

A Synthetic Sandalwood Odorant Induces Wound-Healing Processes in Human Keratinocytes via the Olfactory Receptor OR2AT4

Daniela Busse¹, Philipp Kudella¹, Nana-Maria Grüning^{1,7}, Günter Gisselmann¹, Sonja Ständer², Thomas Luger³, Frank Jacobsen⁴, Lars Steinsträßer^{4,8}, Ralf Paus⁵, Paraskevi Gkogkolou⁶, Markus Böhm⁶, Hanns Hatt¹ and Heike Benecke^{1,9}

As the outermost barrier of the body, the skin is exposed to multiple environmental factors, including temperature, humidity, mechanical stress, and chemical stimuli such as odorants that are often used in cosmetic articles. Keratinocytes, the major cell type of the epidermal layer, express a variety of different sensory receptors that enable them to react to various environmental stimuli and process information in the skin. Here we report the identification of a novel type of chemoreceptors in human keratinocytes, the olfactory receptors (ORs). We cloned and functionally expressed the cutaneous OR, OR2AT4, and identified Sandalore, a synthetic sandalwood odorant, as an agonist of this receptor. Sandalore induces strong Ca^{2+} signals in cultured human keratinocytes, which are mediated by OR2AT4, as demonstrated by receptor knockdown experiments using RNA interference. The activation of OR2AT4 induces a cAMP-dependent pathway and phosphorylation of extracellular signal-regulated kinases (Erk1/2) and p38 mitogen-activated protein kinases (p38 MAPK). Moreover, the long-term stimulation of keratinocytes with Sandalore positively affected cell proliferation and migration, and regeneration of keratinocyte monolayers in an *in vitro* wound scratch assay. These findings combined with our studies on human skin organ cultures strongly indicate that the OR 2AT4 is involved in human keratinocyte re-epithelialization during wound-healing processes.

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INTRODUCTION

The expression of olfactory receptors (ORs) is not restricted only to the nasal epithelium, but it also occurs in various human tissues (Feldmesser *et al.*, 2006; Zhang *et al.*, 2007;

Flegel *et al.*, 2013). Currently, the function of most ectopically expressed ORs remains unknown. However, an increasing number of studies have described physiological roles for ectopically expressed ORs in various human cell types (Kang and Koo, 2012) such as spermatozoa (Spehr *et al.*, 2003; Veitinger *et al.*, 2011), prostate epithelial cells (Neuhaus *et al.*, 2009), and enterochromaffin cells of the gut (Braun *et al.*, 2007). However, the human skin, which functions as the outermost barrier of the body and is in direct contact with the chemical diversity of our environment, has not yet been described to express physiologically functional ORs.

The skin not only has multiple functions as a barrier, such as protecting the body against pathogens and excessive water loss, but it also senses environmental information such as temperature, humidity, and mechanical stimulation (Denda *et al.*, 2007; Ikeyama *et al.*, 2013). Epidermal keratinocytes express a variety of different receptors, such as transient receptor potential channels (Inoue *et al.*, 2002; Chung *et al.*, 2004; Ständer *et al.*, 2004; Denda and Tsutsumi, 2011), ATP receptors (Denda *et al.*, 2002; Inoue *et al.*, 2005; Denda *et al.*, 2012), and endocrinology receptors (Slominski and Wortsman, 2000; Feingold and Denda, 2012), which enable cutaneous chemosensation and contribute to information processing in the skin (Denda *et al.*, 2007). Therefore, it has been suggested that keratinocytes form the forefront of

¹Department of Cellphysiology, Ruhr-University Bochum, Bochum, Germany;

²Department of Dermatology, Competence Centre Chronic Pruritus, University Hospital Münster, Münster, Germany; ³University Hospital Münster, Münster, Germany; ⁴Department of Plastic Surgery, BG University Hospital Bergmannsheil, Ruhr-University Bochum, Bochum, Germany; ⁵Institute of Inflammation and Repair, University of Manchester, Manchester, UK and ⁶Laboratory for Neuroendocrinology of the Skin and Interdisciplinary Endocrinology, Department of Dermatology, University Hospital Münster, Münster, Germany

⁷Current address: Department of Biochemistry, University of Cambridge, Cambridge, UK.

⁸Current address: Evangelisches Krankenhaus Oldenburg, Oldenburg, Germany.

⁹Current address: Center Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB), Göttingen, Germany.

Correspondence: Hanns Hatt, Department of Cellphysiology, Ruhr-University Bochum, Universitätsstrasse 150, 44780 Bochum, Germany.
E-mail: hanns.hatt@rub.de

Abbreviations: CNG, cyclic-nucleotide gated; MAPK, mitogen-activated protein kinase; OR, olfactory receptor; RT, reverse transcriptase; scRNA, scrambled RNA; siRNA, small interfering RNA

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skin surface perception and process information to the nervous system (Denda, 2003; Paus *et al.*, 2006).

We previously showed that the stimulation of keratinocytes with synthetic sandalwood odorants, often exposed to the skin as ingredients of perfumes, emollients, and cleaning agents, results in a transient increase of the intracellular calcium concentration (Sondersorg *et al.*, 2014). This observation raises the question of whether keratinocytes express physiologically functional ORs that detect odorants such as synthetic sandalwood odorants.

RESULTS

OR 2AT4 is expressed in human primary keratinocytes and is specifically activated by synthetic sandalwood odorants

To investigate the general expression of ORs in cultured human primary keratinocytes, we analyzed the mRNA expression of virtually all known ORs via a customized microarray chip (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). We generated a ranking of the expression intensities of ORs in keratinocytes and analyzed the top eight candidates (Figure 1a). For five of these candidate receptors (OR6M1, OR11A1, OR2AT4, OR5V1, and OR6V1), the expression in keratinocytes could be confirmed via standard reverse transcriptase PCR (RT-PCR) analysis (Figure 1b).

In this study, we concentrated on OR2AT4 for functional characterization and deorphanization because we could express this OR as a rhodopsin-tagged construct (Krautwurst *et al.*, 1998; Wetzel *et al.*, 1999) in Hana3A cells (Saito *et al.*, 2004), a HEK293T-derived cell line that stably expresses RTP1L, RTP2, REEP1, and $G_{\alpha_{olf}}$, and supports the robust heterologous expression of various ORs (Saito *et al.*, 2004). Appropriate cell surface localization of the OR2AT4 receptor observed 2 days after transfection via immunocytochemical staining indicated the successful integration of the OR in the Hana3A cell membrane (Supplementary Figure S1a online).

To identify specific ligands, screenings with a mixture of 100 structurally different odorants were performed using the calcium imaging technique as previously described (Wetzel *et al.*, 1999; Spehr *et al.*, 2003; Neuhaus *et al.*, 2009). The deorphanization of OR2AT4 revealed significant activation by Sandalore, a synthetic sandalwood odorant, at a concentration of 1 mM (Figure 1c and d). In addition, the investigation of the molecular receptive field of OR2AT4, using six structurally related sandalwood odorants and natural sandalwood oil, identified only Brahmanol as another potential agonist, inducing Ca^{2+} signals in OR2AT4-transfected Hana3A cells. Sham-transfected Hana3A cells showed no odor-induced Ca^{2+} signals for either of the tested odorants (Supplementary Figure S1b online).

Moreover, the activation of OR2AT4 by Sandalore and Brahmanol was analyzed using a CRE-luciferase reporter gene assay (Adipietro *et al.*, 2012; Wallrabenstein *et al.*, 2013). Sandalore and Brahmanol induced significantly increased luminescent signals, whereas the application of Sandranol (as negative control) showed no effect (Figure 1e). Only three of the eight sandalwood odorants could be tested at low odorant concentrations ($<100\mu M$), as the other odorants irreversibly damaged the cells.

Furthermore, we identified two antagonists for OR2AT4, Oxyphenylon and Phenirat, which blocked Sandalore-induced Ca^{2+} signals in OR2AT4-transfected Hana3A cells when co-applied at equimolar concentrations with Sandalore in calcium imaging experiments (Figure 1d). The application of either antagonist alone at an mM concentration did not elicit Ca^{2+} responses (Supplementary Figure S1b online). Figure 1f illustrates the characterized receptive field of OR2AT4.

Expression of OR2AT4 in skin cells and various tissues

Apart from expression in primary cultured keratinocytes in various passages (P_0 – P_2), OR2AT4 mRNA could be detected in HaCaT cells and in whole human skin (punch biopsy) using standard RT-PCR analysis (Figure 2a). OR2AT4 transcripts were also detected in other skin cells, such as dendritic cells and melanocytes, but not in cells of connective tissue, such as fibroblasts or adipocytes.

For the detection of OR2AT4 receptor proteins, the immunocytochemical staining of cultured keratinocytes and skin specimen was performed using a custom-made antibody. The antibody specificity was verified by immunocytochemical staining experiments with OR2AT4-transfected Hana3A cells (Supplementary Figure S2a online) and the use of a specific OR2AT4-blocking peptide (Supplementary Figure S2b online). Both HaCaT cells and primary keratinocytes showed an obvious expression of the receptor protein (Figure 2b). In normal skin specimens, OR2AT4 could be detected in the keratinocytes of the epidermis (Figure 2c), with basal keratinocytes showing the strongest staining (Figure 2c, left). Control experiments performed using rabbit pre-immune serum showed clearly distinguishable nuclear staining (Figure 2c, right).

Sandalore activates human primary keratinocytes and HaCaT cells by activating the cAMP-dependent pathway

On short-term application (20 seconds) of 500 μM Sandalore in calcium imaging experiments, an increase in the intracellular calcium concentration could be observed in at least 70% of primary human keratinocytes and 95% of HaCaT cells at the fourth application (Figure 3a and b, and Supplementary Figure S3a online). The Sandalore-induced Ca^{2+} signals were significantly sensitized on repetitive stimulation, which is more pronounced in HaCaT cells than in primary keratinocytes (Figure 3c). The activation is dose dependent with an EC_{50} of 430 μM in HaCaT cells at the first application (Figure 3d). Similarly, the activation of keratinocytes or HaCaT cells was observed for 500 μM Brahmanol, which also induced sensitizing Ca^{2+} signals (Supplementary Figure S3b online).

The signal transduction pathway, activated by Sandalore in primary human keratinocytes, was characterized pharmacologically. Calcium imaging measurements under calcium-free conditions showed that the Sandalore-evoked response of keratinocytes depends on extracellular calcium (Figure 3f). Furthermore, the co-application of keratinocytes with the adenylyl cyclase inhibitors MDL-12.330A (40 μM ; Figure 3j) (Siegel and Wiech, 1976) or SQ-22536 (100 μM ; Figure 3g) (Weinryb and Michel, 1974) significantly diminished Sandalore-induced Ca^{2+} signals (Figure 3j).

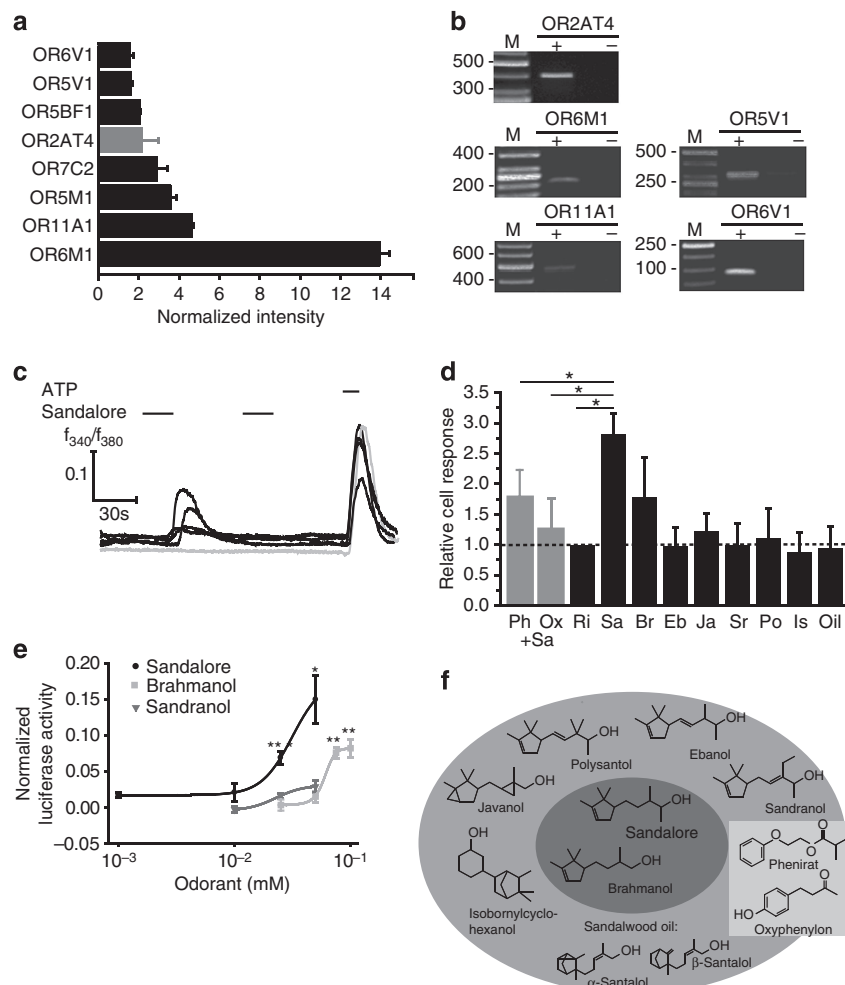


Figure 1. OR2AT4 is expressed in human primary keratinocytes and specifically activated by Sandalore and Brahmanol. (a) Microarray analysis of olfactory receptors (ORs) in primary cultured keratinocytes ($n = 2$ cultures). The mean normalized expression intensities are shown. (b) Reverse transcriptase PCR (RT-PCR) analysis of ORs obtained from microarray data. “+”, + RT, cDNA; “-”, - RT, RNA; M, marker. Numbers, length of fragments (bp). (c) Representative calcium imaging traces of Hana3A cells transiently transfected with OR2AT4 (gray) or a sham-transfected Hana3A control cell (black) that was stimulated with Sandalore (1 mM). (d) Quantification of deorphanization studies of OR2AT4 expressing Hana3A cells. Numbers of responding Hana3A cells to different odorants (1 mM) were compared relative to the number of cells responding to the application of Ringer's solution as a control. Number of measurements (n): Ri, Ringer's solution (23); Sa, Sandalore (22); Br, Brahmanol (20); Ja, Javanol (15); Sr, Srandranol (18); Eb, Ebanol (15); Is, isobornylcyclohexanol (14); Po, Polysantol (14); Oil, natural sandalwood oil (1:10,000) (14); Ph, Phenirat (22); Ox, Oxyphenylol (20). (e) The response of Hana3A cells transiently transfected with OR2AT4 to Sandalore and Brahmanol in a CRE-luciferase-based cell assay significantly differed from that of the mock-transfected controls (not shown). $n \geq 3$ experiments. (f) Molecular receptive field of OR2AT4; inner circle, agonists; outer circle, inactive substances; box, antagonists. For sandalwood oil, the two main components, α - and β -Santalol, are shown. The data are shown as the means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

In contrast, the preincubation of keratinocytes with the phospholipase C-inhibitor U-73122 had no effect on Sandalore-induced calcium signaling (Figure 3i and j). U-73122 functionality was demonstrated by the inhibition of histamine-induced calcium signals in keratinocytes based on a Phospholipase C-dependent pathway (Koizumi and Ohkawara, 1999; Fitzsimons *et al.*, 2002) (Figure 3i).

As the blocker experiments suggested the involvement of cAMP, we directly tested whether Sandalore affects the intracellular cAMP level using a cAMP assay and observed that the application of this odorant increases the cAMP level in HaCaT cells nearly threefold. The dose dependency, with an EC_{50} of $197 \mu\text{M}$ (Figure 3e), was in the same range as that for the Sandalore-induced Ca^{2+} increase. Further downstream

of the signaling cascade, CNG (cyclic-nucleotide gated) channels, for which the subunits CNGB1 and CNGA1 are expressed in keratinocytes and HaCaT cells (Supplementary Figure S3c online, Oda *et al.*, 1997), are involved in the Sandalore-induced pathway, as demonstrated using the CNG-channel inhibitor L-*cis* Diltiazem (Haynes, 1992), which significantly reduced Sandalore-dependent calcium signals (Figure 3h and j).

Sandalore-induced Ca^{2+} signals are mediated by OR2AT4 in HaCaT cells

To confirm that the Sandalore-induced Ca^{2+} increase is mediated by OR2AT4 activation, we reduced the *in vitro* expression level of the receptor using a combination of two small

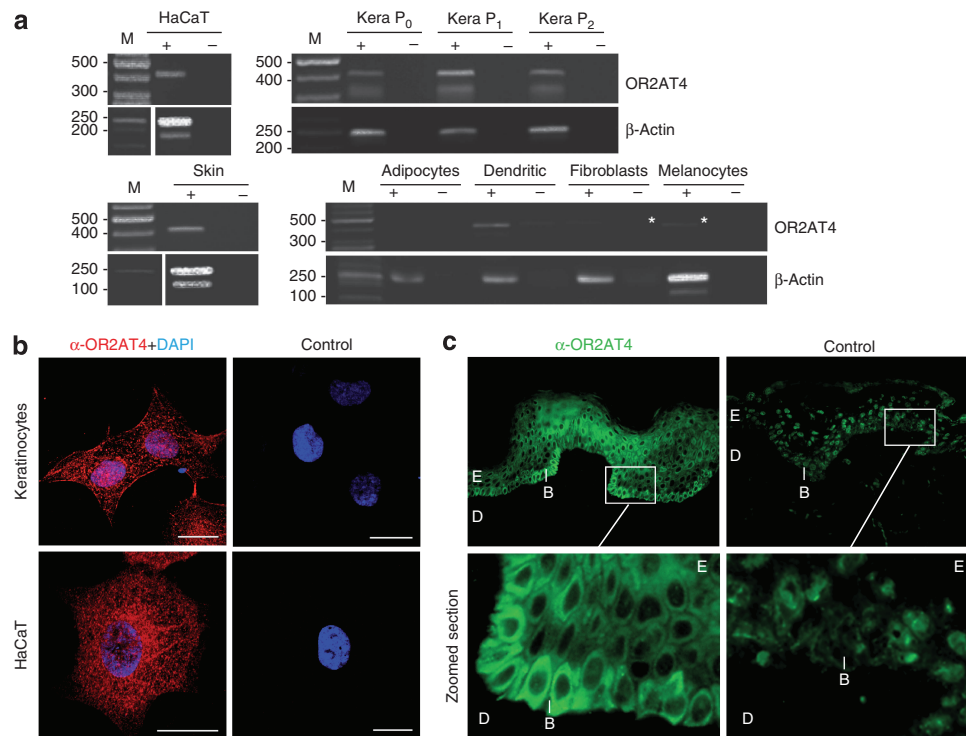


Figure 2. Olfactory receptors (ORs) are expressed in human skin cells. (a) Reverse transcriptase PCR (RT-PCR) analysis of OR2AT4 (top trace) in different human tissues and skin cells. “+”, + RT, cDNA; “–”, – RT, RNA; M, marker. β-Actin (bottom trace), control for RNA quality. Stars, position of weak fragment. Numbers, length of fragments (bp). (b) Immunocytochemical staining of HaCaT cells and primary keratinocytes with an α-OR2AT4 antibody (red). DAPI (4',6-diamidino-2-phenylindole) staining (blue) was used to determine the number and location of cells. Control staining was performed without the primary antibody. Bar = 20 μm. (c) Immunohistochemical staining of human skin slices with α-OR2AT4 antibody. As a negative control, the primary antibody was replaced with rabbit pre-immune serum. Four hundred-fold magnification (top); zoomed section (bottom). B, basal keratinocytes; D, dermis; E, epidermis.

interfering RNAs (siRNA). The inhibitory effect of both siRNA on OR2AT4 expression was confirmed in HEK293 cells by the quantitative evaluation of the siRNA activity using the pmirGlo Dual-Luciferase siRNA Target Expression Vector (Supplementary Figure S4a online).

In calcium imaging experiments, we stimulated HaCaT cells transfected (48 hours) with siRNA or scrambled RNA (scRNA) repetitive with Sandalore and compared Sandalore-induced Ca^{2+} signals in siRNA-expressing with scRNA-expressing HaCaT cells (identifiable by co-expressed GFP, Supplementary Figure S4b online). Strikingly, the quantification of the Ca^{2+} signals revealed that siRNA significantly reduced the Sandalore-mediated Ca^{2+} increase (~80% at the first application; Figure 4a and b).

Furthermore, the co-application of the specific OR2AT4 antagonist Oxyphenylon or Phenirat with Sandalore (1:1, 500 μM) completely blocked Sandalore-induced calcium signals in HaCaT cells (Figure 4c and d). Together with positive siRNA experiments, these results showed that the effects of Sandalore are based on the activation of OR2AT4 in keratinocytes.

In addition, Sandalore-induced Ca^{2+} signals were inhibited in a dose-dependent manner with an IC_{50} of 178 μM for Phenirat and 174 μM for Oxyphenylon (Figure 4e, left). In addition, Sandalore responses were rescued when the agonist concentrations were increased to the fixed amount of the

antagonists (Figure 4e, right), suggesting a competitive mechanism.

Sandalore induced an increased rate of keratinocyte proliferation and migration, and epidermal reepithelialization in a skin organ culture

To examine the physiological function of OR2AT4 in keratinocytes, we first investigated the effect of Sandalore on cell viability using propidium iodide staining. After treatment of HaCaT cells and primary keratinocytes with Sandalore (500 μM) for 5 days, the cells showed no alterations in cell morphology or any apoptotic effects (Figure 5a), but a significant increase (32%) in cell proliferation was observed (Figure 5b). The application of siRNA targeting OR2AT4 abolished the enhanced proliferative effect, thus underlining the specific involvement of OR2AT4 in this physiological process. Unspecific effects could be excluded by the application of scRNA, which did not affect proliferation (Figure 5b).

In addition, long-term stimulation with Brahmanol (500 μM) induced no apoptotic effects on HaCaT cells and primary keratinocytes, but an increased rate of proliferation similar to Sandalore was observed (Supplementary Figure S5a and b online).

Increased keratinocyte proliferation in combination with cell migration is an indicator for reepithelialization in wound healing (Woodley *et al.*, 1993; Gurtner *et al.*, 2008; Shaw and

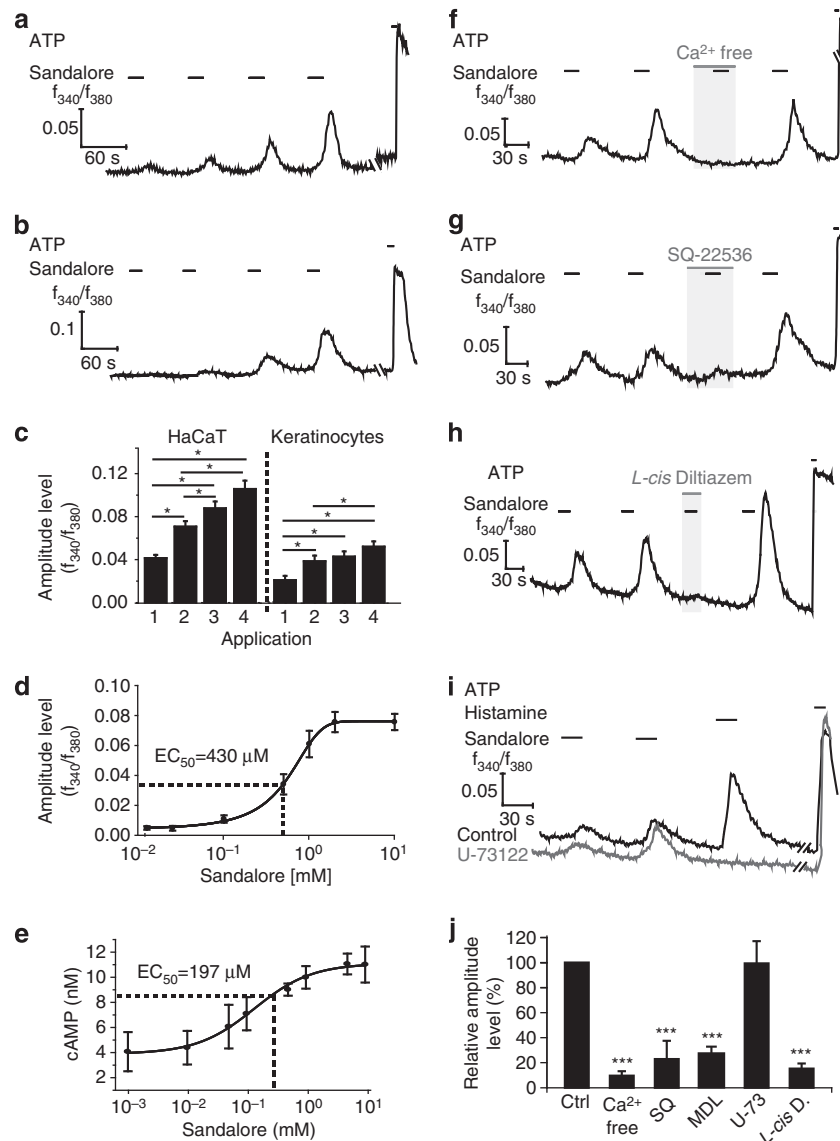


Figure 3. Sandalore induces Ca²⁺ signals in primary keratinocytes and HaCaT cells that depend on a cAMP-signaling cascade. Repetitive stimulation (20 seconds) of (a) HaCaT cells ($n=186$ cells) and (b) primary keratinocytes ($n=195$ cells) with 500 μM Sandalore in calcium imaging experiments. (c) Mean of amplitudes of Sandalore-induced Ca²⁺ signals in keratinocytes and HaCaT cells. (d) Dose–response curve of Sandalore-induced Ca²⁺ signals. The mean amplitudes of stimulated HaCaT cells at the first application in calcium imaging experiments are shown. Number of cells (n): 0.0125 mM (39), 0.025 mM (30), 0.1 mM (46), 0.5 mM (34), 1 mM (63), 2 mM (41), 10 mM (36). (e) Sandalore induced a dose-dependent increase of cAMP in HaCaT cells; $n \geq 3$ experiments. (f) Ca²⁺ free extracellular solution, (g) adenylyl cyclase inhibitor SQ-22536 (100 μM), or (h) cyclic-nucleotide gated (CNG) channel blocker *L-cis* Diltiazem (100 μM) significantly reduced the Sandalore-induced Ca²⁺ signaling in cultured keratinocytes when co-applied with Sandalore (500 μM) in calcium imaging experiments. (i) Keratinocytes reacted to Sandalore after preincubation (10 minutes) with phospholipase C inhibitor U-73122 (10 μM , gray curve; control: black curve (without U-73122)), but these cells did not react to histamine (10 μM). (j) Quantification of Sandalore-induced Ca²⁺ signals of cultured keratinocytes in blocker measurements relative to control measurements. Ctrl, control; Ca²⁺ free, Ca²⁺ free extracellular solution, $n=52$ cells; SQ, SQ-22536, $n=26$ cells; *L-cis* D, *L-cis* Diltiazem, $n=25$ cells; U-73, U-73122, $n=64$ cells; MDL, MDL-12.330A, 40 μM , $n=24$ cells. The data are shown as the means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Martin, 2009; Müller *et al.*, 2012; Plikus *et al.*, 2012). Therefore, we examined the migration of HaCaT cells using an agarose gel assay, which facilitates the examination of chemotactic cell migration in the presence of a certain effector. Compared with 0.1% DMSO (control), the migration of Sandalore-stimulated keratinocytes was significantly increased (40% distance and 50% area) after incubation for 5 days (Supplementary Figure S5c online).

To determine the signaling pathway that mediates the induction of proliferation and migration in HaCaT cells, we used western blot analysis to examine the effect of Sandalore on the phosphorylation of members of the mitogen-activated protein kinase (MAPK) family, which mediate the regulation of many cellular processes, including wound healing (Marshall, 1994; Seger and Krebs, 1995). Indeed, Sandalore increased p38 and Erk1/2 MAPK phosphorylation after 5 minutes of

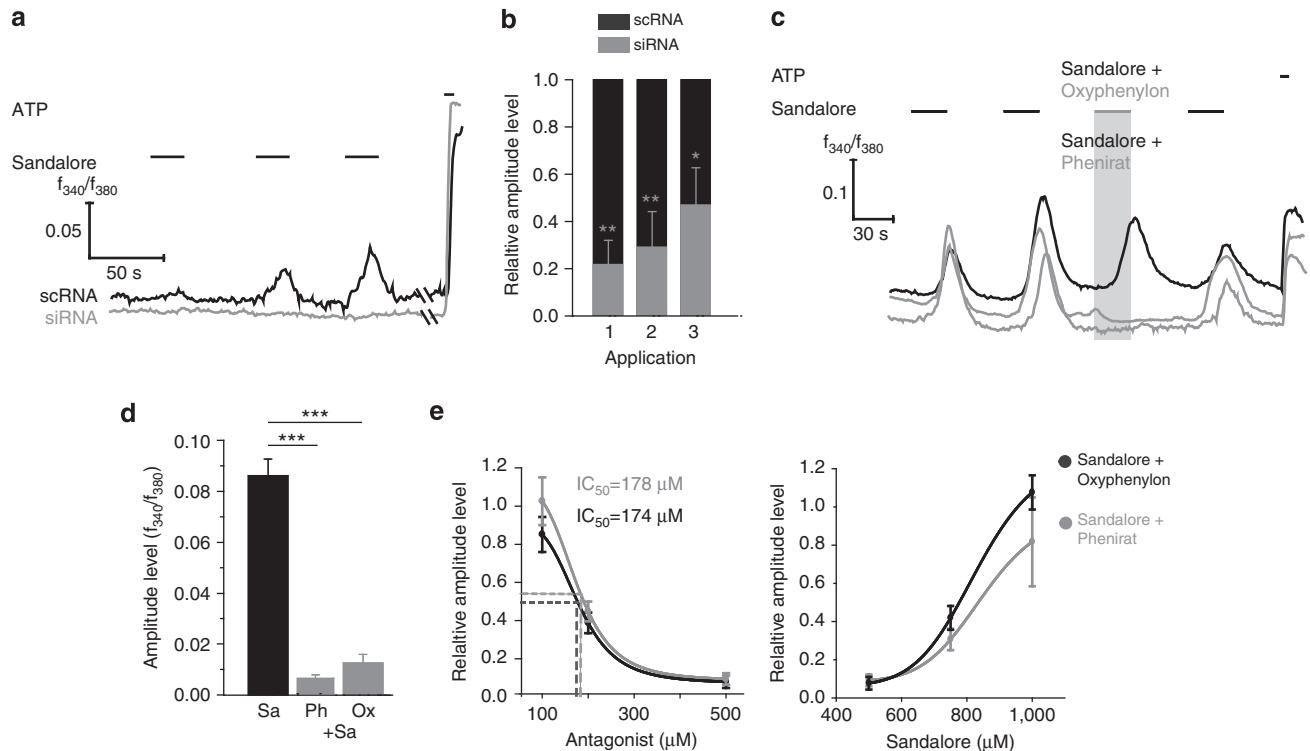


Figure 4. OR2AT4-dependent induction of Ca^{2+} signals in HaCaT cells by Sandalore. (a) Representative calcium imaging trace of a scrambled RNA (scRNA)- (black) and small interfering RNA (siRNA)-expressing HaCaT cell (gray) repetitively stimulated with 500 μ M Sandalore 48 hours after transfection. Control: 0.1% DMSO. (b) Comparison of the Ca^{2+} -signal amplitudes of siRNA-expressing HaCaT cells relative to scRNA-expressing HaCaT cells at 48 hours after transfection. Each Ca^{2+} signal was normalized to ATP prior to calculation. ($n = 18$ cells (siRNA), $n = 17$ cells (scRNA).) (c) Representative calcium imaging trace of HaCaT cells co-stimulated (1:1, 500 μ M) with Sandalore and Oxyphenylol ($n = 43$ cells) or Phenirat ($n = 67$ cells). (d) Quantification of Ca^{2+} signals of co-stimulated HaCaT cells with Sandalore (500 μ M) and antagonists (500 μ M) in calcium imaging experiments. Ox, Oxyphenylol; Ph, Phenirat; Sa, Sandalore. (e) Left: determination of the IC_{50} values of both antagonists by the co-application of different concentrations of antagonists and 500 μ M Sandalore on HaCaT cells in calcium imaging experiments. Right: calcium imaging experiments with fixed antagonist concentration (500 μ M) and increasing Sandalore concentration. Amplitude levels of Ca^{2+} signals are shown relative to control measurements. Number of cells (n): Oxyphenylol 100 μ M (58), 250 μ M (47), 300 μ M (46), 500 μ M (56, 70); Phenirat 100 μ M (35), 250 μ M (27), 300 μ M (37), 500 μ M (22, 23); the data are shown as the means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

continued ligand exposure (Figure 5c). The quantification of western blot experiments showed the significant phosphorylation of Erk1/2 and p38 MAPK on Sandalore stimulation (Figure 5d).

As both MAPKs coordinate cellular migration and proliferation in epithelial wound healing (Sharma *et al.*, 2003; Wang *et al.*, 2006; Shibata *et al.*, 2012), we further investigated whether Sandalore stimulation leads to increased "wound" closure in a monolayer of human keratinocytes using an *in vitro* wound scratch assay. Exposure to Sandalore (500 μ M; 48 hours) induced the significant acceleration of the regeneration rate of the monolayer compared with 0.1% DMSO-stimulated cells (Figure 5e). The same results were obtained with HaCaT cells (Supplementary Figure S5d online). Co-stimulation with antagonist or specific MAPK inhibitors significantly diminished the positively affected "wound" closure in HaCaT cells (Supplementary Figure S11b online). MAPK inhibitors and antagonists alone had no significant effect on "wound" regeneration (Supplementary Figure S5e online).

Next, we assessed the impact of Sandalore on human skin organ cultures as an *ex vivo* model of epidermal wound

healing. To this end, we measured the length and size of the epidermal tongues in full-thickness skin explants after treatment with Sandalore (50 μ M) versus control (0.01% DMSO) for 5 days. Sandalore significantly increased both parameters compared with DMSO-treated skin organ cultures (Figure 5f).

DISCUSSION

The expression of human ORs is not restricted only to the nasal epithelium, but it also occurs in different non-olfactory tissues (Vanderhaeghen *et al.*, 1997; Feldmesser *et al.*, 2006; Zhang *et al.*, 2007; Flegel *et al.*, 2013). However, the functional role of these human ORs has only been determined in a few examples (Spehr *et al.*, 2003; Braun *et al.*, 2007; Neuhaus *et al.*, 2009; Zhang *et al.*, 2012; Zhao *et al.*, 2013).

Strikingly, we could demonstrate that human keratinocytes also express functional ORs. The expression of five ORs could be identified (OR6M1, OR11A1, OR6V1, OR5V1, and OR2AT4) using microarray and RT-PCR analyses. Furthermore, the expression of OR2AT4 could also be validated at the protein level in primary human keratinocytes, HaCaT cells, and skin biopsies using immunocytochemical staining. Deorphanization studies using recombinant OR2AT4

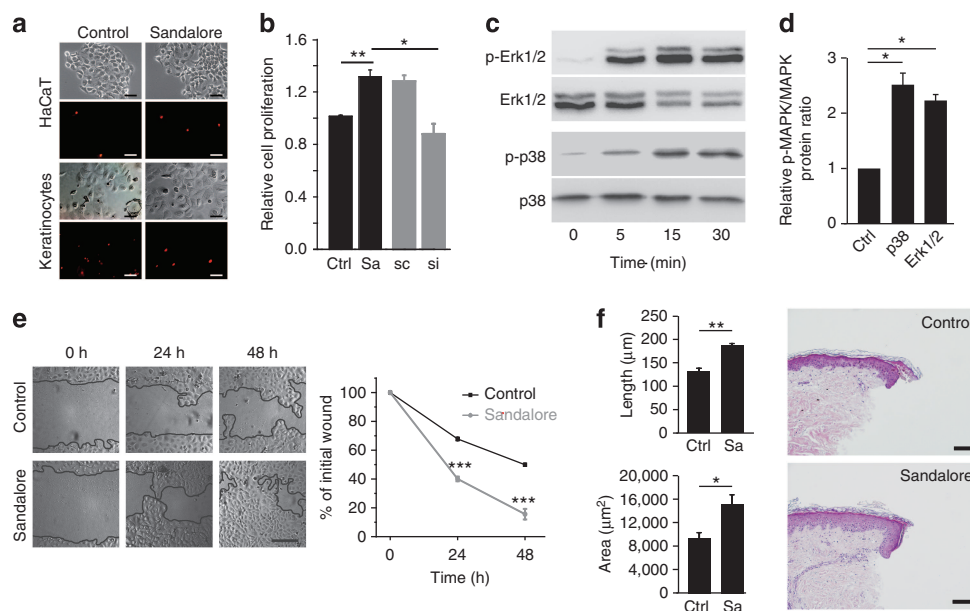


Figure 5. Sandalore stimulation increased keratinocyte proliferation and migration, and epidermal wound healing. (a) Propidium iodide staining of long-term stimulated (5 days) HaCaT cells and primary keratinocytes with 500 μ M Sandalore. Bar = 50 μ m. (b) Treatment of HaCaT cells with Sandalore (Sa; 500 μ M; 5 days) resulted in significantly increased proliferation relative to control conditions (Ctrl). HaCaT cells transfected with OR2AT4-targeted small interfering RNA (siRNA (si)) showed a compensation of the Sandalore-induced proliferative effect. $n \geq 3$ experiments. sc, scrambled RNA. (c) Western blot analysis for the detection of the phosphorylation of members of mitogen-activated protein kinase (MAPK) family in HaCaT cells on stimulation with Sandalore (500 μ M; 0, 5, 15, and 30 minutes). (d) Quantification of phosphorylation of MAPKs in HaCaT cells on stimulation with Sandalore (500 μ M; 30 minutes) relative to unstimulated cells (Ctrl). $n = 3$ experiments. (e) Wound scratch assay with primary cultured keratinocytes in the presence of 500 μ M Sandalore or 0.1% DMSO (control); $n = 3$ experiments. (f) Skin explants were treated with Sandalore (50 μ M) or control (0.01% DMSO, Ctrl) for 5 days. The length and area of the migratory epidermal tongues at the edges of the explants were evaluated after fixation and hematoxylin and eosin staining. $n = 3$ experiments. Bar = 200 μ m. The data are shown as means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

identified the synthetic sandalwood odorants Sandalore and Brahmanol as specific activators. The specificity of the receptor for these two substances was supported using six other structurally related sandalwood odorants that did not activate the recombinant receptor. OR2AT4 might represent a “narrowly” tuned receptor, responding only to a small number of closely related odorants (Saito *et al.*, 2009). This hypothesis is consistent with Bieri *et al.* (2004), who showed that different combinations of synthetic sandalwood odorants induce calcium transients in murine OR neurons, indicating the existence of different sandalwood odorant receptors. However, Sandalore did not activate murine OR neurons, which can be specifically activated by other synthetic sandalwood odorants.

The specific activation of OR2AT4 by Sandalore and Brahmanol might be based on the missing electron-rich structural feature in the carbon chain spacer between the bulky lipophilic moiety and the polar OH group, which could shift Sandalore and Brahmanol into a different subgroup of sandalwood odorants (Bieri *et al.*, 2004).

In the past years, the development of synthetic sandalwood molecules has led to a series of substitutes that are often used in cosmetics, deodorants, and perfumes, because the essential sandalwood oil obtained from the East Asian sandalwood tree (*Santalum album* L.) is quite rare and is therefore an expensive substance. Thus, the identification of “sandalwood-odorant

receptors” will be important to produce new commercially available products and cheaper and biocompatible surrogates.

ORs can be inhibited by specific antagonists, as shown in various studies (Spehr *et al.*, 2003; Oka *et al.*, 2004; Shirokova *et al.*, 2005; Abaffy *et al.*, 2007; Neuhaus *et al.*, 2009), which are good tools for verifying the specific activation of ORs. Here we identified Phenirat and Oxyphenylon, which inhibit Sandalore-induced calcium signals in the recombinant system and in a dose-dependent manner in HaCaT cells. In addition, Sandalore responses were rescued when agonist concentrations were increased to the fixed amount of antagonists. We therefore assume that both odorants are competitive antagonists of OR2AT4.

The agonists of OR2AT4, Sandalore and Brahmanol, induced calcium signals in primary keratinocytes and HaCaT cells. This activation is mediated by OR2AT4, verified by antagonist application and siRNA experiments that significantly reduce Sandalore-induced Ca^{2+} signals in HaCaT cells. The activation of OR2AT4 by Sandalore induced a cAMP-dependent signaling cascade in both HaCaT cells and primary keratinocytes, respectively. The activation of the cAMP-signaling pathway via OR2AT4 also involves a CNG channel further downstream, as demonstrated using the CNG-channel inhibitor L-*cis* Diltiazem. Keratinocytes express transcripts of the α -subunit of the rod CNG channel CNGB1 (Oda *et al.*, 1997). We confirmed this expression using RT-PCR for

HaCaT cells and keratinocytes, and could additionally show the expression of the rod CNGB1 subunit. Together, these subunits form functional heteromeric channels (stoichiometry 3:1) in human rod photoreceptors (Zheng *et al.*, 2002).

Further downstream of the signaling cascade, Sandalore stimulation leads possibly via the cAMP/PKA pathway (Stork and Schmitt, 2002) or multiple pathways, including EGFR (Ansarie *et al.*, 2008) to the phosphorylation of the MAPKs Erk1/2 and p38, and to the increased proliferation and migration of cells. These results are consistent with other studies substantiating the general activation of Erk1/2 and p38 in cell migration and proliferation (Sharma *et al.*, 2003; Wang *et al.*, 2006; Shibata *et al.*, 2012). The acceleration of both processes is often a characteristic of reepithelialization events in wound healing (Woodley *et al.*, 1993; Gurtner *et al.*, 2008; Shaw and Martin, 2009; Müller *et al.*, 2012; Plikus *et al.*, 2012). Consistently, Sandalore stimulation significantly increased the regeneration rate of HaCaT cell monolayers and primary keratinocyte monolayers in an *in vitro* scratch assay. Co-stimulation with the antagonist or specific MAPK inhibitors significantly diminished the Sandalore-induced “wound” closure effect in HaCaT cells, demonstrating the involvement of OR2AT4 and p38 and Erk1/2 in keratinocyte re-epithelialization processes. Apart from cultured keratinocytes, Sandalore stimulation also enhanced epidermal “wound healing” in human skin organ cultures.

In vivo, basal epidermal keratinocytes are responsible for natural wound healing (Odland and Ross, 1968; Stenn and Depalma, 1988; Hosokawa *et al.*, 2005; McGovern *et al.*, 2013). Interestingly, immunostainings of human skin sections showed the strongest expression of OR2AT4 in the basal keratinocyte layer. As a lipophilic low-molecular substance (<500 Da), Sandalore has the potential to easily contact the receptor by penetrating the natural skin barrier (Bos and Meinardi, 2000). Consequently, we assume that the physiological effects shown *in vitro* might be transferable to the intact skin, and OR2AT4 might be used as a therapeutic target in wound healing processes to accelerate reepithelialization. At the preclinical level, this can be tested by the topical application of Sandalore in appropriate vehicles on organ-cultured human skin (Flori *et al.*, 2011).

Sandalore-induced adenylyl cyclase activity not only triggers the proliferation and migration of keratinocytes but also induces the ATP-mediated communication of keratinocytes with adjacent neurons in a coculture approach, as previously demonstrated in our recent study (Sondersorg *et al.*, 2014). Interestingly, there is increasing evidence that skin–nerve interactions might also have an important role in mediating wound healing (Roosterman *et al.*, 2006; Scuri *et al.*, 2010). Thus, we speculate that Sandalore might induce a reciprocal interaction between keratinocytes and sensory neurons, which might enhance wound healing. Novel assays, which have recently become available, will facilitate an assessment of the role of OR-mediated signaling in keratinocyte–neural interactions during wound healing in the human system (Lebonvallet *et al.*, 2012; Lebonvallet *et al.*, 2013).

Apart from keratinocytes, OR2AT4 could also be detected in melanocytes and dendritic cells. Interestingly, melanocytes

are proposed as key sensory cells in the human skin (Slominski *et al.*, 1993; Plonka *et al.*, 2009; Paus, 2013). Therefore, it would be interesting to investigate whether OR2AT4 stimulation with Sandalore exerts the same function in different skin cell types, including human skin and hair follicle organ cultures (Gáspár *et al.*, 2011; Samuelov *et al.*, 2013).

Sandalore, as a synthetic substance, is externally applied to the skin. However, endogenous substances might also exist, representing natural ligands of the receptor, such as androstenedione derivatives in the case of the OR51E2 expressed in prostate cells (Neuhaus *et al.*, 2009). Thus, future studies should identify endogenous substances that also activate the receptor and contribute to wound healing.

Most importantly, our finding that epidermal homeostasis and repair underlies OR2AT4-dependent environmental chemosensation suggests that we have only just begun to identify the non-olfactory, ancestral functions of ORs in epithelial biology, and the novel modulation of important keratinocyte functions by OR2AT4 represents one of multiple ancestral OR functions preserved over a wide stretch of integumental development in human skin.

MATERIALS AND METHODS

Cell culture

Cultures of human primary keratinocytes were kindly provided by Dr Frank Jacobsen (Bergmannsheil, University Hospital Bochum, Germany) and prepared from freshly received material from foreskin circumcisions or as relicts from surgeries. The cultures were established as previously described (Jacobsen *et al.*, 2005). These procedures were approved by the Regional Research Ethics Committee (Ethical Committee of Ruhr-University Bochum), and all subjects provided written informed consent to participate. The study was conducted according to the Declaration of Helsinki Principles.

HaCaT cells represent an appropriate model for human primary keratinocytes with respect to morphological and growth characteristics (Boukamp *et al.*, 1988). The Hana3A cells were kindly provided by H. Matsunami (Duke University Medical Center, Durham, NC). Hana3A cells are a HEK293-derived cell line stably expressing RTP1L, RTP2, REEP1, and $G\alpha_{olf}$, which supports the robust heterologous expression of various ORs (Saito *et al.*, 2004). HaCaT and Hana3A cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 100 units per ml of penicillin/streptomycin (all from Gibco/Invitrogen, Carlsbad, CA).

Calcium imaging experiments

Hana3A cells, HaCaT cells, or primary keratinocytes grown in 35-mm dishes were incubated for 30 minutes at 37°C with Ringer’s solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.3) and 3 μ M Fura-2-AM (Molecular Probes, Eugene, OR). Calcium imaging experiments were performed as described in Spehr *et al.* (2003). Details of blocker experiments are given as Supplementary Information online.

For the Ca^{2+} imaging experiments, Hana3A cells were grown in 35-mm cell culture dishes (50% confluence) and transfected with pcDNA3-OR2AT4 (3 μ g), mRTP1S (0.5 μ g), and $G\alpha_{olf}$ (0.5 μ g) for 48 hours using a standard calcium–phosphate precipitation technique (Zufall *et al.*, 1993). Sham-transfected controls were only transfected with mRTP1S (0.5 μ g) and $G\alpha_{olf}$ (0.5 μ g).

Cell proliferation assay

HaCaT cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After 48 hours, the cells were stimulated with DMEM, DMEM + 0.1% DMSO (control), or DMEM + odorant (500 μM). Cell proliferation was investigated after 5 days using the CyQUANT cell proliferation assay kit (Invitrogen) according to the manufacturer's instructions.

Propidium iodide staining

Dishes with cultured keratinocytes or HaCaT cells were incubated with medium + 500 μM of odorants for 5 days. The cells were washed with PBS^{-/-} and incubated with 25 $\mu\text{g} \mu\text{L}^{-1}$ of propidium iodide (in PBS^{-/-}) for 5 minutes. The dead cells were detected under a fluorescence microscope (Axioskop, Zeiss, Oberkochen, Germany).

Wound scratch assay

The regeneration of wounded monolayers of HaCaT cells and human primary keratinocytes was analyzed using an *in vitro* wound scratch assay. Confluent keratinocytes or HaCaT cells grown in monolayers were scratched using a 20- μL pipette tip, and the residual overgrowing gap of the migrating cells at 24 and 48 hours was measured and expressed relative to the initial scratch area (0 hour). Details of experimental procedure of skin organ cultures are provided as Supplementary Information online.

Skin organ cultures

Skin organ cultures were prepared from tissue samples derived from patients undergoing routine diagnostic and therapeutic procedures within the Department of Dermatology, University of Münster. All experiments were approved by the Ethical Committee of the University of Münster and performed as recently described (Meier *et al.*, 2013). Details of experimental procedure of skin organ cultures are provided as Supplementary Information online.

Statistical analysis

The results are presented as mean \pm SEM and *n* is the number of experiments/cells. The level of significance was set as **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Details of statistical analysis are provided as Supplementary Information online.

Chemical substances, RT-PCR and plasmids, siRNA constructs, pmirGlo vector, CRE-Luciferase assay, immunocytochemistry, western blotting, cAMP-Glo assay, agarose migration assay, and microarray analysis, see details in the Supplementary Information online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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