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Expression of S100A8/A9 in HaCaT keratinocytes alters the rate of cell proliferation and differentiation

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List of abbreviations

AA, arachidonic acid; CFDA, carboxy-fluorescein diacetate succinimidyl ester; FACS, fluorescence-activated cell sorting; ROS, reactive oxygen species

Abstract

S100A8/A9 promotes NADPH oxidase in HaCaT keratinocytes and subsequently increases NFκB activation, which plays important roles in the balance between epidermal growth and differentiation. S100A8/A9-positive HaCaT cells present with a significantly reduced rate of cell division and greater expression of two keratinocyte differentiation markers, involucrin and filaggrin, than control cells. S100A8/A9 mutants fail to enhance NFκB activation, TNFα-induced IL-8 gene expression and NFκB p65 phosphorylation, and S100A8/A9-positive cells demonstrate better cell survival in forced suspension culture than mutant cells. S100A8/A9 is induced in epithelial cells in response to stress. Therefore, S100A8/A9-mediated growth arrest could have implications for tissue remodeling and repair.

1. Introduction

The epidermis is a stratified squamous epithelium that undergoes continuous self-renewal to maintain its protective function. In the course of homeostasis, dividing cells in the basal layer continually execute a program of terminal differentiation, move outwards and are sloughed from the skin surface [1]. Growth factors modulate overall skin homeostasis but the intracellular signaling pathways responsible for controlling the balance between proliferation and differentiation in keratinocytes are largely unknown.

A number of gain or loss of function studies in mice have demonstrated that NFκB and components of this system including IKK-α are important for balancing growth and differentiation in the epidermis [2]. Growth of the epidermis is inhibited in mice over-expressing a constitutively nuclear form of the NFκB subunit p50 in skin cells [3]. Knocking out IKKα results in a severe cutaneous phenotype characterized by incomplete epidermal differentiation [4] and mice with an epidermis-specific deletion of IKK-β develop a severe inflammatory skin disease caused by a TNFα-mediated, αβ T cell-independent inflammatory response that develops shortly after birth [5]. Conversely, over-expression of IκB results in hyperplasia and inflammation, and leads to squamous cell carcinomas [6].

The NFκB family includes the p65, p50 and c/rel proteins, which can homodimerize and heterodimerize among themselves [7]. These proteins are stored latently in the cytoplasm

bound to the inhibitory protein IκB. A number of stimuli including TNFα activate IKKs, kinases that phosphorylate IκB and induce its degradation. The degradation of IκB results in activation and nuclear translocation of NFκB. However, redox mechanisms also play a role in NFκB activation [8]. Recently, we demonstrated that increased levels of reactive oxygen species (ROS) caused by the interaction of the S100 proteins, S100A8 and S100A9, with epithelial NADPH oxidases resulted in increased NFκB activation followed by increased IL-8 gene expression [9]. This study is consistent with the view that NFκB is a redox-sensitive transcription factor.

S100A8 and S100A9 belong to the multigenic S100 protein family, which consists of non-ubiquitous cytoplasmic Ca^{2+}-binding proteins of the EF-hand type [10] that are predominantly expressed in myeloid cells [11]. In phagocytes, S100A8 and S100A9 form heteromeric complexes that bind polyunsaturated fatty acids such as arachidonic acid (AA) in a calcium-dependent manner [12]. S100A8/A9 participates in phagocyte NADPH oxidase activation by transferring AA to membrane-bound Nox2 ($gp91^{phox}$) [13]. AA is an essential factor for NADPH oxidase as it binds to gp91phox, which induces structural changes in cytochrome b_{558}.

Normal S100A8 and S100A9 are expressed at minimal levels in the epidermis. However, their expression is transiently induced in keratinocytes after epidermal injury [14, 15] and UVB irradiation [16], and the proteins are expressed at extremely high levels in psoriatic keratinocytes [17]. Furthermore, their expression is induced by pro-inflammatory cytokines such as TNFα and IL1β. Recently, we presented evidence that a complex of Poly (ADP-ribose) polymerase (PARP-1) and Ku70/80 drives stress response-specific S100 gene expression [18].

The stress response-induced expression of the S100 proteins points to an important role in skin pathology. Therefore, the present study was undertaken to investigate the cellular consequences of S100A8/A9 over-expression in epithelial cells. S100A8/A9-over-expressing HaCaT keratinocytes were analyzed with respect to cellular responses including proliferation, cell survival and differentiation.

2. Material and Methods

2.1 Plasmids

Construction of pVIVO-S100A8/A9 (H103,104,105K106A), pVIVO-S100A8/A9 (T113A), pVIVO-S100A8/A9 and pVITRO-S100A8/A9 was performed as previously described [9]. All constructs were tested by sequencing.

2.2. Cell culture

HaCaT (human keratinocyte cell line) cells were cultured in Dulbecco's modified Eagle's medium (Biochrom AG, Germany) containing 10% heat-inactivated fetal bovine serum (Biowest, France), 100 units of penicillin (Biochrom AG, Germany) and 2mM L-glutamine (Biochrom AG, Germany) at 37°C in a humidified atmosphere containing 5% CO_2. MCF-7 (human breast adenocarcinoma cell line) cells were cultured in 90% RPMI 1640 containing 10% heat-inactivated fetal bovine serum, MEM non-essential amino acids, 1 mM sodium pyruvate and 10 μg/ml human insulin at 37°C in a humidified atmosphere containing 5% CO_2. The Ethical Committee of the University Hospital, Muenster, Germany, approved all the studies described. The research was conducted according to the Declaration of Helsinki Principles.

2.3. Transient expression of S100A8 and S100A9

Transient transfection of HaCaT cells was performed as previously described [9]. Cell viability was determined 12 h post-transfection using the trypan blue exclusion assay. S100A8 and S100A9 expression was confirmed by western blotting using antibodies directed against each protein.

2.4. Luciferase activity assays

NFκB luciferase reporter assays were performed as previously described [9]. Luminescence was determined using a Centro LB 960 luminometer and experiments were performed in triplicate. The luminescence of non-stimulated cells was subtracted from that of TNFα-

stimulated cells, and the data were normalized relative to β-galactosidase activity. The data are presented as mean ± S.E.M.

2.5. Fluorescence-activated cell sorting (FACS) Staining

HaCaT cells were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFDA, C-1157, Molecular Probes). After 24h, CFDA-labeled cells were transfected with pVITRO-S100A8/A9. For transfection, 2x 10^6 CFDA-labeled HaCaT cells were seeded on to a 10 cm plate. Prior to the addition to cells, polyethylenimine (PEI, 408727, Sigma Aldrich) was mixed with 36μg DNA in Opti-Mem (without FBS) and incubated for 30 min at room temperature. After cultivation for various time intervals, cells were fixed using BD Phosflow Fix Buffer I and Perm Buffer III as recommended by the manufacturer.

For FACS analysis, cells were stained at 4°C for 30 min with 1 μg of S100A9-specific rabbit polyclonal antibody and 1μg of various mouse monoclonal antibodies including anti-filaggrin (Dianova, #DLN 09496), anti-involucrin (Dianova, #DLN 07099) and Alexa Fluor 647–conjugated mouse anti-pS529-NFκB p65 (BD, #558422). The cells were washed with wash/staining solution, and 1 μg of secondary Cy3-conjugated goat anti-rabbit antibody (Jackson Immuno Research, #111-166-003) was added with 1μg Alexa 647-conjugated goat anti-mouse antibody (Invitrogen, #A21236); for some experiments, directly-conjugated antibodies were used after incubation at 4°C for 30 min. Cells were washed twice, resuspended in 500 μL wash/staining solution, and analyzed using a FACS Calibur flow cytometer equipped with CellQuestPro software (BD PharMingen). There was no non-specific fluorescence between Alexa 647 and Cy3 staining. Each analysis included at least 10,000 events. Quadrants were set according to negative control staining with mouse IgG1 as an isotype control for monoclonal antibody and rabbit IgG as an isotype for the polyclonal antibody, respectively. Values within dot plots indicate the percentage of cells in the respective quadrants. The relative antigen expression indicated was calculated as the percentage of antigen-expressing cells in either S100A9-positive or negative cell subgroups.

2.6. Quantitative PCR

Total RNA was extracted using an RNA Isolation Kit (Qiagen, Germany) and first strand cDNA was synthesized according to common molecular biological techniques. The mRNA levels of genes of interest were calculated by real-time PCR using specific primers:

IL-8(forward) 5´ CTTGTTCCACTGTGCCTTGGTT-3` and
IL-8(reverse) 5´-GCTTCCACATGTCCTCACAACAT-3´;

The C_T values of genes of interest were normalized to GAPDH. IL-8 gene expression is given in relation to the corresponding IL-8 gene expression in non-stimulated cells: ΔC_T IL-8 (TNFα) - ΔC_T IL-8 (non-stimulated) = $\Delta\Delta C_T$ IL-8 (TNFα).

2.7. Anchorage-independent survival assay

HaCaT cells were transfected with empty pVITRO or pVITRO-S100A8/A9wt expression plasmids. After 24h (or extended incubation intervals) the cells were trypsinized, resuspended in 2 ml medium and added to plates pre-coated with 0.9% agarose (10^4cells per well), with or without TNFα (10-50 ng/ml) and NFκB inhibitor III (10 μmol/L) for 24h. Aliquots of cells were reseeded in fresh medium on tissue culture–treated plastic. Crystal violet (0.005%) staining of re-attached viable cells was carried out 24h later. Crystal violet was extracted with 1 ml 95% ethanol and quantified by the absorbance at 560 nm.

2.8. Stable transfection of MCF-7 cells with S100A8 and S100A9

For stable transfection, MCF-7 cells were seeded in 6-well plates 24h prior to transfection ($2.5x10^5$ cells/well). Two μg of pVITRO-S100A/8A9 plasmid DNA was mixed with 9 μl of Lipofectamine transfection reagent and transfected into the cells according to the manufacturer's instructions. Empty pVITRO vector was transfected as a control. Twenty-four hours after transfection, the cells were trypsinized, diluted, and placed in 96-well plates. Transfected cells were selected with 500 μg/ml hygromycin. Individual hygromycin-resistant clones were screened for S100A8 and S100A9 using western blotting. Ten S100A8/A9-

positive clones were pooled for further characterization. Morphological changes were observed and photographed with a phase-contrast microscope.

2.9. SDS-PAGE and Western Blot Analysis

SDS-PAGE and western blotting were performed according to standard procedures and developed using the ECL PlusTM western blotting detection reagents from Amersham Biosciences.

2.10. BrdU immunohistochemistry proliferation assay

The bromodeoxyuridine (BrdU) immunohistochemistry proliferation assay kit was purchased from Calbiochem Inc. (San Diego, CA). For BrdU assays, MCF7wt and MCF-7/a8a9 (10^7) cells were starved for 48h with medium containing 0.2% FCS followed by the addition of medium containing 10% FCS. After 24h, 1 μM BrdU was added for 16h and the BrdU immunohistochemistry proliferation assay was conducted according to the manufacturer's instructions.

2.11. Data analysis

Data are presented as mean ± S.E.M. Statistical significance between means was established using a Student's t-test, and probability values of $p < 0.05$ were considered to represent significant differences.

3. Results and Discussion

3.1. S100A8/A9-overexpression leads to reduced proliferation in HaCaT keratinocytes

Among the pathways with important roles in the regulation of keratinocyte growth and differentiation is the NFκB pathway. NFκB activation has been demonstrated to induce growth arrest in epithelial cells opposite immune cells [3] and recently our group demonstrated that S100A8/A9 promotes epithelial NADPH oxidase(s) and subsequent increases NFκB activation [9]. Therefore, the effect of S100A8/A9 over-expression on cell proliferation was investigated by analyzing the rate of cell division using the carboxy-fluorescein diacetate succinimidyl ester (CFDA) labeling technique. HaCaT keratinocytes were labeled with CFDA and transfected with pVITRO-S100A8/A9. Cells were cultured for three and 13 days, and cell division / proliferation was monitored using fluorescence-activated cell sorting (FACS). To discriminate between S100A8/A9-expressing and non-expressing cells, intracellular S100A9 was stained using a S100A9-specific polyclonal antibody.

Proliferation of S100A9-positive and S100A9-negative cells after three and 13 days is presented in **Fig. 1**. Dot plots demonstrating CFDA fluorescence versus S100A9 antigen expression in HaCaT keratinocytes cultured for three (**Fig. 1A**) and 13 days (**Fig. 1B**) show the characteristic decline in CFDA fluorescence in S100A9-negative cells indicative of cell division; the degree of shift in S100A9-positive HaCaT keratinocytes was significantly less pronounced by day 13.

Representative proliferation of S100A9-negative HaCaT keratinocytes demonstrated a fairly uniform bell-shaped distribution of fluorescence staining at day three (**Fig. 1C**, gray curve). By day 13 (open curve), the mean fluorescence was strongly shifted to the left, indicative of cell division. However, the degree of the shift was lower in S100A9-HaCaT keratinocytes than in S100A9-negative cells (**Fig. 1D**). The delay in the leftward shift associated with proliferation was evident as early as day three (gray curve) and was pronounced by day 13 (open curve). Therefore, S100A8/A9-expressing HaCaT keratinocytes continued to divide, albeit at a significantly slower rate than S100A8/A9-negative cells. More detailed analysis is presented in **Fig. 1S**.

3.2. S100A8/A9 expression interferes with NFκB activation

To confirm the anti-proliferative effect of S100A8/A9-mediated NFκB activation, whether S100A8/A9 expression affected NFκB p65 phosphorylation was investigated. HaCaT keratinocytes were transfected with pVITRO-S100A8/A9 and cultured for two and six days. For FACS analysis, cells were double-stained with K10-895.12.50 monoclonal antibody, which recognizes the phosphorylated serine 529 in the transactivation domain of the human NFκB p65 subunit, and S100A9-specific polyclonal antibody (**Fig. 2A-C**). Isotype and secondary antibodies revealed no background staining (data not shown).

The results presented in **Figs. 2A-C** demonstrate NFκB p65 phosphorylation detected using FACS in S100A9-positive HaCaT keratinocytes compared with results for S100A9-negative cells. The increase in NFκB p65 phosphorylation was evident as early as day two but was not statistically significant (**Figs. 2A** and **C**). However, by day six the pronounced increase in NFκB p65 phosphorylation was significant (p-value < 0.05; $n = 5$; **Figs. 2B** and **C**).

Importantly, this effect did not rely on the release of S100A8/A9 and its binding to cell surface receptors. This was confirmed by immunoprecipitation using the S100A8/A9-specific monoclonal antibody, 27E10 (data not shown). Furthermore, HaCaT cells were transfected with NF-κB luciferase reporter plasmids and stimulated with various concentrations of exogenous S100A8/A9. However, no increase of luciferase activity was detected (data not shown).

Complementation of HaCaT cells with S100A8/A9 results in a significant increase in NADPH oxidase activity and enhanced NFκB activation [9] in accordance with NFκB being a redox-sensitive transcription factor [8]. For further confirmation, the promoting effect of S100A8/A9 on NFκB activation was analyzed using a NFκB reporter assay. HaCaT keratinocytes were transfected with pVITRO-S100A8/A9 and stimulated with increasing concentrations of TNFα for 3h (**Fig. 2D**). β-galactosidase activity measurements demonstrated that the cells were transfected with comparable efficiency (**Fig. 2D inset**). The luciferase assay revealed that TNFα-induced reporter expression of NFκB was significantly higher in HaCaT cells after complementation with S100A8/A9wt (**Fig. 2D right panel**, black

bars) than in mock-transfected cells (**Fig. 2D left panel**, gray bars). Luciferase activity in mock- and S100A8/A9wt-transfected cells increased with increasing concentrations of TNFα. Optimal NFκB activation was achieved at 50 ng/ml TNFα.

Two distinct features of S100A8/A9 are involved in promoting NADPH oxidase activity: (1) the unique C-tail of S100A9 containing a motif comprising four consecutive basic amino acid residues (His103-His105, Lys106) to which the fatty acid carboxyl-group of arachidonic acid, an essential cofactor for NADPH oxidase activation, is bound [12, 13]; (2) the Thr113 residue of S100A9 that is phosphorylated upon cellular activation [11]. Therefore, HaCaT keratinocytes were co-transfected with NF-κB luciferase reporter plasmid and expression plasmids for S100A8/A9 wild-type (pVIVO-S100A8/A9wt) and the two S100A8/A9 mutants (pVIVO-S100A8/A9-T113A or pVIVO-S100A8/A9-H103-105K106A) followed by stimulation with 50 ng/ml TNFα for 4h (**Fig. 2E**). Co-transfection with β-galactosidase-expressing plasmid was used as an internal control to verify efficiency of transfection.

Western blotting demonstrated similar expression of S100A9wt and mutants in transfected HaCaT cells (data not shown). The β-galactosidase activity measurements demonstrated that the transfection efficiencies were comparable (data not shown). The luciferase reporter assay revealed that TNFα-induced reporter expression was significantly higher in S100A8/A9wt-overexpressing HaCaT cells than in S100A8/A9 mutant-transfected cells ($P < 0.05$) (**Fig. 2E**). Minimal reporter expression was observed if either S100A8/A9 mutant or S100A8/A9 wild type-over expressing HaCaT cells were co-transfected with the negative control plasmid (data not shown). TNFα-induced reporter expression was completely inhibited by the NFκB inhibitor III (data not shown). The data indicate that S100A8/A9-enhanced NADPH oxidase affects downstream signaling including NFκB activation.

IL-8 is expressed at very low levels in resting cells but its production is rapidly induced by pro-inflammatory cytokines, bacterial or viral products and cellular stress, via NFκB activation [9]. Therefore, TNFα-induced IL-8 expression in S100A8/A9wt-over-expressing and S100A8/A9 mutant-transfected cells was investigated using real-time PCR.

As demonstrated in **Fig. 2F**, IL-8 gene expression is increased $n^{(6.5-4.0)} = 5.7$ and $n^{(6.5-3.2)} = 9.8$-fold in S100A8/A9wt-over-expressing HaCaT keratinocytes compared with S100A8/A9 (T113A) or S100A8/A9 (H103-105K106A)-transfected cells, respectively. These data indicate the involvement of both S100 phosphorylation and arachidonic acid-binding in TNFα-induced NFκB activation of HaCaT keratinocytes.

NFκB activity enhances HaCaT cell survival in forced suspension culture [19]. Therefore, mock- and pVITRO-S100A8/A9-transfected HaCaT keratinocytes were compared in the anchorage-independent survival assay (**Fig. 2G**). Consistent with the previous finding, TNFα-treated HaCaT cells presented with enhanced cell survival in forced suspension culture that was abrogated in the presence of NFκB inhibitor III, indicating that cell survival was accomplished by up-regulating NFκB activity. Significantly more S100A8/A9-over-expressing HaCaT cells survived in the absence of TNFα than in mock-transfected cells. This is consistent with the results obtained using NFκB reporter assays and FACS analysis using the anti-phospho p65-NFκB (K10-895.12.50) monoclonal antibody.

3.3. S100A8/A9-overexpression triggers expression of involucrin and filaggrin

Studies on mice have demonstrated an important role for NFκB in keratinocyte proliferation, differentiation and growth arrest (senescence). NFκB is present in all epidermal layers but is present only in the cell nucleus in suprabasal layers, further implicating NFκB in epidermal differentiation [3]. Filaggrin is one of the key proteins that facilitate terminal differentiation of the epidermis and formation of the skin barrier; keratinocytes process profilaggrin into filaggrin and undergo specific changes that mark terminal differentiation [20]. Therefore, the expression of two keratinocyte differentiation markers, involucrin and filaggrin, was investigated in S100A8/A9 over-expressing cells (**Figs. 3A-F**).

After two days' cultivation, 90.0 ± 7.4% of S100A9-expressing cells were positive for involucrin expression whereas 80.8 ± 9.0% S100A9-negative cells expressed involucrin (p-value < 0.05). After six days cultivation, 96.3 ± 2.7% of S100A9-postive HaCaT keratinocytes and 88.2 ± 8.6% of S100A9-negative cells expressed involucrin (p-value < 0.05) (**Figs. 3A and B**).

Approximately 32.8 ± 21.2% of S100A9-positive cells expressed filaggrin after two days (p-value < 0.05) and 52.8 ± 13.8% expressed this protein after six days. In contrast, only 8.7 ± 4.8% of S100A9-negative cells expressed filaggrin after two days and 24.6 ± 10.0% after six days (p-value < 0.05) (**Figs. 3D and E**). These data indicate that S100A9-positive cells probably undergo abnormal early or late phase differentiation. FACS analysis revealed deregulation of early and late differentiation markers.

3.4. MCF-7 cells stably over expressing S100A8/A9 display reduced proliferation rate

To investigate whether S100A8/A9 over-expression caused growth arrest in other cells, several breast tumor cells were analyzed with regard to S100 gene expression [21]. S100A8 and S100A9 were present at extremely low levels in MCF-7 cells; therefore, these cells were chosen for transfection with pVITRO-S100A8/A9 plasmid. After hygromycin selection, MCF-7 cell lines expressing S100A8 and S100A9 (MCF-7/a8a9) were obtained. Western blotting revealed the expression of proteins with molecular weights of approximately 10,000 and 14,000 Da in MCF 7/a8a9 cells, while MCF-7wt cells did not express these proteins (**Fig. 4A**).

The morphology and growth pattern of stable MCF-7/a8a9 cells differed markedly from MCF-7wt controls. MCF-7/a8a9 cells multiplied more slowly than the wild type controls and never reached confluence during the time periods utilized in this study. The majority of the cells displayed reduced cell adherence, with rounded cells present in suspension (**Fig. 4B**).

The CFDA dilution assay confirmed that S100A8/A9 over-expression reduced proliferation (**Fig. 4C and D**). Representative proliferation of MCF-7wt cells demonstrated a shift in the mean fluorescence to the left, indicative of cell division (**Fig. 4C**). However, the mean fluorescence of MCF 7/a8a9 cells demonstrated a delay in the leftward shift associated with proliferation (**Fig. 4D**). These data were confirmed using BrdU cell proliferation assays (**Fig. 4E**), indicating that MCF7-a8a9 cells proliferate more slowly than wild type cells.

In conclusion, the present study demonstrates for the first time that S100A8/A9-over expression affects the proliferation and differentiation of HaCaT keratinocytes. In view of the stress response-induced transient expression of S100A8/A9, it is concluded that S100A8/A9-mediated growth arrest could be important in regulating cellular processes such as wound healing and the prevention of UV-induced skin carcinogenesis. S100A8/A9-mediated growth arrest is required for cell fate decisions, i.e. whether a survival phase is followed by differentiation, proliferation or apoptosis. Recently, Nukui et al. [22] reported that S100A8/A9 stimulates proliferation of normal human keratinocytes and this study demonstrates that intracellular S100A8/A9 functions as a mediator for growth suppression. The study of Nukui et al. [22] provides evidence that S100A8/A9 enhances growth of normal human keratinocytes when acting on the cells exogenously. Therefore, S100A8/A9 plays an ambivalent role with respect to growth regulation of keratinocytes. Further studies are required to elucidate its role in skin biology.

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Figures and legends

Fig. 1: CFDA assay of pVITRO-S100A8/A9-transfected HaCaT cells. HaCaT keratinocytes were labeled with CFDA and transfected with pVITRO-S100A8/A9. FACS analysis was performed as described in section 2.5. No background was detectable when isotype and secondary antibodies were tested during control staining (see **Fig. S1**, right histograms, shaded gray graph). **A, B**) Dot plot of CFDA versus S100A9 antigen expression for day three (**A**) and day 13 (**B**) cultured S100A8/A9-HaCaT keratinocytes. Inset: Numbers within plots denote percentage of cells in the respective quadrants. **C, D**) Histograms of S100A9-negative (**C**) and S100A9-positive cells (**D**) are shown. Cells were gated on $S100A9^+$ or $S100A9^-$ upon staining in **A** and **B**. day 3 = gray curve, day 13 = open curve.

Fig. 2: S100A8/A9 expression interferes with NFκB activation. (**A-C**) To investigate whether S100 expression affects NFκB phosphorylation, HaCaT keratinocytes were transfected with pVITRO-S100A8/A9 and cultured for various time intervals. For FACS analysis, cells were incubated with Abs against S100A9 and pS529- NFκB p65. Further details are described in section 2.5. **A, B**: FACS analysis of two day (**A**) and six day (**B**) cultured S100A8/A9-HaCaT keratinocytes. Inset: Numbers within plots denote percentage of cells in the respective quadrants, and data are representative of five independent experiments with similar expression patterns. (**C**) Data are presented as mean ± SEM. Statistical significance of differences between means was established by a Student's t-test. 2d: ; 6d: .

(**D**) NFκB luciferase reporter assay in mock- (**left panel**) and S100A8/A9wt-transfected HaCaT keratinocytes (**right panel**). HaCaT cells were co-transfected with NFκB luciferase reporter plasmid, empty pVIVO or pVIVO-S100A8/A9wt expression plasmid, as described in section 2.4, and luciferase activity was measured after stimulation with various TNFα concentrations for 3h. Luciferase activity was determined for three independent transfection reactions. Inset: Transfection efficiency was determined by β-galactosidase activity using an enzyme assay kit.

(**E**) Effect of S100A8/A9wt and mutants on NFκB activation. HaCaT cells were co-transfected with various combinations of NFκB -regulated luciferase reporter constructs and

S100A8/A9 expression vectors as indicated. After 48h the cells were treated with 50 ng/ml TNFα for 4 h. Luciferase activity was determined for three independent transfection reactions. Luminescence of unstimulated cells was subtracted from that of TNFα-stimulated cells, and the data were normalized relative to β-galactosidase activities. Asterisks indicate statistically significant differences in S100A8/A9wt- and mutant-transfected cells.

(**F**) Effect of S100A8/A9 and mutants on TNFα-induced IL-8 gene expression. HaCaT cells were transfected with various S100A8/A9 expression vectors as indicated. After 24 h the cells were treated with 50 ng/ml TNFα for 18 h, and IL-8 gene expression analysis was performed as described in section 2.6. C_T values of IL-8 were normalized to GAPDH. IL-8 gene expression is given in relation to the corresponding IL-8 gene expression in non-stimulated cells: ΔC_T IL-8 (TNFα) - ΔC_T IL-8 (non-stimulated) = $\Delta\Delta C_T$ IL-8 (TNFα). IL-8 gene expression was determined for three independent transfection reactions. Asterisks indicate statistically significant differences in S100A8/A9wt- and mutant-transfected cells.

(**G**) Cell survival in forced suspension culture due to enhanced NFκB activation. HaCaT cells were suspended in plates pre-coated with 0.9% agarose and with or without TNFα (10 ng/ml) and the NFκB inhibitor III (10 μmol/L) for 24 h. Aliquots of cells were reseeded in fresh medium on tissue culture–treated plastic. Crystal violet staining of re-attached viable cells was carried out 24 h later. Asterisks indicate statistically significant differences in the S100A8/A9wt- and mutant-transfected cells.

Fig. 3: S100A8/A9 over-expression affects cellular differentiation. HaCaT keratinocytes were transfected with pVITRO-S100A8/A9 and cultured for two (**A, D**) and six (**B, E**) days. For FACS analysis, cells were incubated with Abs against S100A9, involucrin (**A-C**) or filaggrin (**D-F**). The corresponding isotype and secondary antibodies revealed no background staining (data not shown). Inset: Numbers within plots denote percentage of cells in the respective quadrants, and data are representative of seven independent experiments with similar expression patterns. **C, F**: Data are presented as mean ± SEM. Statistical significance of differences between means was established using a Student's t-test. 2d: ; 6d: .

Fig. 4: S100A8/A8 expression leads to reduced proliferation of MCF-7/a8a9 cells. (**A**) Western blot of MCF-7wt and MCF-7/a8a9 cell lysates (50 µg) subjected to SDS-PAGE. S100A8 and S100A9 were detected by western blotting using the corresponding antibodies. (**B**) Morphology of MCF-7wt (**left panel**) and MCF-7/a8a9 cells (**right panel**). The majority of MCF-7/a8a9 cells displayed reduced cell adherence, with some round cells present in suspension. **C, D**: CFDA dilution assay. Cells were labeled with CFDA followed by further cultivation for 12 days. FACS analysis was performed as described in section 2.5. Histograms of MCF-7wt (**C**) and MCF-7/a8a9 cells (**D**) are shown. Data from one representative experiment, of three experiments performed, are presented. Day 2 = gray curve, day 12 = open curve. (**E**) For BrdU assays, MCF-7wt and MCF-7/a8a9 cells (10^7) were starved for 48 h with medium containing 0.2% FCS followed by the addition of medium containing 10% FCS. After 24h, 1 µM BrdU was added for 16h, and the BrdU immunohistochemistry proliferation assay was conducted according to the manufacturer's instructions.

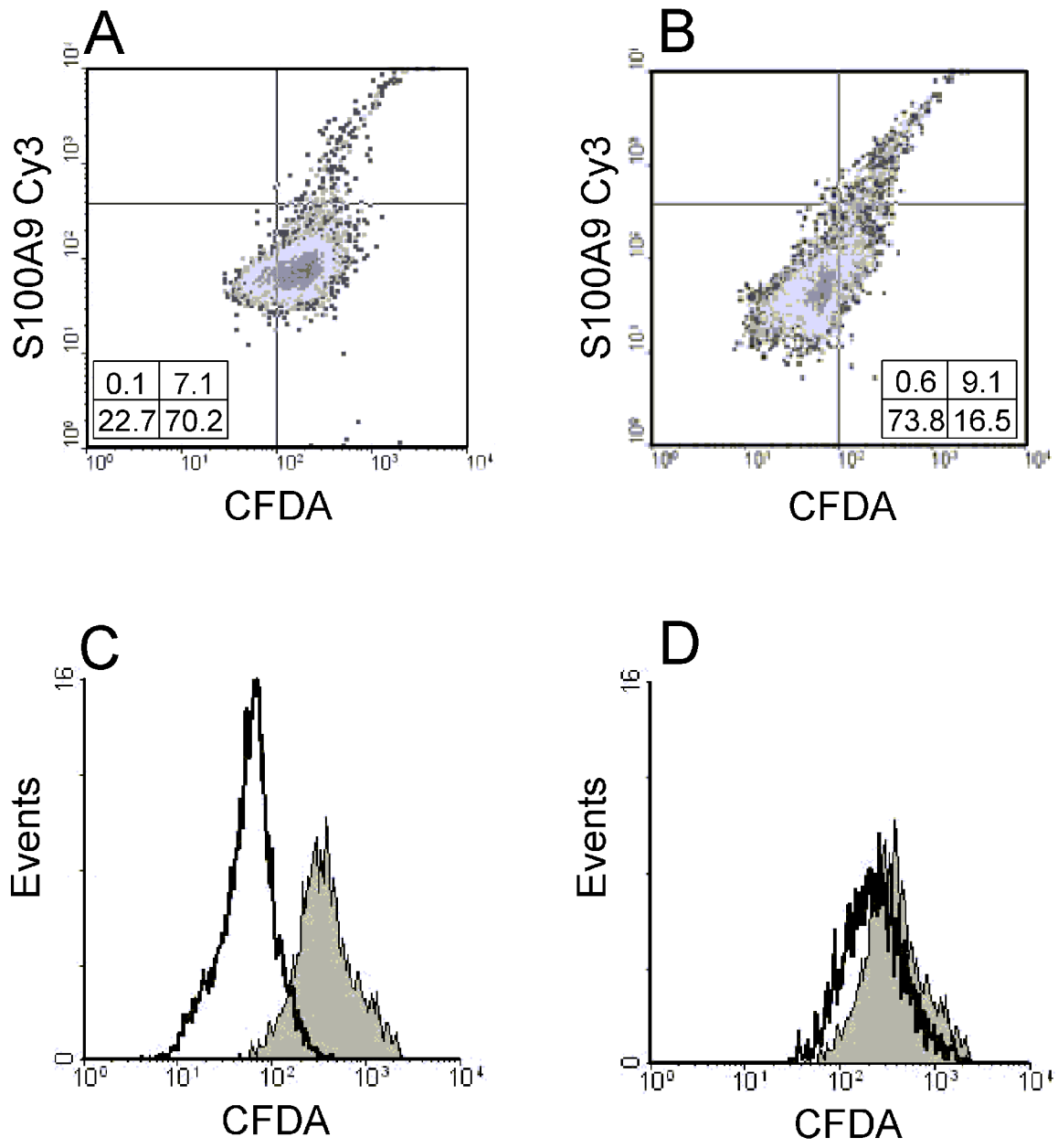

A
S100A9 Cy3
CFDA
0.1
7.1
22.7
70.2
B
S100A9 Cy3
CFDA
0.6
9.1
73.8
16.5
C
Events
16
0
CFDA
D
Events
16
0
CFDA

Figure 1

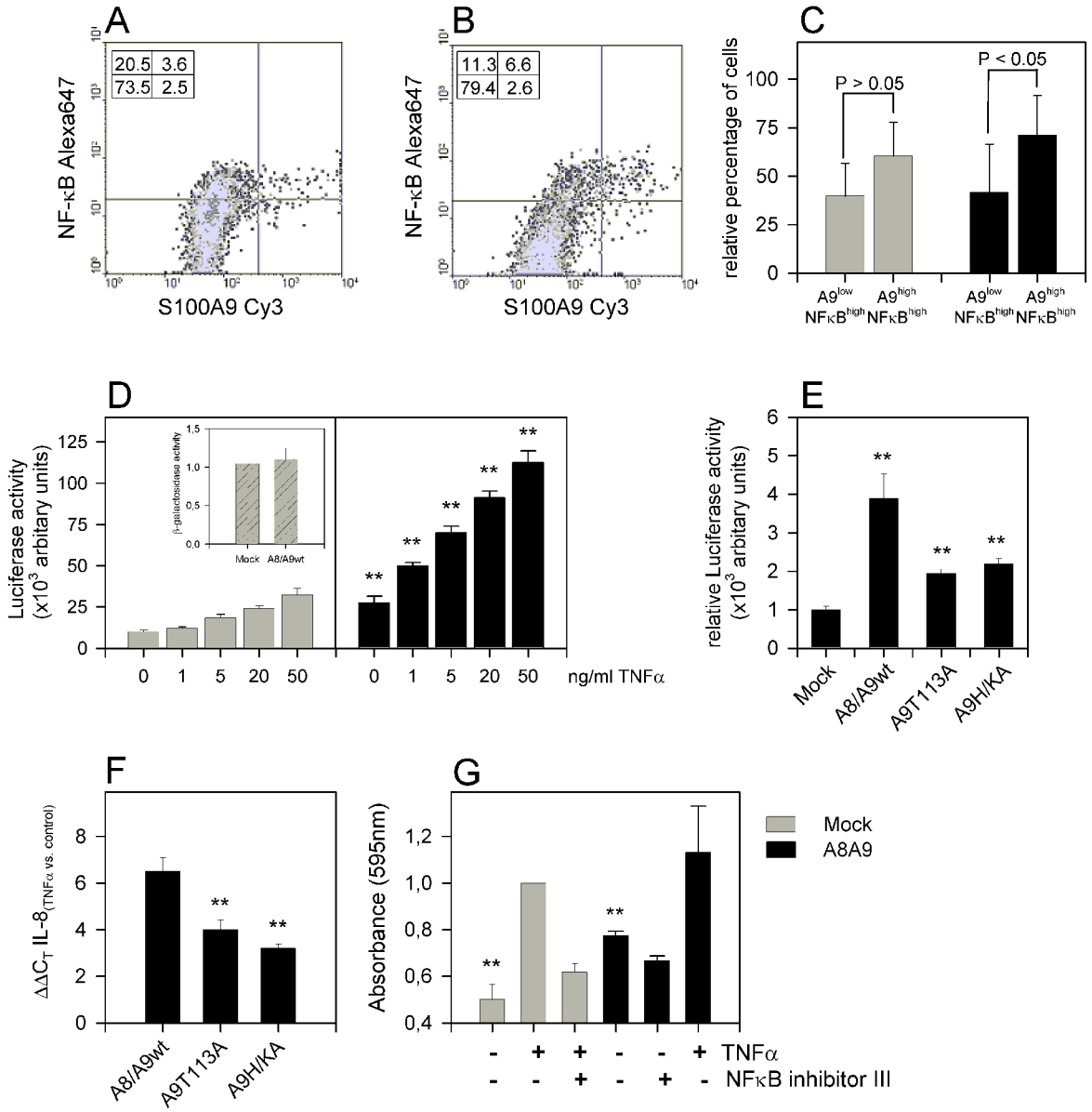
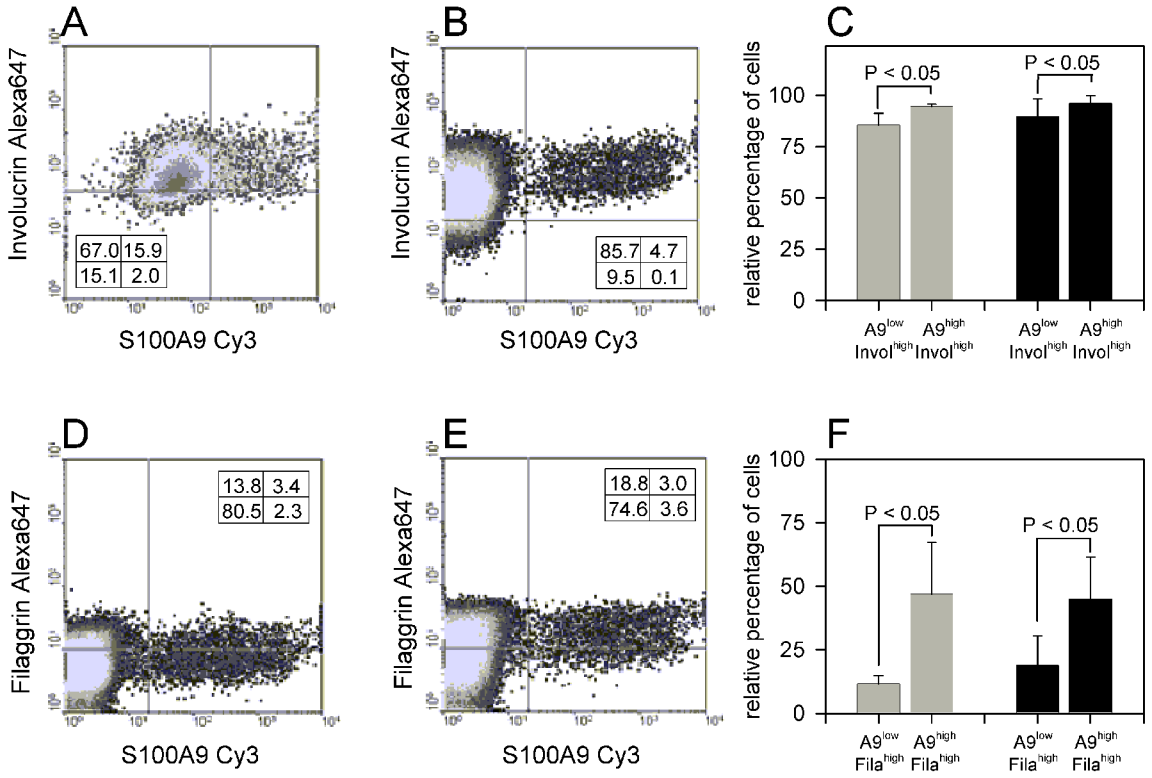

A
B
C
NF-κB Alexa647
S100A9 Cy3
20.5 3.6
73.5 2.5
11.3 6.6
79.4 2.6
relative percentage of cells
P > 0.05
P < 0.05
A9low NFκBhigh
A9high NFκBhigh
D
E
Luciferase activity (x10³ arbitary units)
β-galactosidase activity
Mock
A8/A9wt
ng/ml TNFα
relative Luciferase activity (x10³ arbitary units)
A9T113A
A9H/KA
F
G
ΔΔCT IL-8(TNFα vs. control)
Absorbance (595nm)
Mock
A8A9
TNFα
NFκB inhibitor III

Figure 2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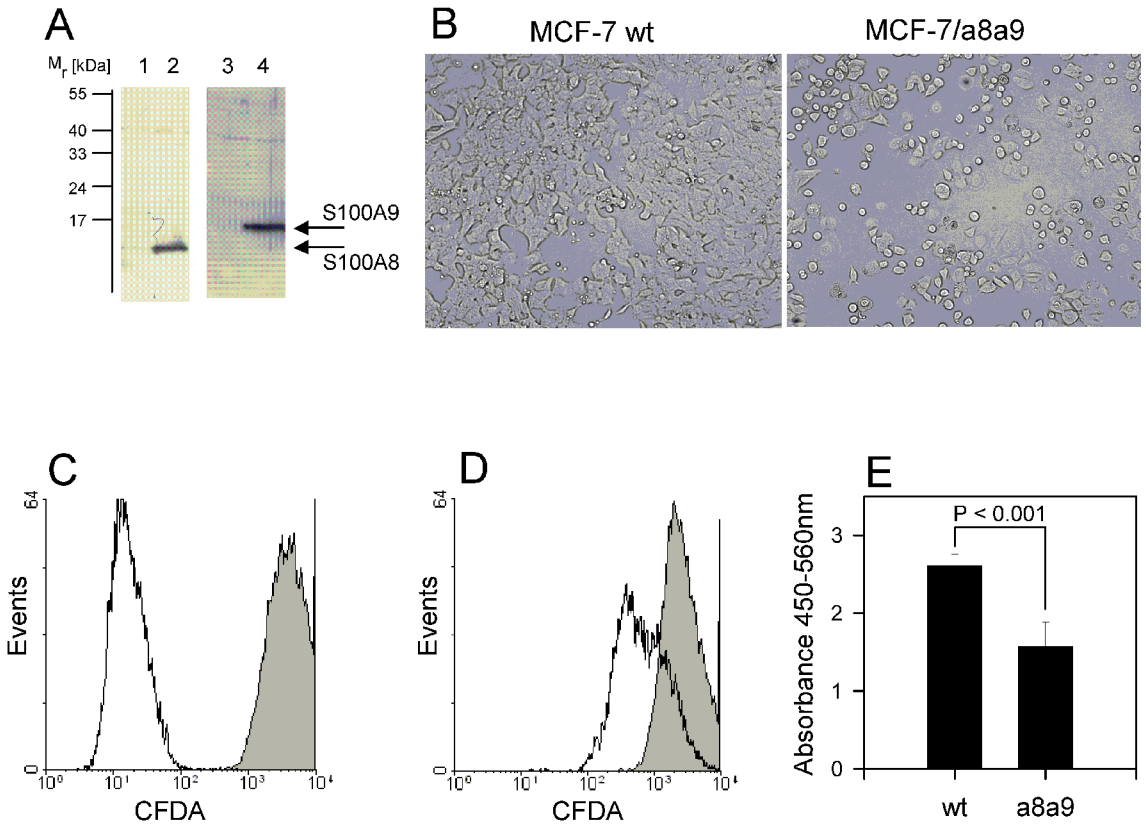
S100A9 Cy3
67.0 15.9
15.1 2.0
B
Involucrin Alexa647
S100A9 Cy3
85.7 4.7
9.5 0.1
C
relative percentage of cells
P < 0.05
P < 0.05
100
75
50
25
0
A9low Involhigh
A9high Involhigh
A9low Involhigh
A9high Involhigh
D
Filaggrin Alexa647
S100A9 Cy3
13.8 3.4
80.5 2.3
E
Filaggrin Alexa647
S100A9 Cy3
18.8 3.0
74.6 3.6
F
relative percentage of cells
P < 0.05
P < 0.05
100
75
50
25
0
A9low Filahigh
A9high Filahigh
A9low Filahigh
A9high Filahigh

Figure 3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55
40
33
24
17
S100A9
S100A8
B
MCF-7 wt
MCF-7/a8a9
C
Events
64
0
CFDA
D
Events
64
0
CFDA
E
P < 0.001
Absorbance 450-560nm
3
2
1
0
wt
a8a9

Figure 4