Abnormalities in the psoriatic non-lesional skin

Ph.D. Thesis

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    Abnormal STAT1 activation in psoriasis
LIST OF ABBREVIATIONS

AKT: v-akt murine thymoma viral oncogene homolog
AP-1: activator protein 1
BSA: bovine serum albumin
COMP: cartilage oligomeric matrix protein
DAPI: 4',6-diamidino-2-phenylindole
ECM: extracellular matrix
EDA+FN: extra-domain-A containing fibronectin
EDTA: ethylene-diamine-tetraacetic
ERK: extracellular signal-regulated kinase
FGF: fibroblast growth factor
FGF7: fibroblast growth factor 7
FGFR2: fibroblast growth factor receptor 2
FN: fibronectin
FRS2: FGFR substrate 2
GAB1: GRB2-associated binding protein 1
GRB2: growth factor receptor-bound protein 2
GWAS: genome-wide association studies
H: healthy
HSPG: heparan sulphate proteoglycan
IFNγ: interferon gamma
Ig: immunoglobulin
IL-1: interleukin-1
IL-6: interleukin-6
IL-8: interleukin-8
IL-12: interleukin-12
IRF-1: interferon regulated factor 1
JAK: Janus kinase
KGF: keratinocyte growth factor
L: lesional
MAPK: mitogen activated protein kinase
MEK1: MAPK/ERK kinase 1
MMP9: matrix metalloproteinase 9
MS: multiple sclerosis
NF-κB: nuclear factor κB
NL: non-lesional
PASI: psoriasis area severity index
PBS: Phosphate buffered saline
PI3: phosphoinositide 3-kinase
PLCγ: phospholipase C gamma
PPIG: peptidyl-prolyl cis-trans isomerase
PSORS: psoriasis susceptibility
RAS: rat sarcoma
RAF: rapidly accelerated fibrosarcoma
RFI: relative fluorescence intensity
SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis
SH2: Src homology 2
SNP: single nucleotide polymorphism
SOCS1: suppressor of cytokine signaling 1
SOS: son of sevenless
STAT1: signal transducer and activator of transcription 1
STAT3: signal transducer and activator of transcription 3
TK: tyrosin kinase
TLR4: Toll-like receptor4
TM: transmembrane
1. INTRODUCTION

1.1. Psoriasis, a complex inflammatory skin disease

Psoriasis is a multifactorial skin disorder affecting about 2-3% of the population worldwide, although it is more prevalent in European, American and Canadian populations (1). The most common form of chronic plaque psoriasis is Psoriasis vulgaris, which is characterized by epidermal hyperplasia, intensified and abnormal dermal angiogenesis and infiltration of inflammatory cells (Figure 1). Altered cellular composition characterized by hyperproliferation of keratinocytes and massive infiltration of immune cells is initiated by secreted cytokines of activated resident immune cells, dendritic cells, macrophages and innate immune cells as well as by keratinocytes themselves in the psoriatic skin (2, 3).

Both genetic risk factors and environmental triggers are known to contribute to the pathomechanism of psoriasis. Psoriasis-susceptibility (PSORS) loci are areas of chromosomes thought to associate with the manifestation or maintenance of psoriasis. At least 15 different PSORS have been identified by linkage analysis in family-based studies, for example PSORS1 is the strongest susceptibility locus known as HLA-C (4, 5). In recent years, genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) in 50 different regions of the human genome that are connected with psoriasis. The function of these polymorphic genes is comprehensive, such as antigen presentation (HLA-Cw6), T cell development and polarization (STAT3), innate immunity (CARD14) and negative regulators of immune responses (SOCS1) (1).

One of the environmental factors involved in psoriasis is the streptococcal infection of the tonsils, where even after the infection is cleared, T cells remain auto-reactive to keratin peptides (6, 7). Acute stressful emotional events like accidents, examinations, sexual assaults or death of relatives have also been related to the onset of the disease. Moreover, a correlation between high stressed group and psoriasis area and severity index (PASI) score has also been established (8, 9). Physical trauma including cuts, scarring, sunburn and Koebner response may also lead to the induction of psoriasis (10, 11). Lifestyle could play a role in modifying psoriasis pathogenesis. Among these factors diet (polyunsaturated fatty acids, gluten and alcohol), smoking and ultraviolet radiation are suspected (12-15).
Several studies focused mainly on the lesional skin and as a result of these efforts now it is well characterized that psoriasis is a chronic, inflammatory skin disease in which both innate and adaptive immune activation play a role in the manifestation and the maintenance of the abnormal skin phenotype (16). However, much less is known about the contribution of resident cells of the tissue to the pathology of the disease. Therefore, we focused on to seek for alterations in tissue responses in psoriasis by investigating the phenotypically normal looking non-lesional (NL) psoriatic skin.

Figure 1. Non-lesional (NL) and lesional (L) psoriatic skin. Psoriatic lesional skin manifests as raised, red scaly and well-demarcated plaques. Non-lesional skin is marked with red. The picture is from the clinical archive of the Department of Dermatology and Allergology, University of Szeged.
1.2. Structure and function of fibronectin

1.2.1. The role of fibronectin-binding integrins

Integrins are cell-surface heterodimeric transmembrane receptors consisting of an α and a β subunit. Integrins are known to play essential roles in connecting the intracellular cytoskeleton with various components of the extracellular matrix (ECM). Through these interactions integrins transduce mechanical and chemical information from the extracellular space into the intracellular environment and regulate specific signal transduction cascades. Fibronectin (FN) is a protein secreted as a soluble dimer and through interactions with integrins they form fibrillar structures. The main FN receptor α5β1 integrin was found to be overexpressed in the NL psoriatic epidermis relative to normal (17, 18). There is evidence that the proper regulation of integrin expression in keratinocytes is essential for normal epidermal homeostasis as shown by the suprabasal overexpression of integrins in transgenic mice resulting in epidermal hyperproliferation and inflammatory reaction (19).

In the steady state of normal human adult epidermis α5 integrin expression of keratinocytes is downregulated. A possible explanation of α5 integrin overexpression in NL skin is the presence of FN and one of its isoforms, the extra-domain-A containing fibronectin (EDA+FN) in the microenvironment of basal keratinocytes (18, 20). The EDA+ domain contains an additional non-classical (non-RGD) binding site (EDGIHEL) for α4β1, α9β1 and αβ7 integrins (Figure 2) (21). α4β1 integrin conveys the EDA-induced stress fibers, myosin light chain phosphorylation, fibrillogenesis, fibronectin synthesis and deposition (22).
1.2.2. Structure and function of EDA+FN and FN

FN is a multifunctional component of the ECM. FN filaments interact with other ECM components including fibrin, fibulin-1, heparin, syndecans, cartilage oligomeric matrix protein (COMP) (23) and collagens (24). Matrix FN provide a base to the cells and regulate the cellular behavior through the transfer of biochemical and mechanical signals. There are two major distinct forms of FN: the plasma and the cellular FN. The secondary structure of FN consist of the following domains: type I, II and III repeats (Figure 2). Type I and II domains are stabilized by disulphide bonds in contrast to the type III domains that consist of seven anti-parallel beta-strand which are arranged into two distinct beta-sheets (25, 26). FN could contain one or two extra Type III domains, named Extra Domain A and B (EDA and EDB, respectively). These two domains could be inserted/excluded through alternative splicing (21). The alternatively spliced ED sequences are lacking from the plasma FN secreted mostly by hepatocytes. In contrast, the cellular form of FN contains the EDA domain and found incorporated into the
ECM of tissues. Several stromal cell types are able to produce cellular fibronectin but the main source of this protein are the fibroblasts. The alternative splicing of FN is regulated by splicing regulator (SR) proteins. The regulatory sequence of EDA is a purin-rich region found within the exon itself (27) and is recognized by splicing factor 2 (SF2) /alternative splicing factor (ASF) (28).

The production of alternatively spliced forms is the highest during embryonic development. Postnatal the amount of the protein is reduced and further decreases with ageing (21). However, elevated amount of the EDA⁺FN is observed during tissue repair, wound healing, angiogenesis and in highly proliferating adult tissues, it can influence cell adhesion, differentiation, cell cycle progression (29-31). The EDA⁺FN is appearing at the basement membrane of NL epidermis compared to normal skin (20, 32). TGFβ1 is known to enhance EDA⁺FN production in wounded tissues (33). Moreover, EDA⁺FN can modulate matrix remodelling by the regulation of cytokine-dependent matrix metalloproteinases (34). EDA⁺FN can activate matrix metalloproteinase 9 (MMP9) and NF-κB production via Toll-like receptor 4 (TLR4) thereby contributing to the manifestation of inflammatory responses (30). In human dermal fibroblasts EDA⁺FN was described to stimulate the expression of pro-inflammatory cytokines including CXCL1-3 (35) and to induce interleukin 8 (IL-8) expression through NF-κB and p38/MK-2 signaling cascade (36).

1.3. The role of KGF in health and disease

1.3.1. Structure and function of KGF

Fibroblast growth factors (FGFs) are small growth factor polypeptides (37). The family of FGF consists of 23 members with a common structural characteristic, although there are only 18 FGFs that are known to serve as a ligand for the four different FGF receptors (38). Most FGFs have an autocrine and/or paracrine function. Fibroblast growth factor 7 (FGF7), also known as keratinocyte growth factor (KGF) is produced by cells of mesenchymal origin and considered to be a major growth factor for keratinocytes. KGF was first isolated by Rubin and colleagues and characterized as a fibroblast produced growth factor of keratinocytes (39). In the skin KGF is produced by dermal fibroblasts and keratinocytes can influence its production through interleukin 1 (IL-1).
KGF is known to induce keratinocyte proliferation and differentiation. Several reports suggest that KGF reduces the ability of keratinocytes to initiate terminal differentiation and undergo programmed cell death. Overexpression of KGF in the basal epidermal cell layer of transgenic mice results in epidermal hyperplasia (40). There is also evidence suggesting that fibroblasts from L and NL skin of psoriatic patients induce keratinocyte outgrowth by producing soluble signals (41). More recently, it has been reported that KGF can induce the expression of α5 integrin and delays the expression of keratin 10 and transglutaminase in keratinocytes (42). Indirect evidence, such as measurement of mitogenic activity, suggests that the specific receptor for KGF on epithelial cells is the IIIb splice variant of fibroblast growth factor receptor 2 (FGFR2) (43).

1.3.2. Structure and isoforms of FGFR2

The FGFR family consists of FGFR1, FGFR2, FGFR3 and FGFR4 encoding for receptors of FGFs. FGFRs are transmembrane-type receptors, composed of an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain. The cytoplasmic domain of FGFRs contains the catalytic protein tyrosine kinase core and regulatory sequences (44). FGFRs are formed in two different ways: by the expression of different splice variants of the same FGFR gene that can form homo- or heterodimers or by the expression of two different FGFR genes that go under hetero-dimerization following ligand binding (39). Alternative splicing in the third immunoglobulin (Ig)-like domain provides increased ligand specificity for different members of the FGFR family (38, 45, 46). The extracellular ligand binding domain of FGFRs consists of either two or three Ig-like domains. Alternative RNA splicing is realized by utilizing one of the two unique Ig-like exons in designated as IIIb and IIIc versions in FGFR1, 2 and 3, with the exception of FGFR4 where no alternative splicing take place (43). FGFR2 has two isoforms FGFR2-IIIb and FGFR2-IIIc. The expression of the different isoforms of FGFR2 is regulated in tissue-specific manner, where isoform IIIb is mainly produced by epithelial cells and the isoform IIIc by mesenchymal cells (47). KGF is known to initiate mitogenic activity most effectively by binding to the FGFR2-IIIb splice variant. KGF can also facilitate cell proliferation through FGFR2-IIIc or FGFR1, but with a much lower efficiency (37). The schematic structure of FGF-FGFR and the formation of the two different FGFR isoforms by splicing are shown in Figure 3.
**Figure 3. FGF-FGFR structure and FGFR splicing.**

*a* The basic structure of the FGF–FGFR complex comprises two receptor molecules, two FGFs and one heparan sulphate proteoglycan (HSPG) chain. FGFs bind with low affinity to cell surface HSPGs (purple) and with high affinity to specific FGFRs. The FGFRs consist of three extracellular immunoglobulin (Ig) domains, a single transmembrane helix and an intracellular split tyrosine kinase (TK) domain. The second and third Ig domains form the ligand-binding pocket and have distinct domains that bind both FGFs and HSPGs. 

*b* Ligand-binding specificity is generated by alternative splicing of the Ig III domain. The first half of Ig III is encoded by an invariant exon (IIIa), which is spliced to either exon IIIb or IIIc, both of which are spliced to the exon that encodes the transmembrane (48) region. Epithelial tissues predominantly express the IIIb isoform and mesenchymal tissues express IIIc. FGFR4 is the only member of the FGFR family that is expressed as a single isoform (38). (Modified figure based on the publication of Turner et al., 2010.)

### 1.3.3. FGF and FGFR signaling network

FGFs transmit their signal through binding to specific FGFRs. FGFs are secreted glycoproteins that are localized to the extracellular matrix and to the cell surface by heparin sulphate proteoglycans (HPSGs) (49). FGFs are released from the extracellular matrix by heparinases, proteases or specific FGF binding proteins and are associated with HPSGs on the cell surface, where they induce the dimerization and autophosphorylation of FGFRs (44). The specificity of FGF-FGFR interaction depends on the alternative splicing of FGFR. FGFs create a ternary complex with respective FGFRs and HPSGs (Figure 4). Following ligand binding FGFR monomers undergo dimerization forming homo- or heterodimers (39). Dimerization leads to conformational change in the receptor triggering the autophosphorylation of the intracellular tail. Phosphorylated tyrosine residues are binding sites to the signal transduction proteins, resulting in the induction or modulation of the four distinct signalling cascades:
phospholipase Cγ (PLCγ), mitogen activated protein kinase (RAS-RAF-MAPK), phosphoinositide-3 kinase (PI3K)- v-akt murine thymoma viral oncogene homolog (AKT) and signal transducer and activator of transcription (STAT) (38, 50).

Activated FGFR2 phosphorylates the FGFR substrate 2 (FRS2) to recruit the son of sevenless and growth factor receptor-bound protein 2 (GRB2) leading to the activation of the RAS-RAF-MEK-MAPK/ERK pathway. AKT-dependent anti-apoptotic signalling cascade is activated by GRB2-associated binding protein 1- PI3K (GAB1-PI3K) in a separate complex. On the other hand, the Src homology 2 (SH2) domain of PLCγ binds and activate the protein kinase C (PKC), which reinforces the activation of MAPK. Finally, the activation of the STAT pathway depends on cellular context (Figure 4).

Figure 4. FGF-FGFR signalling network. Following ligand binding and receptor dimerization, the kinase domains transphosphorylate each other, leading to the docking of adaptor proteins and the activation of four key downstream pathways: RAS-RAF-MAPK, PI3K-AKT, signal transducer and activator of transcription (STAT) and phospholipase Cγ (PLCγ) (green) (38). (Modified figure based on the publication of Turner et al., 2010.)
1.3.4. Autocrine and paracrine signaling roles of KGF and FGFR2 in pathophysiology and therapy

There are several pieces of evidence highlighting the important function of KGF in the processes of wound healing, tissue regeneration and repair. KGF released by stromal cells enrols the appropriate receptor of target epithelial cells thus form paracrine stimuli facilitating epithelial regeneration. The paracrine signaling functions of KGF are explored for their therapeutic potential in mucositis and wound healing (51). Recombinant produced human KGF, Palifermin is already in use to treat mucositis after chemotherapy and radiotherapy (52). Furthermore, KGF can modify the extent of ureteric bud growth during development and the number of nephrons in the kidney (53) and accelerate the repair of renal tubules after chemically induced proximal tubule damage (54).

Genetic alterations of FGFR2 that cause dysregulation of FGFR2 signaling can lead to the development of congenital disorders and acquired diseases including melanoma, Apert syndrome and atopic dermatitis among others (55). FGFR2-IIb knockout mice die after birth due to multiple organ abnormalities including dysgenesis of the skin and agenesis of the lungs and limbs (56). Amino-acid substitutions around the third immunoglobulin-like domain of FGFR2 lead to the alteration of ligand selectivity among different FGFs (57), whereas mutations within the tyrosine kinase domain cause autoactivation of the receptor independently from ligand binding (58, 59). The FGFR2 gene is mutated in Apert syndrome, where ligand specificity experiments showed that the mesenchymally expressed FGF7 is able to bind thereby to activate the mesenchymal splice form of FGFR2 (FGFR2-IIic) (57). Furthermore, genetic alterations in FGFR2 give rise to skin manifestations like melanoma, squamous cell carcinoma and basal cell carcinoma (59).
1.4. The JAK-STAT signaling pathway

1.4.1. The role of the JAK-STAT signaling pathway in inflammation and immunity

The Janus kinase (JAK)-STAT pathway plays an important role in both innate and adaptive immunity and contributes to the manifestation of several inflammatory processes. In mammals the JAK family consists of four tyrosine kinases: JAK1, JAK2, JAK3 and TYK2, while the STAT family has seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. STAT proteins share a similar structural composition: N-terminal domain, coiled-coil domain, DNA binding domain, linking domain, SH2 domain and transcriptional activation domain.

JAK-STAT is activated by over 50 cytokines and growth factors, these cytokines include interferons, colony stimulating factors, several interleukins and hormones (60, 61). Extracellular cytokines or growth factors associate with their corresponding cell surface type I and type II cytokine receptors, ligand binding leads to conformational change and dimerization of the receptor allowing the binding of JAK pairs in the cytoplasm (62). Dimerized JAKs autophosphorilate and become activated enabling STAT binding and phosphorylation. Phosphorylated STAT monomers form dimers that are translocated into the nucleus of the cell and influence transcription of several genes (Figure 5) (63).

The IFNγ-induced route of STAT1 has been well characterized. The regulation of STAT1 signaling by IFNγ takes place via the modulation JAK1 and JAK2 phosphorylation. Furthermore IFNγ activates transcription factors called interferon regulatory factors (IRF) IRF1 and IRF8 via STAT1 (64). IRFs such as IRF1, IRF3, IRF5, IRF7 and IRF8 contribute to the TLR mediated signaling through the critical mediator STAT1 (65, 66). In a TLR4-dependent manner STAT1 phosphorylation can increase the expression of inflammatory mediators such as chemokine C-X-C motif chemokine 10 (CXCL10) and the adhesion molecule ICAM1 by IFNγ and LPS cross-talk (66). Moreover, IRF8 in association with IFNγ and LPS mediate the transcriptional activation of chemokine RANTES (CCL5) (67) leading to attraction of leukocytes to the site of injury of tissues including that of arteries. IFNγ and LPS co-administration triggers macrophage STAT1 pool super-activation and IRF8 expression (68, 69). The newly synthetized IRF8 enhances the crosstalk between IFNγ and TLR inducing further
activation of STAT1 as well as inflammatory molecules and thereby contributing to antimicrobial defense (64).

Figure 5. The schematic illustration of JAK-STAT signaling. (JAK: Janus kinase; STAT: signal transducer and activator of transcription; P: phosphorylation)

1.4.2. Disruption of STAT1 signaling in human diseases

Several studies revealed alterations in the JAK-STAT signaling in chronic skin diseases including psoriasis, atopic dermatitis and melanoma. STAT1 was observed to be localized predominantly in the horny layer in normal skin by immunostaining (70). Impaired phosphorylation of STAT1 and STAT5 was described to cause defective response to IL-2 in melanoma from stage III to IV (71). STAT1 in tumors functions as a suppressor probably by mediating the TNFα triggered apoptotic program (72).

Both loss-of-function and gain-of-function mutations were found in genes coding STAT transcription factors. Strikingly, all of the known STAT1 mutations are coupled with a particular type of pathogen. Patients with STAT1 loss-of-function mutations are more prone to mycobacterial and viral infections, whereas gain-of-function mutations increase susceptibility to fungal infections (73, 74).
2. AIMS

The pathogenesis of psoriasis is still not fully understood, numerous studies have proposed hypotheses regarding to immune dysregulations (3), skin microbiota system (75) or extracellular matrix alterations (76) among others to play essential roles in the development of the disease. A continuously growing body of evidence indicate that the healthy looking NL psoriatic skin represents an unbalanced phenotype and carries structural alterations compared to normal skin making it susceptible for developing the disease. Abnormalities of the basement membrane at the dermal-epidermal junction (DEJ) render the uninvolved skin to become activated and present a chronic wound healing phenotype.

FN is capable to enhance cell cycle entry, without the increase of adhesion, in uninvolved, but not normal keratinocytes, even in the absence of T cell lymphokines (77). The main FN receptor α5β1 integrin was found to be overexpressed in NL psoriatic epidermis relative to normal epidermis (17, 18). KGF together with fibronectin and α5β1 integrin was suggested to play a crucial role in the pathogenesis of psoriasis by influencing inflammation and keratinocyte hyperproliferation. We focus in particular on alterations in NL skin and the role of EDA+FN and KGF in the initiation and progression of psoriasis.

Therefore, we aimed:

- To investigate the expression of KGF, FGFR2, EDA+FN and α5 integrin in healthy and NL psoriatic skin.
- To examine whether KGF has an effect on the EDA+FN and FN production in cultured fibroblasts, keratinocytes and HaCaT cells.
- To determine the expression of different splice forms of FGFR2 in fibroblasts, keratinocytes and melanocytes.
- To identify the signal transduction pathway(s) through which KGF may influence and modulate the expression of EDA+FN and FN in healthy and NL skin derived fibroblasts.
- To study the putative correlation between the activation of STAT1 in healthy, NL and L skin and PASI score of patients.
- To construct an in silico model based on transcriptional regulatory molecules combined with our in vitro results involving KGF and FN signaling.
3. MATERIALS AND METHODS

3.1. Skin biopsies

Patients with moderate-to-severe chronic plaque type psoriasis and healthy volunteers (age 18-60 years) were enrolled into the study. Patients had a medication-free period of at least 4 weeks of systemic and/or at least 2 weeks without local therapy. Participant informed consent was obtained prior surgical intervention. PASI score was determined for all patients by dermatologists. Samples were collected from 16 patients and 25 healthy volunteers for the experiments. Mechanical stress was induced by tape-stripping procedure, and then punch biopsies were taken from uninvolved skin of psoriatic patients (n=6) and from healthy (n=6) subjects from tape-stripped and non-treated skin area from the buttock area at 24 and 48 hours after treatment for all other experiments tissues without the introduction of mechanical stress were used. All tissue collection complied with the Guidelines of the Helsinki Declaration and was approved by the Regional and Institutional Research Ethics Committee (Approval No:2799, 3517).

3.2. Cell culture

Primary keratinocytes, melanocytes and fibroblasts were obtained from healthy participants or psoriatic patients by enzymatic digestion of skin biopsies according to a standard protocol. Briefly, skin specimens were first washed in Salsol A solution (Human Rt, Gödöllő, Hungary) supplemented with 2% antibiotic/antimycotic solution (Sigma-Aldrich). Skin samples were then cut into narrow strips and incubated in Dispase solution (Roche Diagnostics, Mannheim, Germany) overnight at 4°C. The epidermis was subsequently separated from the dermis and incubated in 0.25% trypsin/0.02% EDTA (PAA, Pasching, Austria) for 5 min at 37°C to achieve a single-cell epidermal suspension. Fibroblasts were obtained by incubating the dermis in Digestion Mix (27mg Collagenase, 12.5mg Hyaluronidase and 1mg Deoxyribonuclease/ 10ml DMEM medium) for 2h at 37°C. The cell suspensions were filtered through a 100 µm nylon mesh (BD Falcon, San Jose, CA, USA) and pelleted by centrifugation.

Fibroblasts were grown in low glucose DMEM medium containing 5% FBS. Melanocytes were maintained in Mel-mix medium as previously described (78). Keratinocytes
were grown in keratinocyte serum-free medium (Gibco® Keratinocyte SFM Kit; Life Technologies, Copenhagen, Denmark). HaCaT cells were kindly provided by Prof N.E. Fusenig and cultured in high-glucose DMEM medium containing 10% FBS. All media was supplemented with 1% antibiotic/antimycotic (PAA) and 1% L-glutamine solution (PAA). Fibroblasts, keratinocytes, melanocytes, and HaCaT cells were cultured at 37°C and 5% CO₂ in humidified conditions. Depending on cell growth, the medium was changed every 2–4 days and cells were passaged at 80% confluence. In our experiments, fibroblasts at passage number 5, keratinocytes and melanocytes at passage 3 were used at 70% confluence.

Fibroblasts, keratinocytes and HaCaT cells were cultured in 6-well plates at a starting density of 200,000 cells/well and incubated with 25 ng/mL KGF (ProSpec -Tany TechnoGene Ltd., Rehovot, Israel) for 24 h; untreated cells were used as controls. For KGF signaling pathway blocking experiments the following inhibitors were used: MEK1 inhibitor (PD98059, 20µM), AKT ½ kinase inhibitor (10µM), STAT1 inhibitor (Epigallocatechin gallate, 40µM) and STAT3 inhibitor (Stattic, 4µM), all provided by Sigma-Aldrich, concentrations were applied as previously described (79-81). The pictures were taken using a Nikon Eclipse TS100 inverted microscope (Nikon Incorporation, Melville, USA).

3.3. RNA extraction and real-time quantitative reverse transcription polymerase chain reaction (RT qRT-PCR)

For RNA isolation fibroblasts, melanocytes and keratinocytes were cultured as described above. Cells on 6 well plates were washed once with PBS, then harvested with 0.5 ml TRlzol™ Reagent (Life Technologies). Total RNA was isolated according to the manufacturer’s instructions. RNA concentration was determined from A260 values. All RNA was treated with DNA-free™ reagents (Life Technologies). cDNA was synthesized from 1 µg total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s protocol.

For EDA⁺FN qPCR amplification iQ™ Supermix (from Bio-Rad Laboratories Hercules, CA, USA) was used. The EDA⁺FN primers were constructed as described previously (82). The amplification protocol for EDA⁺FN qPCR was the following: 95°C for 3 min, and then 95°C for 15 s and 56°C for 30 s, repeated 40 times.
FN and 18S rRNA amplifications were carried out with FastStart TaqMan Probe Mastermix (Roche Basel, Switzerland) using the following primers: FN forward 5’-GGAAAGTGTCCCTATCTCTGATACC-3’, reverse 5’-AATGTTGGTGAATCGCAGGT-3’, probe: #33. 18S RNA forward 5’-CTCAACACGGAAACCTCACC-3’, reverse 5’-CGCTCCACCAACTAGAAACG-3’, probe: #77 (probes were from the Roche Universal Probe Library). Primers were designed using Probedfinder for the Universal Probe Library system (Roche Diagnostics, Basel, Switzerland). FN and 18S rRNA amplifications were carried out under the following conditions: 95°C for 3 min, and then 95°C for 15 s and 57°C for 1 min, repeated 40 times.

For FGFR2-IIIb and FGFR2-IIIc, specific custom TaqMan primer/probe sets (Life Technologies, Carlsbad, CA, USA) were developed as described previously (83) and for amplification the following protocol was used: 95°C for 10 min, and then 95°C for 15 s and 60°C for 1 min, repeated 40 times.

RT-PCR experiments were carried out on the LightCycler® 480 System Real-Time PCR machine (Roche Cobas Z 480 Roche Diagnostics). The abundance of each gene products was normalized to the 18S rRNA gene expression in each examined sample. Relative mRNA levels were calculated by the 2^ΔΔCt method. Data are expressed as arbitrary units proportional to the mRNA level.

3.4. Immunofluorescent and immunohistochemistry staining

Human punch biopsies were frozen embedded, stored at -80 °C, then cut into 6 μm sections. Samples were fixed in Fixation/Permeabilization Concentrate and Diluent (eBioscience, San Diego, CA) and washed in PBS. The sections were incubated for 30 min in 1% goat serum in PBS (Sigma-Aldrich, St. Louis, MO, USA). The following primary antibodies were used: FGFR2 (1:100, Bek (C-17), Santa Cruz Biotechnology, Santa Cruz, CA), KGF (1:500, FGF-7 (N-14), Santa Cruz Biotechnology), α5 integrin (1:200, Becton-Dickinson, Franklin Lakes, NJ, USA), EDA+FN (1:500, clone IST9, Abcam, Cambridge, UK), and pSTAT1(Ser727), pSTAT1 (Tyr701), (both 1:400, Cell Signaling Technology, Beverly, MA) overnight at 4°C. Goat anti-rabbit IgG-Alexa Fluor 488, donkey anti-goat IgG-Alexa Fluor 546, goat anti-mouse IgG-Alexa Fluor 647 and Alexa Fluor 546 were used as secondary antibodies (all 1:500, from Life Technologies, Carlsbad, CA) for 2 h, at room temperature. Sections were
incubated without the primary antibody or with isotype control antibody for negative staining controls. Nuclei were stained with DAPI (Sigma-Aldrich).

For immunohistochemistry paraffin embedded section of the three different skin types (mentioned above) were applied at 5 μm thickness and the ABC kit for the appropriate primary antibody (Enzo Life Sciences Inc.) was used according to the guidelines of the manufacturer to develop immunostaining. The counterstain of the cytoplasm and the nuclei was achieved by applying haematoxylin staining.

Cultured human fibroblasts, keratinocytes and HaCaT cells were collected after brief trypsinization (0.25% trypsin solution, Sigma Aldrich) and centrifuged onto a slide using a cytocentrifuge (Cytopro™, Wescor, Logan, UT, USA), then fixed in 2% paraformaldehyde (Sigma-Aldrich). Stainings were carried out, as described above. Images were documented with a Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss Microscopy GmbH, Munich, Germany) equipped with an AxioCam MRm camera and AxioCam MRc camera for transmission light (bright field). Image processing and data analysis were conducted using ZEN 2012 Microscope and Imaging or ImageJ software.

3.5. Flow cytometry

Cells were harvested as described, above fixed in Fixation/Permeabilization Concentrate and Diluent (eBioscience) and resuspended in PBS. Primary antibodies anti-EDA+FN (1:500, clone IST9, Abcam), anti-FN (1:1000, clone IST4, Sigma-Aldrich) were applied for 45 min. Mouse IgG1 isotype antibody (Sigma-Aldrich) was used, for negative as control. Cells were then washed in PBS, and incubated with secondary antibodies as described above. Samples were analysed on a FACS Calibur flow cytometer equipped with 488 and 633 nm lasers (Becton–Dickinson). Data was analysed using CellQuest Pro.

3.6. Statistical analysis

For statistical analysis, Repeated Measures Friedman ANOVA, Wilcoxon or one-way ANOVA were used. Multiple comparisons were done with SigmaPlot software (Systat Software Inc., Chicago, IL, USA). A probability test was used to test for normality, P≤0.05 was considered significant.
3.7. Bioinformatics analysis and model construction

STRING, KEGG database and Reactome were used as a source to create protein-protein interactions and signaling pathways; SABiosciences’ transcription factor binding site data and CisRED (p<0.001) were applied for modeling protein-DNA interactions. Direction of transcriptional regulation was assessed with expression correlation matrix. Data of a microarray study with 48 dermal fibroblast samples carried out by Gabig-Cimińska et al. was downloaded from Gene Expression Omnibus. Probe with the largest interquartile range (IQR) was selected for genes with multiple probes. Coexpression matrix was constructed using MATLAB R2014b (MathWorks Inc., Natick, MA). The genes and proteins related to KGF signaling, fibronectin-signaling, splicing and the regulation of these genes were collected (summarized in Table 1) and used for filtered matrix construction (Table 2). We used Pearson correlation coefficients with p values under 0.05 for the assessment of the direction of transcriptional regulation. If the correlation coefficient was positive it was considered an upregulation, and if it was negative, down regulation was defined.

<table>
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<th>TARGET GENES</th>
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Table 1. List of genes applied for the construction of the co-expression matrix gene model.
Table 2. Co-expression matrix of genes in our model

Red bold characters indicate correlation coefficients with $p<0.05$ between transcription factors and known targets. Red-shaded cells show positive correlation, blue shaded cells show negative correlation. Shade marks the strength of correlation. Cells with thick margin indicate that there is binding site of the given transcription factor in the promoter region of the target gene.
4. RESULTS

4.1. Altered protein expression of $\alpha_5$ integrin, EDA$^+$FN, KGF and FGFR2 in psoriatic NL skin

Healthy skin and psoriatic NL skin were investigated for differences in the expressions of $\alpha_5$ integrin, EDA$^+$FN, KGF and FGFR2 before and 24, 48 hours after tape stripping. Immunofluorescent staining revealed a striking difference in all protein expressions examined even without tape stripping between normal and psoriatic NL skin (Figure 6). Mild mechanical stress introduced by tape stripping of the skin did not lead to any obvious changes in $\alpha_5$ integrin, EDA$^+$FN, KGF and FGFR2 expressions or distributions at 24 and 48 hours after treatment in NL skin. In healthy control sections a slight increase in all protein expression was observed based on immunostaining (Figure 7).

Figure 6. Increased expression of $\alpha_5$ integrin, EDA$^+$FN, KGF and FGFR2 in NL skin without mechanical stress stimuli. Immunofluorescent analysis of $\alpha_5$ integrin, EDA$^+$FN, KGF and FGFR2 expression in H (healthy) and NL (non-lesional) skin (n=6). Bar: 50 $\mu$m
Figure 7. Mild mechanical stress induced by tape stripping has no effect on the expression of α5 integrin, EDA\'FN, KGF or FGFR2 in NL psoriatic skin and only a mild effect on healthy skin. The protein expression of α5 integrin (a), EDA\'FN (b), KGF (c) and FGFR2 (d) was visualized by immunofluorescent staining 24 and 48 hours following tape stripping in H (healthy) and in NL (non-lesional) skin. Bar: 50 µm.
4.2. Determining temporal changes in protein production for downstream analysis

The production of EDA-FN is the highest after 24 hours, whereas the expression of FN and FGFR2 remains similar in the first 48 hours starting at both 40% and 70% confluence of normal human fibroblast.

FN, EDA-FN and FGFR2 protein levels were examined using flow cytometry at 6, 12, 24, 36 and 48 hours in two different states of confluence (40% and 70%) of normal human fibroblasts. FN protein level showed an increasing tendency with time and peaked at 36h however this change did not reach the level of significance. In both 40% and 70% confluence states EDA-FN protein level was significantly elevated at 24h compared to any other measured time points. FGFR2 protein level did not show any change during the first 48h. Protein production was similar at both 40% and 70% confluence in the examined timeframe (Figure 8).

![Graphs showing protein levels over time](image)

Figure 8. Flow cytometry measurement of FN, EDA-FN and FGFR2 protein levels at 6, 12, 24, 36 and 48 hours at 40% and 70% of confluence of normal human fibroblasts. MFI: mean fluorescence intensity. Data are represented as mean±SD (n=3), ** p< 0.01.
4.3. KGF treatment of healthy human fibroblasts leads to the elevation of EDA⁺FN production

EDA⁺FN and KGF are known to stimulate keratinocyte proliferation. KGF induces the expression of the major FN cell surface receptor α₅β₁ integrin. Given the differences in EDA⁺FN and KGF protein levels in healthy and NL psoriatic skin, we set out to investigate a putative regulatory connection between these two molecules. Exogenous treatment of cultured healthy fibroblasts with KGF for 24 hours increased the level of the EDA⁺FN splice variant, but not the total FN protein based on immunostaining and flow cytometry measurements (Figure 9a, b). A similar increase in the EDA⁺FN mRNA level was detected, while the total FN mRNA remained unchanged (Figure 9c). We also investigated this effect of KGF on normal human keratinocytes and on the keratinocyte cell line, HaCaT. As expected, keratinocytes and HaCaT cells expressed the FN and the EDA⁺FN at a very low level compared to fibroblasts (Figure 10). However, upon in vitro KGF treatment these cells did not display detectable changes in mRNA and protein expressions of EDA⁺FN and total FN (Figure 10a-c).
Figure 9. The expression of EDA⁺FN is induced by KGF in normal human fibroblasts. KGF treated fibroblasts and the change in expression in FN and EDA⁺FN at 24 hours. a) Immunofluorescent detection of EDA⁺FN. b) Flow cytometry measurement of EDA⁺FN and FN levels following KGF treatment. RFI: relative fluorescence intensity. c) FN or EDA⁺FN mRNA expression following KGF stimulation. Values are expressed in arbitrary units. Con: control. Data represent the mean±SEM (n=6). Bar: 50 µm.
Figure 10. Exogenous KGF treatment has no effect on EDA+FN expression of keratinocytes and HaCaT cells. The expression of EDA+FN was visualized by immunostaining (a), its protein amount was quantified by flow cytometry (b) and mRNA expression was determined by real-time RT-PCR measurements (c) following 24 hours of 25 ng/ml KGF treatment of healthy human keratinocytes and HaCaT cells. RFI: relative fluorescence intensity. Values are expressed in arbitrary units. Bar: 50 µm.
4.4. Expression of FGFR2 splice variants in fibroblasts, melanocytes and keratinocytes

Two splice variants have been identified for FGFR2 designated as FGFR2-IIIb and FGFR2-IIIc that have different ligand binding preferences. These results were based on mitotic activity measurements (39). KGF has been shown to act on the FGFR2-IIIb variant increasing cellular proliferation (37). We determined the expression of the splice variants in healthy fibroblasts, melanocytes and keratinocytes using specific primers designed for RT-PCR, revealing that melanocytes and keratinocytes expressed the FGFR2-IIIb only, while fibroblasts mainly expressed the FGFR2-IIIc variant (Figure 11 a, b).

![Image](image_url)

**Figure 11.** Human fibroblasts in culture mainly express the FGFR2-IIIc, while melanocytes and keratinocytes the FGFR2-IIIb splice variant of FGFR2. a) RT-PCR products specific for FGFR2-IIIb and – IIIc were run on a 2% agarose gel of cultured human fibroblasts (FB), melanocytes (MC) and keratinocytes. b) FGFR2 mRNA expression by fibroblasts for the two investigated splice variants detected by real-time RT-PCR. Values are expressed in arbitrary units.
4.5. MAPK signaling is involved in the regulation of EDA⁺FN controlled by KGF in healthy fibroblasts

FGF signals (including KGF) are coordinated by four major pathways: the RAS-RAF-MAPK, the PI3-AKT, the STAT and the PLCγ signaling (38, 44). In order to get a better insight into the modulation of FN expression by KGF in cultured fibroblasts derived from healthy skin, we performed blocking experiments targeting key molecules of the signal transduction pathways using specific inhibitors available for MEK1 (MAPK), AKT1/2 (PI3-AKT), STAT1 and STAT3 (STAT) either alone or in combination. After 24h of inhibitory treatment the expressions of FN and EDA⁺FN were determined by flow cytometry and RT-PCR. The KGF induced EDA⁺FN elevation was abolished by the inhibition of MEK1 and returned to the level of control fibroblasts (Figure 12 a). In contrast, blocking AKT1/2 did not have an effect on EDA⁺FN protein expression (Figure 12 c). The inhibition of STAT1 or STAT3 did not influence KGF-mediated changes in EDA⁺FN levels, however, impairing these signaling molecules resulted in a KGF independent elevation of both FN and EDA⁺FN protein levels that was significant in the case of STAT1 inhibition (Figure 12 b).
4.6. Inhibition of the RAS-RAF-MAPK, PI3-AKT and STAT signaling pathways affect fibroblast viability

Next we investigated the effect of blocking the three main signaling pathways (RAS-RAF-MAPK, PI3-AKT and STAT) in fibroblasts using the STAT1, STAT3, MEK1 and AKT 1/2 inhibitors. Cell viability was examined after 24 hours of inhibition by microscopy (based on cellular morphology). Viable cell numbers showed large degree of reduction following STAT1-STAT3, STAT1-STAT3-MEK1 or STAT1-STAT3-AKT1/2 combined treatments (Figure 13). Using STAT1 or STAT3 inhibitors separately did not influence fibroblast viability.
while blocking both STAT proteins simultaneously significantly decreased the survival of fibroblasts however further inhibition of AKT and MAPK pathways did not increase cell death.

Figure 13. Combined blocking of the RAS-RAF-MAPK, PI3-AKT and STAT signaling pathways result in fibroblast cell death. Healthy fibroblasts were detected by microscope following 24h treatment with STAT1, STAT3 alone or in combination with inhibitors for MEK1, AKT1/2 or both. Ten-fold magnification. Con: control, STAT1i: STAT1 inhibitor, STAT3i: STAT3 inhibitor, MEK1i: MEK1 inhibitor, AKT1/2i: AKT1/2 inhibitor. (n=3)
4.7. Abnormal STAT1 activation in psoriatic skin plays a role in the regulation of both FN and EDA+FN

To investigate further, we compared FN and EDA+FN expressions between derived from fibroblasts healthy and psoriatic NL skin 24 hours after STAT1 or STAT3 inhibition in culture. In contrast to healthy controls, blockade of STAT1 did not lead to the elevation of total FN and the EDA+FN splice variant in fibroblasts from NL skin changes upon STAT3 impairment were not significant (Figure 14a).

Therefore, we investigated the phosphorylation pattern of STAT1 at Tyr701 and Ser727 positions that are known to be key amino acid modifications leading to dimerization and influencing activity. Immunofluorescent staining for the phosphorylated Ser727 was highest in lesional psoriatic skin, lower, but clearly detectable in healthy skin, whereas NL skin samples showed the lowest intensity. In two out of four investigated NL samples phosphorylation was undetectable (Figure 14b). Staining for the phosphorylated Tyr701 showed a much less noticeable but similar pattern. In samples of patients where no serine phosphorylation was detected in the NL skin, the staining for the phosphorylated Tyr701 was also not visible in either NL or lesional areas. To elucidate whether differences observed in STAT1 activity among patients correlated with the severity of the disease, PASI scores of donor patients were compared. The patients whose NL skin was negative for phosphorylations of STAT1 had a lower PASI score (12.4 and 17.8), whereas low STAT1 activity in NL skin was associated with a higher PASI score (19.6 and 20).
Immunohistochemistry analysis revealed the presence of pSTAT1 (Ser727) in the both the epidermis and the dermis. STAT1 activity showed a strong correlation with PASI score. High PASI scores (eg. 19.6) were typically coupled with intensive pSTAT1 (Ser727) staining in the lesional skin and in these patients, the NL skin also showed a slight STAT1 activity. In contrast, patients with low PASI score (eg. 9.8) nearly no STAT1 activity was detectable in the NL skin. Indicating that pSTAT1 (Ser727) activation of the NL skin and the PASI scores of patients decreased in parallel. Activation of STAT1 Tyr (701) form decays rapidly and only in samples obtained from patient with high PASI score can be observed, where the presence of both phosphorylated forms is much more intensive (Table 3). We compared NL skin samples near and far from the lesion for the extent of activation for the two different pSTAT1 forms in order to test whether the lesional area has an effect on surrounding NL skin. It was assessed that STAT1 activity is independent from the distance from the lesion indicating a sharp border between the NL and lesional skin (Figure 15). The figure 15. also shows that the strongest STAT1 activation is detected in psoriatic L skin.
Figure 15. STAT1 Ser727 phosphorylated form shows high degree of activation in lesional (L) skin, it is present to a lesser extent in healthy skin, while hardly detectable in non-lesional (NL) skin by immunohistochemistry. NL skin of high PASI patients include active STAT1 in contrast with NL skin of low PASI patients. (n=3)

Table 3. Activation of the pSTAT1 Ser(727) and pSTAT1 Tyr(701) forms in epidermis and dermis of NL and L skin with PASI scores of 3 psoriatic patients. NL: non-lesional, and L: lesional skin, E: epidermis, D: dermis; + (red): high positivity, +/- (yellow): low positivity, - (green): negative
4.8. *In silico* model construction

We constructed an *in silico* model based on our *in vitro* results involving KGF- and FN-signaling and the underlying transcriptional regulation network (Figure 16., Table 1-2.). Our data already suggested the participation of MAPK signaling in KGF induced FN splicing. The generated co-expression matrix implied a potential role of peptidyl-prolyl cis–trans isomerase (PPIG), a protein important in both protein folding and splicing (84), which is regulated by MEK1 induced AP-1. Changes in MEK1 activity could lead to the modulation of FN splicing through PPIG resulting in elevated EDA⁺FN levels. Our model also indicated that STAT3 negatively regulates the expression of MEK1. This suggested inhibitory effect of STAT3 on MEK1 may account for the increased EDA⁺FN production upon STAT inhibition. However, our model did not explain our data on the significant increase in EDA⁺FN and FN following STAT1 inhibition in normal fibroblasts. The schematic summary model of the KGF induced changes on FN based on our results and public databases (*in vivo, in vitro* and *in silico*) of normal skin is presented in *Figure 17.*
Figure 16. *In silico* model of KGF and FN signaling. Green arrow: positive transcriptional regulation; Red arrow with circle arrowhead: negative transcriptional regulation; Arrows with dashed lines: undetermined direction of regulation.
5. DISCUSSION

Despite the massive amount of research on psoriasis the underlying cellular and molecular mechanisms are only partially understood. One of the important characteristics of psoriasis is that the seemingly healthy looking skin of patients already carries certain molecular and structural abnormalities, which are likely to serve as a microenvironment favouring the manifestation of the disease. Previous studies have mainly focused on the characterization of the lesional skin and on differences between NL and lesional skin. However, the examination and comparison of healthy and NL skin in order to gain more knowledge regarding the abnormal processes and properties of NL skin received much less attention. Therefore, we focused our research to study irregularities that are already present in the NL skin.

Psoriatic lesions are characterized by hyperproliferation of keratinocytes, where KGF is known to play an important role. At the protein level both KGF and its receptor FGFR2 were demonstrated to be elevated in lesional skin compared to normal skin (85). An increased expression of both KGF and FGFR2 mRNA were also described by others in NL as well as in lesional tissue (85, 86). KGF stimulates keratinocyte proliferation and it influences terminal differentiation (40, 87). The only known source of KGF in the skin are fibroblasts, however keratinocytes can mediate KGF production in fibroblasts through IL-1 (88, 89), establishing a double paracrine regulatory loop, controlled by the AP-1 subunits c-Jun and JunB and directing the regeneration of the epidermis and maintaining tissue homeostasis in the skin (90). It was reported that microRNA-125 (miR-125) is downregulated in psoriatic lesional tissue (91). miR-125 is known to have a negative influence on keratinocyte proliferation, partially by downregulating FGFR2 expression and driving keratinocytes towards differentiation, which also includes a change in the production of different ECM components (91).

Alterations of the ECM are also part of the pathogenesis of psoriasis and increasing body of evidence indicates that the ECM is equally important under both normal and pathological conditions. By interacting with cell surface receptors (i.e. integrins), the different ECM components provide mechanical and biochemical signals to cells, influencing cellular behavior, affecting cell growth, differentiation, survival and ECM remodelling. ECM defects have been observed in the NL skin including disruption of the laminin layer leading to impaired keratinocyte adhesion to the basement membrane associated with aberrant expression of α5β1 integrin and EDA⁺FN on keratinocytes (18, 92-94). Overexpression of α5 integrin and EDA⁺FN
has already been reported in connection with psoriasis and our group demonstrated that one of the potential sources of EDA\(^+\)FN in the psoriatic NL skin are activated keratinocytes (32). Another report on psoriatic NL skin has shown that EDA\(^+\)FN was localized mainly at the epidermal-dermal junction (20). It is well established that \(\alpha5\) integrin and EDA\(^+\)FN could play an important regulatory role in the abnormal epidermal homeostasis of psoriatic skin (18, 32), and more recent evidence suggests that EDA\(^+\)FN could be crucial in activating immune cells (30, 95). The EDA motif encompasses two non-classical binding sites for \(\alpha4\beta1\) and \(\alpha9\beta1\) integrins (21), receptors present on the surface of T-cells (96, 97) that could enhance T cell migration and accumulation in EDA\(^+\)FN containing tissues, such as the skin. Moreover, both resting and activated Th1 cells but not Th2 cells express EDA\(^+\)FN. Interestingly, activated Th1 cells express lower amount of FN, but with a much higher EDA\(^+\)FN/FN ratio then resting ones (95). EDA\(^+\)FN induces proinflammatory cytokines encoding genes and matrix metalloproteinases in T cells similarly to lipopolysaccharides (30). The EDA\(^+\)FN mediated promotion of inflammatory responses takes place through TLR4 stimulation and leads to the increased expression of the tumour necrosis factor (TNF)-\(\alpha\), IL-1\(\beta\), interleukin-6 (IL-6) and interleukin-12 (IL-12), interleukin-23 (IL-23) and interferon-\(\alpha\) (IFN-\(\alpha\)), contributing to Th1 and Th17 cell mediated cutaneous inflammation (98, 99). These cytokines enhance the expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial expression of E-selectin as well as further recruitment and invasion of T cells into the tissue (100). The newly recruited Th1 cells produce more EDA\(^+\)FN thereby closing the vicious cycle.

EDA\(^+\)FN was also suggested to promote acute inflammation and brain injury following cerebral ischemia in mice (82). EDA\(^+\)FN is normally absent in human plasma, however patients with chronic inflammation and ischemic stroke have high plasma EDA\(^+\)FN. The abnormally regulated overproduction of EDA\(^+\)FN in psoriatic fibroblasts may be responsible not only for maintaining a local chronic inflammation in the skin, but by influencing plasma levels that could play a role in the systemic inflammation that has been described recently in psoriasis (101). The EDA\(^+\)FN rich extracellular milieu in psoriatic skin could be crucial for maintaining the characteristic epidermal changes, as well as the chronic inflammation in psoriatic lesions (for review see (76, 102)). It has been reported that KGF stimulates EDA inclusion in a mouse mammary epithelial cell line (SCp2) (103). Although, the exact regulation of EDA\(^+\)FN splicing
is not completely clear, it is known that TGF-β enhances EDA\(^+\)FN production (104). Our \emph{in silico} model on KGF and FN signaling corroborate our experimental results in suggesting the participation of MEK1 in KGF induced fibronectin splicing through peptidyl-prolyl cis–trans isomerase (PPIG), a protein known to be important in protein folding and splicing (84) and regulated by MEK1 induced AP-1.

Autocrine feedback of several different FGFs through FGFRs exists under both normal and pathological conditions (38, 39, 105). KGF exerts its effect mainly through FGFR2-IIIb, but also via FGFR2-IIIc and FGFR1 (37). Since both FGFR2 and FGFR1 can initiate MEK1 signalling, KGF could potentially modulate fibronectin splicing in a similar autocrine manner as other FGFs, most likely either through FGFR2-IIIc or FGFR1 (39, 106) in fibroblasts. However, the participation of other receptors cannot be excluded either and further research is needed to understand the exact mechanism through which KGF affects FN splicing. Direct blocking of EDA\(^+\)FN seems less feasible as a therapeutic approach, as blocking KGF or its receptors (107).

STAT1 is the founding member of the STAT family and it is activated by interferons (IFNs), and signaling from IL-27, IL-35 and TLRs (108). There is ample evidence implicating IFN\(\gamma\) in the pathomechanism of psoriasis. Aberrant signaling and transcription have been reported for psoriatic keratinocytes with regard to suppressor of cytokine signaling 1 (SOCS1), a negative regulator of IFN\(\gamma\) signaling (109), by supressing STAT1 and IRF-1 (110). The activation of STAT1 by IFN\(\gamma\) induces SOCS3 expression and inhibits STAT3 signaling thereby suppressing Th17 development. On the other hand IL-6 and IL-23 stimuli leads to high SOCS1 expression that inhibits the IFN\(\gamma\)-mediated STAT1 activation and blocks the Th1 direction of T-cell differentiation (111). In regulatory T (Treg) cells the absence of SOCS1 results in STAT1 hyperactivation and an increased secretion of IFN\(\gamma\) and IL-17 (112). STAT1 mediates an antiviral and tumor-suppressor response, whereas STAT3 is known to be a protooncogene (113). STATs can form homo- or heterodimers that can activate different signaling pathways and regulate the expression of different sets of genes. Wan el at. have demonstrated that STAT1 and STAT3 play opposing roles in regulating IFN\(\gamma\) expression in CD4\(^+\) T cells \emph{in vivo} during chronic lymphotic choriomeningitis infection. Similarly, the IL-21 mediated IFN\(\gamma\) and T-box 21 (TBX21) are reciprocally regulated by STAT1 and STAT3. IL-21 mediated STAT1 phosphorylation was increased in CD4\(^+\) cells from patients with STAT1 gain of function
mutations and in patients with autosomal dominant hyper-IgE syndrome. CD8$^+$ T cell cytotoxicity and apoptosis of mantle cell lymphoma are induced by STAT1 following IL-21 stimulation (114, 115). The IL-6-mediated activation of STAT1 is increased in STAT3 deficient mouse embryonic fibroblasts and the IFN$\gamma$-mediated STAT3 activation is enhanced in STAT1 deficient mice highlighting the significance of the balance between STAT1 and STAT3 (116, 117). The processes described above suggest that the STATs are important participants in the maintenance of normal host defence reactions and mechanisms.

The complex role of STAT1 in immune regulation is apparent from animal studies showing that both STAT1$^{-/-}$ and IFN$\gamma$-$^{-/-}$ mice are highly susceptible to experimental autoimmune encephalomyelitis (EAE) (118, 119). It has been demonstrated that in both multiple sclerosis (MS) and in the EAE model $\alpha 5$ integrin and FN could be responsible for the extensive vascular remodelling, characteristic for MS, that takes place during the presymptomatic phase in EAE (120). The regulatory connection between STAT1 and FN described in this paper may explain the more severe disease manifestation in STAT1$^{-/-}$ animals. STAT1 has diverse functions, it inhibits a wide range of genes and it is a functional transcription factor even in the absence of inducer-mediated activation. Among its major targets of negative regulation are genes encoding proteins involved in regulating the extracellular matrix: matrix metalloproteinases (MMPs), perlecan, bullosus phemphigoid antigen-1 (BPAG1)(121). Abnormalities in STAT1 level or activity has been linked to several pathological conditions i. e. STAT1 is activated in SLE and considered to be a major contributor to SLE-associated inflammation (122).

As our results indicate, in normal homeostatic skin tissue a steady state of STAT1 activation is present and this could be partially responsible for downregulating FN and EDA$^+$FN production, since blocking STAT1 in normal fibroblasts results in enhanced production of FN and EDA$^+$FN. We detected the absent suppressive function of STAT1 in psoriatic fibroblasts that could be crucial in the pathomechanism of psoriasis. Using a STAT1 decoy oligodeoxynucleotide (dODN)-containing ointment Wagner et al. were able to inhibit hapten-induced contact hypersensitivity reaction in animal models, indicating that topical STAT1 blockade could be a novel anti-inflammatory therapy in skin inflammation (123). Our data indicate that in psoriasis STAT1 inactivation characterizes the uninvolved skin, thus this type of therapy may well work as a symptomatic therapy for the lesional skin. These results may
help to design new specific therapeutic agents for the treatment of psoriasis that may be more effective and longer lasting in reducing symptoms of the disease, thereby improve the life conditions of psoriatic patients. STAT mutational analysis may have a place in future disease management. The application of STAT inhibitors in inflammatory diseases may have a great potential for future therapies.

Figure 17. Schematic model of investigated molecules playing a role in KGF mediated EDA⁺FN production in healthy skin.
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SUMMARY

In psoriasis the NL skin already contains abnormalities that are likely to serve as a microenvironment favouring the manifestation of the disease. FN and its splice variant the EDA\(^+\)FN are essential extracellular matrix proteins influencing major cellular processes and they are abnormally expressed in psoriatic skin. KGF is overexpressed in psoriatic lesional skin and contribute to keratinocyte hyperproliferation. Given their function, these molecules are likely to play a part in the pathomechanism of psoriasis, therefore, in this study we focused on investigating the production of KGF, FGFR2, FN and EDA\(^+\)FN in healthy and NL psoriatic skin and studied the regulatory mechanisms involving KGF, FN and EDA\(^+\)FN in fibroblasts.

We observed that the psoriatic NL skin displays an overexpression of KGF, FGFR2, \(\alpha_5\) integrin and EDA\(^+\)FN compared to healthy skin. KGF mildly induced only EDA\(^+\)FN, but not FN expression in healthy fibroblasts. Our results revealed that KGF regulated EDA\(^+\)FN production takes place in an autocrine manner through MAPK signaling. Based on these results we designed an *in silico* model to predict key players in a putative regulatory network explaining the effect of KGF on EDA\(^+\)FN production. We provide *in vitro* evidence that STAT1 negatively regulates both FN and EDA\(^+\)FN expressions in healthy fibroblasts and this regulation is compromised in fibroblasts derived from NL psoriatic dermis. We detected active STAT1 in healthy and lesional skin, as reported previously, however, in the NL skin STAT1 activation was close to absent in samples irrespectively from the distance from lesions.

These result led us to the conclusion that the production of FN, EDA\(^+\)FN by fibroblasts and the signaling of STAT1 is abnormally regulated in the psoriatic NL skin, suggesting a crucial mechanism for keeping NL skin in a preactivated state for developing a chronic wound healing phenotype.
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Abnormal regulation of fibronectin production by fibroblasts in psoriasis

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Linked Comment: Pongpairoj and McFadden, Br J Dermatol 2016; 174: 486

Summary

Background Data indicate that in psoriasis, abnormalities are already present in nonlesional skin. Transforming growth factor-β and keratinocyte growth factor (KGF), together with fibronectin and α5β1 integrin, were suggested to play a crucial role in the pathogenesis of psoriasis by influencing inflammation and keratinocyte hyperproliferation.

Objectives To investigate the expression of KGF, fibroblast growth factor receptor (FGFR)2, fibronectin (FN) and extra domain A (EDA)-positive FN in healthy and nonlesional psoriatic skin, and to study the effect of KGF on the regulation of FN and EDA+FN production by fibroblasts.

Methods Healthy, nonlesional psoriatic skin and lesional psoriatic skin were immunostained for α5 integrin, KGF, FGFR2, EDA+FN and signal transducer and activator of transcription (STAT)1. KGF-treated cell cultures were analysed for FN and EDA+FN mRNA and protein by real-time reverse-transcriptase polymerase chain reaction and flow cytometry, respectively. The major downstream signalling of KGF was investigated by blocking experiments using inhibitors of mitogen-activated protein kinase (MAPK) kinase (MEK1), AKT1/2, STAT1 and STAT3.

Results The expression of α5 integrin, EDA+FN, KGF and its receptor FGFR2 is elevated in psoriatic nonlesional skin compared with healthy skin. KGF mildly induced EDA+FN, but not FN expression in healthy fibroblasts through MAPK signalling. Fibroblasts express the FGFR2-IIIc splice variant. STAT1 negatively regulates both FN and EDA+FN expression in healthy fibroblasts, and this regulation is compromised in fibroblasts derived from nonlesional psoriatic dermis. We detected active STAT1 in healthy and lesional skin, similarly to a previous report. However, in the nonlesional skin STAT1 activation was absent in tissues far away from lesions.

Conclusions The production of FN and EDA+FN by fibroblasts and the signalling of STAT1 are abnormally regulated in psoriatic nonlesional skin.

What’s already known about this topic?

- In psoriasis, nonlesional skin already contains abnormalities as a base for the manifestation of the disease.
- Fibronectin (FN) and its splice variant EDA+FN are essential extracellular matrix proteins influencing major cellular processes, and they are abnormally expressed in psoriatic skin.
- Keratinocyte growth factor (KGF) is overexpressed in psoriatic lesional skin and contributes to keratinocyte hyperproliferation.
It is well known that psoriasis is an inflammatory skin disease in which both innate and adaptive immune activation play an essential role in the maintenance of the abnormal skin phenotype.\(^1\) However, it is less well known how much resident cells of the tissue contribute to the pathology. A reasonable approach to seek alterations in tissue responses in psoriasis is to investigate the phenotypically normal-looking nonlesional psoriatic skin.

The main fibronectin (FN) receptor \(\alpha_\beta_3\) integrin was found to be overexpressed in nonlesional psoriatic epidermis compared with normal skin.\(^2,3\) There is evidence that proper regulation of integrin expression in keratinocytes is essential for normal epidermal homeostasis, as shown by the suprabasal overexpression of integrins in transgenic mice resulting in epidermal hyperproliferation and inflammatory reaction.\(^4\) In the steady state of normal human adult epidermis, \(\alpha_5\) integrin expression of keratinocytes is downregulated. A possible explanation for \(\alpha_5\) integrin overexpression in nonlesional skin is the presence of FN and one of its isoforms, extra domain A-containing fibronectin (EDA\(^+\)FN), in the microenvironment of basal keratinocytes.\(^3,5\)

Keratinocyte growth factor (KGF/FGF7) is a member of the fibroblast growth factor (FGF) family produced by mesenchymal cells and considered to be a major growth factor for keratinocytes. Several reports suggest that KGF reduces the ability of keratinocytes to initiate terminal differentiation and undergo programmed cell death. Overexpression of KGF in the basal epidermal cell layer of transgenic mice results in epidermal hyperplasia.\(^6\) There is also evidence suggesting that fibroblasts from lesional and nonlesional skin of patients with psoriasis induce keratinocyte outgrowth by producing soluble signals.\(^7\) More recently, it has been reported that KGF can induce the expression of \(\alpha_5\) integrin, and it delays the expression of keratin 10 and transglutaminase in keratinocytes.\(^8\) Indirect evidence, such as measurement of mitogenic activity, suggests that the specific receptor for KGF on epithelial cells is the IIb splice variant of fibroblast growth factor receptor (FGFR2).\(^9\)

According to our observations, psoriatic nonlesional skin displays a marked overexpression of KGF, FGFR2, \(\alpha_5\) integrin and EDA\(^+\)FN compared with healthy skin. Therefore, in this study we focused on investigating regulatory mechanisms involving KGF and FN. Our results revealed that KGF regulates EDA\(^+\)FN production in an autocrine manner through mitogen-activated protein kinase (MAPK) signalling in healthy fibroblasts. With these results we designed an in silico model to predict a possible regulatory network for the effect of KGF on EDA\(^+\)FN production. We provide in vitro evidence that both FN and EDA\(^+\)FN are negatively regulated by activated signal transducer and activator of transcription (STAT)1 in healthy fibroblasts, and this is in contrast to psoriatic nonlesional skin-derived fibroblasts. Active, phosphorylated STAT1 was found in healthy skin as well as in psoriatic lesions, but not in psoriatic nonlesional skin, suggesting a crucial mechanism for keeping nonlesional skin in a quiescent state to developing a chronic wound-healing phenotype.

### What does this study add?

- KGF and its receptor FGFR2 are overexpressed in psoriatic nonlesional skin. KGF influences EDA\(^+\)FN production in fibroblasts through MEK1 signalling.
- Fibroblasts express the FGFR2-IIIc splice variant of the FGFR2 receptor.
- STAT1 negatively regulates FN and EDA\(^+\)FN production in normal cultured fibroblasts, but not in fibroblasts derived from psoriatic nonlesional skin.
- STAT1 is active in healthy skin and psoriatic lesional skin, but not in nonlesional psoriatic skin.

### Materials and methods

#### Skin biopsies

Patients with moderate-to-severe chronic plaque-type psoriasis and healthy volunteers (age 18–60 years) were enrolled into the study. Patients had a medication-free period of at least 4 weeks without systemic therapy and/or at least 2 weeks without local therapy. Participant-informed consent was obtained prior to surgical intervention. The Psoriasis Area and Severity Index (PASI) score was determined for all patients by dermatologists. Samples were collected from 16 patients and 25 healthy volunteers for the experiments. Mechanical stress was induced by a tape-stripping procedure, then punch biopsies were taken from uninvolved skin from psoriatic lesions, but not in psoriatic nonlesional skin, suggesting a crucial mechanism for keeping nonlesional skin in a quiescent state to developing a chronic wound-healing phenotype.

### Immunofluorescence staining

Human punch biopsies were frozen embedded, stored at –80 °C, then cut into 6-μm sections. Samples were fixed in Fixation/Permeabilization Concentrate and Diluent (eBioscience, San Diego, CA, U.S.A.) and resuspended in phosphate-buffered saline (PBS). The sections were incubated for 30 min in goat serum (Sigma-Aldrich, St Louis, MO, U.S.A.). The following primary antibodies were used, overnight at 4 °C: FGFR2 (1:100, Bek (C-17); Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), KGF (1:500, FGF-7 (N-14); Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), \(\alpha_5\) integrin (1:200, Becton-Dickinson, Franklin Lakes, NJ, U.S.A.), EDA\(^+\)FN (1:500, clone IST9; Abcam, Cambridge, U.K.) and p-STAT1 (Ser727) and p-STAT1 (Tyr701) (both 1:400; Cell Signaling Technology, Beverly, MA, U.S.A.). Goat antirabbit IgG-
Alexa Fluor 488, donkey antigoat IgG-Alexa Fluor 546, goat antimouse IgG-Alexa Fluor 647 and Alexa Fluor 546 were used as secondary antibodies (all 1 : 500; Life Technologies, Carlsbad, CA, U.S.A.) for 2 h at room temperature. Sections were incubated without the primary antibody or with isotype control antibody for negative staining controls. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich).

Cultured human fibroblasts, keratinocytes and HaCaT cells were collected after brief trypsinization (0.25% trypsin solution; Sigma-Aldrich) and centrifuged onto a slide using a cytocentrifuge (Cytopro™; Wescor, Logan, UT, U.S.A.), then fixed in 2% paraformaldehyde (Sigma-Aldrich). Stainings were carried out as described above. Images were documented with a Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss Microscopy GmbH, Munich, Germany) equipped with an AxioCam MRm camera. Image processing and data analysis were conducted using ZEN 2012 Microscope and Imaging (Carl Zeiss) or ImageJ software (http://imagej.nih.gov/ij/).

Flow cytometry

Cells were harvested as described above, fixed in Fixation/Permeabilization Concentrate and Diluent (eBioscience) and resuspended in PBS. Primary antibodies anti-EDA+FN (1 : 500, clone IST9; Abcam) and anti-FN (1 : 1000, clone IST4; Sigma-Aldrich) were applied for 45 min. Mouse IgG1 isotype antibody (Sigma-Aldrich) was used as the negative control. Cells were then washed in PBS and incubated with secondary antibodies as described above. Samples were analysed on a FACSCalibur flow cytometer equipped with 488-nm and 633-nm lasers (Becton-Dickinson).

Statistical analysis

For statistical analysis, repeated-measures Friedman ANOVA, Wilcoxon test or one-way ANOVA were used. Multiple comparisons were carried out with SigmaPlot software (Systat Software Inc., Chicago, IL, U.S.A.). A probability test was used to test for normality; \( P \leq 0.05 \) was considered significant.

Further methods

Detailed information of the materials and methods regarding cell culture experiments, real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and the in silico model are presented in Appendix S1 (see Supporting Information).

Results

Altered protein expression of \( \alpha_5 \) integrin, extra domain A-positive fibronectin, keratinocyte growth factor and fibroblast growth factor receptor 2 in psoriatic nonlesional skin

Healthy skin and psoriatic nonlesional skin were investigated for differences in the expression of \( \alpha_5 \) integrin, EDA+FN, KGF and FGFR2 before and 24 and 48 h after tape stripping. Immunofluorescence staining revealed a striking difference in all protein expressions examined, even without tape stripping, between normal and psoriatic nonlesional skin (Fig. 1). Mild mechanical stress introduced by tape stripping of the skin did not lead to any obvious changes in \( \alpha_5 \) integrin, EDA+FN, KGF or FGFR2 expression or distribution at 24 and 48 h after treatment in nonlesional skin. In healthy control sections a slight increase in all protein expression was observed based on immunostaining (Fig. S1a–d).

![Fig 1. Increased expression of \( \alpha_5 \) integrin, extra domain A-containing fibronectin (EDA+FN), keratinocyte growth factor (KGF) and fibroblast growth factor receptor (FGFR2) in nonlesional skin without mechanical stress stimuli. Immunofluorescence analysis of \( \alpha_5 \) integrin, EDA+FN, KGF and FGFR2 in healthy (left column) and nonlesional skin (right column) (n = 6). DAPI, 4',6-diamidino-2-phenylindole. Bar: 50 \( \mu \)m.](image-url)
Keratinocyte growth factor treatment of healthy human fibroblasts leads to the elevation of extra domain A-positive fibronectin production

EDA^+FN and KGF are known to stimulate keratinocyte proliferation. KGF induces the expression of the major FN cell surface receptor α5β1 integrin. Given the differences in EDA^+FN and KGF protein levels in healthy and nonlesional psoriatic skin, we set out to investigate a putative regulatory connection between these two molecules. Exogenous treatment of cultured healthy fibroblasts with KGF for 24 h increased the level of the EDA^+FN splice variant, but not the total FN protein, based on immunostaining and flow cytometry measurements (Fig. 2a, b). A similar increase in the EDA^+FN mRNA splice variant was detected, while the total FN mRNA remained unchanged (Fig. 2c).

We also investigated this effect of KGF on normal human keratinocytes and melanocytes and on the keratinocyte cell line, HaCaT (Figs S2, S3). As expected, keratinocytes and HaCaT cells expressed FN and EDA^+FN at a very low level compared with fibroblasts, while melanocytes expressed both proteins at a comparable level with fibroblasts (Fig. S3). However, upon in vitro KGF treatment these cells did not display detectable changes in mRNA and protein expression of EDA^+FN and total FN (Fig. S2).

Expression of fibroblast growth factor receptor 2 splice variants in fibroblasts, melanocytes and keratinocytes

Two splice variants have been identified for FGFR2, designated as FGFR2-IIIb and FGFR2-IIIc, which have different ligand-binding preferences. These results were based on mitotic activity measurements. KGF has been shown to act on the FGFR2-IIIb variant, increasing cellular proliferation. We determined the expression of the splice variants on healthy fibroblasts, melanocytes and keratinocytes using specific primers designed for RT-PCR, revealing that melanocytes and keratinocytes expressed FGFR2-IIIb only, while fibroblasts expressed mainly the FGFR2-IIIc variant (Fig. 3).

Mitogen-activated protein kinase signalling is involved in the regulation of extra domain A-positive fibronectin by keratinocyte growth factor receptor in healthy fibroblasts

Fibroblast growth factor signals (including KGF) are coordinated by four major pathways: Ras-Raf-MAPK, phos-
In order to get a better insight into the modulation of which is regulated by MEK1-induced activating protein in silico in vitro 2015 British Association of Dermatologists British Journal of Dermatology (2016) model construction isomerase G Human fibroblasts in culture express mainly fibroblast growth in vitro model based on our results in normal skin (cis–trans, –trans –cis) already suggested the participation of MAPK signalling in KGF-induced FN splicing. The generated coexpression matrix implied a potential role of peptidylprolyl cis–trans isomerase G (PPIG), a protein important in both protein folding and splicing, which is regulated by MEK1-induced activating protein (AP)-1. Changes in MEK1 activity could lead to modulation of FN splicing through PPIG, resulting in elevated EDA FN levels.

In silico model construction

We constructed an in silico model based on our in vitro results involving KGF and FN signalling and the resultant transcriptional regulation network (Fig. 5 and Tables S1, S2). Our data already suggested the participation of MAPK signalling in KGF-induced FN splicing. The generated coexpression matrix implied a potential role of peptidylprolyl cis–trans isomerase G (PPIG), a protein important in both protein folding and splicing, which is regulated by MEK1-induced activating protein (AP)-1. Changes in MEK1 activity could lead to modulation of FN splicing through PPIG, resulting in elevated EDA FN levels.

Our model also indicates that STAT3 negatively regulates the expression of MEK1. This suggested inhibitory effect of STAT3 on MEK1 may account for the increased EDA FN production upon STAT inhibition. However, our model did not explain our data showing a significant increase in EDA FN and FN following STAT1 inhibition in normal fibroblasts. The schematic summary model of KGF-induced changes on FN involving KGF and FN signalling and the resultant transcriptional regulation network (Fig. 5 and Tables S1, S2). Our data already suggested the participation of MAPK signalling in KGF-induced FN splicing. The generated coexpression matrix implied a potential role of peptidylprolyl cis–trans isomerase G (PPIG), a protein important in both protein folding and splicing, which is regulated by MEK1-induced activating protein (AP)-1. Changes in MEK1 activity could lead to modulation of FN splicing through PPIG, resulting in elevated EDA FN levels.

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Abnormal signal transducer and activator of transcription 1 activation in psoriatic skin plays a role in the regulation of both fibronectin and extra domain A-positive fibronectin

To investigate further, we compared the FN and EDA FN expressions between healthy and psoriatic nonlesional skin-derived fibroblasts 24 h after STAT1 or STAT3 inhibition in culture. Changes upon STAT3 impairment were not significant; however, blockade of STAT1 did not lead to the elevation of total FN and EDA FN in fibroblasts from nonlesional skin in contrast to healthy controls (Fig. 4c). Therefore, we investigated the phosphorylation pattern of STAT1 at Tyr701 and Ser727, which are known to be key amino acid modifications leading to dimerization and influencing activity.

Immunofluorescent staining for the phosphorylated Ser727 was highest in lesional psoriatic skin, and lower, but clearly detectable, in healthy skin, whereas nonlesional skin samples showed the lowest intensity. In two of four investigated nonlesional samples phosphorylation was undetectable (Fig. 4d). Staining for the phosphorylated Tyr701 showed a much less noticeable but similar pattern. In samples of patients where no serine phosphorylation was detected in the nonlesional skin, staining for the phosphorylated Tyr701 was also not visible in either nonlesional or lesional areas. To elucidate whether differences observed in STAT1 activity among patients correlated with the severity of the disease, PASI scores of donor patients were compared. The patients whose nonlesional skin was negative for phosphorylations of STAT1 had lower PASI scores (12-4 and 17-8), therefore we were able to take nonlesional samples further away from lesions, at least 10-cm distance. Patients with detectable but low STAT1 activity in nonlesional skin had higher PASI scores (19-6 and 20).

**Discussion**

One of the major characteristics of psoriatic lesional skin is the hyperproliferation of keratinocytes, where KGF is known to play an important role. At the protein level both KGF and FGFR2 were demonstrated to be elevated in lesional skin compared with normal skin. At the mRNA level in both nonlesional and lesional tissue, increased expression of both KGF
and FGFR2 transcripts was also described by others.\textsuperscript{16,17} KGF stimulates keratinocyte proliferation and influences terminal differentiation.\textsuperscript{6,17} The only known source of KGF in the skin is fibroblasts, and keratinocytes can mediate KGF production in fibroblasts through interleukin (IL)-1,\textsuperscript{18,19} establishing a double paracrine regulatory loop, controlled by AP-1 subunits \(c\)-Jun and \(J\)unB, and directing the regeneration of the epidermis and maintaining tissue homeostasis in the skin.\textsuperscript{20}

It was reported that the microRNA miR-125 is downregulated in psoriatic lesional tissue. miR-125 is known to have a negative influence on keratinocyte proliferation, partially by downregulating FGFR2 expression and driving keratinocytes towards differentiation.\textsuperscript{21}

Integrins and their corresponding extracellular ligands also influence cell proliferation and differentiation.\textsuperscript{22} Overexpression of \(\alpha5\) integrin and EDA\(^+\)FN has already been reported in connection with psoriasis, and our group demonstrated that one of the potential sources of EDA\(^+\)FN in nonlesional psoriatic skin is activated keratinocytes.\textsuperscript{23} Another report on nonlesional skin has shown that EDA\(^+\)FN was localized mainly at the dermoepidermal junction.\textsuperscript{5} It is well established that \(\alpha5\) integrin and EDA\(^+\)FN could play an important regulatory role in the abnormal epidermal homeostasis of psoriatic skin,\textsuperscript{3,23} and more recent evidence suggests that EDA\(^+\)FN could be crucial in activating immune cells.\textsuperscript{24,25} The EDA motif encompasses two nonclassical binding sites for \(\alpha4\beta1\) and \(\alpha9\beta1\) integrins,\textsuperscript{26} receptors present on the surface of T cells\textsuperscript{27,28} that could enhance T-cell migration and accumulation in EDA\(^+\)FN-containing tissues, such as the skin.

EDA\(^+\)FN promotes acute inflammation and brain injury following cerebral ischaemia in mice.\textsuperscript{19} EDA\(^+\)FN is normally absent in human plasma; however, patients with chronic inflammation and ischaemic stroke have high plasma EDA\(^+\)FN. The abnormally regulated overproduction of EDA\(^+\)FN in psoriatic fibroblasts may not only be responsible for maintaining a local chronic inflammation in the skin, but may also influence plasma levels that could play a role in the systemic inflammation that has been reported recently in psoriasis.\textsuperscript{30} The EDA\(^+\)FN-rich extracellular milieu in psoriatic skin could be
crucial for maintaining the characteristic epidermal changes, as well as the chronic inflammation in psoriatic lesions.\textsuperscript{31,32}

It has been reported that KGF stimulates EDA inclusion in a mouse mammary epithelial cell line (SCp2).\textsuperscript{33} Although the exact regulation of EDA\textsuperscript{+}FN splicing is not completely clear, it is known that TGF-β enhances EDA\textsuperscript{+}FN production.\textsuperscript{34} Our in silico model of KGF and FN signalling corroborates our experimental results in suggesting the participation of MEK1 in KGF-induced FN splicing through PPIG, a protein known to be important in protein folding and splicing\textsuperscript{14} and regulated by MEK1-induced AP-1.

Autocrine feedback of several different FGFs through FGFRs exists under both normal and pathological conditions.\textsuperscript{10,13,35} KGF exerts its effect mainly through FGFR2-IIIb, but also via FGFR2-IIIc and FGFR1.\textsuperscript{11} As both FGFR2 and FGFR1 can initiate MEK1 signalling, KGF could potentially modulate FN splicing in a similar autocrine manner to other FGFs, most likely either through FGFR2-IIIc or FGFR1\textsuperscript{10,36} in fibroblasts. However, the participation of other unknown receptors also cannot be excluded, and further research is needed to understand the exact mechanism through which KGF affects FN splicing. Directly blocking EDA\textsuperscript{+}FN seems less feasible as a therapeutic approach compared with blocking KGF or its receptors.\textsuperscript{37}

STAT1 is the founding member of the STAT family and is activated by interferons (IFNs), and signalling from IL-27, IL-35 and Toll-like receptors.\textsuperscript{38} There is ample evidence
implicating IFN-γ in the pathomechanism of psoriasis.\textsuperscript{39} Aberrant signalling and transcription have been reported for psoriatic keratinocytes with regard to SOCS1 (suppressor of cytokine signalling 1), a negative regulator of IFN-γ signalling,\textsuperscript{40} and also for STAT1 and interferon-regulated factor 1.\textsuperscript{41} The complex role of STAT1 in immune regulation is apparent from animal studies showing that both STAT1\textsuperscript{−/−} and IFNγR\textsuperscript{−/−} mice are highly susceptible to experimental autoimmune encephalomyelitis (EAE).\textsuperscript{42,43} It has been demonstrated in both multiple sclerosis and in the EAE model that α5 integrin and FN could be responsible for the extensive vascular remodelling, characteristic for MS, that takes place during the presymptomatic phase in EAE.\textsuperscript{44}

The regulatory connection between STAT1 and FN described in this article may explain the more severe disease manifestation in STAT\textsuperscript{−/−} animals. STAT1 has diverse functions: it inhibits certain genes and is a functional transcription factor even in the absence of inducer-mediated activation. Among its major targets of negative regulation are genes encoding proteins involved in regulating the extracellular matrix: matrix metalloproteinases, perlecain and bullous pemphigoid antigen-1.\textsuperscript{45} As our results indicate, in normal homeostatic skin tissue, a steady state of STAT1 activation is present, and this could be partially responsible for downregulating FN and EDA\textsuperscript{+}FN production, as blocking STAT1 in normal fibroblasts results in enhanced production of FN and EDA\textsuperscript{+}FN. The absent suppressive function of STAT1 in psoriatic fibroblasts could be crucial in the pathomechanism of psoriasis. Using a STAT1 decoy oligodeoxynucleotide-containing ointment Wagner et al. were able to inhibit hapten-induced contact hypersensitivity reaction in animal models, indicating that topical STAT1 blockade could be a novel anti-inflammatory therapy in skin inflammation.\textsuperscript{46} Our data indicate that in psoriasis STAT1, inactivation characterizes the uninvolved skin; thus, this type of therapy may well work as a symptomatic therapy for lesional skin.

**Acknowledgments**

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**References**

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Appendix S1. Supplementary materials and methods.

Fig S1. Mild mechanical stress induced by tape stripping has no effect on the expression of α5 integrin, extra domain A-containing fibronectin, keratinocyte growth factor or fibroblast growth factor receptor 2 in nonlesional psoriatic skin and only a mild effect on healthy skin.

Fig S2. Exogenous keratinocyte growth factor treatment has no effect on extra domain A-containing fibronectin expression of keratinocytes and HaCaT cells.

Fig S3. Fibroblasts and melanocytes show higher expression of extra domain A-containing fibronectin and fibronectin than keratinocytes.

Fig S4. Increase of extra domain A-containing fibronectin and fibronectin triggered by exogenous keratinocyte growth factor treatment is not influenced by Akt1/2 inhibition.

Table S1. List of genes applied for the construction of the coexpression matrix gene model.

Table S2. Coexpression matrix of genes in our model.
Abnormal STAT1 activation in psoriasis

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ÖSSZEFoglalás

A STAT (signal transducer and activator of transcription) jelátviteli útvonalak kulcsfontosságú szerepet tölelnek be a pikkelysömör patomechanizmásságában. Kutatácsorunk megállapította, hogy a STAT1 aktivált állapotban van egészséges és tünetes bőrben, ellentétben a tünetmentes bőrben a csökkent mértékű STAT1 aktiváció független a lézióktól való távolságtól. Az aktivált STAT1 korrelációt mutat a PASI (psoriasis area and severity index) értékével, minél magasabb a beteg PASI-ja, annál erősebb az aktivált STAT1 kifejezódése a bőrben. Korábbi vizsgálataink azt mutatták, hogy a STAT1 abnormálisan regulálták a pikkelysömör fibroblasztokban. Ezek az eredmények felvetik annak lehetőségét, hogy a STAT1 új terápiás célpont lehet a betegségben.

Kulcsszavak:
psoriasis - STAT1 - non-lesional skin

Summary

The STAT (signal transducer and activator of transcription) signalling pathway plays a key role in the pathogenesis of psoriasis. Our results indicate that STAT1 is active in both healthy and in psoriatic lesional skin and in contrast to the non-lesional skin where it is mainly inactive. The distance from the lesion does not affect STAT1 activity in the non-lesional skin. The STAT1 activation correlates with the PASI (psoriasis area and severity index) of psoriatic patients. In our previous work we have shown that STAT1 is abnormally regulated in psoriatic fibroblasts. These data indicate that STAT1 could be a potential new target for the therapy of the disease.

Key words:
psoriasis - STAT1- non-lesional skin
lyek a JAK1, JAK2 vagy Tyk2 dimerizációját és autofoszforilálója okozzák (10, 12). A JAK-ok kialakult specifikus kötőhelyeihhez kapcsolódó STAT1 molekulák foszforilálódnak, ezáltal aktiválódnak, és homo- vagy heterodimer formában a sejtmagba transzlokálódnak, ahol növelik számos, az immunfolyamatok szabályozásában részt vevő gén transzkripcióját, így több, mint 65 gén expressziója fokozódik, amely kötőhelybe szerelődő gének 5%-át teszi ki (2, 13, 14). A STAT1 aktivációjához mind a Tyr(701), mind a Ser(727) aminosav foszforilációja szükséges. A Tyr701 foszforilálási engedélyt a STAT1 dimerizációjához, míg a Ser(727) a DNS-kötő affinitásért és a sejtmagba történő transzlokálizációért, így módos az aktivitás kialakulásáért felelős (15, 16).

Korábban Halli és munkatársai leírták az aktivált STAT1 kifejezését pikkelysömörös tünetes bőrben (17). Kutatócsoportunk megállapította, hogy a STAT1 aktív állapotban van jelen az egész és a pikkelysömörös tünetes bőrben egyaránt, ellentétben a tünetmentes bőrrel, ahol jórészt inaktív formában található (18). Jelen vizsgálatok a pikkelysömörös bőr STAT1 aktivitásának részletesebb vizsgálatát célozták meg.

Anyagok és módszerek

Punch biopsziák

Három középsúlyos-súlyos plakk típusú pikkelysömörös beteget és három egészséges donort vontunk be a vizsgálatba a 18-60 éves korosztályból. A 6 mm-es punch biopsziák vétele kezelés nélküli időszakban történt, legalább 4 héttel a szisztémás vagy lokális kezelést követően. A PASI (psoriasis area and severity index) értékét minden betegeknél bőrgyógyász határozta meg. A tünetmentes területekről közvetlenül a lézió mellől (tünetmentes közeli) és a plakkotól 10 cm-re (tünetmentes távoli) vettünk mintákat. A szöveti minták gyűjtése eleget tett a Helsinki deklarációnak és a Népegészségügyi Szakigazgatási Szerv szabályainak (2799, 3517).

Immunhisztokémiai festés

A punch biopszitókat paraffinba ágyaztuk és szobahőmérsékleten tároltuk, majd 5 μm vastagságú metszeteket készítettünk. A metszeteket deparaffináltuk és leszálló alkoholsort alkalmazva rehidrátáltuk a mintákat, ezt követően pH 6-os citrát pufferrel tártuk fel 95 °C-on 10 percig. A metszeteket 20 percig 0,1% H2O2-ot és 0,6% NaN3-ot (Sigma-Aldrich) tartalmazó PBS-sel, majd 30 percig kecske szérumot tartalmazó pufferrel (Invitrogen/Thermo Fisher Scientific) blokkoltuk. Ezt követően pSTAT1 Ser(727) és pSTAT1 Tyr(701), (mindkettő 1:400 hígításban, Cell Signaling Technology) elsődleges ellenanyagokkal egész éjszakán át 4°C-on inkubáltuk. A metszeteket PBS-ben mosott, majd biotinilált kecske anti-nyúl ellenanyaggal inkubáltuk 20 percig. Negativ festési kontrollkont lelődleges ellenanyag nélküli, csupán PBS-sel inkubált mintát használtunk. Az előhívást 3,3’-diaminobenzidin-nél (DAB) végeztük. A sejtmag és a citoplazma festése hematoxilin-eozinnal (Sigma-Aldrich) történt.

Eredmények

A paraffinos mintákon immunhisztokémiai festéssel vizsgált pSTAT1 Ser(727) formája nagyfokú aktivációt mutat tünetes bőrben, egészséges bőrben kisebb mértékben van jelen, míg tünetmentes bőrben szinte alig detektálható. A magas PASI-jú betegek tünetmentes bőrre már tartalmaz aktívat STAT1-et, ellentétben az alacsony PASI-jú tünetmentes bőrrel (n=3).

1. ábra

Immunhisztokémiai festéssel vizsgálva a pSTAT1 Ser(727) formája nagyfokú aktivációt mutat tünetes bőrben, egészséges bőrben kisebb mértékben van jelen, míg tünetmentes bőrben szinte alig detektálható. A magas PASI-jú betegek tünetmentes bőrre már tartalmaz aktívat STAT1-et, ellentétben az alacsony PASI-jú tünetmentes bőrrel (n=3).
mindkét foszforilációs forma aktivációja jóval intenzívebb (1. táblázat). Összehasonlítva a tünetmentes lézióhoz közelezi és attól távoli területekről vett punch biopszik a pSTAT1 formáinak kifejezódését megfigyelhető, hogy a STAT1 aktivitása független a lézióktól való távolságtól (n=3) (1. ábra). Az ábrán az is látható, hogy a STAT1 aktivitása független a lézióktól való távolságtól.

1. táblázat
A táblázat a pSTAT1 Ser(727) és a pSTAT1 Tyr(701) formák aktivációját mutatja tünetmentes és tünetes bőr epitdermiszében és dermiszében, hozzárendelve a 3 vizsgált pikkelysömörös beteg PASI értékeit. TM: tünetmentes, T: tünetes; E: epidermisz, D: dermisz; +/-: erős pozitivitás, -/-: gyenge pozitivitás, -: negatív

<table>
<thead>
<tr>
<th>BETEG</th>
<th>PASI</th>
<th>TM (távoli)</th>
<th>TM (közel)</th>
<th>T</th>
<th>TM (távoli)</th>
<th>TM (közel)</th>
<th>T</th>
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<tr>
<td>1</td>
<td>19,6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
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<td>+/-</td>
<td>+/-</td>
<td>+/+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>9,8</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

A JAK-STAT jelátviteli útvonal proinflammatorikus folyamatainak gátlása kulcsfontosságú lehet az immun-mediatált bőrbetegségek patogenezisében. Hármas fázisú klinikai vizsgálatok folytán kis molekulájú JAK gátlók (tofacitinib, ruxolitinib) potenciális terápiaként való hatékony és biztonságos alkalmazhatóságának megítélésére a pikkelysömör kezelésében. Az I. és II. fázis tanulmányok eredményei döüsszegű javulást mutattak a pikkelysömörös léziók súlyosságának mértékében a placebo csoportban viszonyítva. Számos STAT inhibitorről beszámoltak az irodalomban (24), azonban STAT1 gátlót még csak alatmodellekben használtak. Wagner és munkatársai bapta-indukálta kontakt hiperszenzitivitást gátoltak STAT1 decoy oligodeoxynukleotidot (dODN) tartalmazó krémban.

Eredményeink arra engednek következtetni, hogy a STAT1 molekula kulcsfontosságú lehet a pikkelysömörre való hajlam kialakításában. A pathomechanizmusban bemutatott szerepének pontos felderítése további vizsgálatokat kíván.

A munka elvégzéséhez az OTKA K83277, K105985, K111885, NK105369, PD116992, TAMOP-4.2.4-A/2-11-1/2012-0001 és A2-SZGYA-FOK-13-0001 pályázatok nyújtották segítséget.

IRODALOM


Hazai Hírek

Az MDT Kontakt Dermatitis Munkacsoport 2015. évi ülését a Magyar Dermatológiai Társulat Nagygyűlésén november 28-án tartotta meg.


A szekció ülést követően a Munkacsoport az elmúlt év multicentrikus vizsgálatát beszélt meg, melynek során a hazai épikután sorba bevezetett methilzotiazolin (MI) tesztelés eredményeit értékelte.

A Munkacsoport felhívtá a figyelmet a 2007-től alkalmazott Munkacsoport a kontakt allergiának diagnosztizálásához és kezeléséhez alkalmas metodikák megtalálására. A specifikus testelés alkalmazásával és a standard tesztanyagok alkalmazásával a Munkacsoport megerősítette a kontaktszénzibilis betegek diagnosztizálására és kezelésére a standard metodikák és tesztanyagok előnyeit (dr. Temesvári Erzsébet).


Prof. Dr. Temesvári Erzsébet
eyetemi tanár, a Munkacsoport vezetője