Our research is focused on psoriasis, a chronic inflammatory skin disease of multifactorial origin. Our former results suggested altered skin tissue homeostasis in psoriasis: we have demonstrated that keratinocyte-specific alterations of the normal looking skin of psoriatic patients are key in the initiation of the disease phenotype. In our recent OTKA supported work we aimed to characterize several molecules, which were found to be dysregulated in psoriatic uninvolved epidermis compared to either healthy and involved epidermis. These results are of outmost basic research interest, but can result in the identification of novel targets for therapies.

Results

In our research plan we set two main aims, which will be discussed separately:

AIM I: to examine the role of PRINS non-coding RNA, nucleophosmin and CARD18 (ICEBERG) in the inflammasome activation of keratinocytes and to reveal their contribution to the abnormal stress response of psoriatic uninvolved keratinocytes and to disease pathomechanism.

CARD18 was identified as a negative regulator of inflammasome signaling by a cDNA microarray experiment, comparing the gene expression differences in uninvolved and normal healthy epidermis upon cytokine induction. First we validated the results of the cDNA microarray by real-time RT-PCR. Initial CARD18 expression was higher in psoriatic uninvolved epidermis, however, this high expression could not be further induced by the cytokine mixture of GMCSF, IL-3, IFN-γ, while healthy epidermis reacted to inflammatory stimuli by increased CARD18 expression. Initial characterization showed that CARD18 expression is induced by the differentiation of the cells.

To characterize changes at protein level, CARD18 was stained by immunohistochemistry in healthy, psoriatic involved and psoriatic uninvolved skin samples. In all examined sections, CARD18 appeared exclusively in the epidermis, specifically in the cytoplasm of epidermal keratinocytes. Elevated levels were observed in the psoriatic-involved and uninvolved epidermis compared to the healthy epidermis, confirming our RT-PCR experiments. To see if CARD18 expression is induced in the skin upon mild injury, tape-stripping experiments were carried out. It has long been known that baseline proliferative activity of keratinocytes of uninvolved skin of psoriatic patients does not differ from keratinocytes in skin of healthy individuals; however, the proliferative response to tape stripping or other types of mechanical trauma is significantly higher in psoriatic uninvolved skin compared to healthy skin. CARD18

protein expression was observed in mechanically stimulated skin from healthy and uninvolved skin of psoriatic donors. Punch biopsies were taken before the procedure and two times after tape stripping (24 and 48 h). Compared to untreated sections, CARD18 protein expression level was induced 24 h after tape stripping in healthy, psoriatic uninvolved and psoriatic involved samples, as well. However, the elevation of CARD18 expression in treated and untreated psoriatic uninvolved samples was not as robust as in healthy or psoriatic involved skin. Forty-eight hours after treatment, CARD18 expression remained elevated only in the psoriatic uninvolved samples. These results confirmed that CARD18 expression might be dysregulated in psoriatic skin.

CARD18 was described as a negative regulator of inflammasome signalling. In psoriasis, AIM2 inflammasome activation in keratinocytes was described as a key pathogen factor. AIM2 inflammasome is activated by cytosolic nucleotide fragments, which are highly abundant in psoriatic lesional epidermis. To characterize the functions of CARD18 in keratinocytes we introduced a model of nucleotide fragments induced inflammation, to model psoriasis related inflammatory reactions in keratinocytes. To induce inflammasome activation in keratinocytes, we applied poly(dA:dT) transfection. We found that poly(dA:dT) transformation was able to induce AIM2 inflammasome activation monitored by IL-1ß release of the keratinocytes. However, CARD18 expression could not be induced by poly(dA:dT) treatment alone. To enhance IL-1ß secretion, a TNF-α and IFN-γ priming step is often applied before poly(dA:dT) treatment. Indeed, we found that increased IL-1ß secretion could be observed upon priming, moreover, CARD18 expression was induced in keratinocytes upon this combined treatment. To confirm its functionality, CARD18 was silenced by siRNA based method in keratinocytes. We found that CARD18 silencing resulted in an increased IL-1ß release confirming its potential to negatively regulate AIM2 inflammasome signaling in keratinocytes. However, we suppose that additional feedback loops can play a role in these reactions, since CARD18 silencing resulted in decreased expression of inflammasome members AIM2 and caspase-1.

These results were summarized in the publication: *Göblös et al: Keratinocytes express functional CARD18, a negative regulator of inflammasome activation, and its altered expression in psoriasis may contribute to disease pathogenesis, Mol Immunol. 2016; 73:10-18.*

The non-coding RNA, PRINS was first described by our research group in 2005, as a highly expressed transcript in psoriatic uninvolved epidermis, compared to both healthy and psoriatic

epidermis. Its expression was shown to be altered upon various stressors, and silencing of PRINS resulted in decreased viability of HaCaT kertinocytes, suggesting its role in stress response. Furthermore, PRINS was shown to interact with nucleophosmin (NPM), a chaperone molecule, and be involved in the regulation of NPM translocation upon UVB exposure. We found that poly(dA:dT) exposure decreased the expression of PRINS, thus we analyzed, whether NPM translocation can be observed upon poly(dA:dT) exposure. We found no alteration in the localization of NPM upon poly(dA:dT) treatment compared to untreated control.

To analyze the possible role of PRINS in poly(dA:dT) induced keratinocyte immune reactions, we silenced its expression by a vector based siRNA silencing and created an overexpression construct, where the sequence of AK022045 cDNA, comprising of PRINS, was cloned into the pcDNA3.1(+) vector. We could observe a maximal 30% reduction in PRINS expression upon silencing, but an up to 1000-time induction in PRINS expression upon transfection of the PRINS overexpressing construct. We administered PRINS silencing or overexpression to keratinocytes parallel to poly(dA:dT) transfection to analyze, whether PRINS can regulate inflammasome activation. We found that neither mRNA expression of inflammasome members (AIM2, proIL-1ß, caspase-1), nor secretion of IL-1ß was affected by PRINS silencing or overexpression. These results showed that although PRINS might regulate stress responses of the cells, it is not able to regulate inflammasome activation in keratinocytes.

Recently, PRINS was reported to directly interact with the mRNA of CCL-5, a chemokine also highly expressed in psoriasis. Based on this report we performed an *in silico* analysis, and found, that PRINS might directly interact with the mRNA of several inflammatory cytokines. Based on these results we performed some preliminary experiments. We demonstrated that poly(dA:dT) was also able to induce the expression of inflammatory cytokines not connected to inflammasome activation. Moreover, the overexpression of PRINS inhibited the expression of IL-6 in keratinocytes. Based on these preliminary results we proposed for a new OTKA grant (K124651) to further characterize the predicted interactions. To demonstrate direct binding of PRINS RNA to IL-6 mRNA we plan biophysical experiments by using MicroScale Thermophoresis, and functional experiments on cellular level to see how destroying of the binding site in PRINS affects its ability to regulate IL-6 expression.

The workgroup has received distinguished recognition in the field of long non–coding RNA research: the PI was asked to complete a review article for the prestigious *Pflügers Archiv European Journal of Physiology*. To complete the review we included new bioinformatics data on PRINS. While the initial bioinformatics analysis in 2005 showed that PRINS harbors two *Alu* elements and lies in a region where no genes were described, nowadays, thanks to the increasing gene number in databases, we know that PRINS lies in an intron of KIAA1227, a gene involved in early stages of embryogenesis. A transcription start site was identified 6 kb proximal to the putative 5′ end of the PRINS gene using the ENCODE database. This region is marked by a high density of binding sites for several transcription factors, including GATA2, Fos, HDAC2 and STAT3, and histone modification sites associated with active transcription, such as mono- and tri-methylation of lysine 4 of histone H3 (H3K4Me1/3) and acetylation of lysine 9 and 27 (H3K9Ac, H3K27Ac), suggesting that a strongly regulated active promoter might be associated with the lncRNA. The region adjacent to the 3′ end of the PRINS lncRNA gene also contains histone modification sights, which, due to the close 3′ proximity to the PRINS lncRNA gene, might be an enhancer element. Morover, sequence analysis showed that PRINS includes the AGCCC pentamer with the sequence restrictions at positions −8 (T or A) and −3 (G or C) of a motif which was reported to be crucial for nuclear localization of lncRNAs. Indeed, our former in situ hybridization experiment showed enrichment of PRINS in the nucleolus of keratinocytes.

The review - incorporating these *in silico* findings - was published as *Széll et al: PRINS, a primate-specific long non-coding RNA, plays a role in the keratinocyte stress response and psoriasis pathogenesis, Pflügers Arch - Eur J Physiol. 2016; 468(6):935-943*

AIM II: to identify abnormal mRNA maturation and splicing patterns in human keratinocytes due to altered expression of the splicing regulatory genes PPIG, SFRS18 and LUC7L3.

Our previously performed cDNA microarray experiment demonstrated that the PPIG, SFRS18 and LUC7L3 genes were all differentially expressed in psoriatic uninvolved epidermis upon T-lymphokine treatment when compared to normal epidermis. RT-PCR based validation confirmed that basal expression of PPIG, SFRS18 and LUC7L3 was higher in psoriatic uninvolved epidermis compared to healthy epidermis. As the splicing factors showing altered

mRNA expression in psoriasis are poorly characterized, we examined the pattern of protein expression in healthy, psoriatic uninvolved and involved skin samples. LUC7L3 and PPIG exhibited nucleolar localization while SFRS18 exhibited perinuclear staining. The highest levels of expression of LUC7L3 and SFRS18 were found in psoriatic involved epidermis samples, whereas the levels in healthy and psoriatic non-involved epidermis were relatively modest. In contrast, the expression of PPIG was lower in psoriatic non-involved epidermis than in healthy and psoriatic samples; however, highest expression was also detected in psoriatic involved epidermis samples, as well.

To initially characterize the expression of these molecules, we used synchronized HaCaT and HPV-KER cell lines. Interestingly, the mRNA expression profile of the splicing factors was found to be very similar in synchronized HPV-KER and HaCaT cell lines. Immediately after the synchronized cells were released from cell quiescence and subcultured, an induced mRNA expression was observed (0h) which dropped at 12h. Smaller peak of mRNA abundance was seen at 24h and high levels of expression similar to the 0h time point was observed at 72h to 168h, likely induced by cellular stress processes.

To functionally characterize these splicing regulators in keratinocytes, we analyzed the expression of EDA+ fibronectin, an alternative splice variant of the fibronectin gene, which was described by our research group to be altered in psoriatic skin. The gene-specific silencing of these regulators led to an altered EDA+ fibronectin/fibronectin ratio, demonstrating their functionality in keratinocytes. These results were summarized in a manuscript: *Szlávicz et al: Splicing factors differentially expressed in psoriasis alter mRNA maturation of disease-associated EDA+ fibronectin; the manuscript is under 2nd revision in Molecular and Cellular Biochemistry.*

Furthermore, the effects of LUC7L3 and SFRS18 silencing were studied using whole transcriptome sequencing techniques, in which gene expression changes and altered splicing

patterns have also been identified. Paired-end RNA sequencing (RNA-Seq) revealed several genes with significantly altered expression patterns which had already been found to be associated with psoriasis by our research group: interferon α inducible protein 6 (IFI6), α5 integrin (ITGA5), syndecan 4 (SDC4), and cyclin D1 (CCND1). Notably, the number of genes with alternative exon usage was more frequent than the number of genes with expression changes. With standard cut-off values we found at least 1500 genes, while using stricter cut-off values were found at least 200 genes with altered exon usage. These are caused by alternative splicing, exon skipping or alternative promoter usage. The quantification of splicing alterations was performed using the DEXSeq and Cufflinks programs. The identified transcripts with altered exon usage are involved in several fundamental cellular processes including transcription (CREB1), signal transduction (CDKL2, ITPR2), cell cycle (RCC1, ODF2, CDC14A), cell adhesion (FN1) and ubiquitination (CUL1, HERC6). Validation of these results confirmed the expression changes found by RNA-Seq. Results are currently summarized in a manuscript, describing bioinformatics analysis of the results. In the future we will focus on ubiquitination changes caused by alternative splicing of CUL1, since ubiquitination has not been extensively studied in psoriasis.

EDA+ fibronectin regulation was also studied at signaling level. EDA+ fibronectin expression is regulted by MAPK signaling and STAT1 was shown to negatively regulate both FN and EDA+FN expression in healthy fibroblasts, and this regulation is compromised in fibroblasts derived from uninvolved psoriatic dermis. Moreover, STAT1 and STAT3 signaling is altered in psoriatic uninvolved skin and depend on the severity of the disease. These results were published in two publications: *Gubán et al: Abnormal regulation of fibronectin production by fibroblasts in psoriasis. Br J Dermatol. 2016; 174(3):533-541. and Gubán et al: [Abnormal STAT1 activation in psoriasis] Abnormális STAT1 aktivitás pikkelysömörben. Bőrgyógyászati és Venerológiai Szle. 2016;92(1):18-21. [Hungarian].*

The work related to the project led to the completion of two PhD dissertations and succesfull PhD defense at the Doctoral School of Clinical Medicine, University of Szeged: Göblös Anikó: Characterization of molecules showing altered expression profile in psoriasis Konczné Gubán Barbara: Abnormalities in the psoriatic non-lesional skin

In 2016, Letizia La Rosa, a student from the University of Catania, Catania, Italy visited our laboratory in the frames of the ERASMUS+ progam. She completed her B.Sc. thesis based on the work completed as parts of the project, and succesfully presented her results at the final exam at the University of Catania.

In addition to the work closely connected to the project, we were involved in several co-operative psoriasis-related projects that resulted in the publication papers:

Képíró et al: Genetic risk and protective factors of TNFSF15 gene variants detected using single nucleotide polymorphisms in Hungarians with psoriasis and psoriatic arthritis. Hum Immunol. 2014;75(2):159-162.

Mahil et al: AP1S3 Mutations Cause Skin Autoinflammation by Disrupting Keratinocyte Autophagy and Up-Regulating IL-36 Production. J Invest Dermatol. 2016;136(11):2251-2259.