




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

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**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

facwrtors 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

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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- $\gamma$ , 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  $\geq 4$  weeks without systemic therapy and/or  $\geq 2$  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  $\mu\text{m}$  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  $\mu\text{g}/\text{ml}$  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 IFN $\gamma$ , 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<sub>2</sub> 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™ 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  $\Delta\Delta C_t$  method.

### Immunofluorescent staining

Biopsies were frozen, embedded, stored at  $-80\text{ }^\circ\text{C}$ , and then cut into 6- $\mu\text{m}$  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-carboxamide (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  $\text{cm}^2$  cell culture flasks and maintained in keratinocyte serum-free medium (Gibco<sup>®</sup> Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark) supplemented with 1% antibiotic/antimycotic solution (PAA, Pasching, Austria) and 1% L-glutamine (PAA) at 37  $^\circ\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ . 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  $\alpha$ -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<sup>®</sup> Reagent (Invitrogen Corp., Carlsbad, CA, USA), following the instructions provided in the manual. cDNA was synthesized from 1  $\mu\text{g}$  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. 1a. 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

**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)

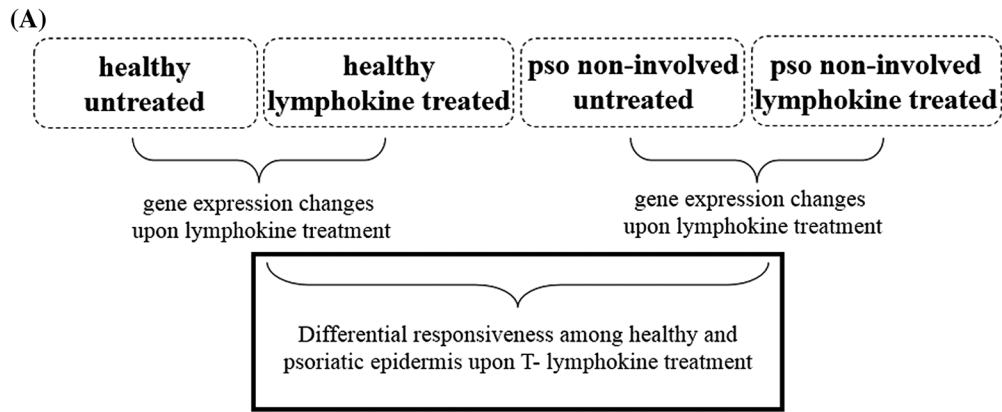
epidermis, whereas *SFRS18* shows only minimal, non-significant elevation in non-involved epidermis. The highest levels of expression of *LUC7L3* and *SFRS18* were found in psoriatic involved epidermis samples. The expression pattern of *PPIG* was different from that of the other splicing factors: *PPIG* 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).

### LUC7L3, PPIG, and SFRS18 expression patterns in synchronized cells

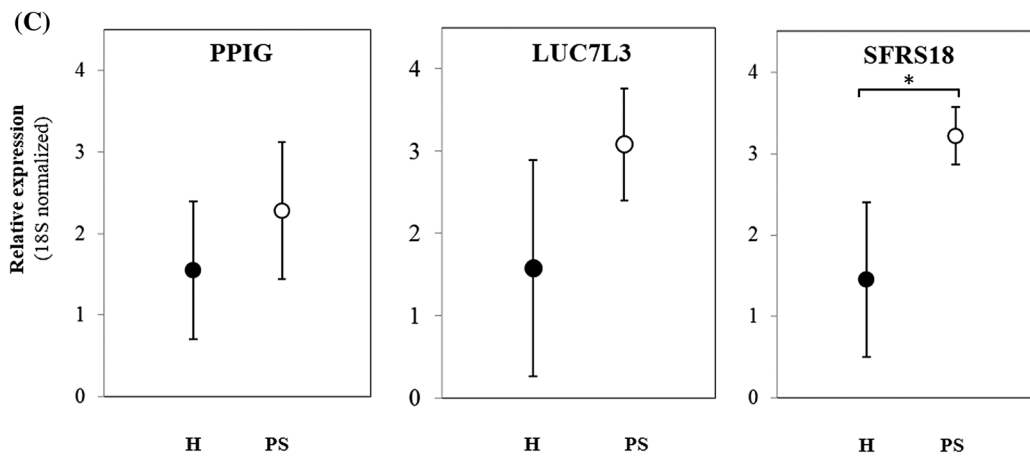
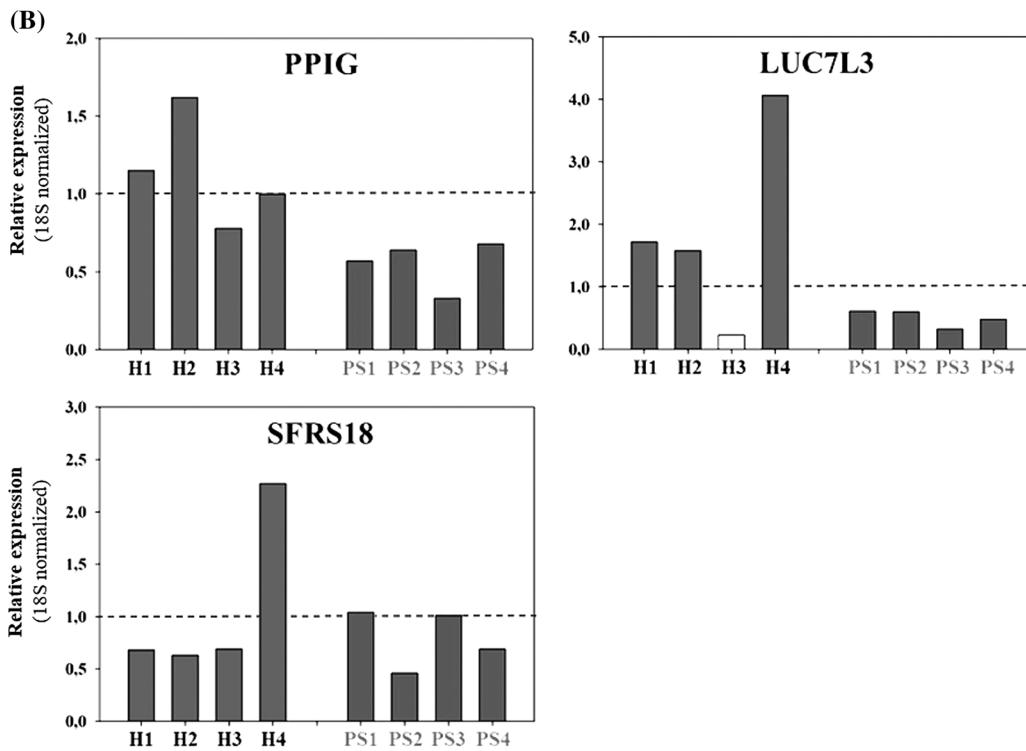
To compare the expression levels in proliferating and differentiating keratinocytes, both mRNA and protein expression patterns of the selected splicing factors were analyzed in synchronized, immortalized cells. HPV-KER cells were synchronized by contact inhibition and in the absence of growth factors [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.

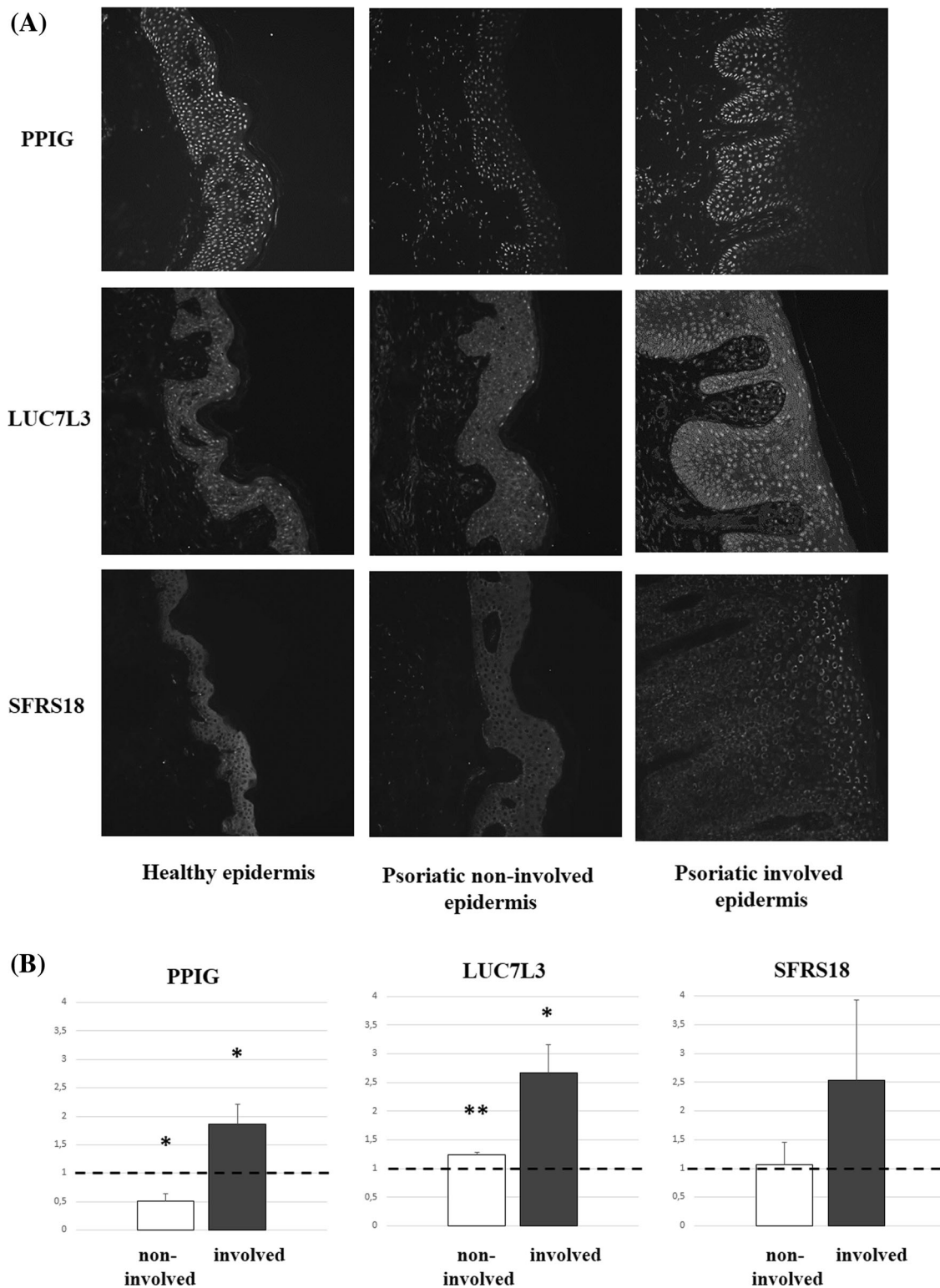
Comparing the expression of the splicing factors during the proliferation and differentiation phases, a very similar mRNA expression pattern was observed for all three splicing factors (Fig. 3a). mRNA expression decreased at 12 h. The first peak of mRNA abundance was seen at 24 h, with steady elevation 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).



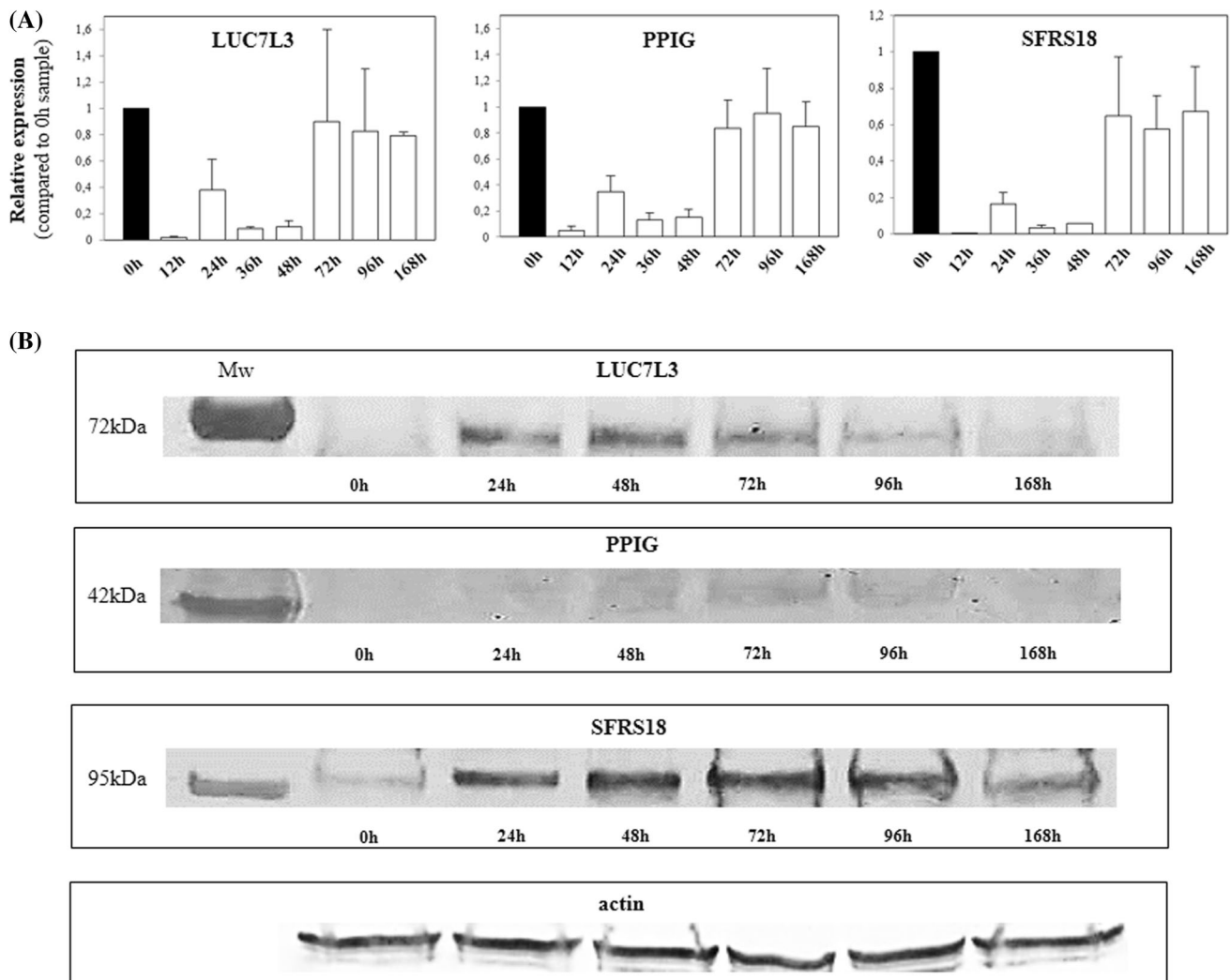
Fold-change>2, p<0.05, 95% CI





**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.  $\alpha$ -actin was used as a loading control

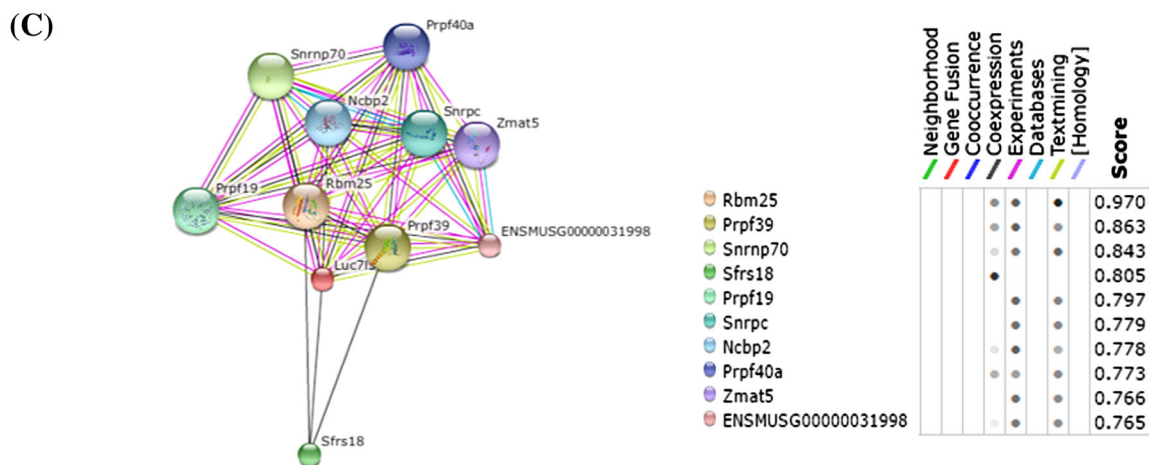
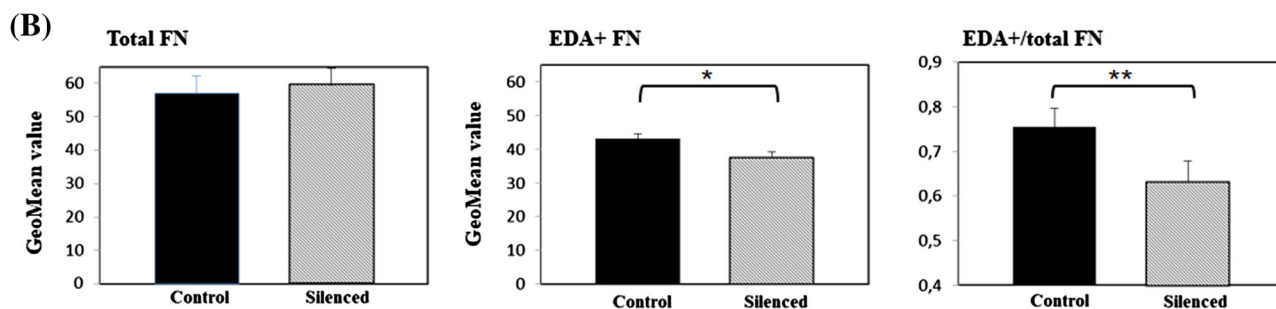
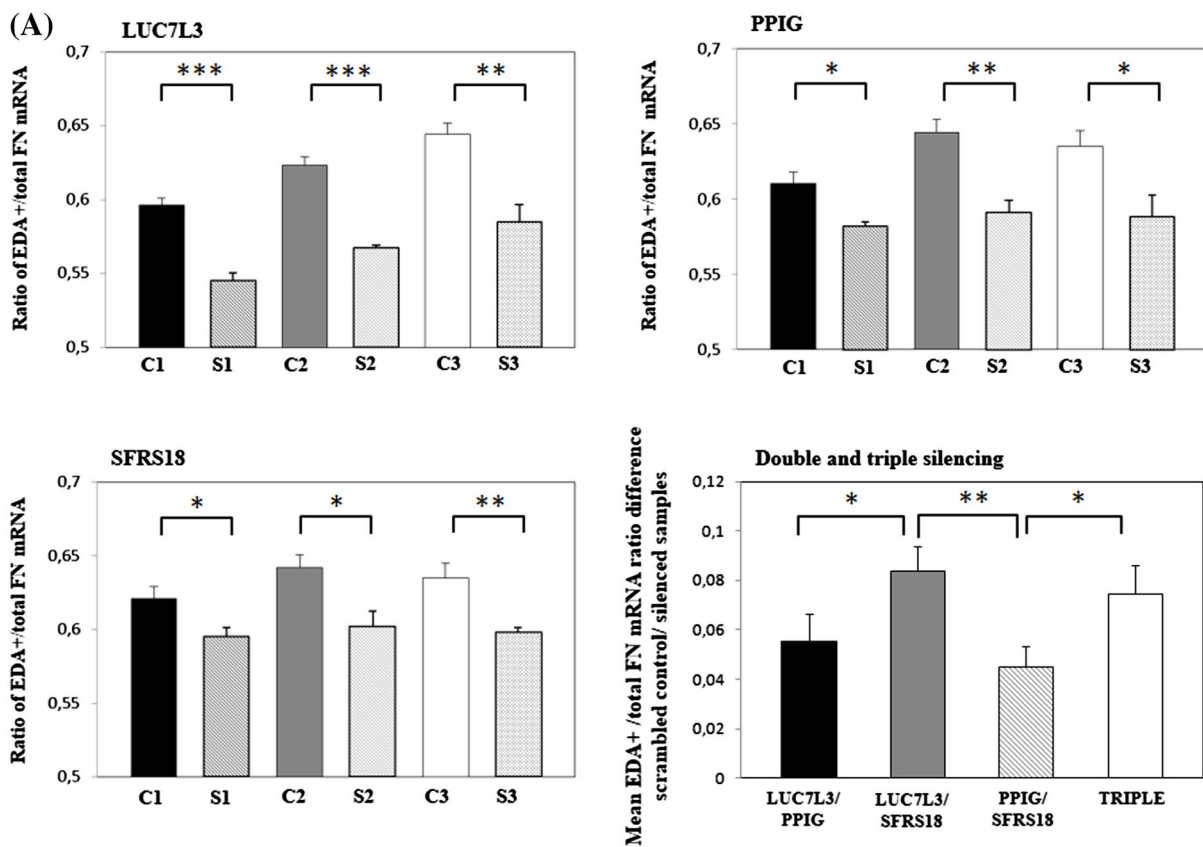
### Silencing of *PPIG*, *SFRS18*, and *LUC7L3* affects fibronectin splicing

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





**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- $\beta$  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  $\alpha 5\beta 1$  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  $\alpha 5\beta 1$  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.

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