NIH Public Access
Author Manuscript
J Invest Dermatol. Author manuscript; available in PMC 2015 January 16.

Published in final edited form as:
J Invest Dermatol. 2014 June ; 134(6): 1579–1588. doi:10.1038/jid.2013.507.

Downregulation of STRA6 Expression in Epidermal Keratinocytes Leads to Hyperproliferation-Associated Differentiation in Both *In Vitro* and *In Vivo* Skin Models

Claudia Skazik[#1], **Philipp M. Amann**[#1], **Ruth Heise**[1], **Yvonne Marquardt**[1], **Katharina Czaja**[1], **Arianna Kim**[2], **Ralph Rühl**[3], **Peter Kurschat**[4], **Hans F. Merk**[1], **David R. Bickers**[2], and **Jens M. Baron**[1]

[1]Department of Dermatology and Allergology, University Hospital, RWTH Aachen University, Aachen, Germany [2]Department of Dermatology, College of Physicians and Surgeons, Columbia University, New York, New York, USA [3]Department of Biochemistry and Molecular Biology, University of Debrecen, Debrecen, Hungary and [4]Department of Dermatology, University Hospital of Cologne, Cologne, Germany

[#] These authors contributed equally to this work.

Abstract

Retinoids are known to affect skin cell proliferation and differentiation and are key molecules that target retinoid and retinoic acid receptors (RXRs and RARs), leading to physiological and pharmacologic effects. Our aim was to elucidate the role of the retinol-binding protein receptor STRA6, mediating cellular uptake of retinol, on skin structure and function. Our results indicate that STRA6 is constitutively expressed in human epidermal keratinocytes and dermal fibroblasts and is regulated via RAR/RXR-mediated pathways. HaCaT (Human adult low Calcium high Temperature) cells with stable STRA6 knockdown (STRA6KD) showed increased proliferation. Consistently, human organotypic 3D skin models using stable STRA6KD HaCaT cells showed a significantly thicker epidermis and enhanced expression of activation, differentiation, and proliferation markers. The effects were reversible after treatment with free retinol. Human skin reconstitution employing STRA6KD HaCaT cells leads to massive epithelial thickening under *in vivo* conditions in SCID mice. We propose that STRA6KD could lead to cellular vitamin A deficiency in keratinocytes. Consequently, STRA6 has a role for regulating retinoid homeostasis and in helping to program signaling that drives proliferation and differentiation of human skin cells. By its influence on hyperproliferation-associated differentiation, STRA6 could also have a role in skin regeneration and could be a target for pharmacological approaches to improve wound healing.

© 2014 The Society for Investigative Dermatology

Correspondence: Jens M. Baron, Department of Dermatology and Allergology, University Hospital, RWTH Aachen University, Pauwelsstraße 30, D-52074 Aachen, Germany. JensMalte.Baron@post.rwth-aachen.de.

CONFLICT OF INTEREST The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

INTRODUCTION

Vitamin A (all-*trans* retinol, atROL) and its biologically active derivatives (retinoids) are important for maintaining physiological processes, including regulation of growth and development, and in skin barrier function (D'Ambrosio *et al*., 2011). A wealth of data exist showing that retinoids have critical regulatory functions in skin and influence keratinocyte differentiation and skin homeostasis (Fisher and Voorhees, 1996). The physiological effects of retinoids are largely controlled by a family of nuclear ligand–dependent retinoic acid receptors (RARα, RARβ, and RARγ) and retinoid X receptors (RXRα, RXRβ, and RXRγ) (Amann *et al*., 2011).

Retinol-binding protein (RBP) is the principal physiological carrier of retinol in the bloodstream for delivery to target tissues (Wolf, 2007). The existence of a specific cell-surface receptor on cells targeted by retinoids, mediating the uptake of retinol from RBP, was described in the 1970s (Heller, 1975; Bok and Heller, 1976; Rask and Peterson, 1976; Chen and Heller, 1977). Since that time there has been increasing evidence for the existence of a cell- and tissue-specific RBP receptor (Sivaprasadarao and Findlay, 1994; Smeland *et al*., 1995; Sundaram *et al*., 1998). Bouillet *et al* (1995) described STRA6 (stimulated by retinoic acid gene 6) as an integral transmembrane protein of unknown function that is inducible by retinoic acid.

A breakthrough was achieved by Kawaguchi *et al* (2007) who showed in bovine retinal epithelial cells that (i) RBP can bind STRA6 with high affinity, (ii) STRA6-transfected cells efficiently take up retinol, (iii) RNAi knockdown of STRA6 suppresses retinol uptake, and (iv) STRA6 is expressed in tissues consistent with its function as an RBP receptor. These results provide strong evidence that STRA6 is a specific RBP receptor mediating the cellular uptake of retinol.

Little is known about the physiological retinoid uptake processes and how they mediate their effects on skin cells. To determine the significance of STRA6 in skin, we analyzed STRA6 expression and its regulation in human skin cells. To approximate *in vivo* conditions we established an organotypic human 3D skin model with stable STRA6KD HaCaT (Human adult low Calcium high Temperature) keratinocytes and used a human skin reconstitution model in mice to investigate the influence of STRA6 on the structure and function of human skin.

RESULTS

STRA6 is constitutively expressed in human skin cells

We could show that various human skin cells constitutively express STRA6 mRNA (Figure 1a and b). By using quantitative real-time (qRT) PCR analysis, we could demonstrate significantly higher STRA6 mRNA levels in normal human epidermal keratinocytes (NHEKs), HaCaT cells, and in normal human dermal fibroblasts (NHDFs) compared with the expression in primary human melanocytes.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

STRA6 mRNA and protein expression increases after stimulation with atRA in the murine keratinocyte cell line PAM212

To determine whether STRA6 is also upregulated in skin cells after stimulation with atRA we treated PAM212 cells with different concentrations of atRA (10^{-6}, 10^{-7}, or 10^{-8} M) for 24 hours. STRA6 mRNA expression increased in a dose-dependent manner after stimulation with atRA compared with untreated cells (Figure 1c). atRA also led to upregulation of STRA6 protein expression, which was confirmed by quantitative evaluation (Figure 1d).

STRA6 mRNA expression increases after stimulation with ligands of the nuclear retinoid acid receptors in NHEKs and NHDFs

To determine which signaling pathways mediate STRA6 gene expression, we examined the effects of different ligands of the nuclear retinoid receptor on STRA6 expression in NHEKs (Figure 2a) and NHDF (Supplementary Figure S1a online). Cells were stimulated with atRA, 9-*cis* retinoic acid (9*cis*RA), 13-*cis* retinoic acid (13*cis*RA), and atROL as prototypic endogenous retinoids (ligands of RAR) and with targretin (LGD1069) as the ligand of RXR, in comparison with ligands of other class II nuclear receptors—phenobarbital, dexamethasone, and benzanthracene—at a concentration of 10^{-6} and 10^{-7} M for 4, 8, and 24 hours. We observed a time-dependent strong increase in STRA6 mRNA expression after stimulation with RAR ligands in the NHEKs of three separate donors (Figure 2a). Similar results were obtained after stimulation of NHDFs but with lesser upregulation of STRA6 mRNA expression (Supplementary Figure S1a online). Furthermore, treatment with ligands of other class II nuclear receptors had no effect on Stra6 mRNA expression.

STRA6 mRNA expression decreases after incubation with LE540, an RAR panantagonist

Next, we blocked retinoid signaling using the RAR panantagonist LE540 (Umemiya *et al*., 1997) after stimulation with the RAR ligands. Retinoid-stimulated STRA6 mRNA expression is significantly downregulated after treatment with LE540 in NHEKs (Figure 2b) and NHDFs (Supplementary Figure S1b online), verifying that STRA6 mRNA expression is likely mediated by RAR.

Proliferation is enhanced in stable STRA6KD HaCaT cells

To assess the influence of STRA6 on the structure and function of human skin cells we established a lentiviral-stable STRA6KD in HaCaT cells. STRA6 mRNA expression in STRA6KD cells was reduced up to 96% (clone K1) and compared with untreated HaCaT cells. STRA6KD cells showed significantly enhanced proliferation. Proliferation of both STRA6KD clones K1 and K2 on day 7 correlated with STRA6 knockdown efficiency (Figure 3a, Supplementary Figure S2 online).

To further study the proliferative capacity of STRA6KD cells we performed an *in vitro* scratch assay. In STRA6KD cells (K1) there was marked acceleration in the rate of closure of the scratch wound as compared with control cells. After 8 hours the gap was ~60% closed in the STRA6KD cells as compared with 16–30% in the control cells (Figure 3b, A1–B1). Immunofluorescence DAPI staining revealed increased numbers of nuclei by closure of the scratch of STRA6KD cells as compared with control cells (Figure 3b, A2–B2). STRA6KD

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

cells showed enhanced expression of keratin 16 (Krt16), a known proliferation marker in keratinocytes (Korge *et al*., 1990; Patel *et al*., 2006) (Figure 3b, A3–B3). These findings confirmed that the observed effects on gap closure were preferentially due to enhanced cell proliferation and not due to any augmentation in migration.

Human 3D skin equivalents constituted with STRA6KD HaCaT cells showed thicker epidermis and enhanced expression of proliferation, activation, and differentiation markers

To further assess the proliferative effects of STRA6KD on morphological structure and function in a complex cell system, we established an organotypic 3D skin model using STRA6KD cells (K1) and HaCaT control cells (Figure 4a). The epidermis of the STRA6KD 3D skin model displayed a significantly thicker epidermis (2.3-fold after 7 days and up to 2.7-fold after 14 days) (Figure 4a, A1–D1; Figure 4b, A1–B1) and immunofluorescence DAPI staining revealed greater cell density in the STRA6KD 3D skin (Figure 4a, A2–D2) as compared with control cells. The cell proliferation marker Krt16 was slightly upregulated in the STRA6KD 3D model after 7 days and even more so after 14 days (up to 18-fold) (Figure 4a, A3–D3, Figure 4b, A2–B2). In addition, the keratinocyte activation marker Krt6 (Freedberg *et al*., 2001) was significantly upregulated (sevenfold after 7 days and up to threefold after 14 days) in the STRA6KD 3D model (Figure 4a, A4–D4; Figure 4b, A3–B3). Expression of the keratinocyte differentiation marker Krt10 (Kopan and Fuchs, 1989; Freedberg *et al*., 2001) was significantly increased in STRA6KD cells (6.8-fold after 7 days and up to 4.3-fold after 14 days) (Figure 4a, A5-D5; Figure 4b, A4–B4). As indicated by Ki67 staining, STRA6KD K1 showed a stronger, and atypically not restricted to the basal layer, proliferation after 7 days. However, Ki67 expression in the higher epidermal layer could not be detected in the 14-day-old STRA6KD model (Figure 4a, A6–D6).

Similar effects on proliferation and differentiation were shown in the 3D model with STRA6KD HaCaT clone K2. Probably because of reduced STRA6 knockdown efficiency in K2 the observed effects were to a lesser extent (Supplementary Figure S3 online).

Human skin reconstitution with STRA6KD HaCaT cells leads to massive acanthotic epithelial thickening *in vivo*

A skin reconstitution assay was utilized according to the cell-sorted skin equivalent (CeSSE, Figure 5a) model in SCID mice as described by Wang *et al*. (2000), with some modifications. Transplanted control HaCaT cells developed a regular stratified epithelium consisting of 8–12 layers of keratinocytes that exhibited normal differentiation features with a prominent stratum granulosum and orthokeratosis (Figure 5b). In contrast, transplanted STRA6KD HaCaT cells resulted in a disorganized, highly acanthotic epidermis with >20 cell layers and elon-gated rete ridges with corresponding lengthened dermal papillae (Figure 5c). Furthermore, we observed a massive parakeratotic stratum corneum covering the exophytic protruding epithelium.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Effects of STRA6KD in human 3D skin equivalents are diminished after incubation with free (RBP-unbound) atROL

To determine whether the proliferative behavior of STRA6KD cells relates to retinol deficiency we performed a retinol rescue experiment with the organotypic STRA6KD 3D model. First, *in vitro* uptake assays were performed to verify that HaCaT control and STRA6KD cells are capable of retinol uptake. As expected, both types of cells were able to take up similar amounts of free (RBP-unbound) atROL in a time-dependent manner (Figure 6a). If the observed effects on skin morphology are mediated by a STRA6KD-induced retinol deficiency, the addition of retinol should rescue the cells. Indeed, incubation with free atROL at a concentration of 10^{-6} and 10^{-7} M leads to reduced epidermal thickness as compared with the untreated STRA6KD 3D model (Figure 6b, A1–D1, A3–D3). Immunofluorescence staining revealed downregulation of Krt16 in the atROL-treated STRA6KD model (Figure 6b, A2–D2, A4–D4). Whereas a slight reduction in epidermal thickness was also seen in the 10^{-6} M atROL-treated HaCaT control model, the control model treated with 'more physiological levels' of atROL (10^{-7} M) showed no clear differences in epidermal thickness compared with the untreated control model. A possible explanation is that, by balancing the retinoid level in STRA6KD HaCaT cells by addition of free atROL, skin development and differentiation normalizes in 3D skin equivalents.

DISCUSSION

Retinoids are important regulators of skin cell proliferation and differentiation and represent a class of drugs that are remarkably useful therapeutically (Niles, 2002; Wang *et al*., 2009). Despite their undoubted importance, little is known about physiological retinoid uptake processes in skin cells. This—to our knowledge previously unreleased—report reveals STRA6 expression and its regulation in skin cells using stable transfected STRA6KD cells. We showed that STRA6 is constitutively expressed in skin cells such as NHEK, HaCaT, and NHDF. Smeland *et al* (1995) previously showed that undifferentiated human keratinocytes have the highest RBP-binding activity among many tested cell types.

The hypothesis that STRA6 expression is driven by atRA is supported by studies in other cell lines (Bouillet *et al*., 1997; Szeto *et al*., 2001). Consistent with these findings, our results indicate that STRA6 expression is upregulated after stimulation with atRA in the murine keratinocyte cell line PAM212 in a dose-dependent manner. Upregulation of STRA6 expression by atRA may be expected to result in potentially excessive cellular intake of atROL, leading to even higher atRA concentrations (Blaner, 2007). Recent cell culture studies have addressed this issue and have shown that STRA6-expressing cells, preloaded with atROL, release more atROL into the culture medium than the cells that do not express STRA6, and indicate that the flux of atROL between RBP and STRA6 is bidirectional (Isken *et al*., 2008). In addition, atROL accumulation depends on the presence of other cellular proteins such as cellular retinol-binding protein and the enzyme lecithin/ retinol-acyl transferase, which catalyzes the esterification of atROL (Isken *et al*., 2008; Kawaguchi *et al*., 2011; Amengual *et al*., 2012; Kawaguchi *et al*., 2012).

In this study we show that STRA6 mRNA expression is strongly upregulated in NHEK and NHDF in a time-dependent manner by RAR ligands and is also slightly increased by the

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

RXR ligand targretin (Wu *et al*., 2002; Tooker *et al*., 2007). Furthermore, LE540, a RAR panantagonist, diminished the upregulation of STRA6 (Umemiya *et al*., 1997). These results indicate that STRA6 expression in skin cells is mediated by RAR/RXR-signaling pathways.

However, the data from *in vivo* measurements of STRA6 expression are partly contradictory. No changes in STRA6 expression were observed after retinoid treatment of chick embryos (Reijntjes *et al*., 2010), and dietary retinoid deficiency had no effect on STRA6 expression in mouse embryos (Kim *et al*., 2008), whereas retinoid-deprived quail embryos do show upregulation of STRA6 (Reijntjes *et al*., 2010). In human skin biopsies STRA6 was only expressed at low mRNA levels (Pasutto *et al*., 2007). We and others (D'Ambrosio *et al*., 2011) speculate that control of STRA6 expression is regulated in a tissue-specific manner at both the gene and protein levels, and depends on the cellular context and the *in vivo* retinoid status.

Interestingly, there are greater differences in responses to retinoids between keratinocytes *in vitro* and keratinocytes *in vivo*. Paradoxically, retinoids often have effects in cultured keratinocytes that are the opposite of those in keratinocytes *in vivo*. Accordingly, we used both *in vitro* and *in vivo* approaches in monolayer cell culture experiments, in organotypic 3D models, and in a mouse model to further define the STRA6-mediated effects.

Keratinocyte differentiation is marked by the expression of differentiation-specific gene products. In human skin, epidermal differentiation occurs via two pathways. Under normal conditions, differentiation is associated with the expression of Krt1, Krt2, and Krt10. There are also additional pathways. For example, differentiation in regenerative skin is associated with increased expression of the hyperproliferation-associated Krt6 and Krt16 markers, whereas embryonic differentiation is characterized by the expression of Krt4, Krt13, and Krt19 (Van Rossum *et al*., 2000). Under normal conditions, Krt13 expression is limited to non-keratinizing squamous epithelia and its expression in skin is, together with the expression of keratins Krt4 and Krt19, assumed to be a sign of reinduction of embryonic differentiation (Van Rossum *et al*., 2000).

Previous studies revealed a strong correlation between Krt6 and Krt16 expression and epidermal hyperproliferation that occurs in skin diseases such as psoriasis and actinic keratosis (Stoler *et al*., 1988). Recently, upregulation of Krt6 and Krt16 was shown in cancer stem cells of squamous cell carcinoma (Schober and Fuchs, 2011). *in vitro* studies of retinoids in keratinocytes have shown that they inhibit cell proliferation (Popadic *et al*., 2008) and decrease the expression of differentiation-associated proteins such as Krt10 and several other gene products associated with the formation of the epidermal barrier (Fisher and Voorhees, 1996; Törmä, 2011). Lee *et al*. (2009) found that the differentiation marker Krt10 and the proliferation marker Krt16 are suppressed in keratinocytes after treatment with atRA. Downregulation of Krt6 and Krt16 was also observed after atRA treatment in cultured skin sebocytes (Zouboulis *et al*., 1991). Conversely, in the absence of atROL or its derivatives, terminal differentiation of keratinocytes is enhanced (Törmä and Vahlquist, 1984). One possible explanation may be that keratinocyte differentiation and keratinization in the skin relates to a functional vitamin A deficiency in the upper layers of the epidermis. The observation that differentiated keratinocytes downregulate the RBP receptor is

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

consistent with such a regulation of differentiation (Smeland *et al*., 1995). If we assume that STRA6KD leads to cellular retinol deficiency because of disturbed retinol influx, similar effects of retinoid deficiency on differentiation and proliferation might occur.

Indeed, STRA6KD HaCaT cells manifest substantially increased proliferation as confirmed by Krt16 upregulation in monolayer experiments. Consistently, human organotypic 3D skin models using stable transduced STRA6KD HaCaT cells showed a significantly thicker epidermis and enhanced expression of activation, differentiation, and proliferation markers (e.g. Krt6, Krt10, Krt16 and Ki67). As mentioned above, the hyperproliferation-associated keratins Krt6 and Krt16 are involved in the differentiation of regenerative skin (Van Rossum *et al*., 2000). Krt6 and Krt16 are upregulated in keratinocytes at the wound edge within 2–6 hours after injury to normal epidermis and other stratified epithelia (Wawersik and Coulombe, 2000). Transgenic mouse skin keratinocytes express human Krt16 at steady-state levels approaching those of endogenous Krt14 and display defects in cell–substratum adhesion, differentiation, and migration but not in their ability to proliferate. In addition, overexpression of human Krt16 has been shown to delay the closure of full-thickness skin wounds *in vivo* (Wawersik *et al*., 2001). Because of its role in alternative hyperproliferation-associated differentiation (Van Rossum *et al*., 2000) STRA6 could also have a role in skin regeneration and could be a target for pharmacological approaches to improve wound healing.

In contrast, retinoids enhance keratinocyte growth *in vivo* but do not suppress keratinocyte terminal differentiation (Fisher and Voorhees, 1996). To approximate *in vivo* conditions we used a reconstituted skin model in a SCID/NOD mouse model. Knocking down STRA6 in keratinocytes resulted in a massively acanthotic epithelium. The epidermal thickening was accompanied by a lack of differentiation with a loss of the granular layer and prominent hyperparakeratosis. These observations support our *in vitro* data that the STRA6 loss in keratinocytes results in increased proliferation and reduced differentiation. Future studies should ascertain whether hyperproliferative skin diseases such as psoriasis are associated with decreased expression of STRA6 in keratinocytes. In addition, the conditional complete knockdown of STRA6 in keratinocytes by using, for example, the Cre-loxP-system may be useful for generating animal models for skin diseases characterized by marked acanthosis, such as psoriasis vulgaris.

AtROL is a small lipophilic molecule that can penetrate biological membranes by passive non-ionic diffusion. As expected, both HaCaT control and STRA6KD cells are capable of free atROL uptake in a time-dependent manner. In a rescue experiment we showed that atROL treatment leads to reduced epidermal thickness in the STRA6KD 3D skin model. An atROL-induced downregulation and normalization of Krt16 expression was shown in the STRA6KD 3D skin model at a similar level to the atROL-treated or untreated HaCaT control model. These results provide evidence that the effects of STRA6KD in human 3D skin equivalents can be diminished after treatment with free atROL.

We propose that STRA6KD causes cellular vitamin A deficiency because of disturbed retinol influx. Consequently, the RBP receptor STRA6 is essential for retinoid supply to maintain retinoid homeostasis and to balance physiological regulatory effects on the

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

proliferation and differentiation of human skin cells. How the mechanisms of retinoid homeostasis are mediated in detail, and to what extent the RBP receptor STRA6 is involved, is not understood completely yet. Nevertheless, it is obvious that the steady-state system of intracellular retinoids is regulated by a complex feedback control system involving the regulation of retinoid influx and efflux that is important for skin homeostasis.

MATERIALS AND METHODS

Cell culture and skin equivalents

Isolation and culturing of NHEK, NHDF, and the keratinocyte cell line HaCaT were performed as described elsewere (Heise *et al*., 2006; Amann *et al*., 2012). Engineering of cocultures mainly leaned toward the publication of Schoop *et al* (1999). In brief, NHDFs were seeded into collagen gels, and, the following day, cells were plated onto the fibroblast-collagen matrix. After 2 days, skin equivalents were lifted to the air–liquid interphase and cultured over a period of 7 or 14 days. For rescue experiments, atROL (10^{-6} or 10^{-7} M) was added every 48 hours after lifting the skin equivalent to the air–liquid interphase.

Stable STRA6 knockdown (STRA6KD) in HaCaT cells

HaCaT cells were transduced with MISSION Lentiviral Transduction particles with the following clone IDs: TRCN0000128299 (clone K1), TRCN0000130376 (clone K2), and TRCN0000129158 (clone K3) (Sigma, Taufkirchen, Germany).

Control cells were transduced with non-target ctrl particles (SHC002V) (Sigma, Taufkirchen, Germany). Stable shRNA–expressing cells were generated by 2 weeks of bulk selection in Puromycin (0.5 μg ml^{-1})-containing growth medium.

RNA isolation and qRT–PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Purified RNA was reversed transcribed with the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Weiterstadt, Germany). TaqMan experiments were carried out on an ABI Prism 7000 sequence detection system (Applied Biosystems, Weiterstadt, Germany) using Assays-on-Demand gene expression products for human STRA6 (Hs00223621_m1) and mouse Mm00486457_m1 according to the manufacturer's recommendations. An Assay-on-Demand product for human HPRT (Hs99999909_m1) and mouse GAPDH (Mm99999915_g1) was used as an internal reference to normalize the target transcripts.

Western blot

Cells were lysed using an MSE Soniprep150 sonicator and denatured by suspension in Laemmli buffer containing 5% mercaptoethanol. Western blot was performed according to the method described by Schiffer *et al* (2003). The primary antibodies used were STRA6 anti-mouse antibody (EB07811; Everest Biotech, Upper Heyford, UK) and b-actin anti-mouse antibody (A5441; Sigma, Taufkirchen, Germany). Detection was performed using

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

horseradish peroxidase–conjugated anti-goat or anti-mouse antibody (Dako, Glostrup, Denmark) and the ECL kit (Amersham, Arlington Heights, IL).

In vivo human skin reconstitution assay in SCID mice

Human skin reconstitution assay was performed according to the CeSSE model as previously described, with modifications (Wang *et al*., 2000).

Briefly, after anesthetizing the mice ($n = 9$), a circular 1-cm-diameter sample of upper back skin was removed. The brim and hat of the silicon chamber (CRD culture chambers, Renner, Dannstadt, Germany) were placed under the edge of the skin around the perimeter of the surgical wound incision. The mixed STRA6KD HaCaT/fibroblast or HaCaT control/fibroblast cell suspension containing 5×10^6 cells of each cell type was transferred into the chamber. After 1 week the silicone chamber was removed and the wound was allowed to dry for 3 additional weeks before biopsies were taken. Reconstituted skin samples were fixed in 4% formalin and embedded in paraffin. Sections measuring 4 μm in thickness were stained with hematoxylin and eosin.

Cellular retinol uptake assay

STRA6KD cells or HaCaT control cells were seeded in 24-well plates and cultured up to 70% confluency. Subsequently, the cells were exposed to radiolabeled atROL (2 μCi ml^{-1}) (Perkin Elmer, Boston, MA). After incubation for 30 or 60 minutes, the radioactive medium was removed, cells were washed with PBS, and lysed by adding Optiphase “Supermix” (Wallac, Turku, Finland) as scintillation fluid. The cell-associated radioactivity was determined by transferring the lysate into a flexible 24-well plate and counting radioactivity using a Wallac 1450 MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland). The values are expressed as counts per minute. The median values of three independent experiments in triplicate were used for statistical data analysis.

Proliferation assay

Cells (5×10^3) were plated in 24-well plates. Proliferation was assayed over 1 week using the alamarBlue reagent (AbD Serotec, Düsseldorf, Germany) according to the manufacturer's protocol.

Scratch assay with STRA6KD cells

Culture inserts (IBIDI, Martinsried, Germany) were placed in chamber slides, and 1.4×10^5 cells were seeded in each chamber of the inserts. The culture inserts were removed when cells reached confluence, leading to a cell-free gap of ~500 mm.

Light microscopy and immunofluorescence

For light microscopy and immunofluorescence 4-μm cryosections were processed as described previously (Neis *et al*., 2010). Primary antibodies (Krt6, Santa Cruz Biotechnology, Santa Cruz, California; Krt10, Dako, Glostrup, Denmark; Krt16, AbD Serotec, Düsseldorf, Germany; DAPI, Applichem, Darmstadt, Germany; Ki67, Dako, Glostrup, Denmark) and fluorochrome-conjugated secondary antibody Alexa Fluor 488/546

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(Molecular Probes, Eugene, Oregon) were used. Relative quantification of Krt6, Krt10, and Krt16 expression in 3D skin equivalents was evaluated with the fluorescence imaging software cell F (Olympus, Hamburg, Germany).

Statistical analysis

Data are given as arithmetical means $\pm_{SEM.}$ The given *P*-values were compared with control. One-way ANOVA with Tukey's multiple comparison test or the unpaired *T*-test was performed with Sigmaplot Version 11 (Erkrath, Germany). Values of $*P<0.05$, $**P<0.01$, and $***P<0.001$ were considered significant and are indicated in the figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by START grants (to J.M.B. and P.M.A.) of the Medical Faculty of the RWTH Aachen University and by a grant (0315226C) from the BMBF.

Abbreviations

9cisRA	9-cis retinoic acid
13cisRA	13-cis retinoic acid
atRA	all-trans retinoic acid
atROL	all-trans retinol
HaCaT	human adult low calcium high temperature
Krt	keratin
LGD1069	targretin
NHEK	normal human epidermal keratinocyte
NHDF	normal human dermal fibroblast
RAR	retinoic acid receptor
RBP	retinol-binding protein
RXR	retinoid X receptor
STRA6	stimulated by retinoic acid gene 6
STRA6KD	STRA6 knockdown

REFERENCES

Amann PM, Luo C, Owen RW, et al. Vitamin A metabolism in benign and malignant skin cells: Importance of lecithin/retinol acyltransferase and RPE65. J Cell Physiol. 2012; 227:718–28. [PubMed: 21465477]

Amann PM, Eichmüller SB, Schmidt J, et al. Regulation of gene expression by retinoids. Curr Med Chem. 2011; 18:1405–12. [PubMed: 21366525]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Amengual J, Golczak M, Palczewski K, et al. Lecithin:retinol acyl transferase is critical for cellular uptake of vitamin A from serum retinol binding protein. J Biol Chem. 2012; 287:24216–27. [PubMed: 22637576]

Blaner WS. STRA6, a cell surface receptor for retinol –binding protein: the plot thickens. Cell Metab. 2007; 5:164–6. [PubMed: 17339024]

Bok D, Heller J. Transport of retinol from the blood to the retina: an audiographic study of the pigment epithelial cell surface receptor for plasma retinol-binding protein. Exp Eye Res. 1976; 22:395–402. [PubMed: 945179]

Bouillet P, Oulad-Abdelghani M, Vicaire S, et al. Efficient cloning of cDNAs of retinoic acid-response genes in P19 embryonal carcinoma cells and characterization of a novel mouse gene. STRA1 (mouse LERK-2/ Eplg2). Dev Biol. 1995; 170:420–33. [PubMed: 7649373]

Bouillet P, Sapin V, Chazaud C, et al. Development expression pattern of STRA6, a retinoic acid-responsive gene encoding a new type of membrane protein. Mech Dev. 1997; 63:173–86. [PubMed: 9203140]

Chen C, Heller J. Uptake of retinol and retinoic acid from serum retinol-binding protein by retinal pigment epithelial cells. J Biol Chem. 1977; 252:5216–21. [PubMed: 560371]

D'Ambrosio DN, Clugston RD, Blaner WS. Vitamin A metabolism: an update. Nutrients. 2011; 3:63–103. [PubMed: 21350678]

Fisher GJ, Voorhees JJ. Molecular mechanisms of retinoid actions in skin. FASEB J. 1996; 10:1002–13. [PubMed: 8801161]

Freedberg IM, Tomic-Canic M, Komine M, et al. Keratins and keratinocyte activation cycle. J Invest Dermatol. 2001; 116:633–40. [PubMed: 11348449]

Heller J. Interactions of plasma retinol-binding protein with its receptor. J Biol Chemistry. 1975; 250:3613–9.

Heise R, Mey J, Neis MM, et al. Skin retinoid concentrations are modulated by CYP26AI expression restricted to basal keratinocytes in normal human skin and differentiated 3D skin models. J Invest Dermatol. 2006; 126:2473–80. [PubMed: 16778795]

Isken A, Golczak M, Oberhauser V, et al. RBP4 disrupts vitamin A uptake homeostasis in a STRA6-deficient animal model for Matthew-Wood syndrome. Cell Metab. 2008; 7:258–68. [PubMed: 18316031]

Kawaguchi R, Yu J, Honda J, et al. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science. 2007; 315:820–5. [PubMed: 17255476]

Kawaguchi R, Yu J, Ter-Stepanian M, et al. Receptor-mediated cellular uptake mechanism that couples to intracellular storage. ACS Chem Biol. 2011; 6:1041–51. [PubMed: 21774515]

Kawaguchi R, Zhong M, Kassai M, et al. STRA6-catalyzed Vitamin A influx, efflux, and exchange. J Membrane Biol. 2012; 245:731–45. [PubMed: 22815070]

Kim YK, Wassef L, Hamberger L, et al. Retinyl ester formation by lecithin: retinol acyltransferase is a key regulator of retinoid homeostasis in mouse embryogenesis. J Biol Chem. 2008; 283:5611–21. [PubMed: 18093970]

Kopan R, Fuchs E. The use of retinoic acid to probe the relation between hyperpoliferation-associated keratins and cell proliferation in normal and malignant epidermal cells. J Cell Biol. 1989; 109:295–307. [PubMed: 2473080]

Korge B, Stadler R, Mischke D. Effect of retinoids on hyperproliferation-associated keratins K6 and K16 in cultured human keratinocytes: a quantitative analysis. J Invest Dermatol. 1990; 95:450–5. [PubMed: 1698888]

Lee DD, Stojadinovic O, Krzyzanowska A, et al. Retinoid-responsive transcriptional changes in epidermal keratinocytes. J Cell Physiol. 2009; 220:427–39. [PubMed: 19388012]

Neis MM, Wendel A, Wiederholt T, et al. Expression and induction of cytochrome p450 isoenzymes in human skin equivalents. Skin Pharmacol Physiol. 2010; 23:29–39. [PubMed: 20090406]

Niles RM. The use of retinoids in the prevention and treatment of skin cancer. Expert Opin Pharamcother. 2002; 3:299–303.

Patel GK, Wilson CH, Harding KG, et al. Numerous keratinocyte subtypes involved in wound re-epithelialization. J Invest Dermatol. 2006; 126:497–502. [PubMed: 16374449]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Pasutto F, Sticht H, Hammersen G, et al. Mutations in STRA6 cause a broad spectrum of malformations including anophthalmia, congenital heart defects, diaphragmatic hernia, alveolar capillary dysplasia, lung hypoplasia, and mental retardation. Am J Hum Genet. 2007; 80:550–60. [PubMed: 17273977]

Popadic S, Ramic Z, Medenica L, et al. Antiproliferative effect of vitamin A and D analogues on adult human keratinocytes in vitro. Skin Pharmacol Physiol. 2008; 21:227–34. [PubMed: 18509257]

Rask L, Peterson PA. In vitro uptake of vitamin A from the retinol-binding plasma protein to mucosal epithelial cells from the monkeýs small intestine. J Biol Chem. 1976; 251:6360–6. [PubMed: 824287]

Reijntjes S, Zile MH, Maden M. The expression of Stra6 and Rdh10 in the avian embryo and their contribution to the generation of retinoid signatures. Int J Dev Biol. 2010; 54:1267–75. [PubMed: 20563989]

Schiffer R, Neis M, Höller D, et al. Active influx transport is mediated by members of the organic anion transporting polypeptide family in human epidermal keratinocytes. J Invest Dermatol. 2003; 120:285–91. [PubMed: 12542534]

Schober M, Fuchs E. Tumor-initiating stem cells of squamous cell carcinomas and their control by TGF-beta and integrin/focal adhesion kinase (FAK) signaling. Proc Natl Acad Sci USA. 2011; 108:10544–9. [PubMed: 21670270]

Schoop VM, Mirancea N, Fusenig NE. Epidermal organization and differentiation of HaCaT keratinocytes in organotypic coculture with human dermal fibroblasts. J Invest Dermatol. 1999; 112:343–53. [PubMed: 10084313]

Sivaprasadarao A, Findlay JBC. Structure-function studies on human retinol-binding protein using site-directed mutagenesis. Biochem J. 1994; 300:437–42. [PubMed: 8002949]

Smeland S, Bjerknes T, Malaba L, et al. Tissue distribution of the receptor for plasma retinol-binding protein. Biochem J. 1995; 305:419–24. [PubMed: 7832754]

Stoler A, Kopan R, Duvic M, et al. Use of monospecific antisera and cRNA probes to localize the major changes in keratin expression during normal and abnormal epidermal differentiation. J Cell Biol. 1988; 107:427–46. [PubMed: 2458356]

Sundaram M, Sivaprasadarao A, Alaten DM, et al. Expression, characterization and engineered specificity of rat epididymal retinoic acid-binding protein. Biochem J. 1998; 334:155–60. [PubMed: 9693115]

Szeto W, Wen J, Tice DA, et al. Overexpression of the retinoic acid-responsive gene STRA6 in human cancers and its synergistic induction by Wnt-1 and retinoic acid. Cancer Res. 2001; 61:4197–205. [PubMed: 11358845]

Tooker P, Yen WC, Ng SC, et al. Bexarotene (LGD1069, Targretin), a selective retinoid X receptor agonist, prevents and reverse gemcitabine resistance in NSCLC cells by modulating gene amplification. Cancer Res. 2007; 67:4425–33. [PubMed: 17483357]

Törmä H, Vahlquist A. Vitamin A uptake by human skin in vitro. Arch Dermatol Res. 1984; 276:390–5. [PubMed: 6542769]

Törmä H. Regulation of keratin expression by retinoids. Dermatoendocrinology. 2011; 3:136–40.

Umemiya H, Fukasawa H, Ebisawa M, et al. Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers. J Med Chem. 1997; 40:4222–34. [PubMed: 9435893]

Van Rossum MM, Mommers JM, Van de Kerkhof PCM, et al. Coexpression of keratins 13 and 16 in human keratinocytes indicates association between hyperproliferation-assoziated and retinoid-induced differentiation. Arch Dermatol Res. 2000; 292:16–20. [PubMed: 10664010]

Wang CK, Nelson CF, Brinkman AM, et al. Spontaneous cell sorting of fibroblasts and keratinocytes creates an organotypic human skin equivalent. J Invest Dermatol. 2000; 114:674–80. [PubMed: 10733672]

Wang F, Kwak HSR, Elbuluk N, et al. Retinoic acid 4-hydroxylase inducibility and clinical response to isotretinoin in patients with acne. J Am Acad Dermatol. 2009; 61:252–8. [PubMed: 19525031]

Wawersik MJ, Mazzalupo S, Nguyen D, et al. Increased levels of keratin 16 alter epithelialization potential of mouse skin keratinocytes in vivo and ex vivo. Mol Biol Cell. 2001; 12:3439–50. [PubMed: 11694579]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Wawersik M, Coulombe PA. Forced expression of keratin 16 alters the adhesion, differentiation, and migration of mouse skin keratinocytes. Mol Biol Cell. 2000; 11:3315–27. [PubMed: 11029038]

Wolf G. Identification of a membrane receptor for retinol-binding protein functioning in the cellular uptake of retinol. Nutr Rev. 2007; 65:385–8. [PubMed: 17867372]

Wu K, Zhang Y, Xu XC, et al. The retinoid X receptor-selective retinoid, LGD1069, prevents the development of estrogen receptor-negative mammary tumors in transgenic mice. Cancer Res. 2002; 62:6376–80. [PubMed: 12438218]

Zouboulis CC, Korge B, Akamatsu H, et al. Effects of 13-cis-retinoic acid, all-trans-retinoic acid, and acitretin on the proliferation, lipid synthesis and keratin expression of cultured human sebocytes in vitro. J Invest Dermatol. 1991; 96:792–7. [PubMed: 1708801]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

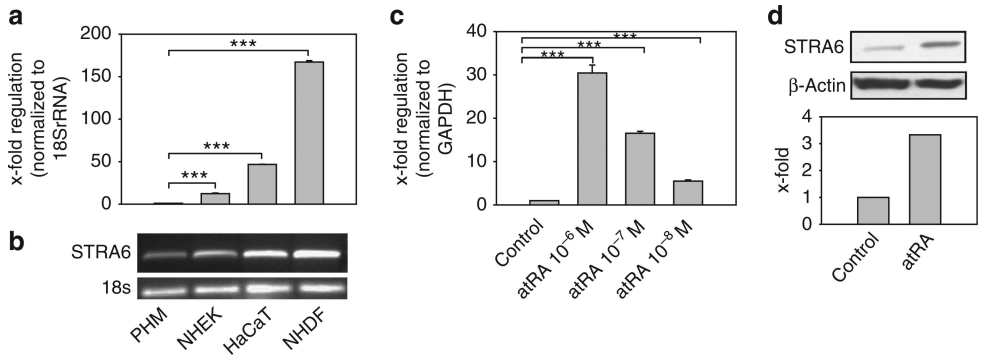

Figure 1. STRA6 is constitutively expressed in skin cells and regulated by all-trans retinoic acid (atRA)

(**a, b**) TaqMan real-time PCR analysis of Stra6 mRNA expression in different human skin cells, primary human melanocytes (PHM), normal human epidermal keratinocytes (NHEK), human adult low calcium high temperature (HaCaT) cells, and normal human dermal fibroblasts (HDF), was performed. qRT–PCR products were separated on 1.8% agarose gel and stained with ethidium bromide. Values of ***P<0.001 were considered significant. (**c, d**) Stra6 mRNA expression increased in the murine keratinocyte cell line PAM212 after the addition of atRA at various concentrations (10^{-6}, 10^{-7}, or 10^{-8} M) for 24 hours. TaqMan real-time PCR analysis was performed (**c**). Stra6 protein expression increased after atRA stimulation in the murine keratinocyte cell line PAM212. Western blot was performed and quantified b-actin was used as loading control (**d**).

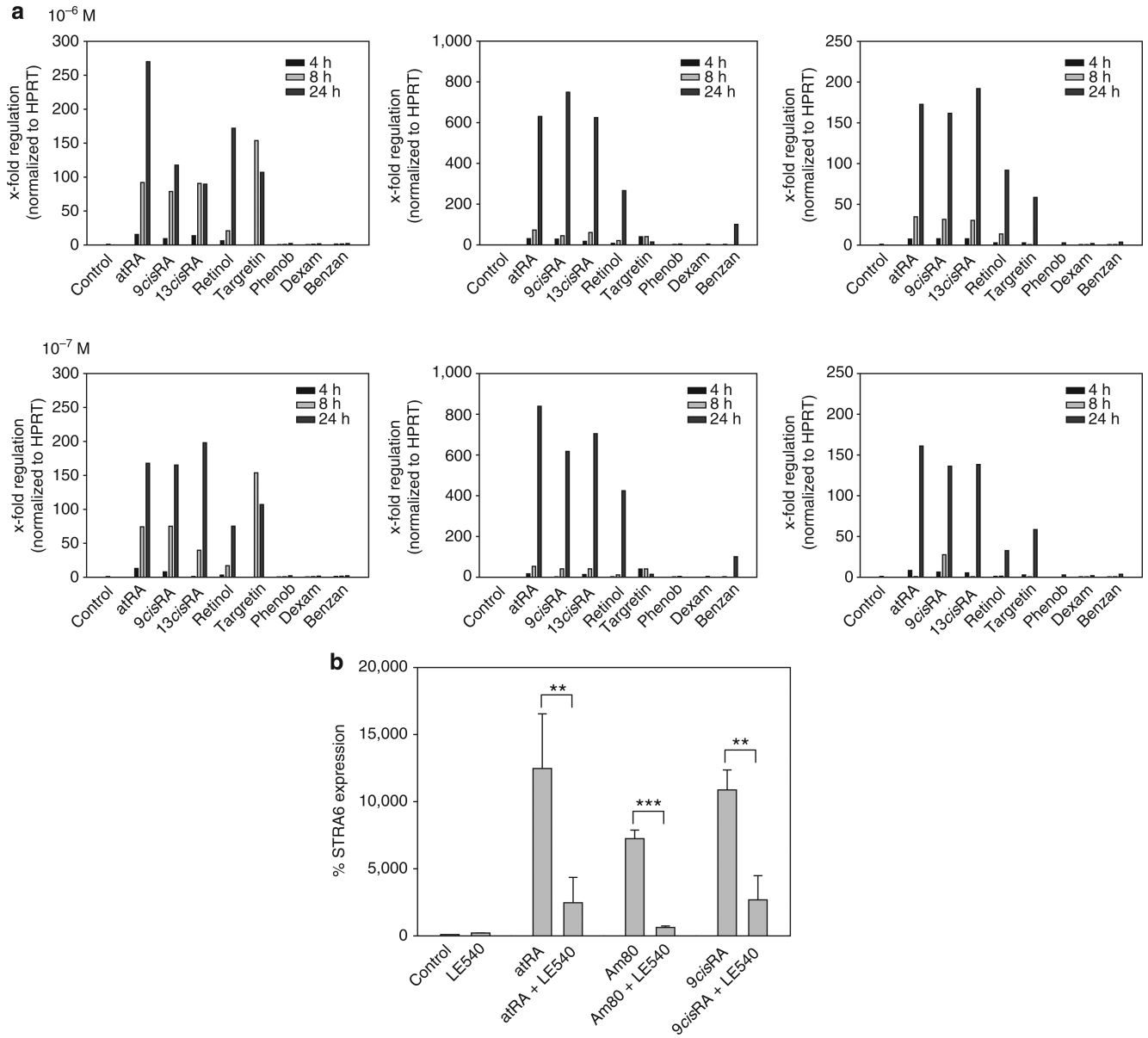

Figure 2. Regulation of Stra6 expression in normal human epidermal keratinocytes (NHEKs)

(**a**) NHEKs of three independent donors were stimulated with different ligands of retinoic acid receptor (RAR) and ligands of other class II nuclear receptors at a concentration of 10^{-6} and 10^{-7} M for 4, 8, and 24 hours. Untreated cells were used as control. TaqMan real-time PCR analysis was performed. (**b**) NHEKs of two independent donors were stimulated with different ligands of RAR at a concentration of 10^{-6} M and LE540, a RAR panantagonist, at a concentration of 2.5 μmM for 24 hours. Untreated cells and cells treated only with LE540 were used as control. Values of $^{**}P<0.01$ and $^{***}P<0.001$ were considered significant.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

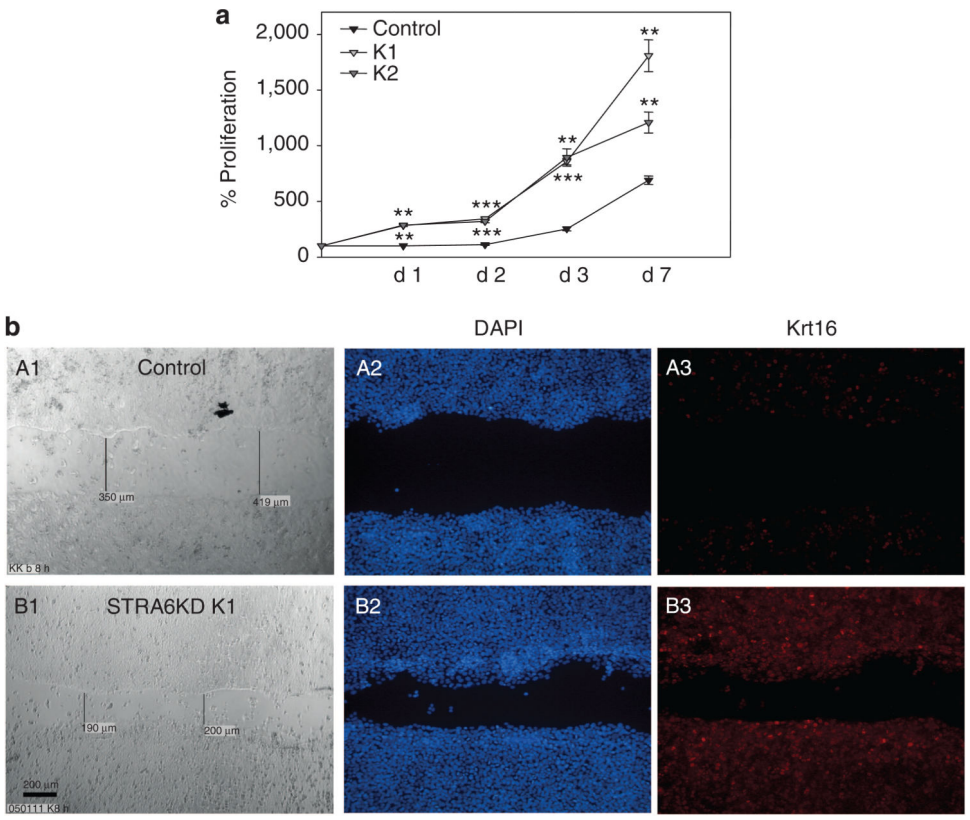

Figure 3. Proliferation is highly upregulated in stable STRA6KD HaCaT cells

(**a**) Proliferation of stable STRA6KD cells K1 and K2 and of control cells transduced with non-target vector was measured using AlamarBlue from day 1 to day 7. Values of ***P*<0.01, and ****P*<0.001 were considered significant. (**b**) Scratch assay: culture inserts (IBIDI) were placed in chamber slides and 1.4×10^5 cells were seeded in each chamber of the inserts. The culture inserts were removed when cells were confluent, leading to a cell-free gap of ~500 mm. After 8 hours the experiment was completed and immunofluorescence staining of DAPI and Krt16 was performed. Scale bar: 6 mm = 200 μm.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

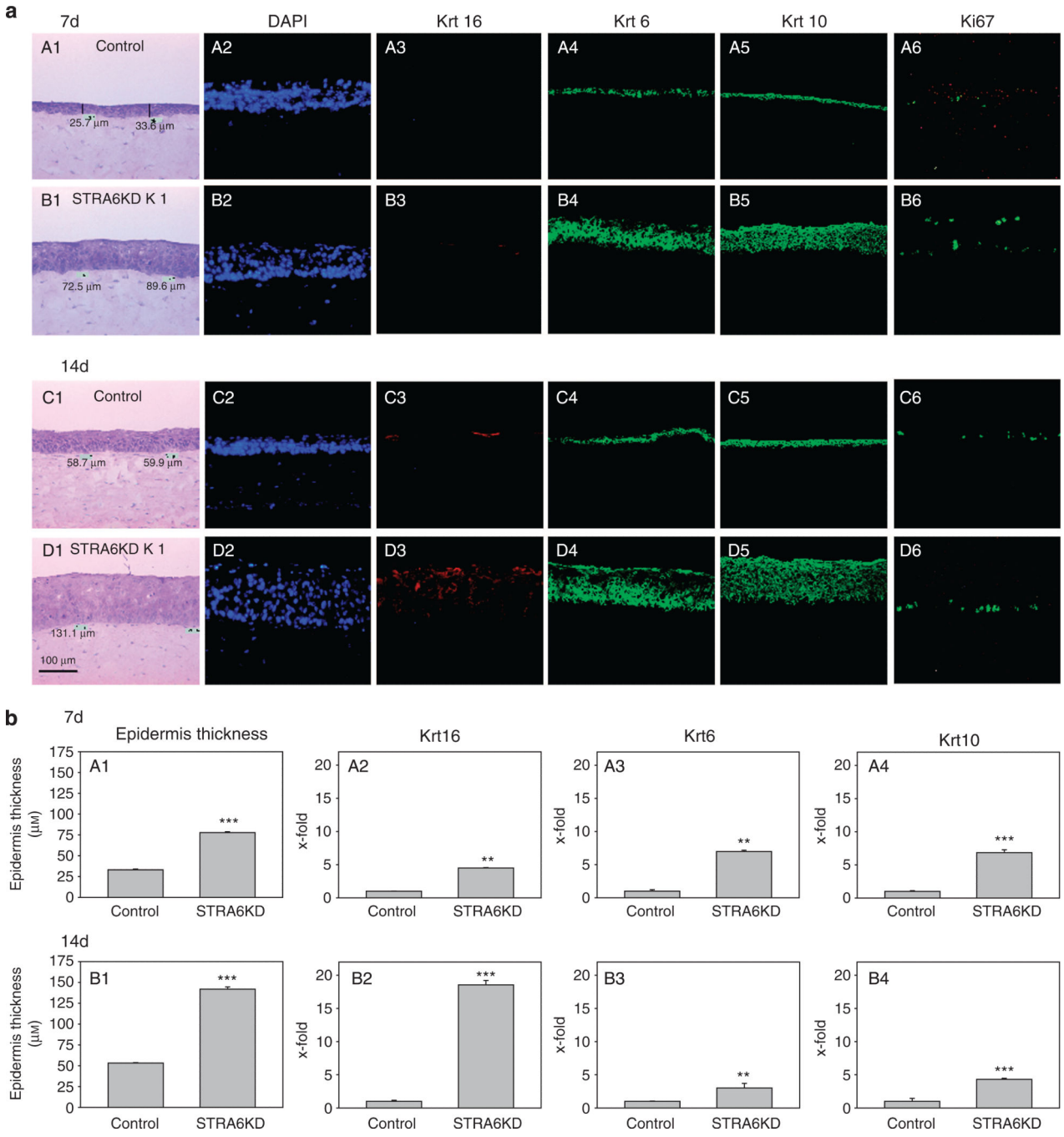

Figure 4. Human HaCaT 3D skin equivalents with STRA6KD showed thicker epidermis and strongly enhanced expression of proliferation, activation, and differentiation markers

(**a**) 3D skin models using STRA6KD cells (A, C)) and HaCaT control cells (B, D) were established and harvested after 7 days or 14 days. Immunofluorescence examination of DAPI (A2–D2), Krt16 (A3–D3), Krt6 (A4–D4), Krt10 (A5–D5), and Ki67 (A6–D6) was performed. Scale bar: 9 mm = 100 µm. (**b**) Epidermal thickness (A1–B1) was measured and data were presented as mean epidermis thickness in mm. For Krt16 (A2–B2), Krt6 (A3–B3), and Krt10 (A4–B4) three slides were determined for STRA6KD and control models. Data

are presented as x-fold to the control. Values of $^{**}P<0.01$, and $^{***}P<0.001$ were considered significant.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

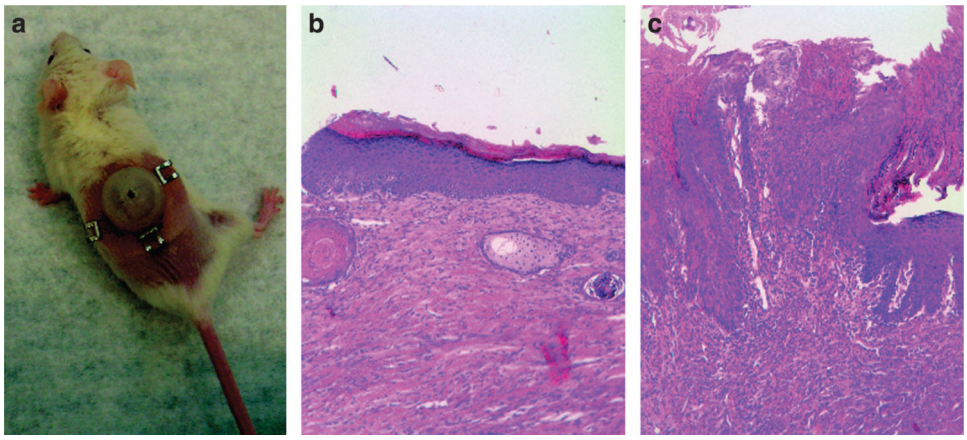

Figure 5. Human skin reconstitution in the cell-sorted skin equivalent (CeSSE) model in NOD/SCID mice

(**a**) Implantation of human skin cells in a silicon chamber implanted on the back of a SCID mouse. Four weeks after cell implantation skin biopsies were taken. (**b**) Epidermis and upper dermis generated using control HaCaT keratinocytes and fibroblasts. (**c**) Epidermis and upper dermis generated using STRA6KD HaCaT cells and fibroblasts.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

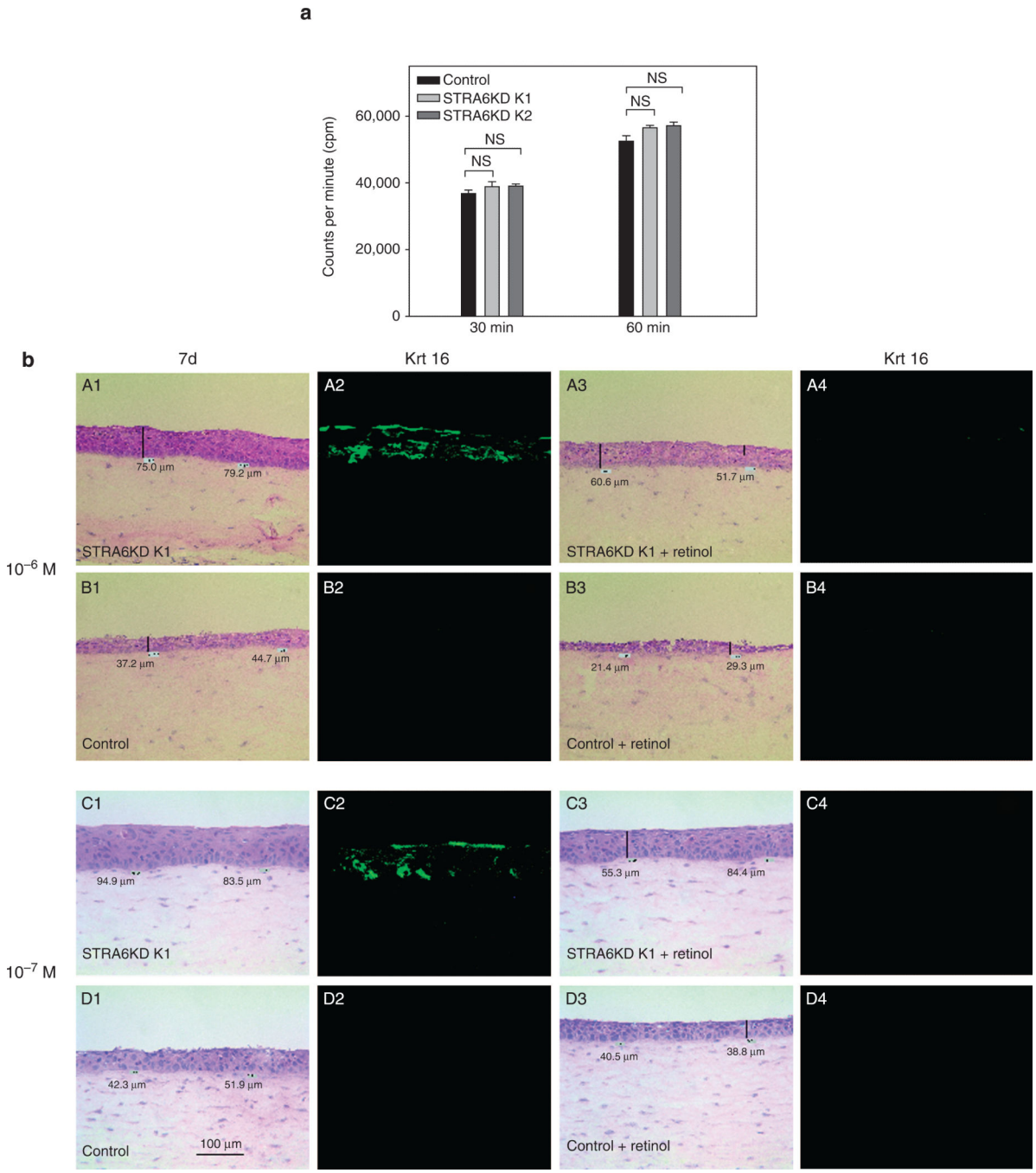

Figure 6. Effects of STRA6KD in human 3D HaCaT skin equivalents are reversed after incubation with all-trans retinol (atROL)

(**a**) HaCaT control cells and STRA6KD cells were exposed to radiolabeled free atROL. After incubation for 30 or 60 minutes, the radioactive medium was removed, and cells were washed and lysed by adding scintillation fluid. The cell-associated radioactivity was determined. The values are expressed as counts per minute and differences between cells were not significant (NS). (**b**) After lifting the skin equivalent to the air–liquid interphase, atROL was added every 48 hours at a concentration of 10^{-6} or 10^{-7} M. STRA6KD and HaCaT control 3D models without atROL treatment were used as control. 3D models were

harvested after 7 days. Immunofluorescence examination of Krt16 (A2–B2) was performed. Scale bar: 9 mm = 100 μm

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript