhibition and in the absence of growth factors [22]. Cells were harvested at various time points after release from cell quiescence, and RNA and protein levels were measured by real-time RT-PCR and western blot analysis, respectively.

Comparing the expression of the splicing factors during the proliferation and differentiation phases, a very similar mRNA expression pattern was observed for all three splicing factors (Fig. 3a). mRNA expression decreased at 12 h. The first peak of mRNA abundance was seen at 24 h, with steady elevation starting from 48 h, a phenomenon that is likely induced by cellular stress processes. This synchronization experiment was repeated using synchronized, immortalized HaCaT cells: the splicing regulators exhibited an identical pattern of gene expression in HaCaT cells that were observed in the HPV-keratinocytes (Suppl. Fig. 1).

The pattern of expression in HPV-KER cells of both RNA and protein was similar. However, protein expression was more even across sampled time points than for mRNA. The highest expression of LUC7L3, PPIG, and SFRS18 protein was detected at 48 h after release from cell quiescence (Fig. 3b).


(A)
healthy untreated
healthy lymphokine treated
pso non-involved untreated
pso non-involved lymphokine treated
gene expression changes upon lymphokine treatment
gene expression changes upon lymphokine treatment
Differential responsiveness among healthy and psoriatic epidermis upon T- lymphokine treatment
Fold-change>2, p<0.05, 95% CI
(B)
PPIG
LUC7L3
SFRS18
Relative expression (18S normalized)
H1 H2 H3 H4 PS1 PS2 PS3 PS4
(C)
PPIG
LUC7L3
SFRS18
*
Relative expression (18S normalized)
H PS

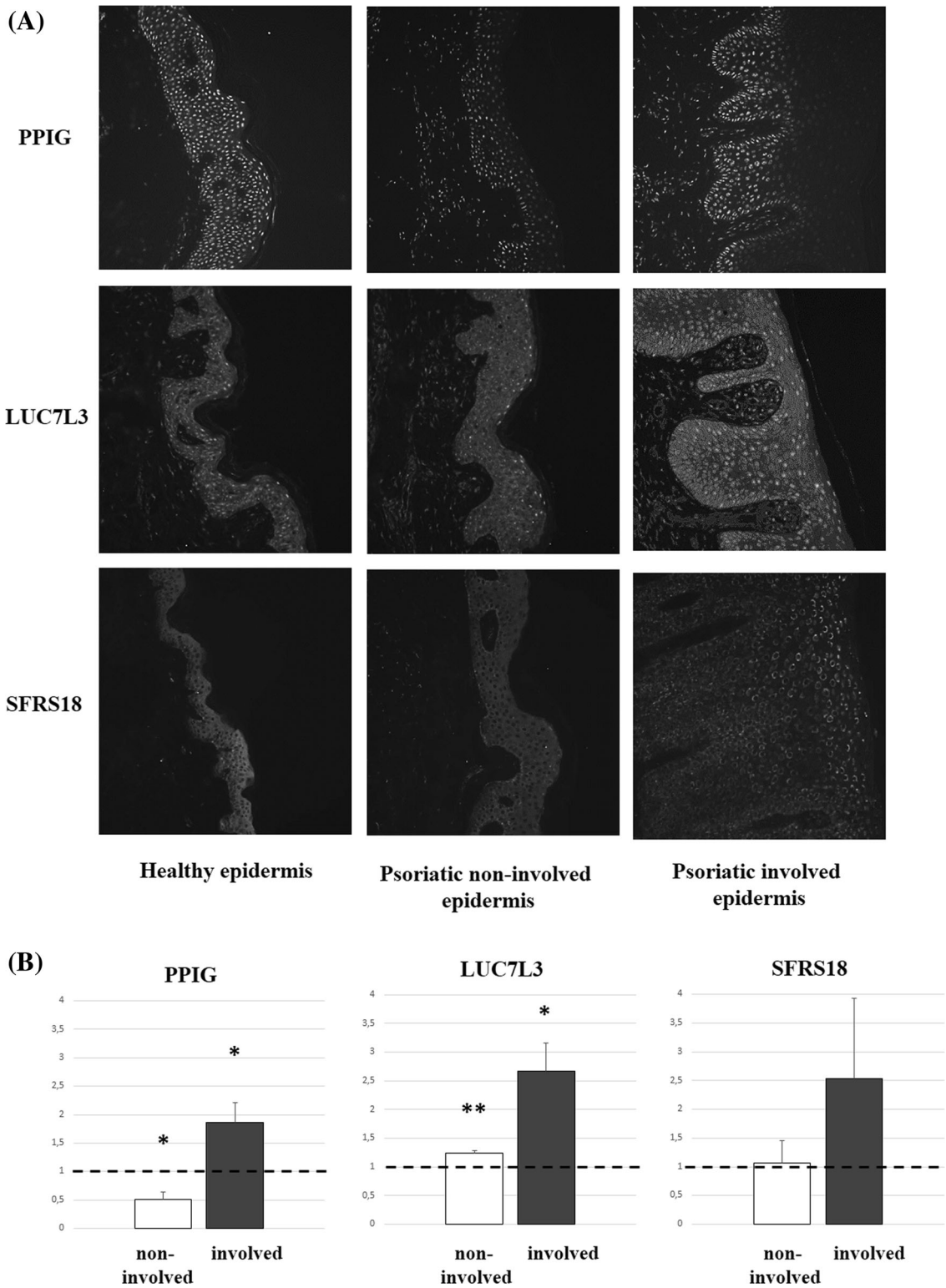


Fig. 2 Immunofluorescent staining of PPIG, LUC7L3, and SFRS18 proteins (**a**) was carried out on healthy ($n = 3$), psoriatic non-involved ($n = 3$), and psoriatic involved ($n = 3$) samples. One representative staining is provided for each protein. Magnification: $\times 40$. Fluorescent intensity measurements of PPIG, LUC7L3, and SFRS18 (**b**) in non-involved and involved epidermis compared to healthy samples are indicated as follows: *dashed lines* represent the unaltered condition, where the ratio of non-involved/healthy and involved/healthy is 1; significance levels are indicated by *asterisks* ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

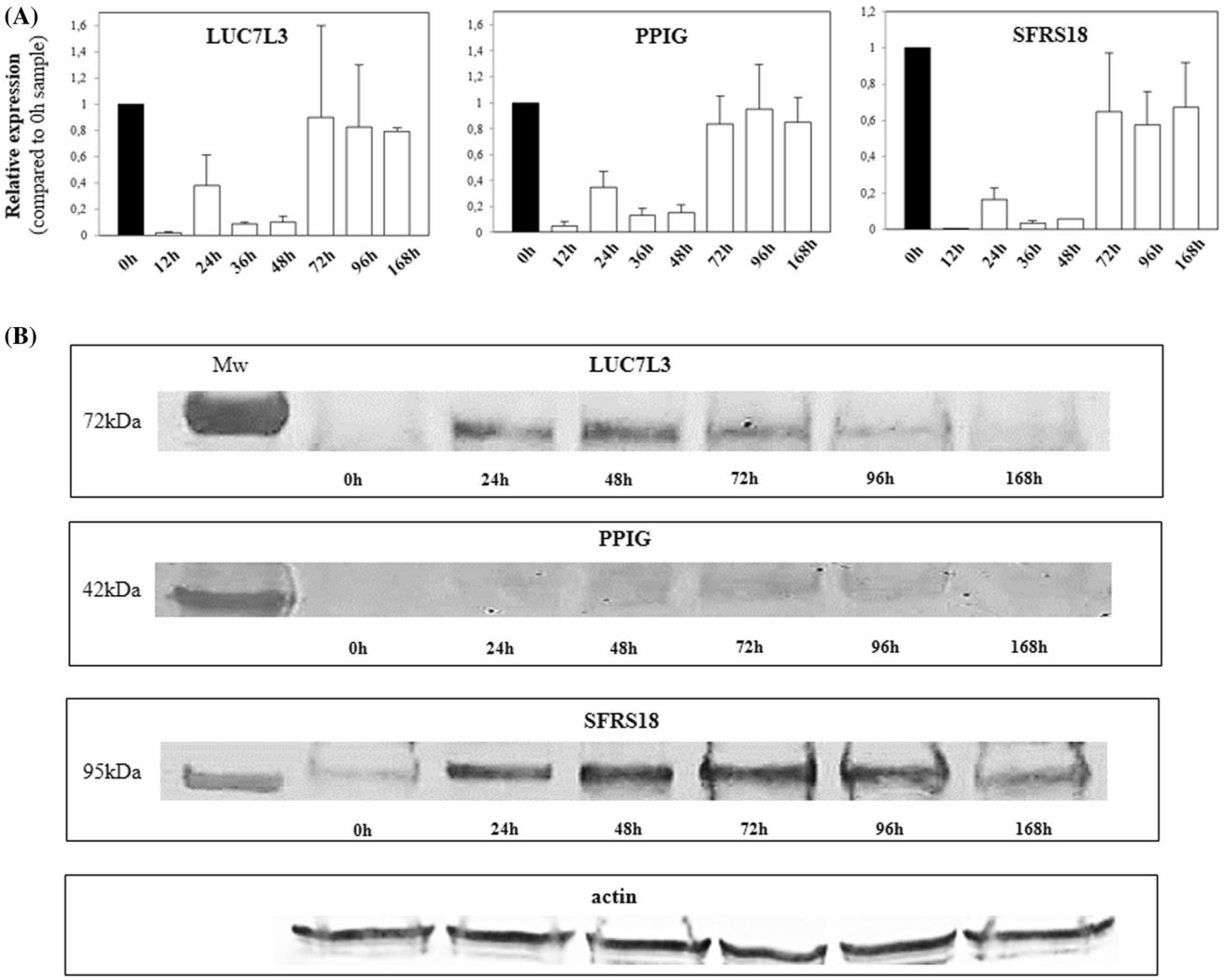


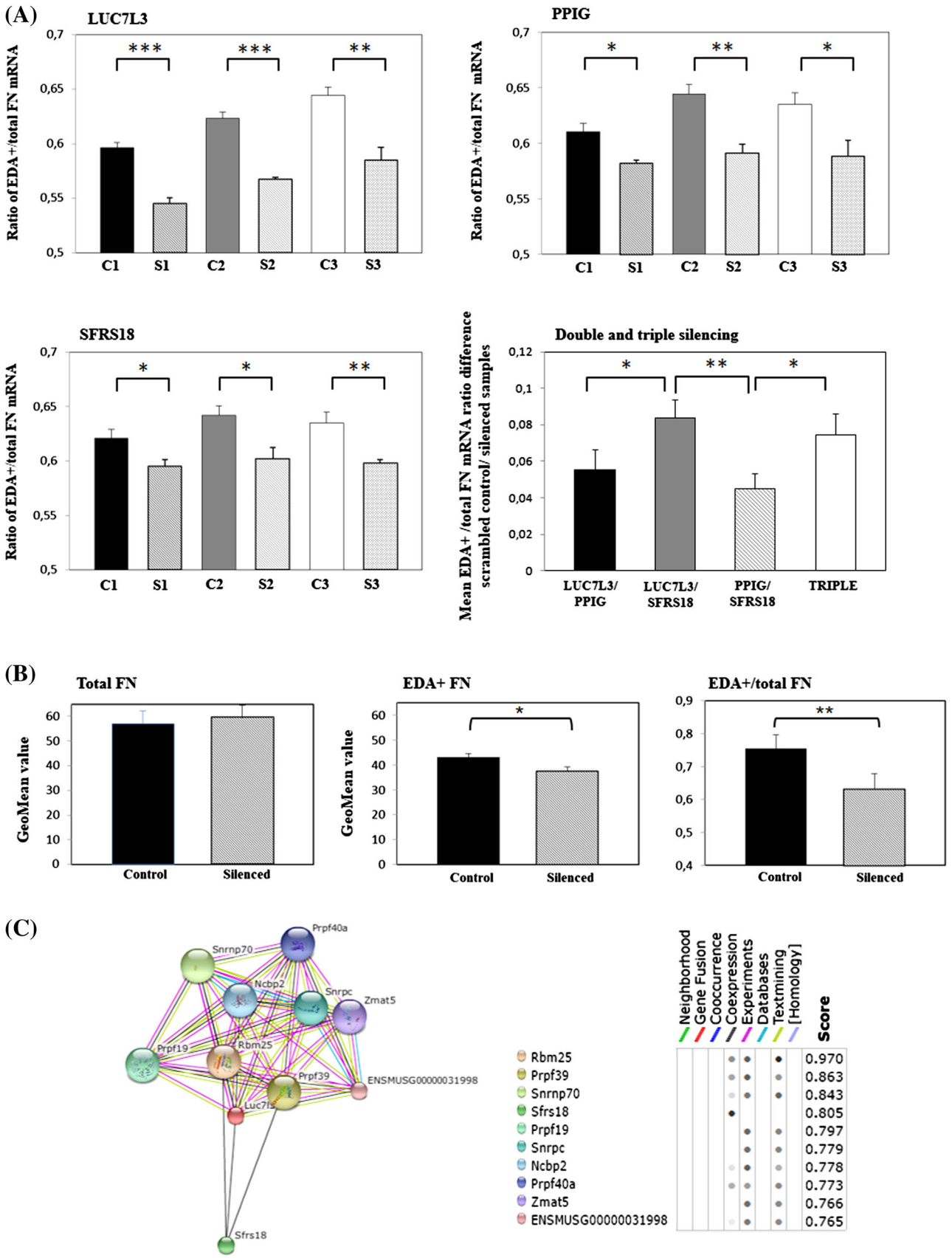
Fig. 3 HPV-KER cells were synchronized by contact inhibition and by withdrawal of supplement, and samples were taken at the indicated time points from the re-passaged (0 h) cells. Changes in mRNA levels (**a**) were followed by real-time RT-PCR and changes in protein levels (**b**) were followed by western blot analysis. The experiment was carried out with three independent series of cell cultures. The mRNA expression data were calculated as the average of these three parallel experiments. One representative western blot is provided for each splicing factor. α-actin was used as a loading control

Silencing of *PPIG*, *SFRS18,* and *LUC7L3* affects fibronectin splicing

To determine the physiological relevance of LUC7L3, PPIG, and SFRS18 in the fibronectin mRNA maturation processes, HPV-KER cells were transfected with siRNA constructs silencing these splicing-factor genes. The EDA+/total fibronectin ratios were compared in scrambled and gene-specific siRNA-transfected cells, and the mean of silencing efficacies was approximately 80% (summarized in Suppl. Fig. 2). Changes in the fibronectin splicing pattern were investigated by RT-PCR, and densitometry analysis was applied as an accurate and semiquantitative method to determine changes in the EDA+/total ratio in response to silencing of the three different splicing factors either alone or in combination.

In the experiments where a single splicing factor was silenced, siRNA transfection of *LUC7L3* has lowered the EDA+/total ratio for fibronectin, and a slight decrease in this ratio was also seen with *PPIG* and *SFRS18* silencing (Fig. 4a). In the scrambled RNA-transfected samples, the relative amount of the EDA+ variant was higher compared to the EDA− fibronectin isoform.

Bioinformatics analysis suggests that LUC7L3 and SFRS18 are interacting partners (Fig. 4c). Therefore, experiments in which two or three genes were silenced were also performed to examine the possible interactions among the splicing factors. The combined silencing of


(A)
LUC7L3
Ratio of EDA+/total FN mRNA
0,7
0,65
0,6
0,55
0,5
***
***
**
C1
S1
C2
S2
C3
S3
PPIG
Ratio of EDA+/total FN mRNA
*
**
*
SFRS18
Ratio of EDA+/total FN mRNA
*
*
**
Double and triple silencing
Mean EDA+ /total FN mRNA ratio difference scrambled control/ silenced samples
0,12
0,1
0,08
0,06
0,04
0,02
0
LUC7L3/ PPIG
LUC7L3/ SFRS18
PPIG/ SFRS18
TRIPLE
(B)
Total FN
GeoMean value
60
50
40
30
20
10
0
Control
Silenced
EDA+ FN
GeoMean value
*
EDA+/total FN
0,9
0,8
0,7
0,6
0,5
0,4
**
(C)
Prpf40a
Snrnp70
Ncbp2
Snrpc
Zmat5
Prpf19
Rbm25
Prpf39
ENSMUSG00000031998
Luc7l3
Sfrs18
Neighborhood
Gene Fusion
Cooccurrence
Coexpression
Experiments
Databases
Textmining
[Homology]
Score
Rbm25 0.970
Prpf39 0.863
Snrnp70 0.843
Sfrs18 0.805
Prpf19 0.797
Snrpc 0.779
Ncbp2 0.778
Prpf40a 0.773
Zmat5 0.766
ENSMUSG00000031998 0.765

◀**Fig. 4** HPV-KER cells were transfected with specific silencing constructs for *LUC7L3*, *PPIG,* and *SFRS18* (**a**) and the ratio of EDA+ fibronectin mRNA was calculated in each repeated experiment (S1, S2, S3). The changes in this ratio were compared to that of the scrambled RNA-transfected controls (C1, C2, C3) in each experiment. Comparison of the effect of double and triple silencing of the *LUC7L3*, *PPIG,* and *SFRS18* genes to the scrambled RNA-transfected controls revealed that the double silencing of *LUC7L3* and *SFRS18* resulted in the most robust changes in the EDA+/total ratio of fibronectin mRNA. Effect of triple silencing did not differ significantly from that of *LUC7L3* and *SFRS18* double silencing. Columns represent means of three independent experiments (+SE), all performed in triplicate. Significance levels are indicated by asterisks ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Total fibronectin and EDA+ fibronectin were detected by flow cytometry in HPV-KER cells ($n = 5$) in which both *LUC7L3* and *SFRS18* were silenced (**b**). Silencing did not affect the amount of total fibronectin, whereas a significant ($p < 0.05$) decrease in the amount of the EDA+ fibronectin was detected after silencing, and this decrease resulted in significant ($p < 0.01$) changes in the EDA+/total ratio of fibronectin. Bioinformatics analysis revealed a predicted functional interaction between the LUC7L3 and SFRS18 splicing regulators (**c**)

LUC7L3 and *SFRS18* leads to the most robust change in the EDA+/total ratio of fibronectin (Fig. 4a). Notably, silencing of all three splicing factors had no additional effect on the ratio compared to the double silencing of *LUC7L3* and *SFRS18*.

The effect of silencing was also examined at the protein level. Total and EDA+ fibronectin amount was measured with flow cytometry using the most effective combination of double *LUC7L3* and *SFRS18* silencing. Similar to the mRNA experiments, the immortalized HPV-keratinocytes expressed high levels of EDA+ fibronectin protein. This double silencing significantly diminished the quantity of EDA+ fibronectin, without decreasing the total amount of fibronectin (Fig. 4b). Taken together, these results demonstrate that silencing of the splicing factors decreased the EDA+/total fibronectin ratio both at mRNA and protein level.

Discussion

The importance of fibronectin as an excellent model for studying splicing events as well as the importance of various fibronectin isoforms—including variants containing the EDA and EDB domains—is increasingly being acknowledged. It was previously shown that EDA+ fibronectin is overexpressed in samples of psoriatic non-involved epidermis [5]. Some years later, our research group reported that both normal cultured keratinocytes and HaCaT cells produce the oncofetal EDA+ fibronectin isoform. Moreover, it has also been proven that keratinocytes from psoriatic non-involved epidermis are sources of EDA+ fibronectin, while the expression of this isoform is minimal in healthy keratinocytes. These data suggest that EDA+ fibronectin might initiate a signal transduction process that sensitizes keratinocytes to the mitogenic signals of immune cells [4].

In our recently performed cDNA microarray experiment, we aimed to reveal that novel molecular factors make it prone to form psoriatic lesions. From this work, we identified three splicing factors that participate in the molecular processes of mRNA maturation. In addition to regulation of splicing, PPIG is responsible for the normal folding of proteins, and LUC7L3 has been implicated in cisplatin resistance in tumor cells [27–29]. PPIG and SFRS18 are physical interactors of the splicing factor and the desmosome-associated protein, pinin [27, 30].

Our study opens the questions whether LUC7L3, PPIG, and SFRS18 are involved in the mRNA maturation process of fibronectin and whether the altered expression of these splicing factors can be linked to the documented presence of EDA+ fibronectin in psoriasis. Although EDA+ fibronectin is known to be involved in several biological processes, the regulation of its synthesis is not completely understood. TGF-β is known to promote inclusion of EDA domain [31–33]. Other research groups reported that the SR-rich protein SF2/ASF exerts a similar effect, whereas heterogeneous ribonucleoprotein particles inhibit its inclusion [8, 34, 35]. According to the latest findings, keratinocyte growth factor (KGF) slightly increases the formation of the EDA+ variant, and STAT1 induces exclusion in fibroblasts [36].

Based on our results, LUC7L3 might have a direct regulatory role on EDA+ domain inclusion: similarly to the EDA+ fibronectin, its protein level is also increased in non-involved epidermis. SFRS18 showed only a minimal tendency towards elevation. PPIG amounts are decreased in non-involved epidermis compared to healthy controls, and this expression pattern resembles that which we previously observed for STAT1, another component of the EDA+ fibronectin-related molecular pathway. Moreover, similar to STAT1, PPIG proved to be part of the functional molecular network involving KGF and fibronectin signaling [36].

Another relevant outcome of our current study is that all three splicing factors exhibit the highest level of staining in psoriatic involved epidermis, with approximately 2- to 3-fold elevation in involved epidermis compared to non-involved samples. These data indicate that the examined splicing factors could influence the mRNA maturation of several other molecular components involved in the inflammatory and proliferative processes of psoriatic lesions.

In addition to tissue staining, results from in vitro characterization of splicing regulator expression also support the congruent pattern detected in psoriatic involved

epidermis. In synchronized, immortalized keratinocytes, remarkable similarities in the expression patterns were observed both at the RNA and protein levels. Genetic linkage does not account for these similarities, as the *LUC7L3*, *SFRS18,* and *PPIG* genes are located on different chromosomes. Instead, the corresponding mRNAs might possess common transcription-factor binding sites and upstream regulators. Based on western blot analysis and immunofluorescent staining results, we conclude that expression of the three splicing factors is marked in both immortalized keratinocytes and involved epidermis. This finding supports that elevated amounts of LUC7L3, PPIG, and SFRS18 might be responsible for the shortened and abnormal maturation of keratinocytes that is a characteristic of psoriatic epidermis and is also typical of the immortalized HPV-KER cells.

The influence of the LUC7L3 splicing regulator on the formation of EDA+ fibronectin was also confirmed by in vitro experiments. We demonstrated that immortalized HPV-KER cells express the oncofetal EDA+ isoform of fibronectin in higher amounts than the EDA− isoform. *LUC7L3* silencing resulted in the decrease of the EDA+/total ratio for fibronectin, indicating that the relative amount of EDA− fibronectin is elevated in response to *LUC7L3* silencing. Similar results were obtained with silencing of *PPIG* and *SFRS18*, although the changes in the EDA+/total fibronectin ratios were more moderate than with *LUC7L3* silencing. These data suggest that the three splicing factors promote exon inclusion of the EDA domain. This finding is in accordance with previous work showing that another type of SR protein, SF2/ASF, supports the inclusion of the EDA element [34, 35].

Double silencing of *SFRS18* and *LUC7L3* was the most effective in decreasing the EDA+/total ratio of fibronectin, as expected from the results of bioinformatics analysis. Moreover, LUC7L3 and SFRS18 exhibited a similar pattern of expression during immunofluorescent staining. To analyze whether the changes in the EDA+/total fibronectin ratio detected by PCR are similar at the protein level, flow cytometric measurements were performed in cells in which both *SFRS18* and *LUC7L3* were silenced. The EDA+/total ratio for fibronectin protein decreased in these experiments. The quantity of total fibronectin was constant before and after silencing, and the ratio changes were thus due to increased production of the EDA− fibronectin isoform via an altered splicing mechanism. In addition to SF2/ASF, other SR-rich proteins have also been shown to promote inclusion of the EDA domain. This finding is of special interest, considering that SR proteins usually interact with enhancer splicing elements, inducing exon inclusion [37–39].

Several previous studies indicate that alternative splicing changes the binding characteristics of fibronectin radically [6, 8, 13, 40]. The α5β1 integrin receptor is one of the most important interacting partners for fibronectin, and the inclusion of the EDA domain has been shown to increase affinity towards this receptor [6, 40, 41]. However, a contradicting report demonstrated that the EDA domain does not bind to α5β1 integrin [8]. It is also well documented that EDA+ fibronectin binds to the TLR4 receptor, which is part of the innate immune system [6, 33, 42]. It would be a very interesting new approach to study the changes of TLR4-mediated signal transduction in response to the altered expression of splicing factors.

Taken together, LUC7L3 might—through inclusion of the EDA domain and, thus, elevation of EDA+ fibronectin abundance in the epidermis—contribute to an autocrine loop by which psoriatic keratinocytes maintain hyper-responsiveness towards proliferative signals. Although the amounts of SFRS18 did not show elevation in the non-involved epidermis, in vitro studies support the suggestion that the presence of this protein could facilitate the effect of LUC7L3. All three splicing regulators are notably elevated in psoriatic involved epidermis, indicating that the mRNA maturation of fibronectin is influenced by these proteins, and that these regulators could also have multiple roles in disease development.

In future work, it would be interesting to clarify the upstream regulatory elements maintaining the highly synchronous expression of these three splicing factors and whether these elements are altered in psoriasis. Moreover, identification of additional mRNAs with altered expression and/or splicing patterns under the regulation of the LUC7L3, PPIG, and SFRS18 splicing factors might lead to the identification of novel molecular patterns in psoriasis pathogenesis.

Acknowledgements The study was supported by OTKA K105985, OTKA K111885 and TÁMOP-4.2.2.A-11/1/KONV-2012-0035, GINOP-2.3.2-15-2016-00015 research Grants.

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Mol Cell Biochem
DOI 10.1007/s11010-017-3105-y

ERRATUM

Erratum to: Splicing factors differentially expressed in psoriasis alter mRNA maturation of disease-associated EDA+ fibronectin

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Erratum to: Mol Cell Biochem DOI 10.1007/s11010-017-3090-1

The original article was published with error in an abstract. The word "facwwtors" in the seventh sentence should read as "factors".

The online version of the original article can be found under doi:10.1007/s11010-017-3090-1.

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Published online: 02 August 2017

III.

World Journal of Dermatology

Online Submissions: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
doi:10.5314/wjd.v3.i3.50

World J Dermatol 2014 August 2; 3(3): 50-57
ISSN 2218-6190 (online)

What have we learned about non-involved psoriatic skin from large-scale gene expression studies?

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Supported by OTKA NK77434, OTKA K 83277, OTKA K105985 and TÁMOP-4.2.2.A-11/1/KONV, TÁMOP-4.2.2-B-10/1-2010-0012; the Bolyai Foundation of the Hungarian Academy of Sciences (to Kornelia Szabo)
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Received: December 29, 2013 Revised: May 22, 2014
Accepted: May 28, 2014
Published online: August 2, 2014

Abstract

Psoriasis is a chronic inflammatory skin disorder; its genetic background has been widely studied in recent decades. Recognition of novel factors contributing to the pathogenesis of this disorder was facilitated by potent molecular biology tools developed during the 1990s. Large-scale gene expression studies, including differential display and microarray, have been used in experimental dermatology to a great extent; moreover, skin was one of the first organs analyzed using these methods. We performed our first comprehensive gene expression analysis in 2000. With the help of differential display and microarray, we have discovered several novel factors contributing to the inherited susceptibility for psoriasis, including the EDA+ fibronectin splice variant and PRINS. The long non-coding PRINS RNA is expressed at higher levels in non-involved skin compared to healthy and involved psoriatic epidermis and might be a factor contributing cellular stress responses and, specifically, to the development of psoriatic symptoms. This review summarizes the most important results of our large-scale gene expression studies.

Key words: Non-involved psoriatic skin; Differential display; cDNA microarray; EDA+ fibronectin isoform; PRINS long non-coding RNA; mRNA maturation

Core tip: Large-scale gene expression studies, including differential display and microarray, have provided valuable data on the molecular background of psoriasis pathogenesis. This review summarizes the most important results of the available literature and our large-scale gene expression studies obtained from the clinically non-involved psoriatic skin: we identified the EDA+ fibronectin splice variant as an autocrine proliferation signal for psoriatic hyperproliferative keratinocytes and PRINS, a long non-coding regulatory RNA. We believe that the characterization of new candidate genes and proteins might establish new therapeutic approaches, which may allow treatment of already existing psoriatic lesions as well as non-involved psoriatic skin by affecting molecular aberrancies, and may lead to the development of prophylactic interventions.

Szlavicz E, Szabo K, Bata-Csorgo Zs, Kemeny L, Szell M. What have we learned about non-involved psoriatic skin from large-scale expression studies? *World J Dermatol* 2014; 3(3): 50-57 Available from: URL: http://www.wjgnet.com/2218-6190/full/v3/i3/50.htm DOI: http://dx.doi.org/10.5314/wjd.v3.i3.50

INTRODUCTION

Psoriasis is a hyperproliferative inflammatory skin disorder affecting approximately 2%-3% of the European population[1]. However, in some other parts of the world, this disease is almost unknown: *e.g.*, in Africa the occurrence of psoriatic cases is remarkably rare[2]. The exact trigger of the disease is still obscured and the subject of several investigations. Inherited and environmental factors (*e.g.*, mechanical trauma, UV exposure, stress) are responsible for the development of psoriatic symptoms[2,3].

In the most typical cases, hyperproliferative psoriatic plaques are formed on the skin of the knees, elbows and the scalp. In addition, the disorder can affect skin annexes and joints. In the case of some patients with severe psoriasis, the entire body is covered with lesions. Severe psoriasis is often associated with metabolic syndrome; hence, psoriasis patients also have elevated cardiovascular and stroke risks[2]. Unfortunately, psoriasis has a negative effect on the patient's quality of life due to serious psychosocial and emotional stress[4]. A number of emerging arguments support the idea that psoriasis is a systemic disorder rather than simply a skin disease. Psoriasis has many common features with chronic autoimmune inflammatory disorders, such as progressive arthritis. Moreover, psoriasis and autoimmune syndromes often share common genetic loci[5-7]. Similarities are especially evident when psoriasis is compared to chronic inflammatory bowel disorders, such as Crohn's disease, where internal barriers are involved[8,9].

Similarly to autoimmune disorders, immune-activation plays an important role in psoriasis: the development of the characteristic erythematosus, demarcated and scaly lesions is related to the abnormal functioning of the cellular immune system[10-14]. Cytokines produced by aberrantly functioning T-lymphocytes are able to stimulate keratinocytes, which show an elevated sensitivity to these proliferative signals[14,15]. However, it is still unknown whether the primary triggers of the disease phenotype are the professional immune cells or the keratinocytes. Inherited susceptibility of keratinocytes has been partially established. Identification and characterization of these factors may greatly facilitate the understanding of the molecular background of psoriasis. Large-scale gene expression profiling methods developed and used in the 1990s might be useful tools to answer these exciting questions.

DAWN OF THE LARGE-SCALE GENE EXPRESSION STUDIES: DIFFERENTIAL DISPLAY AND SAGE

In recent years, we and others have tried to characterize molecular factors responsible for the hyper responsiveness of keratinocytes to various stimuli[8,12,14,16]. To reveal these processes, researchers need suitable and powerful methods that can detect more than one possible target. Previously, altered expression of only a few candidate genes or proteins was possible. The development of large-scale gene expression analysis methods marked a significant breakthrough in this field. With the help of microarrays and their predecessors, differential display (DD) and the serial analysis of gene expression (SAGE), gene-expression patterns of serial samples can be compared for large data sets.

For DD, gene expression profiles are analyzed for pairs of corresponding sample sets. The most important steps of the method are the isolation of total RNA from the samples and its reverse transcription into cDNA. Subsequently, cDNA is amplified, subjected to gel electrophoresis and, after the expression pattern has been compared, bands representing differentially expressed genes are cut out from the gel and the DNA content is cloned into a plasmid vector. It should be mentioned that the DD method has some limitations due to the relatively frequent incidence of false positive results. Hence, the results must be validated using an independent technique. Validation is usually carried out by reverse Southern blot analysis, followed by sequencing the differentially expressed transcripts[17-19]. The great advantage of DD is that it is an "open ended" analysis system, allowing unannotated differentially expressed transcripts to be identified.

Another sequence-based approach, SAGE, was developed at the Oncology Center of the Johns Hopkins University by Velculescu and his co-workers. Changes in gene expression patterns are detected by sequencing reverse transcribed cDNAs. Application of short oligonucleotide sequence tags allows quantitative changes to be monitored, in addition to the qualitative analysis[20,21].

MICROARRAYS

Microarrays provide more extended and comprehensive methods for analyzing gene expression profiles than DD and SAGE. The biggest advantage of this approach is that it allows thousands of genes to be measured simultaneously. Moreover, complex regulatory networks can be assessed[22,23]. In contrast to DD and SAGE, microarrays are a "closed" analysis system, allowing only known sequences to be screened. The introduction and widespread use of microarrays has facilitated advances in several branches of science, including experimental dermatology. In fact, the skin was one of the first human organs to be analyzed with this technique[24-27].

Microarray technologies rely on complementarity for sequence-specific recognition of the DNA segments[28]. Most commonly used probes are cDNAs derived from bacterial libraries and BACs or oligonucleotides. Long oligonucleotide probes (50-120 nt) might support a higher degree of specificity and sensitivity than short (15-25 nt) probes[22]. The probes are fixed to a solid support, such as glass or plastic that are referred to as "chips" in common laboratory jargon[23].

For microarray experiments, total RNA is isolated

from samples and reverse-transcribed with fluorescent dyes such as Cy3 and Cy5 or with radioactive isotope to label the synthesized cDNA. After hybridizing the labeled probes to the chips for approximately 16-24 h, the chips are washed and the fluorescence is scanned with a confocal microscopy. Data are then analyzed using specially developed software. Like DD, this method can identify false positives and, therefore, must be validated by RT-PCR, northern blot analysis or RNAse protection assay[22,28].

The outstanding advantage of microarray techniques is the simultaneous investigation of thousands of genes and, thus, the possibility to explore novel molecular pathways. This technique can be a powerful tool in tumor and biomarker research and may serve as the basis of personalized therapies[27].

LARGE-SCALE GENE EXPRESSION STUDIES OF PSORIASIS: IDENTIFICATION OF MOLECULAR FACTORS CONTRIBUTING TO PATHOGENESIS

The use of large-scale gene-expression analysis methods has been fruitful for experimental dermatology. Microarrays have been used to study several disorders, such as melanoma, atopic dermatitis and autoimmune skin diseases. In the past decade, DD and microarray techniques have been widely employed alone or in combination with other methods in psoriasis research[29].

Gene-expression profiling of peripheral blood cells and epidermis samples from healthy, psoriatic involved and psoriatic non-involved skin proved to be a powerful tool for the characterization of aberrant molecular patterns in the disease[30]. The results of cDNA microarrays supported previous findings and were useful to describe novel pathways implicated in psoriasis pathogenesis. Psoriasis research was dominated by the so-called "immune theory" for many years, and microarray studies further proved the involvement of genes related to inflammation and immune responses. One of the earliest microarrays identified several inflammation- and immune-related genes (*IL4R*, *CD2*, *CD24* and *INF-γ* induced genes) that were not previously reported to contribute to the pathogenesis of this disorder[30,31]. Moreover, Oestreicher *et al*[31] performed a longitudinal analysis in which they characterized changes in gene expression in response to recombinant human IL-1 or cyclosporine in therapy responder and non-responder populations. A study from Zhou, which compared samples from healthy, involved psoriatic and non-involved psoriatic skin biopsies further supported the involvement of the activated T-cell product INF-γ and transcription factors induced by this pro-inflammatory lymphokine[30,32]. The role of IL-17 signaling was also demonstrated in large-scale gene-expression studies[13]. In addition, Gudjonsson *et al*[3] emphasized the role of altered innate immune functions in psoriasis. Differential expression of genes encoding chemokines and their receptors were also described by several research groups[32-34].

Other important cellular pathways related to psoriasis regulate epidermal keratinocyte proliferation and apoptosis. The implication of *PPAR-δ*, *mTOR*, *NFκB*, *BCL-2* and *BAX* expression was verified for these mechanisms[35-37]. In a study of Wnt pathways responsible for stem cell proliferation and differentiation, Reischl *et al*[38] found that only *Wnt5a* expression was higher in psoriatic involved skin than in non-involved samples. In addition, actin cytoskeleton organization can be affected: the *CCNA2* gene is responsible for the G_2/M transition in the cell cycle and affects intracellular cytoskeleton organization and cell migration[39,40].

The clinical association of psoriasis and metabolic syndrome is a well-known phenomenon. Gudjonsson and co-workers were able to show that lipid metabolism pathways were altered in psoriatic non-involved epidermis compared to healthy samples[3]. In this comparison, it was proven that lipid metabolism genes were down-regulated in non-involved skin samples as compared to healthy skin and further down-regulation was identified in psoriatic involved skin[3]. Romanowska *et al*[35] studied the role of PPARδ, a transcription factor participating in metabolic and inflammatory processes, in psoriasis. PPARδ exerts proangiogenic and antiapoptotic affects and is suspected to be involved in the enhancement of keratinocyte proliferation[35].

Most recently, bioinformatic meta-analyses were performed using publicly available databases of psoriasis-related microarray data. In one of the first microarray meta-analysis, Tian *et al*[41] analyzed the result of five previous cDNA microarrays experiments. In a subsequent meta-analysis, Manczinger *et al*[40] compared differentially expressed genes of psoriatic involved and non-involved epidermis. The findings of these two meta-analyses agreed and showed that the most important components of the molecular networks related to psoriasis are factors implicated in cell proliferation and immunomodulation. Importantly, these meta-analyses confirmed that several differentially expressed transcripts were also involved in metabolic disturbances, such as impaired glucose tolerance, insulin tolerance and atherosclerosis[40,41].

It is important to note that most of the large-scale gene expression studies for the identification of molecular patterns in psoriasis pathogenesis have compared the gene expression profiles of psoriatic involved and non-involved skin or psoriatic involved and healthy skin. This research provided extremely valuable data for the molecular events of psoriasis[40]. Much less information is available, however, on differentially expressed genes in normal epidermis compared to psoriatic non-involved epidermis. We and others believe that identifying aberrantly expressed genes and molecular patterns in non-involved psoriatic epidermis is important for understanding this disease.

DIFFERENTIAL DISPLAY AND MICROARRAY EXPERIMENTS OF OUR RESEARCH GROUP, FOR THE IDENTIFICATION OF NOVEL MOLECULAR FACTORS OF PSORIASIS

Our research group performed the first comprehensive gene-expression analysis for psoriasis in 2000 to compare psoriatic non-involved epidermal samples with control healthy epidermis. This approach allowed early and inherited molecular factors to be studied in detail and allowed novel susceptibility factors to be revealed. This study identified two known transcripts that were differentially expressed: *RAB10*, an oncogene that belongs to the small GTPase superfamily, and fibronectin, a well-known extracellular matrix component[42]. Our subsequent studies focused on the role of fibronectin in the pathogenesis of psoriasis.

Fibronectin is a complex glycoprotein composed of repetitive modules[43]. At least 24 differentially spliced variants of this gene have been described, and the presence of certain variants depends on age, developmental state and cell type[44]. Alternative processing involves three preferred sites: extra domain A (EDA), extra domain B and extra type homology B[43,45]. The splice variants containing the EDA domain play a crucial role in embryonic development and wound healing. However they are detectable only in modest amounts in adult normal tissues[44,46,47]. Because it is also abundantly expressed in different types of tumors, it is referred to as the oncofetal fibronectin splice variant[48]. Interestingly, in the brain, an organ in which fibronectin is poorly expressed, the inclusion of the EDA domain is abundant in young adults (88% as compared to fetal level) and decreases with age to 33%[46].

The presence of the EDA+ fibronectin variant is associated with several pathological conditions and is suspected to participate in the development of psoriasis as well. The oncofetal fibronectin form was found to be present in a higher ratio at the dermal-epidermal junction of psoriatic non-involved skin compared to healthy normal skin[14,49]. Unlike the conventional variant, the oncofetal EDA+ fibronectin form interacts with the α5 integrin subtype, instead of α2 and α3, and, as a result, its effect on cellular signaling processes is more robust. α5β1 integrin receptors were shown to be upregulated in both non-involved and involved psoriatic skin[14,50].

In addition, other authors reported that the EDA+ fibronectin variant is co-localized with CD11c+ macrophages. It was suggested that these cells might contribute to the production of the oncofetal variant; however, because of their relatively low number, they are likely not to be the most important source[49]. Based on our results we supposed that keratinocytes themselves might produce EDA+ fibronectin and, as an autocrine molecular factor, may contribute to the induction and maintenance of keratinocyte hyperproliferation in psoriasis[16].

We have also performed *in vitro* experiments to understand the role of EDA+ fibronectin in the regulation of keratinocyte proliferation. Subsequently, RT-PCR was carried out using immortalized HaCaT cells. Our results indicated that, after serum starvation and contact inhibition, the highest level of EDA+ fibronectin expression could be detected in the highly proliferative HaCaT cells, and the ratio of EDA+/EDA- fibronectin produced by the keratinocytes might well be a potent mitogen signal in cell cycle regulation. In contrast to fibroblasts and normal human keratinocytes, the ratio was altered in this cell line. Flow cytometry supported the RT-PCR results. The results of the HaCaT cell line experiments indicated that keratinocytes themselves might produce the oncofetal fibronectin variant[16].

In addition to proteins with known functions, the DD experiment identified a novel transcript: the corresponding gene was subsequently named psoriasis-susceptibility-related RNA gene induced by stress (PRINS, accession number AK022043). During the structural investigation of PRINS, we found that the gene consists of two exons containing several stop codons, which prevent the formation of a longer open reading frame. *In silico* sequence comparison supported the hypothesis that PRINS functions as a non-coding RNA molecule, rather than serving as a template for protein translation. In addition, PRINS contains two repetitive *Alu* sequences and has 70% sequence similarity with the *Tetrahymena thermophyla* G8 small nucleolar non-coding RNA[42].

In a quantitative RT-PCR analysis, we demonstrated that PRINS is expressed at higher levels in non-involved skin compared to healthy and involved psoriatic epidermis. Our *in vitro* experiments performed on synchronized HaCaT cells showed that PRINS expression dropped significantly when the cells were released from cell quiescence and the cells started to proliferate actively[42]. These data suggested that PRINS might be a factor disposing keratinocytes to hyperproliferation and contributing to the development of psoriatic symptoms. The exact role of PRINS is still unknown, but it is very possible that it plays an important role in cellular stress responses. Silencing PRINS did not affect the survival of the cells; however under certain stress conditions (such as serum starvation) the cells died at a much higher rate when the expression of PRINS was down-regulated[42,51]. Consequently, the PRINS-silenced cells became more vulnerable, supporting the cellular-stress response hypothesis. Moreover, our research group later showed that the G1P3 antiapoptotic protein might be regulated by the PRINS non-coding RNA[52].

Since then, we have identified nucleophosmin as one of the possible cellular interacting partners of PRINS. Nucleophosmin is a phosphoprotein which is a member of the p53 pathway, and its movement in fibroblasts, cancer cells and keratinocytes is triggered by ultraviolet (UV) exposure[53]. We also demonstrated that silencing PRINS prevents nucleolar-cytoplasmic shuttling of nucleophosmin.

This result indicates that PRINS might physically interact with the nucleophosmin protein and that the abnormal functioning of the PRINS-nucleophosmin ribonucleoprotein complex may contribute to psoriasis pathogenesis[54].

Taken together, we consider the identification of novel factors implicated in the early molecular defects in psoriasis pathogenesis-the EDA+ fibronectin splice-variant and the PRINS non-coding RNA-the most significant outcomes of our DD experiments. Due to the success of the DD, we attempted to identify novel psoriasis susceptibility factors using newly available cDNA microarray technology for large-scale gene-expression analysis. In particular, we aimed to identify molecular patterns that are responsible for the differential reactivity of normal healthy epidermis and psoriatic non-involved epidermis.

Organotypic tissue cultures were created from four healthy and four psoriatic non-involved skin samples. Half of the samples were treated with a mixture of T-cell lymphokines, containing IL-3, IFNγ and GM-CSF, cytokines previously described to be implicated in the T-cell response and the formation of psoriatic plaques[10]. After three days of treatment, the dermis and epidermis were separated. Total RNA was isolated from the epidermis, reverse transcribed and used to perform the cDNA microarray experiment. Based on the results, we selected genes that showed an altered gene expression in response to the lymphokine treatment[12].

We identified 61 transcripts that exhibited altered gene expression. Of these, eleven had been demonstrated earlier to contribute to psoriasis. Using bioinformatics tools, such as Gene Ontology and Ingenuity pathway analysis, we demonstrated that most of these molecules are implicated in two important intracellular pathways: "apoptosis" and "metabolism of small molecules and lipids." Real-time RT-PCR validation experiments revealed that many of these genes are already upregulated in non-involved psoriatic epidermis, and the lymphokine treatment did not further increase expression. In contrast, expression of these genes was inducible in healthy samples. These data indicate that keratinocytes in psoriatic non-involved epidermis are in a presensitised status, which explains their altered response to different triggering stimuli[12].

Among the differentially expressed genes, we also identified members of the serine-arginine rich (SR) proteins SR splicing factor 18 (SFRS18), peptidylprolyl isomerase G (PPIG) and luc-7 like 3 (LUC7L3), which regulate mRNA splicing. It was previously described that these proteins interact with pinin and SR-related nuclear protein[55-60]. Splicing is a post-transcriptional regulatory process and one of the most important sources of mRNA diversity, permitting the production of different mRNAs from the same DNA template. Splicing dysfunction has been shown to be involved in several disorders, and some novel therapeutic modalities have been designed to repair them[61-63].

Our research group has previously demonstrated that the fibronectin splice variants containing the EDA domain is implicated in the pathogenesis of psoriasis. This suggests the interesting question whether the identified splicing genes, *LUC7L3*, *PPIG* and *SFRS18*, contribute to the production of the EDA+ fibronectin variant. We are currently investigating the role of *LUC7L3*, *PPIG* and *SFRS18* splicing regulatory genes in the production of EDA+ fibronectin, and we aim to identify further differentially spliced mRNA variants contributing to psoriasis pathogenesis.

CONCLUSION

Taken together, recent comparisons between psoriatic non-involved and involved epidermis dominated large-scale gene expression studies related to psoriasis. Relatively few studies have focused on the comparison of gene expression differences between healthy and psoriatic non-involved epidermis samples. Nonetheless, we believe that these experiments are valuable for identifying factors that increase the risk for developing psoriatic plaques. In our microarray studies, we identified several novel candidate genes and molecular patterns that might contribute the formation of typical lesions. The altered expression of EDA+ fibronectin and that of LUC7L3, PPIG and SFRS-18 suggests that some kind of splicing anomalies have an important role in the development of psoriatic symptoms. The exploration of cellular networks related to RNA-maturation processes gave us a deeper insight into the molecular pathogenesis of psoriasis and investigation of the splicing machinery might be a very new approach in this field. Results of wide-scale gene expression studies have provided pioneering advances in psoriasis research as well as in the recognition of different types of non-coding RNAs, including *PRINS*. This RNA is a long non-coding RNA (lncRNA), and most lncRNAs have been identified in their involvements in the central nervous system and certain tumors[64-69].

The last decade has seen a rapid evolution in large-scale gene expression profiling methods. Techniques, such as RNA-Seq and digital gene expression profiling, provide an even greater resolution and wider dynamic range compared to either DD or cDNA microarray. Advancement of methods based on next-generation sequencing has accelerated the accumulation of data, and processing the results requires huge efforts. Thus, validation and interpretation of these newly discovered factors is a very important challenge. Identification of new candidates might establish new therapeutic approaches, which may allow treatment of already existing psoriatic lesions as well as non-involved psoriatic skin by affecting molecular aberrancies, and may lead to the development of prophylactic interventions.

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P- Reviewer: Husein-ElAhmed H, Kita K, Lonchin S
S- Editor: Song XX **L- Editor**: A **E- Editor:** Lu YJ