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Histamine induces proliferation in keratinocytes from atopic dermatitis patients

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Abstract

Background—Epidermal hyperproliferation resulting in acanthosis is an important clinical observation in atopic dermatitis and its underlying mechanisms are not completely understood by now.

Objective—Since elevated levels of histamine are present in lesional skin, we investigated the effect of histamine, especially with regard to H4R activation, on the proliferation of human and murine keratinocytes.

Methods—The expression of H4R on human and murine keratinocytes was detected by real-time PCR. Keratinocyte proliferation was evaluated by different *in vitro* cell proliferation assays, scratch assays and measurement of epidermal thickness of murine skin.

Results—We detected H4R mRNA on foreskin keratinocytes and on outer root sheath keratinocytes; H4R mRNA was more abundant in keratinocytes from patients with atopic dermatitis as compared to non-atopic donors. Stimulation of foreskin keratinocytes, atopic dermatitis outer root sheath keratinocytes and H4R transfected HaCaT cells with histamine and H4R agonist resulted in an increase of proliferation, which was blocked with the H4R-specific antagonist JNJ7777120. Abdominal epidermis of H4R-deficient mice was significantly thinner and the *in vitro* proliferation of keratinocytes derived from H4R-deficient mice was lower

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Conflict of Interest
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compared to control mice. Interestingly, we only detected H4R expression on murine keratinocytes after stimulation with lipopolysaccharide and peptidoglycane.

Conclusion—The H4R is highly expressed on keratinocytes from atopic dermatitis patients and its stimulation induces keratinocyte proliferation. This might represent a mechanism that contributes to the epidermal hyperplasia observed in atopic dermatitis.

Keywords

Histamine; Keratinocyte; Proliferation; Histamine 4 receptor; Human; Mouse; Atopic dermatitis

INTRODUCTION

Atopic dermatitis affects up to 20% of the population and its incidence is steadily rising.[1] Moreover, the therapeutic interventions for atopic dermatitis are limited, because the disease is not fully understood yet. Atopic dermatitis is a multifactorial disease whose development can be caused by numerous trigger factors and involves the interaction of different cell types, on one hand of the skin resident keratinocytes and on the other hand of immune cells present in the skin or infiltrating the skin upon inflammation.[2] Keratinocytes have been shown to release a set of mediators, among them chemokines and cytokines, which are important for the attraction of immune cells to atopic dermatitis lesions.[3] In turn the expression of cell surface receptors is upregulated on keratinocytes in response to signals from the surrounding immune cells.[4] Apart from the involvement in the immunological response, keratinocytes contribute to the pathology of atopic dermatitis by a change in their growth characteristics. Thickening of the epidermis is evident in chronic lesions of eczema as indicated clinically by lichenification and histologically by acanthosis. It has been shown previously that this epidermal hyperproliferation might in part be due to the inflammatory mediators TGF-α and GM-CSF.[5, 6] Histamine is another important inflammatory mediator that is present at high levels in the skin of atopic dermatitis patients[7] and plays an important role in disease pathology.[8] Keratinocytes were already shown to express the histamine 1 receptor (H1R) and H2R and histamine stimulation modulates the expression of inflammatory mediators, such as interleukin-6 (IL-6), IL-8 and CCL5 as well as cell surface molecules MHC-II and ICAM-1.[4, 9] The previously performed work with regard to histamine and keratinocytes did not include the most recently described H4R.[10, 11] The H4R was shown to be involved in the pathogenesis of atopic dermatitis by modulating the function of various immune cells present in the skin, i.e. T cells[12] and dendritic cells[13], however the expression and function of this receptor on keratinocytes was not identified yet.

Here we investigated the function of the H4R on human and murine keratinocytes with regard to proliferation. Importantly we found higher expression of the H4R on and higher proliferation in response to its stimulation in keratinocytes derived from patients with atopic dermatitis. Accordingly we found that the H4R in murine neonatal keratinocytes is expressed after the mimicry of inflammatory conditions by treatment with lipopolysaccharide (LPS) and peptidoglycane (PGN).

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METHODS

Animals

Male and female wildtype and H4R knockout mice of age 20 – 22 weeks were used. All animals were healthy and were housed in groups of six mice per cage at 22 °C with a 12 h light/dark cycle. Water and a standard diet (Altromin, Lage/Lippe, Germany) were available ad libitum. H4R knockout mice were kindly provided by Johnson and Johnson Pharmaceutical Research and Development (New Brunswick, NJ, USA). The H4R knockout mice were generated by Lexicon Genetics (Woodlands, TX, USA), as described previously[14] and were cross breed with BALB/c from Charles River (Sulzfeld, Germany) to obtain heterocygote mice for the H4R. These mice were used to obtain homocygote H4R knockout mice and their respective wildtype.

Reagents

Various histamine receptor ligands were used in this study: histamine (agonist for all histamine receptors, Alk-Scherax, Wedel, Germany), 4-methylhistamine (4-MH, H4R agonist), 2-pyridylethylamin (2-Pyr, H1R agonist), amthamine (Amtha, H2R agonist) (all agonists from Tocris Bioscience, Bristol, UK), JNJ777120 (JNJ, H4R antagonist, Tocris), levocetirizine (Levo, H1R antagonist, UCB, Anderlecht/Brüssel, Belgium) and ranitidine (Rani, H2R antagonist, Biomol, Hamburg, Germany).

For stimulation of mouse keratinocytes LPS (0111:B4) and PGN were used (both from Sigma-Aldrich, Munich, Germany).

Keratinocyte isolation and culture

Human primary neonatal keratinocyte (nKC) cultures were prepared from foreskin as described previously.[15] The foreskin was cut into pieces and incubated over night at 4 °C in 2.4 U dispase II (Roche, Mannheim, Germany). The next day the epidermis was separated from the dermis and placed for 20 min at 37 °C in EDTA (0.02 %)-trypsin (0.05 %) solution (PAN-Biotech, Aidenbach, Germany). After stopping the trypsin reaction by addition of fetal calf serum (PromoCell, Heidelberg, Germany), the cell suspension was filtered through a sterile gauze (40 μm) and washed two times in PBS. The obtained single cell suspension of nKC was incubated in serum free growth medium, Keratinocyte Growth Medium 2 Kit (PromoCell, Heidelberg, Germany), at 37 °C in a humidified atmosphere containing 5 % CO_2. Adult human keratinocytes were isolated from the outer root sheath from plucked hair (orsKC) as described previously.[16] Briefly, the hairs were placed in dishes with a feeder layer of 3T3 fibroblasts that had been treated with mitomycin C (Roche, Mannheim, Germany), the medium was changed every 2-3 days and when sufficient orsKC had outgrown, they were selectively trypsinized and passaged further.

For experiments KC in passage 3-6 cultured in hydrocortisone (HC) and epidermal growth factor (EGF) free medium were used.

Murine primary neonatal keratinocyte cultures were prepared according to Caldelari and Müller.[17] Skin samples of neonatal wildtype (BALB/c) and histamine H4R knockout mice

were collected and incubated overnight in dispase II (5 mg/ml, Sigma-Aldrich, Munich, Germany) at 4 °C. Skins of two to five mice were pooled for one experiment. On the next day the epidermis was peeled off the dermis and incubated in trypsin/EDTA 0.125 %/0.05 % (Biochrom, Berlin, Germany) for 30 minutes at room temperature. The trypsin/EDTA was inactivated with CnT-07 (CELLnTEC, Bern, Switzerland) plus 10 % FCS (PAA, Pasching, Austria) and the epidermis was washed three times in CnT-07 medium. A single cell suspension was obtained by gentle rubbing and pipetting. Keratinocytes were seeded at a density of $1*10^6$ cells in rat-tail collagen coated (Roche, Mannheim, Germany) 25 cm^2 flasks (Nunclon, Thermo Fischere Scientific, Braunschweig, Germany) and cultured at 37 °C and 5 % CO_2. Cell passages 1-3 were used for experiments.

Transfection of HaCaT keratinocyte cell line

To generate HaCaT cells[18] stably expressing H4R we used a retroviral gene transfer and expression System (Clontech, Mountain View, CA, USA) based on retroviral transduction method described in Ory *et al*.[19] In brief: The human H4R was amplified from pcDNA3-HR4 using the following primers: aaaaaaccggtgccaccatgccagatactaatagcacaa (sense primer) and aaaaaggatccttaagaagatactgaccgactg (antisense primer). The amplicon was digested with AgeI and BamHI and cloned into the retroviral pQCXIN transfer vector (Clontech) that allows bicistronic expression of the gene of interest in combination with a neomycin resistance gene. The generated pQCXIN-H4R construct was confirmed by sequencing of the open reading frame and used for transfection of the 293gpg retrovirus packaging cell line. Retrovirus containing supernatants of the transfected 293gpg cells were used to transduce HaCaT cells according to the manufacturer's protocol (Clontech). H4R expressing, neomycin resistant cells were selected with 800 mg/l G-418 from Calbiochem (Merck4Biosciences, Darmstadt, Germany).

mRNA isolation, reverse transcription and qRT-PCR

Murine keratinocytes were stimulated for 24 h with 50 μg/ml LPS 0111:B4 and 50 μg/ml PGN when they reached confluence. Murine and human keratinocytes were washed in PBS and lysed for RNA isolation using Mini RNA Isolation II Kit (Zymo Research, Orange, USA) and reverse transcription was performed with the First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Real-time quantitative PCR was performed on a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) using SYBR Green with Quantitect primer assays for human glyceraldehyde-3-phosphate dehydrogenase GAPDH (QT01192646), human H1R (QT00199857), human H2R (QT00210378), human H3R (QT00210861) and human H4R (QT00032326) as well as murine GAPDH (QT00199388) and murine H4R (QT00135884) according to the manufacturer's instructions (Qiagen, Hilden, Germany). The following PCR settings were used: an initial activation step of 15 minutes at 95 °C with ramp 20 °C/second was followed by three-step cycling (45 cycles): denaturation 15 seconds, 94 °C; annealing 20 seconds, 55 °C; extension 20 seconds, 72 °C (all three with ramp 2 °C/second). Melting curve analysis was performed from 60–90 °C with ramp 20 °C/second. The amount of target genes relative to the reference GAPDH was quantified using the Relative Quantification Software (Roche Molecular Biochemicals). To visualize the amplification products after completion of the PCR run, agrose gel

electrophoresis was performed with 2 % agarose (Roth, Karlsruhe, Germany) in 1× Tris-Borat-EDTA buffer (Roth).

Cell proliferation assays

Human keratinocytes were seeded in 96-well plates at a density of $1.5*10^4$ cells per well and treated with 10μM histamine or 10μM agonist (2-Pyr, Amtha or 4-MH). For blocking experiments the cells were incubated with 10μM specific antagonist (Levo, Rani or JNJ7777120) 30 min before the addition of the agonists. The proliferation of human keratinocytes was measured at different time points using CellTiter96 non-radioactive proliferation assay (MTT assay, Promega) according to the manufacturer's recommendations or by thymidine incorporation test. After one day in culture, keratinocytes were pulsed with tritiated thymidine (0.5~Ci/well) for approx. 16 hours. The decay as a measure for the thymidine incorporation during cell division was counted in a liquid scintillation counter (Wallac System1409). The proliferation index was defined by the ratio of mean counts per minute of stimulated to unstimulated cultures.

Murine wildtype and H4R knockout keratinocytes were seeded in collagen coated 96-well plates (Greiner, Frickenhausen, Germany) at a density of $2*10^4$ cells per well and cultured until 70 % confluency. Cells were grown for 12, 24 and 48 h and cell proliferation was measured by BrdU-incorporation (BrdU colorimetric assay, Roche, Mannheim, Germany) according to the manufacturer's recommendations.

Scratch assay

Scratch assays are regularly used to investigate wound closure mediated by cell migration and proliferation and they have been used before to evaluate the proliferative capacity of keratinocytes.[20] Keratinocytes or HaCaT cells were grown in 6-well plates until they reached a confluence of around 90-100 %. A scratch was set in the middle of the well with a standard 1000 μl pipette tip and a photograph was taken. After 24 h the cells were stained with CFSE, photographs were taken and the scratch thickness was evaluated by a group of blinded observers and ranked from 0 (no closure of the scratch) to 6 (complete closure of the scratch).

Evaluation of murine epidermal thickness

The abdominal hair of mice was removed using depilatory cream (Veet, Zurich, CH). Mice were sacrificed 24 h later and the abdominal skin of the mice was separated. Skin specimens were fixed in Bouin's solution, and paraffin sections (6 μm) were prepared and stained with hematoxylin and eosin. The sections were examined histologically by a blinded investigator. Epidermal thickness (distance from the basal membrane to the stratum corneum) of ten randomly selected areas was measured using Axiovision 4.8 software (Carl Zeiss, Jena, Germany). The stratum corneum itself could not be measured, since it was partially or completely tom off during sectioning.

Statistical analysis

For statistical evaluation of normal distributed data the paired t-test (Figures 1, 2 and 4) and for not normal distributed data the Mann-Whitney test (unpaired, non-parametric, Figures

3A and 5) or Wilcoxon matched pairs signed rank test (paired, non-parametric, Figure 3B-C) was used; a p-value below 0.05 was regarded as significant. $p<0.05$ is depicted with * and $p<0.005$ with **. The program GraphPad Prism® version 3.02 (GraphPad Software, Inc, San Diego, USA) was used for statistical analysis.

Ethics

The animal experiments have been approved by the LAVES, Oldenburg, Germany (AZ.G33.9-42502-04-10/0252).The investigation of the role of histamine receptors in human allergic skin inflammation was approved by the local ethics committee of the Hannover Medical School (Vote Nr. 4253) and was conducted according to the Declaration of Helsinki Principles.

RESULTS

Neonatal keratinocytes express the H4R and its stimulation induces proliferation

LightCycler real-time PCR experiments showed that human primary neonatal keratinocytes isolated from foreskin express the histamine H4R on mRNA level (see Figure 1A). We also detected expression of H1R mRNA but no H2R or H3R mRNA (see Figure 1B). Stimulation with histamine or the H4R agonist 4-MH induced proliferation of keratinocytes (1.5 to 2-fold increase) as shown with two different cell proliferation assays (MTT and thymidine incorporation). The induction of cell proliferation was most pronounced after 48h of treatment (see Figure 1D) and was induced by histamine at concentrations between 10^{-4} M and 10^{-8} M (see Figure 1C). Since 4-MH is known to bind not only H4R, but also H2R[21], we performed experiments in the presence of the selective H4R antagonist JNJ7777120 to show that keratinocyte proliferation was induced by selective H4R stimulation (see Figure 1E). To investigate the role of H1R or H2R we incubated the keratinocytes with specific H1R and H2R agonists and antagonists and show no significant involvement of these receptors in induced proliferation (see Figure 1F).

H4R-transfected HaCaT keratinocytes respond to histamine stimulation with proliferation

The keratinocyte cell line HaCaT (spontaneously immortalized keratinocyte cell line (18)) does express low to undetectable levels of H4R mRNA and was therefore stably transfected with a plasmid expressing the H4R or a control vector, respectively. The H4R transfected HaCaT cells showed a marked increase of H4R expression (see Figure 2A). Futhermore HaCaT cells expressed the H1R mRNA but no H2R or H3R mRNA (see Figure 2B). H4R-transfected HaCaT cells responded to histamine treatment with proliferation while non-transfected and control-transfected HaCaT cells did not show induction of proliferation (see Figure 2D). Histamine dose-dependently induced the increase of proliferation at concentrations between 10^{-4} M and 10^{-6} M (see Figure 1C). The H4R antagonist JNJ7777120 blocked the 4-MH induced proliferation in H4R-transfected HaCaT cells (see Figure 2E). Incubation of H4R-transfected HaCaT cells with specific H1R and H2R agonists and antagonists showed no influence of these receptors on proliferation (see Figure 2F).

H4R is highly expressed on and triggers proliferation of keratinocytes derived from patients with atopic dermatitis

We investigated the expression level of H4R on keratinocytes derived from the outer root sheath (orsKC) from healthy individuals and patients with atopic dermatitis or psoriasis. The patients with atopic dermatitis showed higher H4R mRNA expression on orsKC as compared to healthy controls; for psoriasis no significant difference was detected (see Figure 3A). Keratinocytes from patients with atopic dermatitis but not from psoriasis also expressed significantly more H1R mRNA compared to healthy donors (see Figure 3A). In the proliferation assay orsKC derived from psoriasis patients showed higher basal proliferation, but their proliferative capacity was not changed by histamine. Atopic dermatitis orsKC responded to stimulation with histamine or a H4R agonist with approximately 1.5 fold higher proliferation as compared to non-stimulated controls (see Figure 3B). The H4R antagonist JNJ7777120 blocked histamine-induced proliferation in orsKC derived from atopic dermatitis (see Figure 3C).

Closure of scratching wounds is accelerated upon histamine stimulation in H4R-expressing keratinocytes

Stimulation of keratinocyte monolayer cultures of cells that express high H4R levels, i.e. H4R-transfected HaCaT cells and orsKC derived from atopic dermatitis patients, with histamine or the H4R agonist 4-MH resulted in faster closure of scratching wounds (see Figure 4A, 4B and 4C). In non- or eGFP-transfected HaCaT cells and orsKC from healthy individuals histamine did not influence the rate of wound closure (see Figure 4B and 4C). The H4R antagonist JNJ7777120 blocked the effect of histamine and 4-MH (see Figure 4A).

H4R knockout mice show reduced epidermal thickness and decreased *in vitro* proliferation of keratinocytes

Histological analyses revealed that the epidermis of healthy abdominal skin of H4R knockout mice is significantly thinner than the epidermis of wildtype mice. Wildtype epidermis was 21 ± 4.6 μm (mean ± SD) thick, while the epidermis of H4R knockout mice was only 15.9 ± 2 μm (mean ± SD) thick (see Figure 5A). Proliferation of neonatal murine keratinocytes was analysed by a colorimetric BrdU assay after 12, 24 and 48 h of culture. H4R knockout keratinocytes showed a trend towards a reduced basal proliferation compared to wildtype keratinocytes at each time point (see Figure 5B). Stimulation with histamine did not modulate the proliferation of wildtype and H4R knockout keratinocytes (data not shown.)

Murine keratinocytes express H4R under stimulation with LPS and PGN

Expression of the H4R mRNA could not be detected in untreated murine neonatal keratinocytes isolated from healthy wildtype mice. Stimulation of murine keratinocytes with the inflammatory molecules LPS and PGN for 24 h induced the expression of the H4R (see Figure 6). In contrast, in human keratinocytes the stimulation with PGN, PolyIC, LPS or a combination of PGN and LPS did not result in an increased expression of H4R (data not shown).

DISCUSSION

Changes in epidermal proliferation and differentiation are observed in inflammatory skin diseases such as atopic dermatitis and psoriasis and represent an important disease phenotype.[22, 23] Jensen et al demonstrated that in lesional skin of atopic dermatitis patients the proliferation is 5-fold increased and even in non-lesional skin keratinocytes show a 2-fold increase of proliferation compared with healthy individuals.[24] Accordingly, other studies report an enhanced expression of the proliferation-associated keratins 6 and 16 in skin of atopic dermatitis and psoriasis patients.[25] The mechanisms underlying the keratinocyte hyperproliferation are not fully elucidated yet, although several local skin mediators have been shown to be involved. Especially growth factors and cytokines, such as TNF-α, IFN-γ and IL-1 are involved in the growth of keratinocytes.[26] The cytokines IL-21 and IL-23 were recently shown to induce keratinocyte proliferation and epidermal hyperplasia associated with psoriasis.[27, 28]

In the present study we analysed the influence of histamine on keratinocyte proliferation, since this mediator is present at elevated levels in skin of patients with atopic dermatitis and psoriasis.[7, 29] Moreover, previous studies showed histamine-induced proliferation of different cell types, among them hepatoma cells through H3R stimulation[30], hematopoietic stem cells via H2R stimulation[31] and airway smooth muscle cells.[32] There is also evidence that histamine influences the proliferation of different cell types in the human skin: histamine inhibits the proliferation of skin fibroblasts through activation of H1R and protein kinase C.[33]

In the present study we show that histamine can increase the proliferation of human keratinocytes from atopic dermatitis patients via the H4R, which might be relevant in epidermal hyperplasia in atopic dermatitis. We found increased proliferation of neonatal keratinocytes and H4R-transfected HaCaT cells in response to histamine or a H4R agonist. Furthermore, we observed by histological analysis that H4R knockout mice have a thinner epidermis compared to the corresponding wildtype mice. Macroscopically, no skin abnormalities could be detected. The *in vitro* proliferation potential of keratinocytes derived from H4R deficient mice was lower compared with keratinocytes from wildtype controls, thus correlating with the epidermal phenotypes observed. Surprisingly, H4R expression on murine neonatal keratinocytes was only detectable after stimulation with LPS and PGN, and not on untreated cells. In contrast, we did not observe up-regulation of H4R mRNA in human keratinocytes after stimulation of toll-like receptors. The lack of H4R mRNA expression in normal murine neonatale keratinocytes complicates the interpretation of the differences that we observed in H4R knockout mice with regard to epidermal thickness and basal proliferative capacity. Moreover, in contrast to other studies, we did not observe proliferation of murine keratinocytes in response to histamine. Maurer et al. showed that histamine stimulates the proliferation of keratinocytes in epidermal sheets obtained from 6-8 weeks old C57BL/6 mice.[34] Explanations for the differences could be the fact that Maurer et al. used telogen skin of adult mice, which displays the lowest rate of basal keratinocyte proliferation under organ culture conditions in the murine hair cycle and is therefore most sensitive for the detection of even discrete stimulatory effects.[34] The proliferative activity of murine neonatal keratinocytes is high at birth[35], and murine neonatal keratinocytes might be

therefore not sensitive for the detection of stimulatory effects. Beside the cellular activation of the keratinocytes (i.e. anagen versus telogen skin), also the local microenvironment most likely influences the proliferation responses of epidermal cells [36]. The influence of proliferation-modulatory extracellular signals like the local cytokine network can not be studied in isolated keratinocyte populations. This might explain the differences in findings by Maurer et al. (1997) as well as our *ex vivo* data (i.e. decreased thickness of epidermis in H4R knockout mice) and the fact that histamine stimulation did not modulate the proliferation of neonatal keratinocytes *in vitro*. Moreover, in the experimental protocol used in Maurer et al inflammatory conditions upregulating H4R expression might have been induced, for example as reaction to the hair removal. However, why H4R knockout keratinocytes showed a lower basal proliferation in the absence of any external stimulus compared to wildtype keratinocytes still needs to be investigated. Autocrine secretion of factors that modulate keratinocyte proliferations like TGF-α and GM-CSF might be decreased in H4R knockout keratinocytes.[5, 6] In another study, Lin et al showed that also other histamine receptors are involved in murine keratinocyte proliferation; they observed that H1R and H2R antihistamines stimulate *in vivo* murine epidermal proliferation in adult albino hairless (Skh1) mice.[37] This even further complicates the area, since both histamine as well as antihistamines can modulate epidermal proliferation, probably depending on the experimental system. From our studies we have the impression that the H4R knockout mouse does not represent a good model for further *in vivo* investigation of the findings from human keratinocytes and it is unclear if the H4R represents a functional receptor on murine KC.

An important observation that we made in *in vitro* experiments using human keratinocytes is that H4R stimulation induced proliferation in keratinocytes derived from atopic dermatitis patients, but not in cells from psoriasis patients or donors without skin disease. The reason for this difference in the response to histamine might be explained by the observation that keratinocytes from atopic dermatitis express higher levels of H4R, while the receptor is relatively low expressed on healthy control and psoriasis keratinocytes. A study describing differences in H4R expression in Chinese atopic dermatitis patients compared to healthy controls due to polymorphisms in the HRH4 gene[38] provides a hint that regulation on the genetic level might be involved. However, the reason for the higher expression of the H4R on atopic dermatitis keratinocytes still needs to be elucidated and will be subject to future studies.

It was noted already previously, that the epidermal hyperplasia in atopic dermatitis and psoriasis might be triggered by different mechanisms. For example, IL-21 and IL-23 were shown to induce epidermal hyperplasia only in psoriasis.[26, 27] Similarly, the inducer of keratinocyte proliferation PPAR-δ is upregulated in lesional psoriasis skin, but not in atopic dermatitis.[39] Here we identified histamine as an inducer of orsKC proliferation selectively in atopic dermatitis, but not in psoriasis.

Interestingly, we found a difference in the response to histamine depending on the keratinocyte source. In contrast to healthy nKC, healthy orsKC derived from the hair root did not show increased proliferation upon stimulation with histamine. Since both cell types express the H4R, the variation should be in the response to histamine stimulation or in the

proliferative capacity of the keratinocytes themselves. However, at the moment we can only speculate on an explanation for the difference in histamine response of nKC and orsKC, both of healthy origin.

Keratinocyte proliferation can be induced in a paracrine or autocrine manner by various mediators. For example fibroblast growth factor 7 (FGF-7) and insulin-like growth factor I (IGF-I) secreted by fibroblasts and melanocytes act as paracrine proliferation inducers.[33, 40] On the other hand, TGF-α and GM-CSF are synthesized by keratinocytes themselves and promote their proliferation in an autocrine manner.[5, 6] Since histamine is mainly secreted by mast cells, it regulates keratinocyte proliferation in a paracrine manner, if we assume that histamine directly affects keratinocyte proliferation. It can however not be excluded that it is an indirect effect via the upregulation of other mediators, such as GM-CSF, which is known to be upregulated in keratinocytes upon histamine stimulation[41] and is by itself an inducer of keratinocyte proliferation.[6] Therefore, further studies are required to investigate the level of endogenous histamine and other factors (like GM-CSF, TGF-α and endothelin) produced in human and murine keratinocytes.

Recent studies have investigated the effect of histamine on keratinocyte differentiation, a process closely linked to keratinocyte proliferation. In a murine model it was shown that H1R and H2R antihistamines enhance epidermal differentiation as well as epidermal proliferation; the involvement of the H4R was not investigated, since it was not detected in mouse keratinocytes. [37] In human *in vitro* keratinocyte cultures and skin equivalents it was described that histamine reduces keratinocyte differentiation dependent on H1R stimulation, while experiments with histamine receptor agonists and antagonists showed no effect of H4R on keratinocyte differentiation.[42] This study together with our results indicates that histamine mediates keratinocyte growth by two different receptors: proliferation via the H4R and differentiation via the H1R. However, it might still be worth to investigate the differentiation capacity of keratinocytes derived from atopic dermatitis patients in response to histamine and H4R stimulation, since the previous data on differentiation was obtained from healthy keratinocytes.[42]

Summarizing, we demonstrate that the H4R is expressed on human keratinocytes. Interestingly the receptor expression is significantly increased in atopic dermatituis patients compared with healthy individuals or patients with psoriasis. Stimulation of the H4R resulted in induction of proliferation of human keratinocytes. The hyperproliferation of keratinocytes in response to histamine in patients with atopic dermatitis might play an important pathophysiological role and based on our results H4R antagonists could represent a new strategy for the therapy of atopic dermatitis.

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Abbreviations

2-Pyr	2-pyridylethylamine
4-MH	4-methylhistamine
Amtha	amthamine
CCL	chemokine (C-C motif) ligand
CFSE	carboxyfluorescein succinimidyl ester
DC	dendritic cell
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony-stimulating factor
Hist	histamine
HC	hydrocortisone
HxR	histamine receptor x
ICAM	intercellular adhesion molecule
IFN	Interferon
IL	interleukin
Levo	levocetirizine
LPS	lipopolysaccharide
MHC-II	major histocompatibility complex
nKC	neonatale keratinocyte
orsKC	outer root sheat keratinocyte
PBMC	peripheral blood mononuclear cells
PGN	peptidoglycane
PPAR-δ	peroxisome proliferator-activated receptor
Rani	ranitidine
SD	standard deviation
TGF	transforming growth factor
TNF	tumor necrosis factor

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Key Messages

"The H4R is highly expressed on human keratinocytes isolated from atopic dermatitis patients and on murine keratinocytes cultured under inflammatory conditions."

"Stimulation of the H4R induces proliferation of human keratinocytes isolated from atopic dermatitis patients."

"H4R stimulation might play a role for epidermal acanthosis observed in chronic eczema"

"H4R-knockout mice have thinner abdominal epidermis compared with wildtype mice."

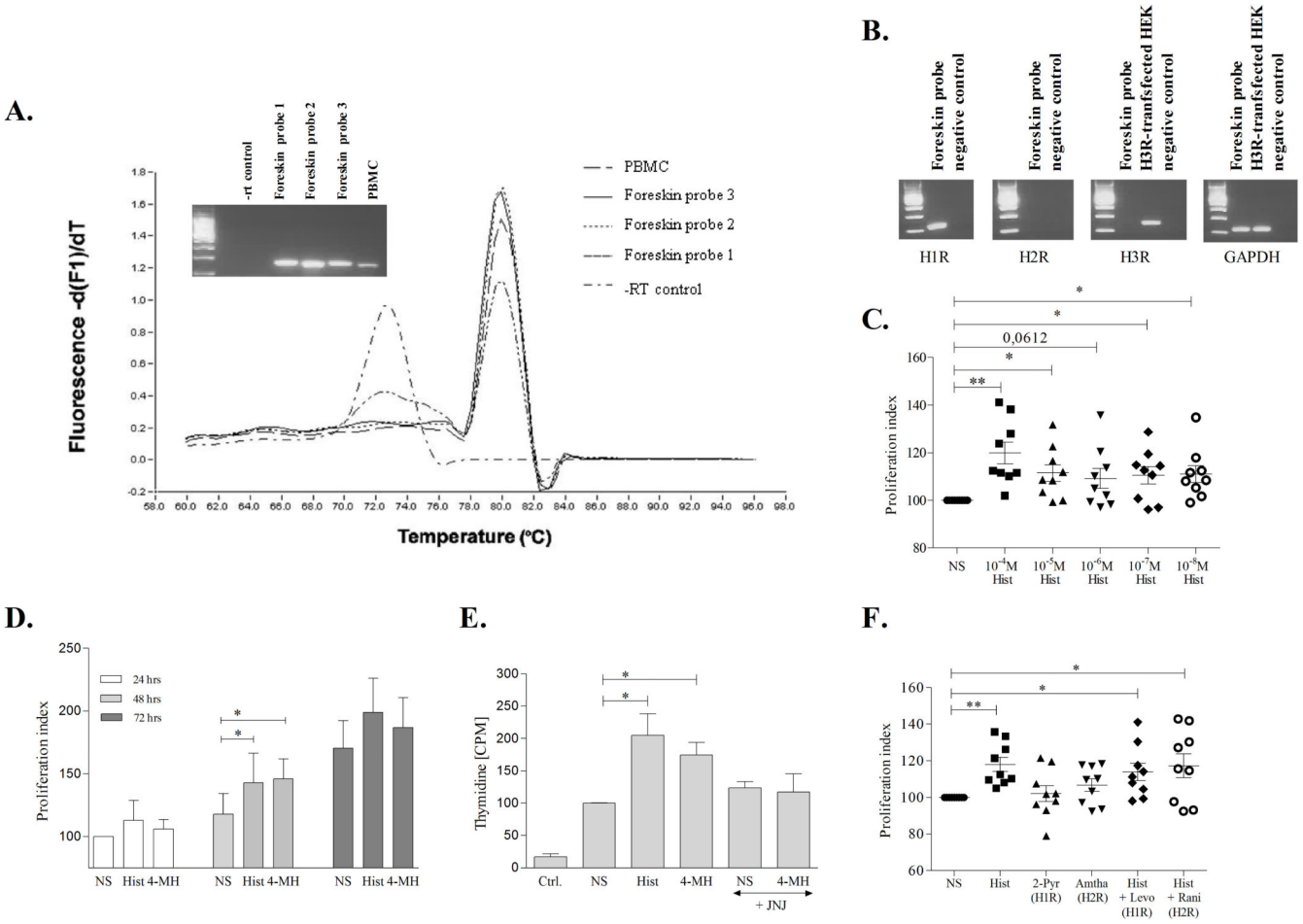

Figure 1. Histamine induces proliferation in human foreskin keratinocytes

Representative amplification products and melting peaks of three independent real-time PCR experiments are shown (A, B). Foreskin keratinocytes were treated with different histamine concentrations (C) and stimulated with histamine or H4R agonist (4-MH) for different time points (D). The H4R antagonist JNJ7777120 (JNJ) abolished the H4R agonist-induced effect (E). H1R and H2R agonists and antagonists had no influence on proliferation (F). Mean and SEM of 4-8 experiments are depicted.

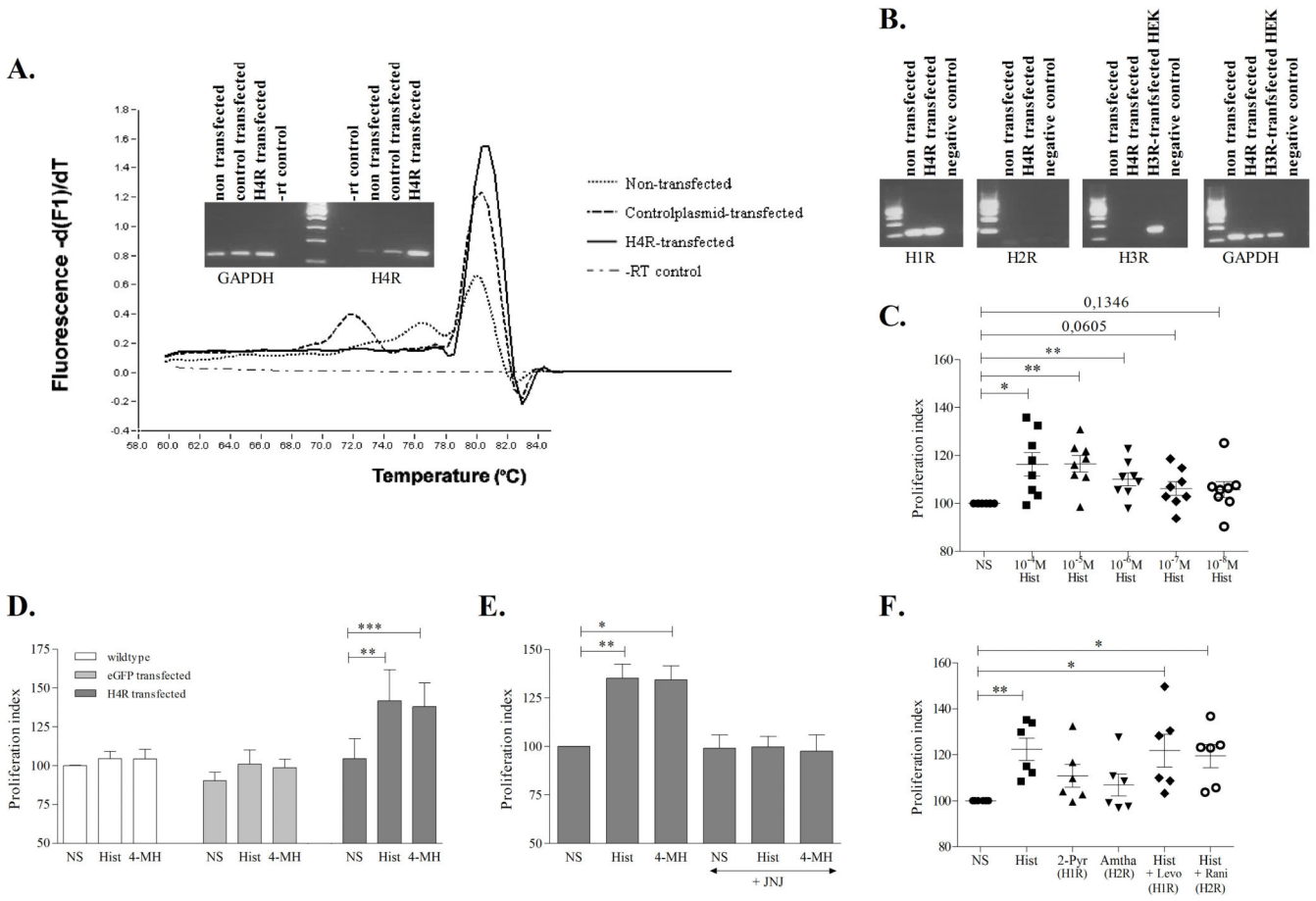

Figure 2. Histamine induces proliferation in H4R-transfected HaCaT keratinocytes

Representative amplification products and melting peaks of five independent real-time PCR experiments are shown (A, B). H4R-transfected HaCaT cells were treated with different histamine concentrations (C). 48 h stimulation with histamine and H4R agonist (4-MH) induced proliferation of H4R-transfected HaCaT keratinocytes as determined by MTT assay (D). The H4R antagonist JNJ7777120 abolished the H4R agonist-induced effect (E), whereas H1R and H2R agonists and antagonists had no influence (F). Mean and SEM of 6-12 experiments are depicted.

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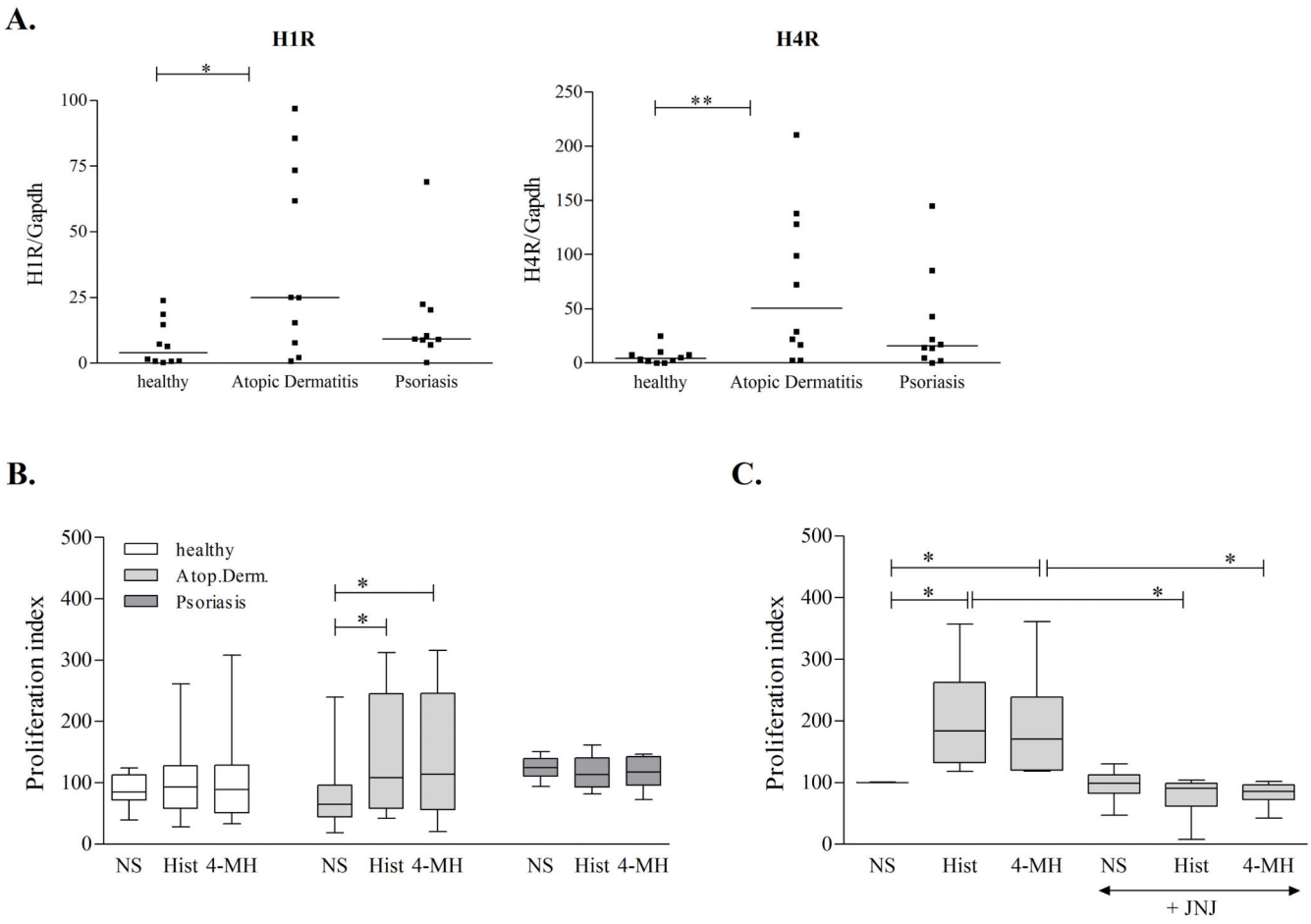

Figure 3. Keratinocytes derived from atopic dermatitis express high levels of H4R and respond with proliferation

Outer root sheath keratinocytes derived from atopic dermatitis showed higher H1R and H4R levels as healthy controls and psoriasis (A). 48 h stimulation with histamine and H4R agonist (4-MH) induced proliferation of atopic dermatitis keratinocytes (B). The H4R antagonist JNJ7777120 (JNJ) abolished the H4R agonist-induced effect in atopic dermatitis keratinocytes (C). Median and quartiles of 6-8 experiments are depicted.

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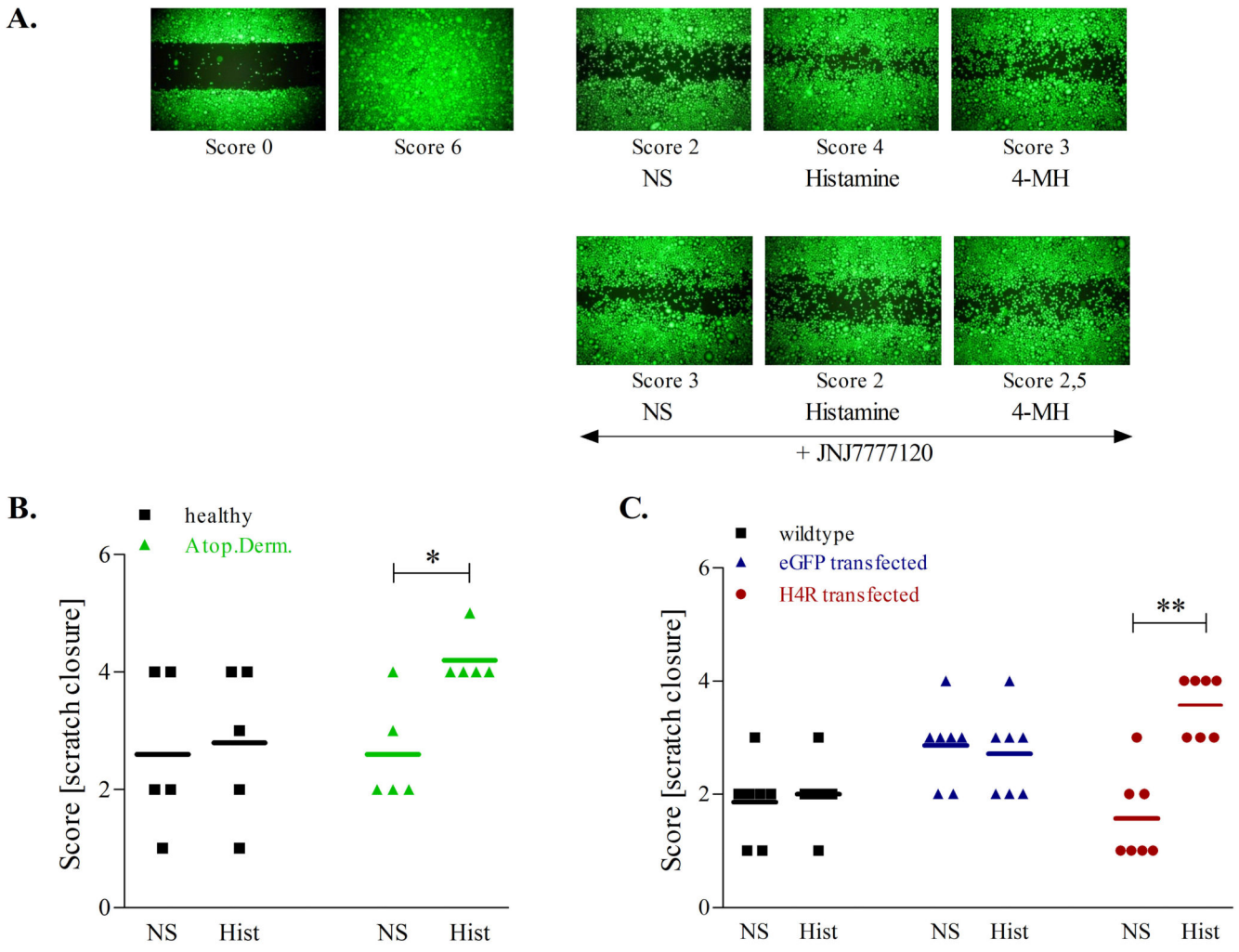

Figure 4. Histamine induces fast scratch-wound healing in high-H4R expressing keratinocytes and H4R-transfected HaCaT cells

In atopic dermatitis keratinocytes the scratch-wound was better closed after 24 h stimulation with histamine or the H4R agonist (4-MH) as compared to non-treated controls (A, B). In contrast, histamine did not modify wound healing in healthy keratinocytes (B). Histamine induced faster scratch-wound healing in H4R-transfected HaCaT keratinocytes, but not in non- or control-transfected cells (C). Individual values and median of 5-7 experiments are depicted.

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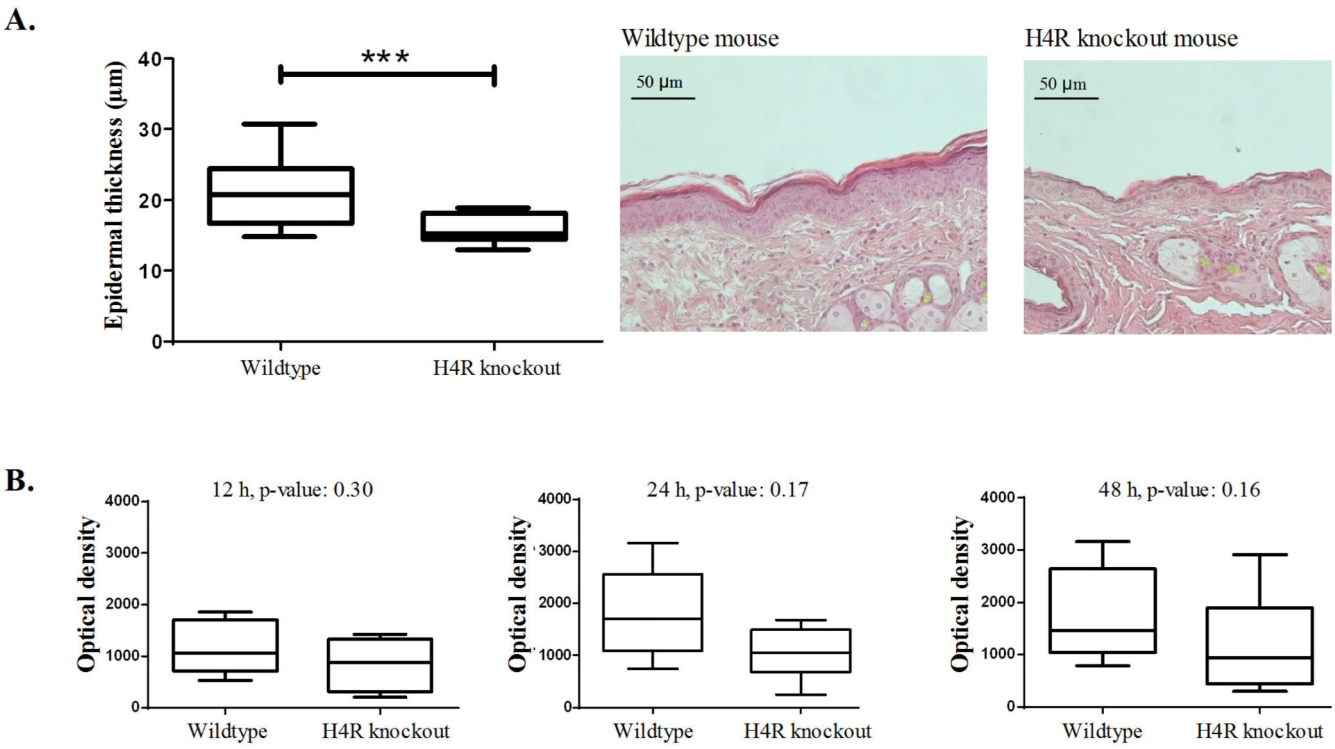

Figure 5. H4R deficient mice show decreased epidermal thickness and keratinocytes derived from H4R deficient mice have lower *in vitro* proliferative capacity

Biopsies were taken from abdominal skin of wildtype and H4R-deficient mice and epidermal thickness was measured (A). Neonatal keratinocytes from H4R knockout and wildtype mice were cultured for 12, 24 and 48 h and cell proliferation was determined by BRDU incorporation (B). Mean and SEM of 5 (12 h), 6 (24 h) and 7 (48 h) experiments are shown.

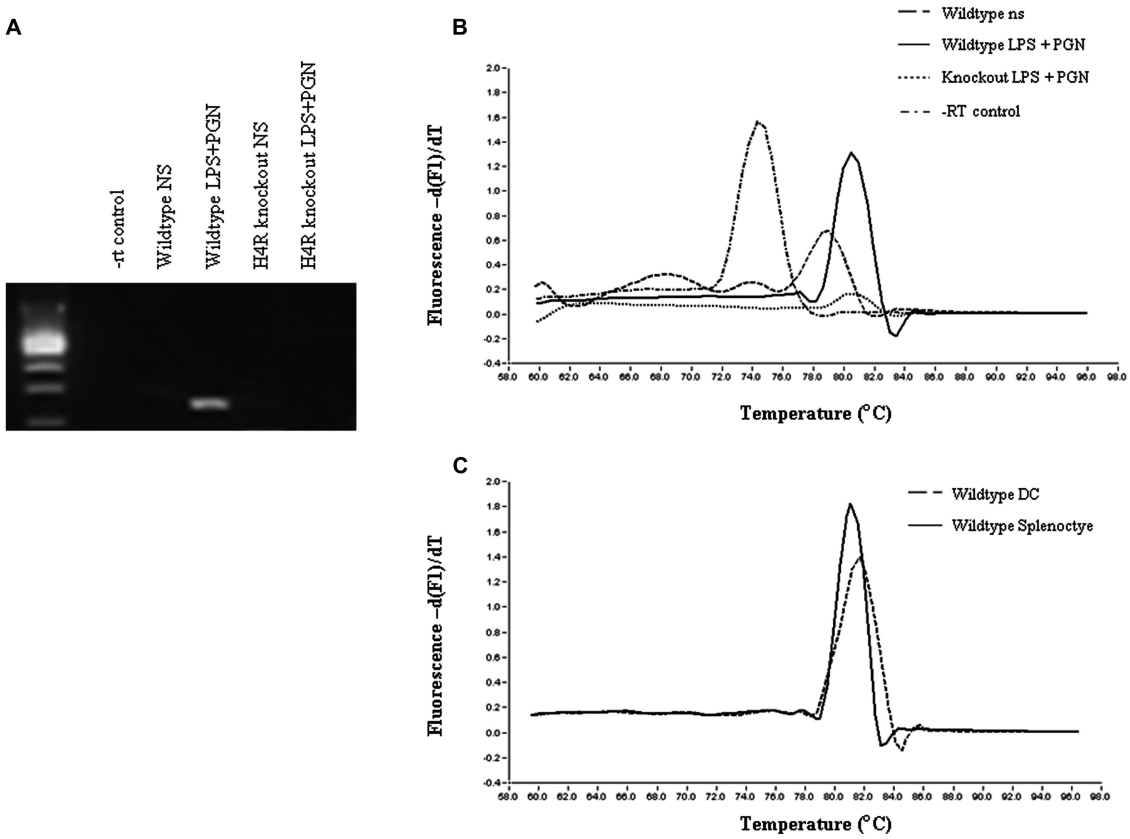

Figure 6. LPS and PGN induce expression of the H4R in murine neonatal keratinocytes

Representative amplification products and melting peaks of three independent experiments are shown (A, B). As a control H4R was amplified from wildtype mice spleen and dendritic cell (DCs) mRNA samples, showing the same melting peak as the treated keratinocytes (C).

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