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

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