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FRA1 mediates the activation of keratinocytes: Implications for the development of psoriatic plaques☆

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ABSTRACT

In this study we investigated the role of FRA1, a transcription factor from the AP-1 family, in the regulation of keratinocyte characteristics important for the development of psoriatic plaques. FRA1 is characterized by elevated expression in the skin of psoriasis patients, thus leading us to predict it to be one of the major regulators of keratinocyte phenotype during the development of psoriatic lesions.

Pathway analysis of RNAseq data allowed us to identify FRA1-mediated signaling cascades leading to the manifestation of the most prominent skin characteristics of the disease: the development of inflammation, epithelial-mesenchymal transition, activation of metalloproteases, and keratinocyte proliferation and migration.

We have confirmed that FRA1-overexpressing keratinocytes produce elevated amounts of proinflammatory cytokines and active matrix metalloproteases, leading to the induction of the autoinflammatory loop and paracrine activation in neighbor cells. Therefore, the elevated expression of FRA1 and its altered transcriptional regulation in the skin of patients with psoriasis is an important driving factor in the development of psoriatic plaques.

1. Introduction

Psoriasis is an immune-mediated skin disease that affects about 2–3% of the world population [1]. The most distinctive hallmarks of psoriasis are cutaneous lesions that represent the areas of hyperproliferation and aberrant differentiation of epidermal cells, as well as altered immune profiles and enhanced vascularization of skin.

Being a complex pathology, psoriasis was actively investigated in order to identify the key molecular alterations leading to the development of the disease. Over time the central paradigm of the disease shifted from keratinocyte-specific alterations to lipid alterations in psoriatic skin, and from immune cell population changes to barrier abnormalities or alterations of skin microbiome [2–4].

Intensive investigation of psoriasis-associated cytokines and immune profiles has led to an important breakthrough in recognizing that the pathology is driven by Th17 immune cell hyperactivation, as well as other alterations in the immune profile. Consequently, a new therapeutic approach has been developed – “biological drugs”, such as antibodies against TNFa or interleukin blockers (ustekinumab, secukinumab, ixekizumab, guselkumab).

However, keratinocytes seem to be important intermediates of the disease, not only responsible for the visible skin manifestations and structural changes, but also involved in the production of many psoriasis-associated cytokines, chemokines and antimicrobial molecules (IL-1α, IL-1β, IL-6, IL-8, IL-23, TNF-α, S100A8, S100A9) [5,6]. The role of keratinocytes is highlighted by the mouse models of the disease with keratinocyte alternations, such as constitutively active *Stat3* expression, inducible *S100A7/A15* overexpression [7], or inducible epidermal deletion of Jun proteins [8] that result in psoriasis-like phenotypes. Besides, recent investigations of CARD14 mutations associated with the development of the disease highlight the importance of keratinocyte signaling in the pathology [9].

The role of keratinocytes in the skin manifestations of psoriasis should not be underestimated - this type of cells is capable of producing many psoriasis-associated cytokines, chemokines and antimicrobial molecules that mediate active migration of immune cells to the site of a lesion during the progression of the disease. Furthermore, even after successful treatment and stable remission of the disease skin cells in the

Abbreviations: EMT, epithelial–mesenchymal transition; RNAseq, next generation sequencing of the transcriptome; ECM, extracellular matrix; qPCR, quantitative polymerase chain reaction; FDR, false discovery rate

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area of the former lesion are characterized by gene expression alterations – the “molecular scar” of the disease [10].

If we characterize psoriasis by aberrant activation of keratinocyte proliferation and migration as well as altered ECM remodeling and wound healing, similarities are found to the endpoints of epithelial–mesenchymal transition (EMT). EMT is a sequence of molecular alterations that lead to phenotypical and physiological changes in epithelial cells. A defining characteristic of EMT is transdifferentiation of differentiated epithelial cells leading to the loss of epithelial phenotypes and cell polarity, and consequently the acquisition of a mesenchymal phenotype. It widely occurs during cancer invasion and metastasis, under inflammatory stress as well as during normal physiological processes like wound healing and embryonic development.

Whether or not EMT transition plays any role in psoriasis is an important question. Recent studies [11,12] have shown that many of the EMT markers are characterized by altered expression profiles in psoriatic keratinocytes (namely vimentin (VIM), fibronectin (FN), plasminogen activator inhibitor 1 (SERPINE1), cytokeratin 10 (KRT10), etc.).

One of the major regulators of proinflammatory cytokine and chemokine gene expression as well as keratinocyte proliferation is the transcriptional factor AP-1 [13–15]. The expression patterns of AP-1 proteins (JUN, JUNB, JUND, FOS, FOSB, FRA1, FRA2) in epidermis vary depending on the epidermal layer and the differentiation state of keratinocytes, implying an important role of AP-1 in the regulation of keratinocyte proliferation and differentiation [16]. Knockouts of various members of the AP-1 superfamily in mice keratinocytes led to disruption of the epidermal homeostasis. For example, JunB$^{-/-}$ mice were characterized by increased epidermal production of *IL-6* and *G-CSF,* skin ulcers and impaired wound healing, while mice with inducible epidermal inactivation of Jun and JunB developed a psoriatic phenotype and so on [16].

Our previous research had shown that FRA1 is the most evidently overexpressed AP-1 protein in lesional skin of patients with psoriasis [17]. This protein is widely associated with different types of cancer [18,19], and recent research has shown it to be an important regulator of EMT-associated gene expression in colorectal cells [20,21] and prostate epithelial cells [22].

In order to evaluate the importance of FRA1-mediated transcriptional regulation in keratinocytes in psoriasis we have created the *FRA1*-overexpressing keratinocyte cell line HaCaT-F, and evaluated their psoriasis-associated characteristics.

2. Materials and methods

2.1. Ethics statement

Procedures were conducted according to the Declaration of Helsinki principles. Informed written consent was obtained from human subjects under protocols approved by the local Ethics Committee of the Vavilov Institute of General Genetics, Russian Academy of Sciences.

2.2. Patients and samples

The patients in this study were adults (older than 18) of both sexes, 5 females and 5 males, ranging from age 24 to 55 y., all unrelated Caucasian individuals with the plaque form of psoriasis. Two 4-mm punch biopsy specimens were taken from the skin of the patients, one from the lesional area of the skin (LS sample) and another from the non-lesional area of skin 3–4 cm apart from the lesion, in an area that did not have any visual signs of psoriasis (NL sample). Patients did not obtain any systemic or PUVA/UV treatment for 1 month before the biopsies. All biopsy samples were immediately transferred to liquid nitrogen until RNA extraction.

2.3. RNA isolation and reverse transcription

TissueLyser LT homogenizer (Qiagen, Germany) was used to homogenize the biopsy specimens. Total RNA was extracted with the ExtractRNA reagent (Evrogen, Russia) according to the manufacturer's protocol. Isolated RNA was dissolved in RNase-free water. Concentration of the samples was evaluated with a Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA) and Qubit RNA BR Assay Kit (Thermo Scientific, Waltham, MA). Reverse transcription was performed with 1 μg of total RNA and a MMLV reverse transcription kit (Evrogen, Russia) using oligo(dT)-primers according to the manufacturer's instructions.

2.4. Cells and antibodies

HaCaT cells were generously provided by Dr. E. Vorotelyak, Russia; HEK293T cells were generously provided by Dr. M. Lagarkova, Russia. Cells were cultured in DMEM high glucose (Thermo Scientific, Waltham, MA), supplemented with 2 mM L-glutamine (Paneco, Russia) and 10% fetal bovine serum (Thermo Scientific, Waltham, MA). Cells were routinely tested for mycoplasma infection using a MycoReport kit (Evrogen, Russia). All the experiments were performed in triplicates, except for the medium transfer assay performed in IncuCyte in eight replicates.

For immunofluorescence, Western blotting and IL-17 receptor neutralizing we have used primary antibodies directed to: β-actin (#4970, Cell Signaling), TNFa (#orb11495, Biorbyt), IL-8 (ab#7747, Abcam), IL-17 (#ab77171, Abcam), IL-17R (MAB177-100, R&D Systems); secondary peroxidase-conjugated goat-anti rabbit AffiniPure antibodies (#111-035-003, Jacson ImmunoResearch) or goat-anti rabbit highly cross-adsorbed secondary antibodies conjugated with Alexa Fluor 594 (#A11037,Thermo Fisher Scientific).

Foe immunohistochemistry we have used primary antibodies directed to: phospho-FRA1 (#NBP1-47757, Novus Biologicals); non-phospho-FRA1 (#5841, Cell Signaling).

2.5. Lentiviral production and cell transduction

FRA1 cDNA was amplified by polymerase chain reaction with high-fidelity DNA polymerase Tersus (Evrogen, Russia) from a p6599_MSCV_IP_N_HAonly_FOSL1 plasmid that was a gift from Peter Howley (Addgene plasmid #34897) with the following primers hFOSL1_fwd_EcoRI GAATTCATGTTCCGAGACTTCGG; hFOSL1_rev_EcoRI GAATTCTCACAAAGCGAGGAGGG. Amplified *FRA1* cDNA was consequently cloned into FU-tet-o-hOct4 (a gift from Konrad Hochedlinger (Addgene plasmid #19778)) instead of the *hOct4* sequence.

To generate HaCaT-based cells capable of inducible overexpression of FRA1, a modified Tet-On system consisting of two vectors was used: FU-tet-o-FRA1 and FUdeltaGW-rtTA-IRES-puro. The latter was a modification created by I. Chestkov, Vavilov Institute of General Genetics RAS, based on the vector FUdeltaGW-rtTA (a gift from Konrad Hochedlinger (Addgene plasmid #19780)), containing reverse tetracycline transactivator rtTA. Lentiviral particles were produced in HEK293T cells by the cotransfection with 3rd generation lentiviral packaging constructs (pMDLg/pRRE, pRSV-Rev plasmids were gifts from Didier Trono (Addgene plasmids #12251, #12253), pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454)), and target vectors FU-tet-o-FRA1 and FUdeltaGW-rtTA-IRES-puro, as described [23]. Transfection was performed with Metafectene Pro transfection reagent (Biontex, Germany) according to the manufacturer's protocol.

Supernatants containing the viral particles were collected 40 to 48 h post-transfection, filtered through a 0.45 μm filter, and used to transduce wild type HaCaT cells. As the lentiviral vectors confer puromycin resistance, selection of stable transfectants was performed by passaging the cells in full medium with puromycin antibiotic (0,8 μg/ml) for 4 weeks. Inducible overexpression of FRA1 was verified by qPCR 48, 72

and 96 h after doxycycline induction (1 μg/ml) and by Western blotting (ab#5841, Cell Signalling, Boston, MA, USA).

2.6. Quantitative real-time PCR

Total RNA from cells was isolated using ExtractRNA reagent (Evrogen, Russia). cDNA was synthesized using MMLV cDNA Synthesis Kit (Evrogen, Russia) according to the manufacturer's instructions, using 1 μg of each RNA sample per reaction. Primer and probe design, synthesis, and verification were performed by DNK-Sintez (Russia). Primer and probe sequences: FRA1, GeneID 8061, Pr+ 5′CACCCTAG CCAATGTCTCC3′, Pr− 5′CACCCTAGCCAATGTCTCC3′, TaqMan probe FAM-CGCGATCAGAAGAAGTTGCTGGAGTTGGATGTGGGATCGCG-BHQ1; MMP1, GeneID 4312, Pr+ 5′CGGTTTTTCAAAGGGAATAAGT ACT3′, Pr− 5′TCAGAAAGAGCAGCATCGATATG3′, TaqMan probe FAM-AATGTGCTACACG GATACCCCAAGGACA-BHQ1; MMP2, GeneID 4313, Pr+ 5′CGCTCAGATCCGTGGTGAG3′, Pr− 5′TGTCACGTGGCGT CACAGT3′, TaqMan probe FAM-TTCTTCTTCAAGGACCGGTTCATT TGG-BHQ1; MMP12, GeneID 4321, Pr+ 5′TCGCCTCTCTGCTGATGA CAT3′, Pr− 5′GTCAGGATTTGGCAAGCGTT3′, TaqMan probe FAM-CATTCAGTCCCTGTATGGAGACCCAA-BHQ1; KRT16, GeneID 3868, Pr + 5′ACCTGAGGAACAAGATCATTGC3′, Pr− 5′ATGCTCATACTTGGTC CTGAAG3′, Sybr-Green assay; KRT10, GeneID 3858, Pr+ 5′AAATCAG ATTCTCAACCTAACAACTG3′, Pr-5′GCTACCTCATTCTCATACTTC AGC3′, Sybr-Green assay; VIM, GeneID 7431, Pr+ 5′CTGAACCTGAG GGAAACTAATCTG3′, Pr− 5′GTTTCGTTGATAACCTGTCCATCTC3′, Sybr-Green assay; SLUG, GeneID 6591, Pr_ 5′CGAACTGGACACACATA CAGTG3′, Pr− 5′GAGCTGAGGATCTCTGGTTGT3′, Sybr-Green assay; FN1 GeneID 2335, Pr+ 5′GCGAGAGTAAACCTGAAGCTG3′, Pr− 5′ GGAGTCTTTAGGACGCTCATAAG3′, Sybr-Green assay; TNFa, GeneID 7124, Pr+ 5′TCTTCTCGAACCCCGAGTGA3′, Pr− 5′CCTCTGATGGCA CCACCAG3′, TaqMan probe FAM-TAGCCCATGTTGTAGCAAACCCTCA AGCT-BHQ1; IL8, GeneID 3576, Pr+ 5′CTTGGCAGCCTTCCTGATTT3′, Pr− 5′TTCTTTAGCACTCCTTGGCAAAA3′, Sybr-Green assay; SERPINE1, GeneID 5054, Pr+ 5′CCTAGAGAACCTGGGAATGAC3′, Pr− 5′GAGGCTCTTGGTCTGAAAGA3′, Sybr-Green assay; B2M, GeneID 567, Pr+ 5′GTGCTCGCGCTACTCTCTC3′, Pr− 5′TGTCGGATGGATGAAAC CCAG3′, Sybr-Green assay; GUSB, GeneID 2990, Pr+ 5′TGGTTGGAG AGCTCATTTGGA3′, Pr− 5′CTCTCTCGCAAAAGGAACGC3′, Sybr-Green assay; GAPDH, GeneID 2597, TaqMan GAPDH Control Reagents (Thermo Scientific, Waltham, MA). All qPCR reactions were performed using an Eco Real-Time PCR System™ (Illumina, San Diego, CA, USA) with either a qPCR mix-HS High ROX Kit (Evrogen, Russia) for reaction with probes or a qPCR mix-HS High ROX + SybrGreen Kit (Evrogen, Russia), 400 nM forward and reverse primer, 250 nM of probe and 2 ng of cDNA in a total volume of 10 μl. All PCR reactions were performed in triplicate. Quantification was performed using the comparative $2-\Delta\Delta CT$ method and 3 reference genes – *GAPDH, GusB* and *B2M*.

2.7. Immunohistochemistry

Histological sections were prepared from biopsies taken from lesional and visually normal skin (nonlesional sample) fixed in 10% buffered formalin and embedded in paraffin. Sections were subjected to immunohistochemical analysis using the following antibodies: primary mouse monoclonal FRA1/FOSL1 Antibody (OTI12F9) #NBP1-47757 (Novus Biologicals, Littleton, CO), and primary rabbit monoclonal phospho-FRA1 (Ser265) (D22B1) #5841 (Cell Signaling, Netherlands). Primary antibodies were detected with a Novolink detection system and DAB Chromogen (Leica Biosystems, Milton Keynes, UK). Images were captured using the Zeiss Axio Imaging system (Zeiss, Germany) and Zeiss AxioCam digital camera (Zeiss, Germany).

2.8. Zymography

Metalloproteinases were detected and characterized by zymography. All the experiments were performed in 3 technical and 3 biological replicates. Wild-type HaCaT and HaCaT-F cells were cultivated in full DMEM medium, supplemented with 10% FBS. Cells were stimulated by 1 mg/ml doxycycline, 24 h later the medium was changed for DMEM without FBS and a mix of psoriasis-associated cytokines (100 ng/ml IL-17, 20 ng/ml TNFa, 25 ng/ml IFNg). The conditioned medium was collected 48 h later, centrifuged for the removal of cell debris, and adjusted for protein concentration (2 μg or 5 μg for the detection of MMP2 and MMP9 respectively). 12% SDS-polyacrylamide separating gel with 40 μg /ml of gelatin (40% *bis*-acrylamide, 1.5 M Tris-HCL pH 8.8, 10% w/v ammonium persulfate, 0.04% v/v TEMED, 10% v/v SDS and distilled water to reach final volume) and 4% SDS-polyacrylamide staking gel (40% v/v bisacrylamide, 1 M tris-HCL pH 6.8, 10% w/v ammonium persulfate, 0.1% v/v TEMED, 10% v/v SDS and distilled water to reach final volume) was prepared. The samples in non-reducing buffer (0.5 M Tris–HCl pH 6.8, 10% v/v glycerol, 4% v/v SDS, and 0.05% w/v bromophenol blue) were loaded on a gel and electrophoresis was carried out at 4 °C at 90 V for approximately 2.5 h. Gels were washed 2 times in 2.5% v/v Triton-X 100 for 15 min, incubated on incubation buffer (Tris–HCl 50 mM, CaCl2 10 mM, NaCl 50 mM, pH 7.6) for 16–18 h at 37 °C, stained by 1% v/v Coomassie brilliant blue R-250 solution for 10 min under gentle shaking, and then destained with 25% v/v ethanol and 8% v/v acetic acid solution. Areas of proteolytic activity were then visible as clear bands against the blue background of stained gel. Images were captured using an Epson Perfection 1650 (Seiko Epson Corporation, Japan) and analyzed with ImageJ software [24].

2.9. Scratch assay and wound healing

WT HaCaT cells or HaCaT-F cells were seeded on culture plates and after reaching confluency were scratched with a sterile blue 1000 μl tip and the cell debris was removed by two washes with warm PBS (Thermo Scientific, Waltham, MA). In order to minimize the variation caused by the difference in the width of the scratches the assay was performed on cell culture plates with a 2-mm grid (Corning) and the width of the scratches for both assessed cells and control cells amounted to the width of a single grid (2 mm). 72 h post-scratch the images were captured using the Zeiss Axio Imaging system (Zeiss, Germany) and Zeiss AxioCam digital camera (Zeiss, Germany).

Next the experiment was repeated; the cells were plated on 6-well plates and 72 h after wounding cells were fixed with 96% ice-cold EtOH and stained with 0.5% crystal violet staining solution for 5′RT. Then the staining solution was removed, the plates were rinsed with distilled water and the images of the wells were captured using an Epson Perfection 1650 (Seiko Epson Corporation, Japan) and analyzed with ImageJ software.

For the medium transfer assay, HaCaT-F and WT HaCaT cells ("donor cells") were seeded in the full DMEM supplemented with 10% FBS and 24 h later they were stimulated with DOX (1 μg/ml) and psoriasis-associated cytokines (100 ng/ml IL-17, 20 ng/ml IFNg, 25 ng/ml TNFa). After 24 h of stimulation the medium was changed for the full DMEM supplemented with 10% FBS. At the same time the "recipient cells" were seeded; a total of $35 * 10^3$ WT HaCaT cells were seeded per well of 96-well plate and allowed to adhere overnight. 72 h after stimulation the medium from the "donors" was collected, centrifuged, filtered through a 0,22 μm filter and mixed as 2:1 with fresh medium containing FBS. The scratch was performed on the "recipients" using a 96-pin IncuCyte WoundMaker (Essen BioScience, Ann Arbor, MI), cells were washed twice with warm PBS for debris removal, then the wells were filled with 200 μl of conditioned medium mix, transferred from either WT or HaCaT-F cells, and the plate was placed into the Incucyte machine (Essen BioScience, Ann Arbor, MI). A conditioned medium from the same cells without stimulation was used as a control (WT ctrl and HaCaT-F ctrl). Hourly images were captured with the 10×/1.49 NA objective lens. Using the metrics from the IncuCyte

ZOOM 2015 software, the closure was evaluated in dynamics for 60 h, and the percent of size of the wound + -SEM was used for comparison.

2.10. Western blotting and immunofluorescence

Wild-type HaCaT and HaCaT-F cells were cultivated in full DMEM medium supplemented with 10% FBS. Cells were FBS-starved for 24 h, then stimulated by 1 μg/ml doxycycline (HaCaT + DOX) or a 1 μg/ml doxycycline plus a mix of psoriasis-associated cytokines (100 ng/ml IL-17, 25 ng/ml TNFa, 20 ng/ml IFNg) (HaCaT + DOX + cyto). 24 h later the cells were washed with warm PBS and the medium was changed for full DMEM with FBS. 24 h later the medium was changed to the FBS-free medium. 72 h after the stimulation 4 ml of the cell medium was collected, supplemented with Protease Inhibitor Cocktail 4 MammCell/Tissue Plus (#A7757, Applichem) and concentrated by centrifugation at +4 C to the volume of 500 μl with Amicon Ultra-4 10 K centrifugal filters (#UFC801008, Merck/Millipore) according to the manufacturer's instructions. The cells were trypsinised and collected with RIPA buffer supplemented with Protease Inhibitor Cocktail 4 MammCell/Tissue Plus (#A7757, Applichem). Western Blotting was performed as previously described [58] with several modifications: the samples were loaded on the 4–12 Bis-Tris gel (40% bis-acrylamide, 1.5 M Tris-HCL pH 8.8/0.5 M tris-HCL pH 6.8, 10% w/v ammonium persulfate, 0.04% v/v TEMED, 10% v/v SDS and distilled water to reach final volume) at equal amounts for each of the experiments, between 5 and 10 μg protein per lane (depending on the antibody) and separated by electrophoresis with 200 V until full separation as indicated by the protein ladder (PageRuler Plus Prestained 10–250 kDa Protein Ladder, #266191, Thermo Scientific). The proteins on the gel were transferred to the 0.45 μm PVDF Transfer Membrane (#88518, Thermo Scientific) or 0.2 μm Low-Fluorescence PVDF Transfer Membrane (#22860, Thermo Scientific) by electroblotting (BioRad Criterion Blotter) at 80 V for 2.5 h. Blots were blocked for 1 h RT using 5% nonfat dried milk powder (#A0830,0500, Applichem) and incubated overnight at 4 °C with primary antibodies, diluted in Tris-buffered saline with 5% nonfat dry milk and 0.1% Tween 20. The blots were probed with peroxidase-conjugated secondary antibodies and visualized using Pierce ECL Chemiluminescent Substrate (#32209, Thermo Scientific) according to the manufacturer's instructions. The Western blots were visualized manually using UltraCruz autoradiography film (#SC-201697, SantaCruz Biotechnology) and radiography developer and fixer Renmed plus (#10850 and #10854, VIPSMED, Russia). The blots were digitalized using an Epson Perfection 1650 (Seiko Epson Corporation, Japan).

Immunofluorescence was performed using a previously described method [57]. The images were captured using the Zeiss Axio Imaging system (Zeiss, Germany) and Zeiss AxioCam digital camera (Zeiss, Germany) and AxioVision Rel. 4.2 software.

2.11. Statistical analysis

The quantification of qPCR results was performed using the comparative $2-\Delta\Delta CT$ method vs 3 reference genes – GAPDH, GusB and B2M. Before plotting the qPCR data the mean and standard deviation of the mean were computed. The resulted points are represented on the corresponding plots.

Normal distribution and statistical comparisons between groups were determined using the Shapiro-Wilk test, Student *t*-test (two tailed), or two-way analysis of variance with a Bonferroni posttest where appropriate. For all statistical tests, the variance between each group was determined, and probability values of $p < 0.05$ were considered statistically significant.

To analyze the differential expression of genes (DEGs) between lesional and non-lesional skin samples we used our previously published data acquired from the SOLiD RNA-seq experiment. The raw sequencing data was processed with the DESeq R package to compute the RPKM values for genes in every sample. Next, we implied Log fold-change comparison of mean RPKM values for every group. To identify significant DEGs we used the ± 1.5 Log FC with < 0.05 adjusted p.value cut-off.

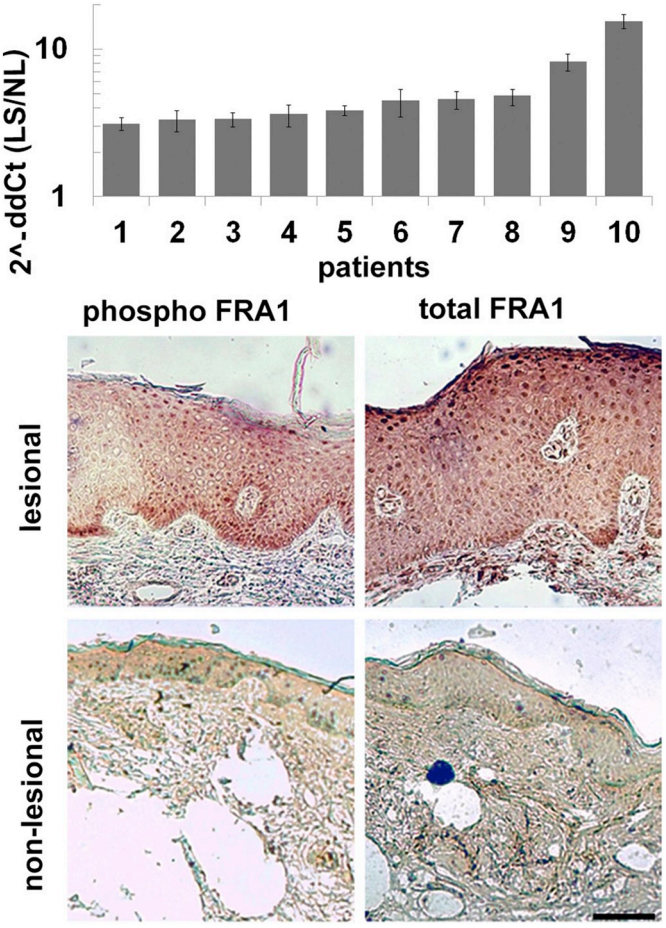

Fig. 1. FRA1 is overexpressed in lesional skin of psoriatic patients. The expression of FRA1 was evaluated in lesional and nonlesional skin of the same patients using qPCR. a. *FRA1* gene expression in lesional skin compared to non-lesional skin samples of the same patients (mean ± SD, $p < 0.05$) b. Immunohistochemical analysis of FRA1 protein abundance in lesional and non-lesional biopsies of psoriatic skin. Left panel – antibodies against phospho-FRA1; right panel – antibodies against non-phospho-FRA1 (representative photos of 5 skin pairs).

Wound closure dynamics were analyzed using the IncuCyte WoundMaker. Wound sizes for every group of replicates were compared using the Wilcoxon signed-rank test with threshold of significant difference between wound sizes at p.value < 0.05. The wound closure data is presented as mean + standard error of the mean.

3. Results

3.1. FRA1 is overexpressed in psoriatic skin

In order to evaluate the abundance of FRA1 mRNA and protein in skin of patients with psoriasis we have performed qPCR analysis of *FRA1* expression in 10 pairs of lesional and non-lesional skin samples of patients with psoriasis (Fig. 1a) as well as immunohistochemical analysis of 5 pairs of biopsies (Fig. 1b). The analysis has shown elevated mRNA and protein levels of FRA1 in all the analyzed lesional samples compared to the non-lesional skin samples.

Table 1
Top FRA1-mediated processes, important for psoriasis (enrichment of the MetaCore pathway database with the expression data from GSE78023).

Process	Genes with differential expression in psoriatic plaques	p-Value
Cell cycle progression	*LMNB1, IL-1b, HSPA2, GPRC5A, NOR1, CCNB1, CCNB2, HURP, PKC, ZBTB16, RARA, EPGN, HSP70, HNF3a, EREG, AURKA Aurora-A, PLK1, WNT, HNF3, BTC, TRIM21, CDK1, CDC6, CCNA2, ORC1L, RRM2, CCNE1, CDC45L*	2.388E−06
Immune response_IL-17/23 signaling	*IL1B, CCL20, IL-17, GRO-1, iNOS, DEFB4A, DEFB4B, GCP2, ICAM1, CCL2, IL-8, NGAL, IL-17F, MMP-9, CCL7, STAT3, RORC, IL-17F, SOCS3*	1.227E−06
Cell adhesion	*ITGAL, ITGB2, ICAM1, PIK3R5, IL-8, IL8RB, PLCB2, CCL5, LGALS3, SELL, CCL8, MMP-9, XCL1, XCL2, SELE, PLAUR, GRO-1, GCP2, CCL2, PLAT, ACTA2, VAV-1, TIMP3, MMP-9, COL3A1*	4.654E−02
Cytoskeleton remodeling	*TUBB6, KRT6A, KRT6C, CDK1, PLAUR, PPARD, PLAT, WNT, WIF1, ACTA2, VAV-1, MRLC, iNOS, PLCB2, PLCB4, ACM3*	2.679E−02

3.2. FRA1 is the regulator of the psoriasis-associated pathways

In prior research [GSE78023, 15] we have performed RNA-seq of 14 paired biopsies from lesional and non-lesional skin of psoriatic patients, and identified 1564 differentially expressed genes (Fold Change ≥ 1.5, FDR < 0.05). Using MetaCore software [25] we have performed the pathway enrichment analysis with differentially expressed genes and then performed the identification of the transcription regulators of the enriched pathways.

Among the pathways enriched with FRA1 target genes (identified through the published data) we have chosen those associated with keratinocyte proliferation, migration, and inflammation development (Table 1). Thus pathway analysis allowed us to identify the genes expressed in keratinocytes that contribute to the disease and could be directly or indirectly regulated by FRA1. We have divided them to the marker groups according to the psoriasis-associated function: 1) proliferation/differentiation (*KRT10, KRT16; VIM; MMP-12; MMP-1; MMP-2*) [26,27]; epithelial-mesenchymal transition (EMT) *(SLUG; SERPINE1; FN1; VIM; MMP-12; MMP-1; MMP-2)* [11,28,29]; inflammation *(TNFa, IL-8; MMP-12; MMP-1; MMP-2)* [30,31].

3.3. FRA1 inducible overexpression leads to activation of gene signatures associated with inflammation, proliferation and EMT of keratinocytes

It is an interesting question, why FRA1 is upregulated in the site of psoriatic inflammation. According to the published data [13], the stimulation of keratinocytes with proinflammatory cytokines such as IL-1, IL-17, IFNg, TNFa, lead to the activation of FRA1-mediated transcriptional regulation. Our experiments on keratinocytes have shown that FRA1 overexpression could both be the reason of IL-17A elevated production by keratinocytes, or the consequence of the proinflammatory environment present in site of psoriatic lesion (Figs. S1 and S2). When we stimulated keratinocytes with recombinant human IL-17 alone or the mix of prionflammatory cytokines (IL-17, IL-6, TNFa), the expression of FRA1 was elevated. Addition of the neutralizing antibodies blocking the IL-17 receptor reduced the level of FRA1 overexpression (Fig. S1).

In order to evaluate the impact of *FRA1* overexpression on the mRNA levels of selected target genes in keratinocytes we have created HaCaT-F, a HaCaT-based cell line capable of inducible *FRA1*-overexpression based on a modified Tet-on Dox-system [32]. HaCaT-F cells were grown in a full medium and induced by doxycycline (DOX, 1 μg/ml) for 48, 72 or 96 h, then *FRA1* expression was evaluated using quantitative real-time PCR (Fig. 2a). 72 h after induction *FRA1* expression in target cells was > 100-fold higher than in control HaCaT cells.

Expression alterations of FRA1 target genes were measured 72 h after the induction of *FRA1* overexpression. qPCR analysis (Fig. 2c) revealed altered expression of all the marker groups analyzed. Among proliferation/differentiation markers cytokeratin 10 (*KRT10,* marker of keratinocyte differentiation [27]) was down-regulated, cytokeratin 16 (*KRT16,* promotes keratinocyte proliferation [27]) was up-regulated, and matrix metalloprotease gene expression was also up-regulated (*MMP-1, MMP-2* and *MMP-12,* MMPs could activate keratinocyte proliferation in psoriatic plaques via the remodeling of ECM and progressive loosening of cell adhesion and tight junctions between the cells). Vimentin expression was not altered significantly (*VIM*, a member of the intermediate filament family normally expressed in cells of mesenchymal origin) and was shown to accumulate in the proliferative/migratory rim of the growing keratinocyte colonies [33].

All the inflammation marker genes were characterized by elevated expression in FRA1-overexpressing cells (*TNFa*, pro-inflammatory cytokine and important regulator of immune cells as well as activator of keratinocytes; *IL8*, pro-inflammatory cytokine produced by keratinocytes and chemotactic for neutrophils and T lymphocytes, *MMP-1,-2* and *-12* – MMPs that process precursor forms of cytokines and chemokines into the active pro-inflammatory forms). All the analyzed genes that mediate EMT of keratinocytes, except for *VIM,* were overexpressed in HaCaT-F cells (*SLUG* (*SNAI2*), regulator of epithelial-mesenchymal transformation during development and a transcriptional repressor of E-cadherin [34]; *SERPINE1* (*PAI-1*), that negatively regulates plasmin-dependent matrix degradation, preserving a stromal scaffold permissive for keratinocyte motility [35]; *FN1*, a glycoprotein involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense, and metastasis). We have also evaluated the protein levels of inflammation-associated markers by Western blotting and immunofluorescent analysis (Fig. S4).

Considering IL-17 to be the one of the main psoriasis-associated cytokines, we have evaluated the levels of IL-17 protein production by HaCaT-F cells. FRA1-overexpressing keratinocytes have shown elevated IL-17 protein production compared to WT keratinocytes (Fig. S2).

3.4. FRA1 is the activator of both MMP expression and activity in keratinocytes

We have used a zymography assay to evaluate the connection between FRA1-mediated overexpression of MMPs and their activity. The samples of conditioned medium from cells were loaded on a polyacrylamide gel, supplied with gelatin. We have performed densitometric analysis of the zymograms using the ImageJ software. Roughly 1.5 more gelatinase activity for both MMPs in FRA1-overexpressing cells was identified (Fig. 2b, Fig. S5).

3.5. FRA1 stimulates keratinocyte motility and wound healing

To support the hypothesis of FRA1-directed development of psoriatic plaques we have performed a scratch assay [36]. The monolayers of WT HaCaT cells or HaCaT-F cells were scratched with a sterile blue 1000 μl tip. As shown in Fig. 4, HaCaT-F cells were characterized by enhanced migration capability and 72 h post-scratch they had closed the “wound”, unlike the WT HaCaT (Fig. 3a).

3.6. FRA1 could mediate the activation of the autoinflammatory loop in psoriasis

Next, the ability of FRA1-overexpressing cells for paracrine activation of the neighbor cells was tested. We suggested that under cytokine

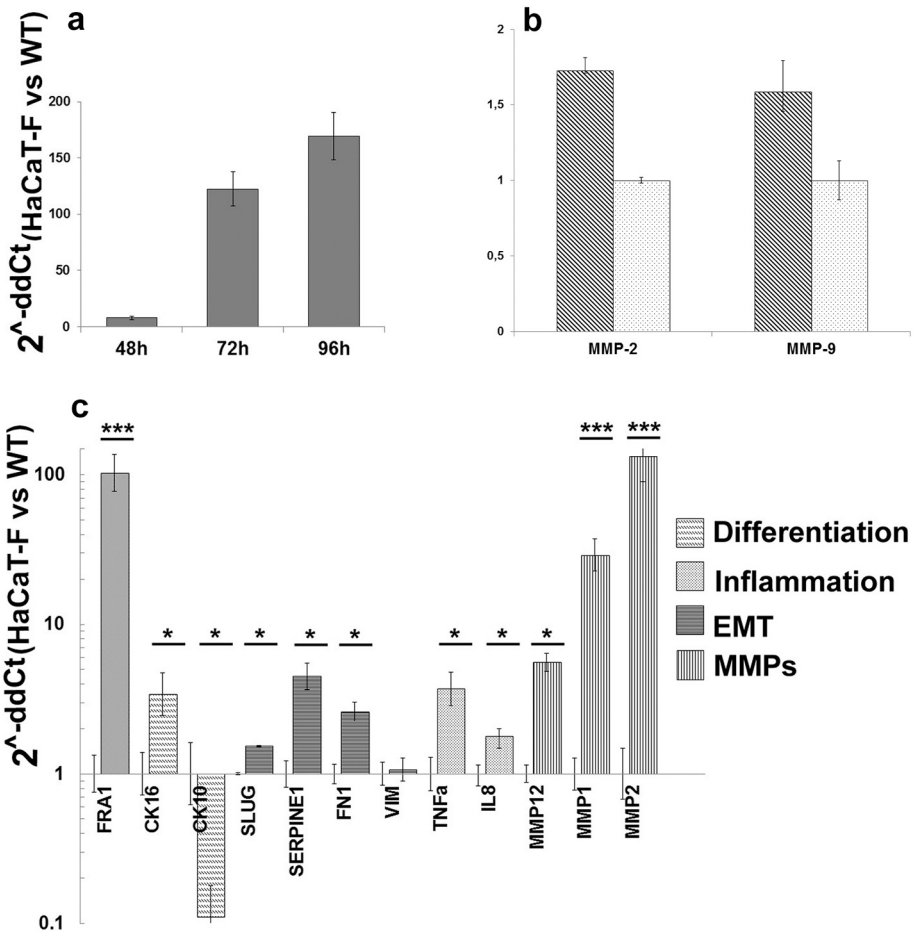

Fig. 2. FRA1 overexpression stimulates keratinocytes to produce proinflammatory cytokines, metalloproteases and EMT marker proteins. a. *FRA1* expression in HaCaT-F cells vs WT HaCaT cells after doxycycline induction (mean ± SD, $p < 0.05$). b. Differential gelatinase activity of MMP-2 и MMP-9 in HaCaT-F (dark grey) and WT HaCaT cells (light grey) (mean ± SD, $p < 0.05$). c. Expression alterations of different groups of marker genes in HaCaT-F cells relative to the levels in WT cells (mean ± SD, * corresponds to $p < 0.05$; *** corresponds to $p < 0.01$). WT, wild type.

storm apparent in blood of psoriasis patients the overexpression of FRA1 in keratinocytes could lead to the amplification of skin manifestations of the disease.

In order to test the hypothesis, HaCaT-F and WT HaCaT cells ("donor cells") were stimulated with DOX (1 μg/ml) and psoriasis-associated cytokines (100 ng/ml IL-17, 20 ng/ml IFNg, 25 ng/ml TNFa) for 24 h, then the medium was changed for the full DMEM supplemented with 10% FBS. At the same time the "recipient" WT HaCaT cells were seeded on a 96-well plate. 72 h later a scratch was performed on the "recipients" and the conditioned medium was transferred from either WT or HaCaT-F cells. A conditioned medium from the same cells without cytokine and DOX stimulation was used as a control (WT ctrl

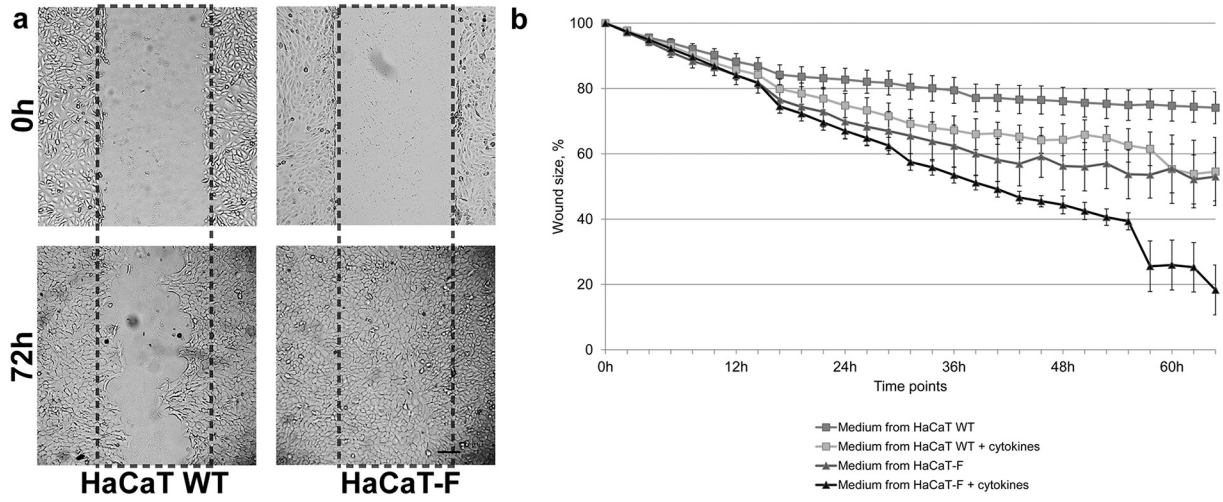

Fig. 3. Wound closure of keratinocyte monolayers is enhanced by FRA1 overexpression. a. Representative microscopic photographs of "wound" closure by monolayers of HaCaT-F cells and WT HaCaT cells at 0 h and 72 h post-scratch. WT, wild type b. Wound closure (the size of the wound) dynamics by monolayers of WT HaCaT cells, stimulated with conditioned medium from: WT cells without cytokines (WT ctrl), WT cells stimulated with proinflammatory cytokines (100 ng/ml IL-17, 20 ng/ml IFNg, 25 ng/ml TNFa) - (WT induced), HaCaT-F cells without cytokines (HaCaT-F ctrl), and HaCaT-F cells stimulated with proinflammatory cytokines (HaCaT-F induced) (mean ± SEM vs the size of the initial wound). WT, wild type.

and HaCaT-F ctrl). The closure was evaluated in dynamics for 60 h, and the percent of size of the wound + -SEM was used for comparison (Fig. 3b). The results of the experiment supported the hypothesis: wild type cells supplemented with the medium from FRA1-overexpressing cells closed the wound more actively than the cells supplemented with the medium from WT cells, either treated by cytokines or untreated.

To test if the main reason of the wound closure is proliferation or the elevated migration of cells, we have pretreated the "recipient" cells with cell cycle inhibitor mitomycin C (Fig. S6) before the stimulation with the conditioned medium from "donor" cells. The pretreatment of the recipient cells had slightly inhibited the wound closure, leveling down the difference between the variants of the "donor" medium. It seems that the activation of proliferation is the main paracrine effect in FRA1-overexpressing keratinocytes, and the elevated migration plays less role in the observed phenotype. However, the possibility of the elevated migration by FRA1-overexpressing cells shouldn't be withdrawn, because the skin is a complex organ and more complicate interactions between the cell types and the produced mediators are present in site of inflammation.

This result highlights the role of FRA1 in the enhanced proliferation and migration of keratinocytes, production of elevated amounts of proinflammatory cytokines that altogether could exacerbate the development of psoriatic plaques under the fire of proinflammatory cytokines present in skin of patients with psoriasis.

4. Discussion

FRA1 is overexpressed in keratinocytes in sites of indicative psoriasis manifestations – psoriatic plaques (Fig. 1) both at the mRNA and protein level. Inducible overexpression of FRA1 in the HaCaT keratinocyte cell line induced the elevated expression of markers of inflammation (*IL-8* and *TNFa*), proliferation and dedifferentiation (downregulated *KRT10*, upregulated *CK16* and *MMP*'s) and to the state that mimics epithelial to mesenchymal transition (elevated *SLUG, SERPINE1, FN1, MMP*'s) (Fig. 4).

As it was shown recently [11], psoriatic epidermal keratinocytes can be characterized as cells with an intermediate phenotype of type 2 EMT characterized by upregulation of *VIM, FN* and *PAI-1* (*SERPINE1*), downregulation of *KRT10*, loss of epithelial phenotypes and cell polarity, and the acquisition of a mesenchymal phenotype. The results we obtained with HaCaT-F cells support the hypothesis that FRA1 overexpression is sufficient for the development of the expression profiles mirroring that detected in psoriatic keratinocytes.

The important role of this transcription factor in psoriasis is at least partly explained by its potency to regulate matrix metalloprotease activation (Fig. 3) and consequent ECM remodeling. FRA1 is known to drive MMP activity in a vast majority of normal and cancer tissues: pulmonary epithelium [18], endothelium [37], liver tissue [38], skeletal muscle [39], breast cancer cells [40,41], melanoma [42] (Tower et al., 2003), renal carcinoma cells [43], colorectal cancer cells [44], as well as many others. The prominent role of matrix metalloproteases in the migratory or invasive potential of human epidermal cells has been well documented [45–47]. MMP processing of ECM components can yield bioactive fragments. For example, MMP-2 and MMP-9 expose a cryptic epitope within collagen IV that promotes angiogenesis, and ECM proteins themselves may be the source for bioactive cleavage products such as perlecan, laminin or fibronectin [48].

Cell-ECM interactions not only regulate cell adhesion and migration, but also affect cell growth and differentiation, and modulate both receptor and intracellular signaling pathways. Psoriasis is associated with drastic remodeling of cell-cell and cell–extracellular matrix interactions and basement membrane remodeling [49]: the main changes observed in psoriatic plaques, such as increased vascular permeability and immune infiltration, vasodilation and development of epidermal thickening (acanthosis) with regular elongation of rete ridges are closely linked to ECM remodeling. Besides, psoriasis-associated hyperproliferation of keratinocytes may be mediated by the loss of tight intercellular connections, as well as by loss of the attachment to the basement membrane. ECM degradation also releases non-covalently bound growth factors and cytokines and thereby increases their bioavailability, including release of VEGF and TGF-β [48].

Furthermore, matrix metalloproteases activities are not restricted to the degradation of ECM: they are important mediators of cytokine and chemokine processing from pro-forms into the active forms that could lead to further amplification of psoriatic inflammation [50]. For example, gelatinases MMP-2 and MMP-9 process IL-1 beta precursor into biologically active mature form [51] and MMP-9 potentiates interleukin-8 about tenfold by aminoterminal processing [52].

One of the important pathways active in psoriasis is EGF signaling. It was shown [18] that phospho-FRA1 induces EGFR phosphorylation in an MMP-dependent manner, and an EGFR-specific inhibitor is able to block Fra1-enhanced cell motility and invasion. A possible route of FRA1-dependent amplification loop of psoriatic hyperproliferation of keratinocytes is via MMP-dependent EGFR overexpression leading to ERK activation with consequent activation of FRA1, binding to the consensus sites in MMP promoter areas and overexpression of MMPs. This hypothesis is supported by the data that MMPs as well as EGFR inhibitors block FRA1-induced motility and invasion of lung cancer cells.

FRA1-mediated shift in keratin profiles of cells (Fig. 2) also plays its role in the development of psoriatic plaque: KRT8 regulates keratin reorganization and migration [53], KRT10 impacts proliferation of basal keratinocytes and cell size [54], and KRT16 regulates epithelial inflammation [55]. In normal epidermis keratin expression patterns are restricted to certain layers of skin, indicating the proliferative/differentiated state of a cell: keratins 5 and 14 are expressed in the basal layer of epidermis, marking actively proliferating cells; keratins 1 and 10 are accumulated at the suprabasal (spinous) layer; keratins 6, 16, and 17 normally are expressed in the epidermis of glabrous skin, oral mucosa, and several appendages, and are induced in skin under stress conditions [55].

Another characteristic of psoriatic phenotype of keratinocytes is active production of chemoattractive cytokines and chemokines [5]. FRA1-overexpressing keratinocytes were characterized by enhanced production of important proinflammatory mediators TNFa and IL8 (Fig. 2). It was shown [56] that TNFa itself could induce EMT via activation of AP-1 signaling and subsequent expression of the EMT regulator ZEB2, and could activate both the PI3K/Akt and MAPK/ERK pathways, which act upstream of AP-1. Considering the potential of TNFa to activate *FRA1* expression it could be another driver of the autoamplification loop and reinforcement of inflammation in psoriatic plaques.

FRA1-overexpressing keratinocytes are prone to proliferation, migration and active wound closure (Fig. 3). Such a phenotype could be considered a two-edge sword, leading to wound healing in normal conditions and to the development of psoriatic plaques under the inflammatory environment. Besides, our experiments confirmed the hypothesis of paracrine amplification of keratinocyte activation (Fig. 3). Such paracrine stimulation could target not only keratinocytes, but also the immune cells – activate resident dendritic cells and attract neutrophils and T cells to the site of inflammation. We have summarized different roles of FRA1 in psoriatic plaque development at the scheme (Fig. 4).

5. Conclusions

In summary, our investigations provide the support for assigning FRA1 as a regulator of psoriasis-associated phenotype in keratinocytes. FRA1 overexpression leads to the activation of keratinocytes, amplification of the inflammation as well as proliferation, and altogether to the development of psoriatic plaques. The exact reasons of FRA1 overexpression in skin of psoriasis patients are still to be investigated, but it

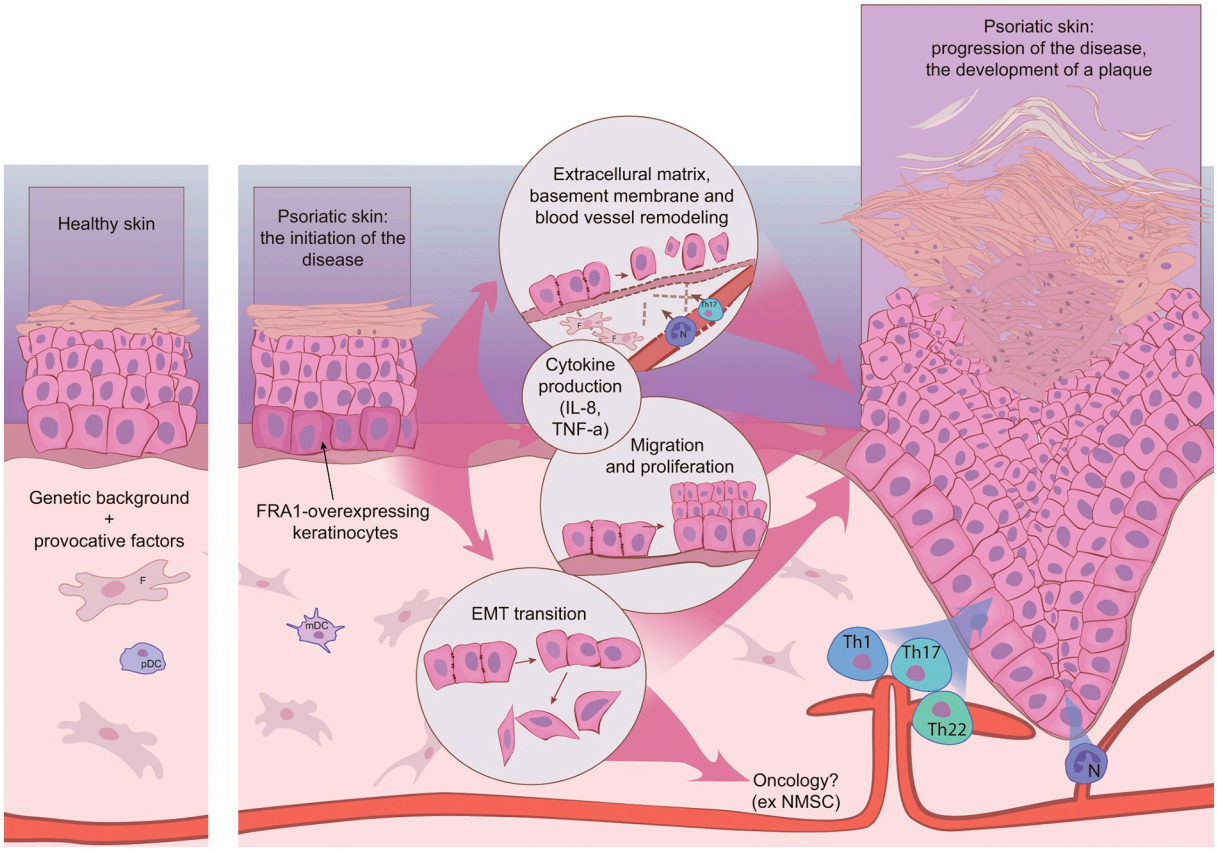

Fig. 4. The proposed model of FRA1 participation in the various steps of the development of psoriatic plaques. FRA1 overexpression in psoriatic keratinocytes could induce the development of psoriatic plaques in a several ways: through the enhancement of keratinocyte proliferation and the inhibition of the cell differentiation; through the elevated production of proinflammatory cytokines and chemokines; through the elevated ECM, basement membrane and vessel wall remodeling with the consequent migration of the immune cells at the proinflammatory background; and, through the progressive acquisition of the EMT phenotype of cells.

is possible that the development of FRA1-modulating topical therapeutic agents could be a promising approach for the management of psoriasis manifestations on skin.

Conflict of interest

The authors state no conflict of interest to declare.

The funding sources had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.09.016.

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