E-FABP induces differentiation in normal human keratinocytes and modulates the differentiation process in psoriatic keratinocytes in vitro

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Abbreviations: E-FABP: epidermal-fatty acid binding protein; KSC: keratinocyte stem cells; TA: transit amplifying cells; PM: post mitotic; K: keratin

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ABSTRACT

Epidermal fatty acid-binding protein (E-FABP) is a lipid carrier, originally discovered in human epidermis. We show that E-FABP is almost exclusively expressed in post mitotic (PM) keratinocytes, corresponding to its localization in the highest suprabasal layers, while it is barely expressed in keratinocyte stem (KSC) and transit amplifying (TA) keratinocytes. Transfection of normal human keratinocytes with recombinant (r) E-FABP induces overexpression of K10 and involucrin. On the other hand, E-FABP inhibition by siRNA downregulates K10 and involucrin expression in normal keratinocytes through NF-κB and JNK signaling pathway. E-FABP is highly expressed in psoriatic epidermis, and it is mainly localized in stratum spinosum. Psoriatic PM keratinocytes overexpress E-FABP as compared to the same population in normal epidermis. E-FABP inhibition in psoriatic keratinocytes markedly reduces differentiation, while it upregulates psoriatic markers such as survivin and K16. However, under high calcium conditions, E-FABP-silencing downregulates K10 and involucrin, while survivin and K16 expression is completely abolished. These data strongly indicate that E-FABP plays an important role in keratinocyte differentiation. Moreover, E-FABP modulates differentiation in psoriatic keratinocytes.

Key words: E-FABP, keratinocytes, psoriasis, skin, subpopulations

INTRODUCTION

Epidermal differentiation is a physiological mechanism characterized by morphological changes and by the expression of a variety of markers. Changes in keratinocyte differentiation are associated with variation in lipid composition of epidermal layers (1). During this process, keratinocytes start to express lipid carriers, including high amounts of Epidermal-Fatty Acid Binding Protein (E-FABP) (2). E-FABP belongs to the family of FABP proteins and represents the epidermal subtype. E-FABP is not exclusively expressed in epidermis (3,4), but it is also detected in adipose tissue, endothelial cells, brain, liver, kidney and mammary tissue (5). E-FABP fulfills different roles, including fatty acid transport, control of fatty acids metabolism and cell migration (6). It also regulates cytokine productions (7), and it is related to all-trans retinoic acid sensitivity in cancer cells (8, 9). E-FABP is capable of binding other types of long-chain fatty acids and transporting them from the inner plasma membrane to different cell compartments (10). When fatty acids are transported to the nucleus, binding of FABPs to transcription factors of the PPAR family, may activate the differentiation process (10,11,12). E-FABP is involved in the differentiation mechanism of several cell types, such as T-helper cells, neural cells and mouse keratinocytes (13, 14, 15).

Psoriasis is an immuno-mediated, hyperproliferative disease characterized by abnormal differentiation of epidermis that also displays an altered calcium metabolism and a defective response to extracellular calcium gradients (16). E-FABP is overexpressed in psoriatic keratinocytes as compared to normal cells (3). Yet, a functional role of E-FABP in human keratinocyte differentiation both in normal and psoriatic epidermis remains to be determined.

Here, we show that either silencing or overexpressing E-FABP markedly affects keratinocyte differentiation. We also demonstrate that E-FABP is more expressed in post mitotic (PM) cells from psoriatic epidermis than in the same subpopulation from normal skin. Finally, silencing E-FABP alters differentiation in psoriatic keratinocytes.

METHODS

Cell culture

Human keratinocytes were obtained from neonatal foreskin, adult and psoriatic skin, and cultured in keratinocyte growth medium (KGM) (Lonza, Basel, Switzerland) as described previously (17). To induce differentiation cells were grown to 30-40% confluency and treated with 1.8 mM calcium chloride.

Keratinocyte subpopulations were obtained as previously shown (18,19). Briefly, total keratinocytes were first allowed to adhere to human type IV collagen-coated dishes for 5 minutes, to obtain a population enriched in keratinocyte stem cells (KSC). Keratinocytes adhering overnight were considered TA cells, as previously reported (19). Non adhering cells represent a population of terminally differentiated, post-mitotic (PM) keratinocytes.

Transfection of normal and psoriatic keratinocytes

Human keratinocytes, either normal or psoriatic, were plated in antibiotic free KGM medium. After 24 hours, cells were transfected with 75 nM FABP5 or scrambled siRNA (Thermo Scientific, Denver, CO), on-Target plus smart pool human FABP5 or on-Target plus siControl non-targeting pool, combined with Lipofectamin 2000 and Opti-MEM (both from Invitrogen, Paisley, UK), according to the manufacturer's instructions. Cells were transfected twice and used 48h later for Western Blotting. Psoriatic and normal human keratinocytes transfected with FABP5 siRNA were either treated or untreated with calcium 1.8mM immediately after transfection. Cells were lysed or MTT assay was performed on the cells 48h after treatment.

The protein delivery was performed on Gene Pulser System (Bio-Rad Laboratories Inc., CA). Normal human keratinocytes were grown under subconfluent conditions in KGM medium, detached from the dish and resuspended in 60 μl serum-free DMEM with (+rE-FABP) or without (-rE-FABP) 5 μg of rE-FABP, or with the same amount of a 18.8 kDa control protein (Cayman Chemicals, Ann Arbor, MI). Both recombinant proteins were purified and delipidated by the manufacturer (Cayman Chemicals) before use. The cell suspensions were transferred into two 0.1 cm sterile cuvettes (Bio-Rad), and left in ice for 10 minutes. The applied current was 100V, 250 μFD, 7.0 ms for +rE-FABP sample, 100V, 250 μFD, 6.9 ms

for control protein and 100V, 250 μFD, 6.4 ms for -rE-FABP sample. The treated cells were left in ice for 10 minutes and either lysed (0h) or transferred into 3X6 cm well plates. To each plate 5 ml of KGM medium was added and cells were lysed 48h after.

MTT assay

Cells were plated in a 96-well tissue culture plate (5,000 cells per well), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed at 48 and 120 hours after transfection. Proliferative cells were detected by incubating with MTT (Sigma-Aldrich) solution at 37°C for 4 hours. The formazan dye produced after DMSO solubilization was evaluated by a multiwell scanning spectrophotometer at 540 nm. The results are expressed as viability percentage, as compared to control. Results are calculated as the mean ± SD of three different experiments. Student's t-test was performed for comparison of the means.

Western blotting analysis

Total lysates from lesional and non-lesional psoriatic epidermis, and healthy epidermis were obtained as follows. Punch biopsies were washed with PBS and treated with dispase II (Roche, Basel, Switzerland) overnight at+4°C. The day after, epidermal sheets were separated from the dermis and homogenized in RIPA buffer. The study was conducted in accordance to the Declaration of Helsinki Principles, approved by the local Ethics Committees of the participating institutions. A total of 12 untreated psoriatic patients and 21 healthy controls were enrolled in the study.

Cultured keratinocytes were washed with PBS and harvested on ice in RIPA buffer at different time points or buffer for phosphorylated proteins (50mM Tris-HCl pH 8, 1% NP40, 0.5% sodium deoxycholate, 150mM NaCl, 0.1% SDS, 1mM Na3VO4, 5mM NaF). Western blotting was performed as previously described (18). Membranes were first incubated in blocking buffer then overnight at 4°C with primary anti-human FABP5 goat antibody (Ab) (1:1,000; R&D Systems, Inc. Minneapolis, MN), anti-human K10 rabbit Ab (1:50,000; Epitomics, Burlingame, CA), anti-human involucrin mouse mAb (1:6,000; Sigma-Aldrich), anti-human survivin rabbit Ab (1:1,000; Novus Biologicals, Littleton, CO), anti-human K16 mouse Ab (1:200; AbD Serotec, Kidlington, UK), anti-human S100A7 mouse Ab (1:400; Abcam, Cambridge, UK),

anti-human mouse NF-κB mAb (1:200; S.Cruz, Biotechnologies, Santa Cruz, CA, USA), anti-human rabbit P-NF-κB Ab (1:200; S.Cruz, Biotechnologies), anti-human rabbit active JNK1 Ab (1:5000; Promega, Madison, WI, USA), anti-human rabbit IKB-α Ab (1:500; Millipore, Billerica, MA, USA). After further washing in PBS/Tween 20, the membranes were incubated with peroxidase conjugated anti-goat IgG (1:5,000, Santa Cruz Biotechnologies), or anti-mouse IgG (1:2,000, Dako, Glostrup, Denmark) or anti-rabbit IgG (1:3,000, Dako) for 45 minutes at room temperature. Membranes were washed and developed using the ECL chemiluminescent detection system (Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, England). This experiment was repeated three times and the membranes were reprobed with a mouse monoclonal antibody to β-actin (1:3,000, Sigma-Aldrich) to assess total protein loading. The band intensity was quantitatively determined using ImageJ software, and protein levels intensity was normalized to β-actin expression.

Immunofluorescence

Keratinocytes were detached, fixed in 4% buffered PFA for 20 minutes and cytospun onto glass slides, as previously described (20). For cytoplasmic and nuclear staining, keratinocyte subpopulations were incubated with anti-FABP5 (1:50; ProteinTech Group) or anti-K10 (1:100, Epitomics) or anti-involucrin (1:200, Sigma Aldrich) or anti-ki67 Ab (1:200, Epitomics). Cells were then incubated with Alexa Fluor 546 anti-rabbit or Alexa Fluor anti-mouse 488 (Invitrogen Corporation). After three washes in PBS, slides were stained for 5 minutes with 1 μg/ml Dapi (Sigma-Aldrich), washed and covered with coverslips. Micrographs were taken on a Confocal Scanning Laser Microscopy (Leica TCS4D) (Leica, Exton, PA). Quantification of immunofluorescence staining was performed by analyzing six representative fields for each staining sample. Scoring was done by means of cell counting.

Real Time PCR

Total RNA was extracted from keratinocytes using TRI Reagent method performed as described by the manufacturer (Sigma-Aldrich). One microgram of total RNA extracted was reverse transcribed as described by the manufacturer (Roche). Nucleotide sequences of the oligomers used (MWG Biotech, Ebersberg, Germany) were: E-FABP-DP 5'-3': ATGGCCACAGTTCAGCAGCTG, RP 5'-3':

CAGGTGACATTGTTCATGAC; involucrin-DP 5'-3': GGACTGCCTGAGCAAGAATGTG, RP 5'-3': TAAGCTGCTGCTCTGGGTTT; K10-DP 5'-3': CCTTCGAAATGTGTCCACTGG, RP 5'-3': CAGGGATTGTTTCAAGGCCA; GAPDH-DP 5'-3':ACATCGCTCAGACACCATG, RP 5'-3':TGTAGTTGAGGTCAATGAAGGG. SYBR green Taq-DNA polymerase mixture (Roche, Basel, Switzerland) was used for Real Time PCR using an ABI 7500 (Applied Biosystems, Foster City, CA, USA). The differences in cycle number past the threshold (ΔCt) is reflective of differences in the initial template concentration in the different samples tested. Data are expressed as fold change relative to GAPDH. PCR was carried out at least three times for each sample and the experiment was performed in triplicate. Data from each sample were compared with stem cells or healthy PM cells, as calibrators, using the Sequence Detection Software, version 1.2.3, according to the Relative Quantification Study method (Applied Biosystems).

Statistical analysis

The Student's t-test was used to compare the average intensities of western blot bands, average viabilities and average cell counts. One or two asterisk indicate a significant difference, $0.01<p<0.05$ and $p<0.01$ respectively.

RESULTS

E-FABP is expressed in normal human post mitotic keratinocytes

As previously shown, E-FABP is expressed in the higher spinous layers with a weak pattern that intensifies moving upward to the outermost layers, cosistent with increased cell differentiation (Fig. S1a). To further evaluate E-FABP localization in epidermis, we separated keratinocyte subpopulations as previously shown (14) and analyzed E-FABP expression by western blotting (Fig. 1a), and E-FABP, K10, involucrin expression by Real Time PCR (Fig. 1c). E-FABP was mostly expressed in PM keratinocytes, while it was weakly detectable in TA and absent in KSC, both at the mRNA and protein level. This was also confirmed by immunofluorescence performed on keratinocyte subpopulations, analyzed immediately after separation. KSC expressed no E-FABP, involucrin or K10, while only few TA cells blandly expressed E-FABP. On the other hand, almost all PM cells expressed E-FABP and involucrin, while K10 was barely detected (Fig. 1b). Finally, K10 and involucrin were mostly expressed by PM cells, and weakly stained in KSC or TA cells (Fig. 1c)

E-FABP modulates differentiation in normal human keratinocytes through NF-kB and JNK1

Because E-FABP is expressed in PM cells, we asked whether this protein could be involved in keratinocyte differentiation. Increased E-FABP protein levels in keratinocytes cultured in presence of high calcium concentration or under confluent conditions have been previously shown (4). However, a direct correlation between RNA and protein levels has not been analyzed. We induced differentiation in human keratinocytes either by adding calcium to the medium or by growing cells to confluency. Under calcium-induced keratinocyte differentiation, E-FABP levels increased in a time-dependent manner up to 96h, both at the mRNA (Fig. S1f) and protein level (fig. S1b and d), in parallel with K10 and involucrin expression. E-FABP was also overexpressed in confluent keratinocytes from 24h up to 72h, as compared to pre-confluent cells, both at the mRNA (fig. S1g) and protein level (Fig. S1c and e). Similarly, both involucrin and K10 expression increased in confluent keratinocytes (Fig. S1c,e and g).

Although E-FABP is modulated during keratinocyte differentiation, the ability of E-FABP to stimulate human keratinocyte differentiation is not completely understood. We transfected normal human

keratinocytes with a recombinant E-FABP protein (rE-FABP). rE-FABP was successfully delivered to the cells, as confirmed by the 18kDa band, corresponding to the exogenous protein, at 0h. At the same time point, a band corresponding to endogenous E-FABP was equally expressed in -rE-FABP, +rE-FABP cells and cells transfected with the control protein (Fig. 2a). On the other hand, at 48h, no band corresponding to rE-FABP was visible, while a 10 kDa band appeared, possibly indicating a degradation or a modification of the recombinant protein. At this time point, cells transfected with rE-FABP overexpressed K10 and involucrin, while the same cells transfected with a control protein fail to induce K10 and involucrin increase (Fig. 2b).

Because E-FABP induces a modulation of differentiation-marker expression, we wanted to confirm this finding by silencing E-FABP protein. We transfected human keratinocytes with a FABP5-specific siRNA in presence or absence of calcium chloride. As shown in figure 2d, E-FABP protein was successfully downregulated by siRNA delivery up to 72h after transfection. E-FABP knockdown induced a reduction of K10 and involucrin. In presence of calcium, scramble siRNA but not E-FABPsiRNA induced up-regulation of K10 and involucrin expression. Moreover, E-FABP down-regulation induced no changes in cell viability at 48 and 120h after transfection (Fig. S2a). Similarly, confocal microscopy confirmed the reduction of K10 in siRNA treated cells, and revealed that proliferation rate was not affected by E-FABP silencing, as shown by the unchanged expression of Ki67 (Fig. 2c). A semi-quantitative analysis of K10 and Ki67 staining is reported in figure S2b. To further investigate the role of E-FABP in differentiation, we studied NF-kB/JNK1 signaling pathways in human keratinocytes transfected with scramble siRNA or E-FABP siRNA, in presence or absence of calcium. As shown in Fig. 2d and S2c, E-FABP silencing reduced the levels of activated NF-κB expression (represented by the phosphorylated form of the protein, P- NF-κB) but not of total NF-κB. The effect on P-NF-κB was even more evident in presence of calcium. On the other hand, E-FABP downregulation increased IkBα expression, in particular in presence of calcium. We also evaluated active-JNK1 expression in siRNA treated keratinocytes and found that decreased levels of In addition, E-FABP siRNA reduced active-JNK1 expression, while calcium failed to induce JNK1 activation. Altogether these data suggest that lack of E-FABP decreases differentiation markers through NF-kB and JNK signaling pathway.

E-FABP expression in psoriatic keratinocyte subpopulations

Psoriasis is characterized by altered keratinocyte differentiation, and E-FABP expression appears to be increased in psoriatic epidermis. E-FABP was more expressed in psoriatic skin as compared to healthy skin *in vivo* (Fig. S3a and b). This was also confirmed by western blotting, showing that lesional psoriatic epidermis expresses higher levels of E-FABP, as compared to non-lesional psoriatic or healthy epidermis, partially mimicking the expression of involucrin (Fig. 3a and b). Consistently, in psoriatic sections, E-FABP and involucrin were mostly detected in high spinous layers (Fig. S3b). Moreover, S100A7 and K16 were exclusively expressed in psoriatic epidermis, their expression partially overlapping E-FABP localization in the high spinous layers (Fig. S3b). In addition, cytoplasmic survivin, a marker of KSC, was expressed in the basal layer of normal epidermis, while it was expressed also in suprabasal psoriatic keratinocytes with an almost exclusively nuclear pattern (Fig. S3a and b).

To further evaluate the differential expression of E-FABP in normal and psoriatic epidermis, we separated keratinocyte subpopulations and evaluated E-FABP expression by Real Time PCR and western blotting. At the mRNA and protein levels, E-FABP was mostly expressed in PM psoriatic keratinocytes, while it was almost undetectable in KSC and TA cells (Fig. 3c, f and S3c respectively). This was in line with increased mRNA levels of K10 and involucrin in psoriatic PM keratinocytes as compared to KSC and TA cells (Fig. 3d and e). In addition, by comparing equal amounts of PM cell lysates from healthy and psoriatic skin, we found that psoriatic PM cells expressed significantly higher levels of E-FABP as compared to normal PM cells both at the protein (Fig. 3g and S3d) and mRNA level (Fig. 3h).

E-FABP modulates differentiation and psoriatic markers in psoriasis

Although psoriatic keratinocytes are difficult to culture (21), low calcium and serum-free conditions allow to culture psoriatic cells for multiple passages (22,16). Under these conditions, low passage psoriatic keratinocytes expressed higher levels of K10, K16 and S100A7 than normal keratinocytes (Fig. 4a). To further evaluate E-FABP role in psoriasis, we treated psoriatic keratinocytes with a siRNA specific for E-FABP, with or without high calcium concentration. In absence of calcium, E-FABP silencing induced a slight decrease of K10 and involucrin expression, while psoriatic markers survivin and K16 were up-regulated. Upon high calcium conditions, in E-FABP siRNA treated psoriatic keratinocytes, K10 and

involucrin expression were further down-regulated, survivin was markedly reduced, while K16 expression was completely abolished (Fig. 4b and c).

DISCUSSION

In this work, we show that E-FABP is abundantly expressed in PM keratinocytes both in healthy and psoriatic epidermis. Moreover, decreased E-FABP expression deranges keratinocyte differentiation in normal and psoriatic cells. E-FABP is localized in suprabasal layers of healthy epidermis, with increased expression in the spinous and granular layers. The lipid composition of cell membranes changes during keratinocyte differentiation, increasing from suprabasal layers to the more differentiated stratum granulosum and corneum. It has been previously suggested that E-FABP is a potential marker of TA keratinocytes in human epidermis (5). However, the method by O'Shaughnessy and colleagues does not distinguish between TA and PM subpopulations, including both cell subsets in the Non-Adherent pool of keratinocytes. By separating KSC from TA and PM cells based on β1-integrin expression levels, as previously reported (18,19), we show that E-FABP protein is highly expressed in PM cells, while it is barely detected in TA and KSC. Indeed, E-FABP is expressed in the highest epidermal layers and PM keratinocytes are terminally differentiated cells that do not proliferate *in vitro* (18). This result indicates the possibility of a tight correlation between E-FABP function and the state of keratinocyte differentiation. Keratinocyte differentiation is induced by high-calcium conditions and in confluent cells. We show that, when keratinocytes are induced to differentiate, E-FABP mRNA and protein increase, along with a higher expression of involucrin and K10 protein, suggesting that not only E-FABP is mainly localized in differentiated cells *in vivo*, but it also increases when differentiation is promoted in culture.

It remained to be determined whether E-FABP is able to induce differentiation in human epidermis (23). In the neural system, E-FABP stimulates PC12 cells differentiation by inducing neurite extension (14). There is also evidence that E-FABP is actively involved in the differentiation program of murine keratinocytes (4,24). E-FABP deficient mice display defects in water barrier functions, albeit having normal skin morphology and no major defects in other organs (23, 25). This seems to be in contrast with a possible role for E-FABP in keratinocyte differentiation. However, keratinocytes obtained from these mice show reduced expression of differentiation markers such as K1, involucrin and loricrin, in absence of exogenous ligand, and decreased susceptibility to calcium-induced differentiation, as compared to E-FABP +/+ cells (15). These findings suggest the presence of still unknown compensatory mechanisms in

mice lacking E-FABP expression, which lead to normal development of epidermis. In line with E-FABP involvement in the differentiation process of keratinocytes, it has been recently shown that, once bound to a specific ligand, E-FABP is able to physically interact with the transcription factor PPARβ, thus activating the differentiation program in mouse keratinocytes (13). However, to date no data on human keratinocytes indicate an active role of E-FABP in keratinocyte differentiation. In this paper, we show that administration of rE-FABP to human keratinocytes induces an increased expression of K10 and involucrin. The fact that E-FABP is able to act in absence of ligand could be accounted for by some residual fatty acids in the media. Treatment of keratinocytes with rE-FABP also results in the appearance of a smaller 10kDa E-FABP protein. This may be due to the degradation of the recombinant protein itself (26), or the product of a post-translational modification of rE-FABP. The role of E-FABP in keratinocyte differentiation was confirmed by silencing with specific E-FABP siRNA. As expected, E-FABP inhibition induced a down-regulation of both K10 and involucrin, indicating that E-FABP stimulates human keratinocyte differentiation. E-FABP depletion does not influence keratinocyte viability that appears to be slightly affected by decreased differentiation, in line with previous findings (15). In agreement with recent reports in mice showing that reduced NF-kB activity is responsible for decreased K10 expression in E-FABP -/- mouse keratinocytes (15), we report that we report that repression of differentiation induced by E-FABP silencing is associated with a reduction of activated NF-kB expression in normal human keratinocytes. However, calcium treatment did not influence NF-kB expression, as previously suggested (27).

In mouse epidermis, activation of PPAR-β/γ signaling, which is mediated also by E-FABP binding, reproduces a psoriasis-like phenotype (26). Psoriasis is a skin disease with defects in cell differentiation and proliferation. Here we show that E-FABP is abundantly expressed in psoriatic epidermis as compared to normal skin, as previously reported (4,28). In normal human skin, E-FABP is mainly expressed in granular layers, with a decreased staining from upper to lower spinous layers. By contrast, in psoriatic skin, E-FABP is highest in spinous layers, thus behaving as other differentiation markers, such as involucrin and K10, suggesting a possible involvement of E-FABP in the altered differentiation process observed in psoriasis. It has been shown that increasing the proliferation time period of TA

keratinocytes generates a psoriatic phenotype, implying that psoriasis possibly originate by alteration of TA cells (29). On the other hand, in the present study, psoriatic PM keratinocytes express higher levels of E-FABP than normal PM cells. This suggests that E-FABP identifies keratinocytes that have concluded the differentiation program and that do not contribute to the proliferative compartment of the skin. Recent works show that cultured psoriatic keratinocytes retain some characteristics of the disease under optimized culture conditions (30). We show that psoriatic keratinocytes at early passages, under low-calcium and serum-free conditions, express K16 and S100A7. Downregulation of E-FABP in these cells reduces involucrin and K10 expression, thus showing that E-FABP mediates differentiation also in psoriatic cells. Not only E-FABP mediates psoriatic keratinocyte differentiation, but E-FABP depletion also up-regulates both K16 and survivin, which are overexpressed in psoriatic cells i*n vivo*. Thus, E-FABP seems to play an important role in the dysregulated epidermal homeostasis observed in psoriasis. This effect is completely abrogated when cells are treated with high calcium. Psoriatic keratinocytes in culture display an inborn defect in calcium metabolism, thus being not responsive to high extracellular calcium (16). E-FABP down-regulation further reduces psoriatic keratinocytes sensitivity to calcium, leading to decreased differentiation and to reduced expression of psoriatic markers. All together, these data suggest that E-FABP modulation may alter calcium metabolism and uptake in psoriasis.

We can conclude that E-FABP is able to induce normal human keratinocyte differentiation, and that its up-regulation could partially be involved in the altered differentiation mechanism in psoriasis.

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Conflict of Interest

The authors state no conflict of interest.

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LEGENDS FOR ILLUSTRATIONS

Figure 1. E-FABP distribution in human keratinocytes

(**a**) Keratinocytes subpopulations from adult healthy individuals were isolated as described in Material and Methods and cultured in KGM for one week. Protein extracts from KSC, TA and PM cells were immunoblotted with anti-E-FABP antibody. Western blotting is representative of three independent experiments. β-actin was used as a loading control. (**b**) Keratinocyte subpopulations were separated, immediately fixed and stained. Confocal microscopy analysis of involucrin, K10 and E-FABP in KSC, TA and PM keratinocytes. Bars= 50 μm. (**c**) Normal human keratinocytes were cultured as in **a**. RNA was extracted and E-FABP, K10 and involucrin mRNA were analyzed by Real Time PCR. KSC were used as calibrators. Student's *t*-test was used for comparison of the means: *p<0.05 and **p<0.01

Figure 2. E-FABP induces differentiation in normal human keratinocytes

(**a**) Normal human keratinocytes were transfected with or without rE-FABP, and lysed at 0h. (**b**) Cells were also transfected with or without rE-FABP and with or without control protein, and lysed at 48h after transfection. Western blotting for K10, involucrin and E-FABP expression was performed. (**c**) Representative picture of Ki67 and K10 staining in scrambled and E-FABP siRNA treated keratinocytes 48h after transfection. Bars= 20 μm. (**d**) Human keratinocytes were transfected with E-FABP or scrambled siRNA in presence or absence of calcium chloride. Cell lysates were analyzed 72h after calcium addition for E-FABP, K10, involucrin, active-JNK1, P-NF-κB, total NF-κB and IkBα expression. β-actin was used as a loading control.

Figure 3. E-FABP expression in psoriatic keratinocytes subpopulations

(a) Total lysates from normal, lesional (L) and non-lesional (NL) psoriatic epidermis were analysed by western blotting for involucrin and E-FABP expression. (**b**) Relative intensity of the results of **a.** Keratinocytes subpopulations from lesional psoriatic epidermis were separated and cultured for one week. Cells were lysed for RNA extraction and Real Time PCR was performed on cDNA for E-FABP **(c)**, K10 (**d**) and involucrin (**e**) expression. GAPDH was used as a loading control. Psoriatic KSC were used

as calibrators. (**f**) Cells were also lysed and analysed by western blotting for E-FABP protein expression. Lysates from PM cells obtained from normal and psoriatic epidermis were compared by western blotting (**g**) and Real Time PCR (**h**), for E-FABP expression. Healthy PM cells were used as calibrators. Student's *t*-test was used for comparison of the means: ***p*<0.01

Figure 4. E-FABP silencing reduces differentiation in psoriatic keratinocytes

(a) Total keratinocytes from lesional psoriatic and normal human epidermis were cultured under low calcium, serum-free conditions for one week. Cells were then harvested and lysed for western blotting. **(b)** Psoriatic keratinocytes were transfected either with E-FABP or scramble siRNA, and cultured with or without high calcium. Cell lysates were collected 48h after calcium addition and analyzed by western blotting. β-actin was used as a loading control. **(c)** Relative intensity of the results of **b.**

Figure S1.

(a) Immunohystochemical analysis of normal adult human skin for E-FABP expression. On the left: negative control. Bars= 70 μm. **(b)** Normal human keratinocytes were cultured under subconfluent conditions with or without high calcium. Protein were extracted and western blotting analysis was performed for involucrin, K10 and E-FABP expression. (**c**) Normal human keratinocytes were cultured under preconfluent or confluent conditions and cells were lysed at the indicated time points. Western blotting was performed on the cell lysates. β-actin was used as a loading control. (**d**) Quantitative representation of the results of **b**. (**e**) Quantitative representation of the results of **c**. (**f**) Normal human keratinocytes were cultured as in **b** for 48h. RNA was extracted and E-FABP, K10 and involucrin mRNA was analyzed by Real Time PCR. Control was used as calibrator. (**g**) Normal human keratinocytes were cultured under pre-confluent or confluent conditions as in **c**. RNA was extracted, retro-transcribed and amplified by Real Time PCR for the evaluation of E-FABP, K10 and involucrin expression. Subconfluent cells were used as calibrator. Student's *t*-test was used for comparison of the means: **p*<0.05 and ***p*<0.01

Figure S2. (a) Viability of normal human keratinocytes transfected with E-FABP or scrambled siRNA has been evaluated by MTT assay at the indicated time points. **(b)** Scrambled and siRNA treated keratinocytes were stained for Ki67 and K10 48h after transfection. Percentage of Ki67 and K10 positive cells is represented (see Fig. **2c**). **(c)** Relative intensity of the results in Figure **2d.** Student's *t*-test was used for comparison of the means: *p<0.05 and **p<0.01

Figure S3. (a) Staining of survivin, K10, involucrin, E-FABP, S100A7 and K16 in normal human skin. (**b**) Staining of the markers as in **a** in lesional psoriatic skin. Bars= 70 μm. (**c**) Relative intensity of the results in Figure **3f.** (**d**) Relative intensity of the results in Figure **3g.** Student's *t*-test was used for comparison of the means: *p<0.05 and **p<0.01

SUPPLEMENTARY MATERIALS

Immunohistochemistry

Healthy and psoriatic skin biopsies were 4% buffered formalin fixed and paraffin embedded. 4μm tissue sections were cut and deparaffinized in xylene and rehydrated in graded alcohol. The slides were first boiled in citrate buffer, pH6, for 20 minutes in a standard pressure cooker. Expression of survivin, E-FABP, K10, involucrin, K15, K16, S100A7 was examined immunohistochemically by incubating the samples for 1h at room temperature with the following antibodies: FABP5 antibody (1:50, ProteinTech Group Inc, Chicago, IL), cytokeratin 10 antibody (1:100, Epitomics), involucrin antibody (1:200, Sigma-Aldrich), cytokeratin 16 antibody (1:30 AbD Serotech), S100A7 antibody (1:50, Abcam), survivin antibody (1:100, Abcam). After three 5-minute washes in PBS, slides were incubated with secondary antibody for 45 minutes at room temperature followed by incubation with naphtol phosphate substrate for 10 minutes as suggested by the datasheet (Lab Vision Corporation, Fremont CA). Negative controls were obtained by omitting primary antibody.