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MicroRNA-146 and cell trauma downregulate expression of the psoriasis-associated atypical chemokine receptor ACKR2

(Running Title: microRNA regulation of ACKR2 expression).
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Abstract

Chemokines are the principal regulators of leukocyte migration and are essential for initiation and maintenance of inflammation. Atypical chemokine receptor 2 (ACKR2) binds and scavenges proinflammatory CC-chemokines, regulates cutaneous T-cell positioning, and limits the spread of inflammation in vivo. Altered ACKR2 function has been implicated in several inflammatory disorders, including psoriasis, a common and debilitating T cell–driven disorder characterized by thick erythematous skin plaques. ACKR2 expression is abnormal in psoriatic skin, with decreased expression correlating with recruitment of T cells into the epidermis and increased inflammation. However, the molecular mechanisms that govern ACKR2 expression are not known. Here, we identified specific psoriasis-associated microRNAs (miRs) that bind ACKR2, inhibit its expression, and are active in primary cultures of human cutaneous cells. Using both in silico and in vitro approaches, we show that miR-146b and miR-10b directly bind the ACKR2 3’-UTR and reduce expression of ACKR2 transcript and protein in keratinocytes and lymphatic endothelial cells, respectively. Moreover, we demonstrate that ACKR2 expression is further downregulated upon cell trauma, an important trigger for the development of new plaques in many psoriasis patients (the Koebner phenomenon). We found that tensile cell stress leads to rapid ACKR2 downregulation and concurrent miR-146b upregulation. Together, we provide, for the first time, evidence for epigenetic regulation of an atypical chemokine receptor. We propose a mechanism by which cell trauma and miRs coordinately exacerbate inflammation via downregulation of ACKR2 expression and provide a putative mechanistic explanation for the Koebner phenomenon in psoriasis.

Introduction

Chemokines are members of a large family of chemotactic cytokines that are the primary in vivo regulators of leukocyte migration. Chemokines are central to the pathogenesis of inflammatory diseases(1,2) and interact with leukocytes through members of the 7-transmembrane spanning family of G-protein
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coupled receptors(3) to orchestrate the recruitment of inflammatory cells into, and within, tissues. Chemokines and their receptors are broadly categorised as being either inflammatory or homeostatic according to the in vivo contexts in which they function. Importantly, in addition to the classical signalling chemokine receptors, there exists a subfamily of chemokine-binding 7-transmembrane spanning molecules which are referred to as atypical chemokine receptors (ACKRs). The ACKRs are promiscuous in their ligand binding, tend to be expressed on stromal cells and are unable to mediate typical chemokine-induced signalling responses following ligand binding(4-6). We have a particular interest in one of these; Atypical Chemokine Receptor 2 (ACKR2; previously known as D6), which is a high-affinity receptor for multiple inflammatory CC-chemokines(5,7,8). ACKR2 does not mount classical signalling responses following ligand binding(9) but internalises ligands and targets them for intracellular degradation(10,11). Thus ACKR2 functions as a scavenger of pro-inflammatory chemokines and its dysfunction has been implicated in numerous inflammatory diseases(6). In addition, ACKR2 is involved in regulating a range of inflammation-dependent developmental processes(12,13).

ACKR2 expression is elevated in many human inflammatory conditions including rheumatoid arthritis(14), systemic sclerosis(15) and psoriasis(16). Psoriasis is a common systemic inflammatory disease, with profound effects associated with both excess morbidity and mortality(17,18). Psoriasis is typically characterised by clearly demarcated thick erythematous skin plaques with white adherent scales surrounded by extensive areas of apparently normal looking (unaffected) skin. Psoriatic plaques tend to preferentially develop in areas undergoing repeated trauma such as the skin on the elbows and knees(19). Additionally, the Koebner phenomenon is frequently reported in patients with psoriasis, whereby relatively simple skin trauma, of unaffected skin, leads to the rapid development of psoriatic plaques in the vicinity of the preceding trauma(20).

In healthy skin, ACKR2 is primarily expressed by dermal lymphatic endothelial cells and keratinocytes. ACKR2 expression in these cells helps to compartmentalise tissue inflammatory responses to insult and infection by controlling the position of inflammatory leukocytes(21-23). We have recently shown that the spread of psoriasisiform inflammation to unaffected cutaneous sites is restricted by selective up-regulation of cutaneous ACKR2 in the unaffected epidermis. At these sites, high ACKR2 expression in keratinocytes limits local chemokine activity and suppresses entry of T-cells into the epidermis thereby protecting against the development of plaques in uninvolved skin. In contrast, skin in which ACKR2 expression is relatively reduced is associated with enhanced inflammatory chemokine activity, increased numbers of infiltrating T-cell in the epidermis and the emergence of inflammatory plaques(24). The factors that trigger nascent plaque development in psoriasis are not well understood, although our previous data suggest that one such factor includes simple skin trauma, which induces down-regulation of epidermal ACKR2(16).

Despite the importance of epidermal ACKR2 in regulating psoriasiform inflammation, and its transcriptional response to cutaneous trauma, the molecular mechanisms by which ACKR2 expression is regulated in keratinocytes are not understood. Here, by utilising a combination of *in silico* and *in vitro* approaches, we identify two psoriasis-associated microRNAs that are upregulated by trauma in primary cultures of human keratinocytes. We show that the identified microRNAs bind the ACKR2 3’UTR resulting in decreased expression at the transcript and protein level. As such, this is the first demonstration of known disease-associated miRNAs regulating atypical chemokine receptor expression and thereby modulating positioning of inflammatory leukocytes within the skin. Importantly, our study highlights a novel molecular mechanism by which trauma leads to the development of new plaques in psoriasis (the Koebner phenomenon).

**Results**

Three psoriasis-associated microRNAs are predicted to bind the ACKR2 3’-UTR: MicroRNAs have emerged as the most abundant class of gene regulators, and have been implicated in a range of inflammatory disease processes. They predominantly act as negative regulators of gene expression at a post-transcriptional level(25).
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MicroRNAs bind their mRNA target 3’-untranslated region (3’-UTR) which leads to mRNA degradation. Typically, one microRNA binds multiple mRNA targets, and the same mRNA 3’-UTR can be targeted by numerous microRNAs; this promiscuity introduces a significant degree of complexity in microRNA/target interactions and subsequent regulation of gene expression(26). Recent studies have shown that many microRNAs are differentially expressed in psoriasis, with a large number being significantly overexpressed in the psoriatic plaques(27). Given their abundance as negative regulators of gene expression, microRNAs are thus plausible regulators of ACKR2 expression. Accordingly, we used the bioinformatics database TargetScan to identify possible microRNA targets on the ACKR2 3’-UTR. As ACKR2 is present throughout mammals, results were filtered such that only microRNAs that are broadly conserved amongst vertebrates would be included, in order to increase the likelihood of identified microRNA species being biologically relevant. Using this search strategy 16 microRNAs were identified that were both 1) predicted to bind the 3’-UTR of human ACKR2, and 2) broadly conserved amongst vertebrates (the top 10 hits are shown in Figure 1a). To further focus in on those microRNAs of relevance to psoriasis pathogenesis, we compared the list of microRNAs that are predicted to bind the 3’-UTR of ACKR2 with microRNAs previously shown to be differentially expressed in psoriatic plaques, where ACKR2 expression is reduced relative to the surrounding tissue(16). In this way, we identified 3 microRNAs that were present in both lists; miR-10, miR-146 and miR-203. Notably, each of these miRNAs had a particularly high \textit{in silico} likelihood of regulating ACKR2 (Figure 1a,1b). Additionally, these 3 microRNAs have each been shown to play roles in skin homeostasis and inflammation(28-30). They are thus plausible epigenetic regulators of ACKR2 expression and were selected for further evaluation.

\textbf{miR-146b and miR-10b reduce the expression of ACKR2 mRNA in primary human KC and LEC respectively:} Keratinocytes (KC) and lymphatic endothelial cells (LEC) are the main ACKR2 expressing cells in skin(16,31). In order to ensure that microRNAs could be successfully transfected into primary KC, cells were transfected with miR-146b (most strongly upregulated miRNA in psoriatic plaques; Figure 1A) for 24h and the expression of 2 IFN- \textalpha- induced genes known to be downregulated by miR-146b (IRAK1 and TRAF6) determined by Q-PCR (Figure 2a). The data show a clear ability of miR-146b to downregulate levels of both transcripts thus demonstrating that microRNA could be successfully delivered into the cytoplasm of KC. Primary KCs from at least two separate healthy donors were used for all subsequent experiments. Transfection of KC with miR-146b significantly suppressed ACKR2 mRNA expression however miR-10b and miR-203 had no significant effects on ACKR2 transcript levels (Figure 2b).

\textbf{miR-146a and b are differentially expressed in psoriasis, suggesting non-redundant roles in this context(27), although our \textit{in silico} analyses predicted that miR-146a and miR-146b both bind the ACKR2 3’-UTR (Figure 1a). In order to determine whether any differences exist between miR-146a and miR-146b in the regulation of ACKR2 expression the two were directly compared (Figure 2c). Both miR-146a and miR-146b mediated a similar reduction in ACKR2 mRNA expression in KC after 24 hours with no significant differences between the two variants. Thus, both miR-146a and miR-146b downregulate ACKR2 expression however miR-10b and miR-203 had no significant effects on ACKR2 transcript levels (Figure 2b). These data demonstrate that psoriasis-associated micro-RNAs are capable of regulating ACKR2 expression in keratinocytes and lymphatic endothelial cells.

\textbf{miR-146b/miR-10b bind directly to the ACKR2 3’-UTR:} To determine whether miR-146b and miR-10b reduce ACKR2 transcripts through direct interaction with the ACKR2 3’-UTR, the 3’-UTR of ACKR2 was cloned into a dual luciferase reporter vector (the pmiRGLO vector). The ACKR2 3’-UTR containing putative miRNA target sites, were inserted immediately 3’ of a PGK promoter-driven Firefly luciferase gene to evaluate ACKR2 3’-UTR dependent microRNA interactions on transcription. This construct was transfected into HEK293 cells and stable clones selected. These clones were then confirmed as...
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being amenable to microRNA transfection by assessing IRAK1 expression which was significantly reduced following miR146b transfection (Figure 3a). Having demonstrated that HEK cells could be transfected with functional microRNAs of interest, we next determined whether miR-10 and miR-146b transfection modulated luciferase activity (Figure 3b). Both miR-10 and miR-146b transfection significantly downregulated luciferase activity in HEK cells, although dual transfection with both miR did not result in an additive decrease. Thus, both miR-10 and miR-146b can mediate a decrease in transcript levels through direct interactions with the ACKR2 3' UTR.

miR-146b transfection of KC and miR-10b transfection of LEC reduced ACKR2 protein expression: Next we determined the effect of miR-146b on ACKR2 protein expression and distribution in primary KC and LECs. For reasons that are not immediately obvious, we have been unable to successfully apply our functional ACKR2 assay(31) to primary keratinocytes. We have therefore relied on immunofluorescence analysis to measure ACKR2 protein levels. Fully confluent human KC monolayers (Figure 4a with isotype control shown in 4b) were transfected with miR-146b for 48 hours and ACKR2 protein expression determined by immunocytochemistry. Bright green punctate cytoplasmic staining, typical of ACKR2 expression in other cell types(11,32), was observed in KC transfected with scrambled miR control (Figure 4c). This staining was more marked in the peri-nuclear region and often in an asymmetrical fashion in keeping with higher ACKR2 levels in the endoplasmic reticulum. This immunofluorescence staining pattern is as expected and we have previously published that the majority of ACKR2 protein is found within intracellular vesicles that traffic to, and from, the cell surface(11,32). In contrast, when monolayers of KC were transfected with miR-146b, cells exhibited a loss of ACKR2 staining throughout the cytoplasm, although the peri-nuclear staining was still evident (Figure 4d).

To investigate the effect of miR-10b on ACKR2 expression in human LECs, LECs that had been grown as confluent monolayers were transfected with miR-10b and ACKR2 protein expression determined, by immunofluorescence, after 48 hours (Figure 5a with isotype control shown in 5b). In keeping with the known difference in ACKR2 transcript level, the level of ACKR2 protein staining was less intense in LEC compared to KCs. The granular staining in LEC was again more marked to one side of the perinuclear region (akin to what was observed in KC), which became especially apparent at higher magnification (Figure 5c). Importantly, ACKR2 staining was more pronounced and more granular in scrambled miR control transfected LECs, compared to miR-10b transfected LECs, in which staining was not higher than background autofluorescence (Figure 5b,d).

Taken together, immunofluorescent staining of cultured cell monolayers demonstrated that transfection with miR-146b in KC and miR-10b in LEC led to a reduction in ACKR2 staining in both cell types by 48 hours.

Tensile cell trauma of cytokine-treated KC leads to a rapid reduction in ACKR2 mRNA expression: Next, we examined the possible relevance of our finding for cutaneous inflammatory responses such as those typical of psoriasis. Psoriatic patients have elevated cutaneous ACKR2 expression in unaffected skin, which mouse models suggest may protect from further lesion development(24). Superficial trauma of unaffected skin in psoriatic patients can trigger psoriatic plaque development (Koebnerisation) concurrent with ACKR2 expression downregulation(16,33,34). This phenomenon is a feature of many psoriatic patients, where lesions have a particular predilection for sites that undergo repeated stretching in daily life (e.g. knees, elbows, skin folds)(20). Thus, we wanted to determine if tensile trauma downregulated ACKR2 in cultured keratinocytes in a cell-autonomous manner, and if this correlated with increased miR-146 activity. In order to specifically determine the effect of tensile cell trauma on ACKR2 expression, a reductionist in vitro model was developed. The model developed was based on the FlexCell™ International FX5000 machine, whereby primary human cells were grown on silicone membranes. The membrane was subsequently subjected to repeated bi-axial cyclical tension in vitro, with a pre-determined degree of tension, stretch wave-form, and cycle number (this model is summarised in
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Supplementary Figure S1). Neither KCs nor LECs adhered to uncoated silicone, and so KC were grown on Collagen I coated silicone while LECs were grown on Fibronectin coated silicone. This was found to be associated with stable cell attachment over a 12-hour stretch cycle for each cell type.

To examine the effect of tensile trauma on KC ACKR2 expression, confluent KC monolayers were subjected to stretching for 12h during which the membrane was stretched by 15% every 0.8 seconds (0.8Hz), which is not atypical for skin sites such as elbows that are exposed to daily repeated stretching. Cells were rested for a further 12h before lysis to enable gene expression and ACKR2 expression determined by QPCR. The data obtained (Figure 6a) showed that stretching of non-inflamed KC did not result in altered ACKR2 expression. However, these primary human cells are derived from healthy donors and are grown in the absence of typical psoriasis-associated factors. Psoriasis patients have markedly elevated T-cell cytokines, including IFNγ, that correlate with elevated ACKR2 in unaffected skin(16,24). Therefore, in order to better model human psoriasis, where KCs exist in the context of systemically elevated T-cell cytokines, KCs were treated with tissue culture supernatant from activated human T-cells or recombinant IFNγ (both of which upregulate ACKR2 expression(16,24)) and then exposed to tensile stress for 12h at 0.8Hz. In contrast to non-treated KC (Figure 6a), tensile trauma of KC pre-treated with T-cell supernatants, led to a significant decrease in ACKR2 expression (Figure 6b). This was also the case for IFNγ pre-treated KC, suggesting that KC with elevated ACKR2 levels, such as those found in psoriasis patients, display tensile stress-induced downregulation of expression. Interestingly, treatment of the T-cell supernatant with IFNγ neutralising antibodies did not diminish this effect, suggesting that soluble T-cell products other than IFNγ can mediate the observed effect (Figure 6b). In contrast to KC, there was no effect on ACKR2 expression upon flexing of LECs that were stimulated with T-cell supernatant (Figure 6c). Thus, the response of inflamed KC and LEC to tensile stress appears to be different, even when cells have been similarly pre-treated.

To determine whether there was a possible link between these observations and alterations in miR-146b expression, miR-146a/b expression levels were quantified by QPCR in inflamed KCs. Both miR-146a and miR-146b were upregulated in KC that had been treated with tissue culture supernatant from activated human T-cells, compared to resting non-inflamed KC (Figure 6d, grey bars). Importantly, when these cells were subjected to tensile trauma, this effect was dramatically amplified; there was an over 100-fold induction of miR-146a and a lesser (albeit significant) induction of miR-146b (Figure 6d, black bars). A similar observation was found for KC pre-treated with recombinant IFNγ and then subjected to stretching, although in this case miR-146b was more strongly induced than miR-146a (Figure 6e). Taken together, these data suggest that KC exposed to psoriasis-associated T-cell cytokines markedly upregulate miR-146a/b expression concurrent with ACKR2 downregulation upon stretching and provides a putative mechanistic link that explains the Koebner phenomenon in psoriasis.

Discussion

The atypical chemokine receptor ACKR2 is expressed in barrier tissues, including the skin, and functions as a high-capacity scavenger of pro-inflammatory CC-chemokines. In vivo, and specifically in the context of psoriasiform inflammation, we have shown that ACKR2 restricts T-cell entry into the epidermis and limits inflammation. Additionally, ACKR2 expression is deficient in psoriatic plaques in humans, which may enable uncontrolled inflammation and thereby plaque formation. The mechanism by which ACKR2 downregulation occurs has, until now, not been described. Indeed, although some pro-inflammatory cytokines are known to increase expression of ACKR2 (although notably not the psoriasis-associated cytokine IL17; 16,24), little is known about the molecular mechanisms that control atypical chemokine receptor expression. Here we identify microRNAs that are both differentially expressed in psoriasis and predicted to target the ACKR2 3'-UTR. Experimental evidence is provided that miR-146 and miR-10 bind directly to the ACKR2 3'-UTR, and lead to a downregulation of ACKR2 at transcript and
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protein level in keratinocytes (KC) and lymphatic endothelial cells (LEC) respectively.

It is notable that we see, at best, a 50% reduction in luciferase activity, or ACKR2 transcript levels, in cells transfected with miR-146 species. It is widely accepted that this level of transcript knockdown represents a typical response to miR-mediated suppression and that more extensive depletion of target mRNA levels is not commonly seen(35,36).

We have previously shown that mild trauma (tape-stripping, which induces a mild degree of epidermal damage and tensile stress) to uninvolved psoriatic skin leads to rapid transcriptional down-regulation of ACKR2 which, we propose, allows chemokine-dependent inflammation to become established and contributes to the Koebner phenomenon and the development of plaques(16). The Koebner phenomenon is reported in psoriasis, as well as a range of other skin diseases, whereby skin trauma (for example scratching and tape-stripping) triggers the appearance of the underlying disease at those sites(20). However, tape stripping induces several types of damage to skin, including disruption of skin barrier function, as well as tensile stress during the rapid removal of the tape adherent to skin. Additionally, it is notably that psoriatic plaques have a predilection for areas undergoing repeated tension, e.g. elbows, knees, skin folds. To specifically mimic tensile trauma to keratinocytes in vitro, we used a cell-flexing device to induce tensile stretch across the length of the keratinocytes. Flexing of resting keratinocytes, that express very low ACKR2 levels, did not alter transcript levels. In comparison, flexing of keratinocytes pre-treated with conditioned medium from activated human T-cells significantly reduced elevated ACKR2 expression. This was also seen with stretching of IFNγ-treated keratinocytes. Together our results demonstrate that, while keratinocyte trauma does not alter ACKR2 expression under ‘resting’ conditions, it does nonetheless significantly reduce T-cell induced ACKR2 expression levels, thus mimicking what is seen in uninvolved human psoriatic skin upon trauma(16). Despite being well described, a mechanism for the Koebner phenomenon (Koebnerisation) has not been elucidated. Our findings presented here provide a novel molecular mechanism by which Koebnerisation might be induced in unaffected psoriatic skin, potentially through the upregulation of miR-146 and its interaction with ACKR2 transcripts. To this end we defined the impact of flexing on miR-146 expression in T-cell supernatant treated keratinocytes. These analyses revealed a marked induction of miR-146a (and to a lesser extent miR-146b) in flexed, compared to static, T-cell treated keratinocytes. These data therefore indicate that increased expression of miR-146 following mild trauma to keratinocytes, and in psoriatic plaques, is able to counteract the inductive effects of activated T-cell products on ACKR2 expression. Thus, these findings also provide a plausible mechanistic explanation for the Koebner phenomenon in inflammatory skin diseases such as psoriasis and may have wider implications for non-cutaneous inflammatory diseases in which tensile strength is a contributing factor e.g. rheumatoid arthritis.

Experimental Procedures

MicroRNA in silico selection: MicroRNAs that were predicted to bind to the 3'-untranslated region (UTR) of ACKR2 were identified using TargetScan version 6.2 (www.targetscan.org). MicroRNAs with the highest likelihood of binding the ACKR2 3’-UTR (as indicated by the total context, CT score) and that were concurrently identified as being upregulated in psoriatic plaques in humans were selected for further study(27,37).

Cell culture: Primary healthy human keratinocytes (KC) and lymphatic endothelial cells (LEC) were purchased (PromoCell, Heidelberg, Germany) and cultured as per the manufacturer’s instructions in KCGM2 and ECMV2 medium respectively (PromoCell, Heidelberg, Germany). All in vitro experimental work was conducted at Passage 3-4, at 70% confluence (averaged across the vessel). All cells were maintained at 37°C in a humidified tissue culture incubator with 5% CO2. Cells were grown in the presence of 1% Penicillin/Streptomycin (Sigma, UK) and 0.1% Gentamycin solution (50mg/ml, Sigma, UK). Experiments in primary human cells were generally repeated using cells from at least 2 separate cell donors. HEK293 cell lines were kindly donated by Dr K. Hewit, and were grown in
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DMEM (Sigma, UK) to which was added 50ml Foetal Calf Serum and 5ml L-glutamine per 500mls (Sigma, UK). Data representative of 2 or more independent experiments, with a minimum of 3 replicates for each experiment.

**microRNA transfection of cells:** Cells were transfected with Lipofectamine RNAiMax as per manufacturer’s instructions (ThermoFisher, UK). Cell culture medium was changed to antibiotic free equivalents 24h prior to transfection, and maintained antibiotic free throughout the transfection process. Cells were transfected at 70% confluence. microRNAs, scrambled microRNA control and miR inhibitors (miRvana, ThermoFisher, UK) were transfected as per the manufacturer’s in 50μl Opti-MEM (reduced serum medium, ThermoFisher, UK). Transfection was allowed to occur at 37°C for 24 hours before the cells were lysed (or the medium was changed for prolonged incubation). At this concentration, the microRNAs had no detectable effect on cell viability.

**RNA extractions and Quantitative PCR:** RNA was extracted and purified on RNeasy Micro columns, or miRneasy columns (Qiagen, UK) with on-column DNAse digestion (DNase, Qiagen, UK) as per manufacturer’s instructions. Whole tissue samples were homogenized in Qiazol with stainless steel beads using a TissueLyser LT (Qiagen, UK). 1 μg total RNA was reverse transcribed using nanoscript RT or RT2 kits as per manufacturer’s instructions (PrimerDesign, UK). For samples intended for microRNA QPCR, 440ng total RNA was reverse transcribed using miScript II RT kit as per manufacturer’s instructions (Qiagen, UK). Samples were diluted 1:5 in nuclease-free water prior to being used as a template for QPCR. Gene transcripts were quantified by quantitative PCR analysis using Perfecta SYBR Green master mix as per manufacturer’s instructions (Quanta, UK). Samples were analyzed in quadruplicate on a 384-well Applied Biosystems 7900HT platform (LifeTechnologies). ACKR2 transcript levels were normalized to human TATA Binding Protein (TBP). The sequences for the Q-PCR primers were as previously reported(16,31,38) except for TRAF6 and IRAK1. For samples intended for microRNA quantification, QPCR was performed using the miScript SYBR Green PCR kit, with primers for miR-146a and 146b normalised to RNU6B expression (all Qiagen UK, and as per manufacturer’s instructions), and analyzed on a 384-well Applied Biosystems 7900HT (Life Technologies). Results for microRNA were analysed according to the ΔΔ-method, rather than semi-absolute methods as used for mRNA.

**Luciferase microRNA assay:** The 3’-untranslated region (UTR) of ACKR2 was cloned into the dual luciferase system containing the pmiRGLO vector (Promega, UK). The 3’-UTR region was sequenced to ensure putative microRNA binding sites were intact (Eurofins, UK). The plasmid was transfected into HEK293 cells and stable transfectants generated through selection with 0.8mg/ml G418 (Promega, UK). Non-ACKR2 containing pmiR vector was transfected as a control. HEK293 cells were donated by Dr K Hewit, and grown in DMEM (Sigma, UK) with added penicillin/streptomycin as per above. Several clones were identified for further testing, and two of these clones were transfected with the relevant microRNAs/controls, cells lysed and Firefly luciferase activity normalised to Renilla luciferase (as per manufacturer’s instructions, Promega, UK). pmiR containing HEK293 cells (i.e. with no ACKR2 3’-UTR) and native HEK293 cells were used as controls in the assay, that was repeated using two separate HEK293 clones.

**Fluorescent immunocytochemistry:** Cells were cultured in 4-well chamber-slides (ThermoScientific Nunc, or BD Falcon). Slides were washed in Phosphate Buffered Saline (PBS) without calcium (Sigma) and fixed with 100% methanol. Slides were washed and blocked with 20% normal horse serum in PBS with 0.05% Tween (Sigma), followed by an Avidin/Biotin block. Slides were stained for ACKR2 using Sigma Prestige anti-human ACKR2 IgG antibody (Sigma, UK) raised in rabbit in 2.5% normal human serum and 2.5% horse serum (Vector Laboratories, UK) in PBS-Tween (PBST), overnight at 4°C and then stained with a secondary antibody (biotinylated anti-rabbit IgG raised in goat, with 5% human serum). Slides were washed, incubated with Avidin-D fluorochrome conjugated.
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with FITC (Vector Laboratories, UK) and mounted with Vectamount containing DAPI (Vector Laboratories) prior to visualisation through confocal microscopy (LSM510, Zeiss).

**Tensile stress of keratinocytes:** For FlexCell experiments, primary human keratinocytes were grown in BioFlex 6-well plates pre-coated with Collagen I (FlexCell International, USA) for KC, or plain BioFlex 6-well plates coated with Fibronectin overnight prior to use (Sigma, UK) for LEC. Cells were subjected to tensile stress at 15% effective stretch, 0.8Hz, ½ sine waveform using the FlexCell FX-5000 Cell Tension System (FlexCell International). Cells were stretched for 12 hours, and rested for a further 12 hours, prior to lysis. All cells were grown and subjected to tension in a humidified incubator at 37°C with 5% CO₂.

**T-cell isolation and stimulation:** Human T-cells were grown from CD14-depleted human buffy coats from healthy donors, and stimulated with Concanavalin A (5ng/ml, Sigma, UK), and grown in RPMI (LifeTechnologies, UK) with 5% human AB serum and gentamicin (Sigma) in the presence of IL-2 (20 units/ml, Peprotech, UK) from day 4. Cells were purified on Ficoll-Paque after 8 days (GE Healthcare) and grown in the presence of IL-2 alone for 4 days, before being activated using CD2/CD3/CD28 beads as per manufacturer’s instructions at a 1:2 bead:cell ratio (Miltenyi Biotec, UK) for 24 hours prior to the activated supernatant being removed for downstream applications. Where T-cell supernatant was to be used for KC/LEC stimulation, T-cells were grown in serum-free keratinocyte medium KCGM2 or ECMV2 with added supplements during the 24-hour activation period (PromoCell, Germany). T-cells used for migration assays were similarly activated albeit at a 1:4 bead:cell ratio for 48 hours prior to use.

**Statistical analyses:** Student’s t-test, One-way ANOVA, two-way ANOVA and correlation tests were performed in Prism Version 7.0 (GraphPad Software Inc), with multiple comparison tests as appropriate. P<0.05 was deemed significant. All data are n≥3 and are representative of at least 2 independent experiments, and given as Mean ± SEM unless otherwise stated.

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**Author contributions:** KS, CSM. ADB and GJG conceived the study. KS and FS carried out experimental work and MKS provided essential advice, insights and reagents central to the pursuit of the project. All authors were involved in writing the manuscript and all approved the final version for submission.
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Figure legends

Figure 1. *In silico* analyses identified several psoriasis-associated of putative ACKR2-3’-UTR interacting microRNAs.
(a) Summary of microRNAs predicted to bind the ACKR2 3’-UTR with associated microRNA-specific data from previous psoriasis publications. The 10 microRNAs most likely to target ACKR2 are included in the diagram, with relevant findings in two papers given in the right hand column; miR-10, miR-146 and miR-203 were all differentially regulated in psoriasis.(27,37).
(b) Three microRNAs (miR-10, miR-146 and miR-203) are both predicted to bind the ACKR2 3’-UTR and are differentially expressed in psoriasis. Diagrammatic representation of microRNAs that are predicted to bind the ACKR2 3’-UTR in silico, and that have been shown to be differentially expressed in psoriasis by microarray studies.

Figure 2. miR-146 and miR-10 transfection reduced AKCR2 transcripts in KC and LECs respectively.
(a) Absolute quantification of IRAK/TRAF6 mRNA (previously validated miR-146b targets) in primary healthy human keratinocytes that were stimulated with 100ng/ml of human recombinant IFNγ prior to transfection.
(b) Absolute quantification of ACKR2 transcripts following transfection of KC with; (i) miR-146b; (ii) miR-10; (iii) miR-203.
(c) Absolute quantification of ACKR2 transcripts following transfection of KC with miR-146a and miR-146b.
(d) Absolute quantification of ACKR2 transcripts following transfection of LECs with; (i) miR-10; (ii) miR-146b; and (iii) miR-203.
In all cases, cells were transfected for 24 hours and left for a further 24 hours prior to lysis and RNA extraction. microRNAs added at 10nM, control is scrambled microRNA.
Representative experiments conducted in cells from various cell donors. Significance was assessed using Student’s t-test * P<0.05, ** P<0.01, except for (d) where significance was assessed using one-way ANOVA with Tukey’s post-test. * P<0.05, ** P<0.01, *** P<0.005

Figure 3. miR-146b and miR-10b functionally interact with the ACKR2 3’-UTR.
(a) miR-146b can be successfully transfected into HEK293 cells. Absolute quantification of IRAK1 mRNA as assessed by QPCR in HEK293 cells following transfection with miR-146b as compared to scrambled control.
(b) ACKR2 3’-UTR was cloned into the downstream UTR of a firefly luciferase reporter and interaction with transfected miRs determined (bioluminescence inversely proportional to microRNA 3’-UTR binding) and normalised to Renilla luciferase. Results are representative from two different luciferase expressing HEK293 clones, following transfection with miR-10b and miR-146b (singly and in combination) and scrambled miR control. Statistics: one-way ANOVA. * P<0.05, ** P<0.01, *** P<0.005, **** P<0.0001

Figure 4. Transfection of KC with miR-146b reduced cytoplasmic ACKR2 protein distribution.
(a) Representative bright-field image of KC grown as confluent monolayer
(b) Representative immunofluorescence of KC grown as confluent monolayers, stained with isotype control antibody.
(c,d) Representative immunofluorescence microscopy images of confluent monolayers of KC 48 hours after transfection with (c) scrambled miR control or (d) miR-146b.

Figure 5. Transfection of LEC with miR-10 reduced ACKR2 protein expression throughout the cytoplasm.
(a) Representative bright-field image of LEC grown as confluent monolayer
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(b) Representative immunofluorescence of LEC grown as confluent monolayers, stained with isotype control antibody.
(c,d) Representative immunofluorescence microscopy images of confluent monolayers of LEC 48 hours after transfection with (c) scrambled miR control or (d) miR-10. White arrows indicate asymmetric distribution of ACKR2.

Figure 6. Effect of tensile stress on ACKR2 expression by primary human keratinocytes (KC).
(a-c) Absolute quantification of ACKR2 mRNA, normalised to TBP in keratinocytes;
(a) Healthy human primary KC that remained static, or were subjected to tensile stress (flexed) at 0.8Hz for 12h, and then allowed to rest for 12h prior to cell lysis and RNA extraction.
(b) Healthy human primary KC that remained static, or were subjected to tensile stress (flexed) at 0.8Hz for 12h, and then allowed to rest for 12h prior to cell lysis and RNA extraction. KC were pre-treated with either 1) tissue culture supernatant from activated human T-cells (1:8 dilution in fresh medium), 2) 100ng/ml recombinant human IFNγ, or 3) tissue culture supernatant from activated human T-cells plus neutralising anti-IFNγ antibodies, overnight prior to flexing at 0.8Hz for 12 hours. Black bars; non-flexed static controls, grey bars flexed cells. Significance was assessed using One-way ANOVA.
(c) Treatment of healthy primary LEC overnight with tissue culture supernatant from activated human T-cells (diluted 1:8 with fresh medium) prior to flexing.
(d,e) Effect of tensile stress on miR-146 expression in inflamed keratinocytes. Fold change in miR-146a and miR-146b expression as assessed by QPCR, and normalised to scrambled miR treated static KCs. KC were treated for 16 hours with either; (d) tissue culture supernatant from activated human T-cells (1:8 dilution in fresh medium); or (e) recombinant IFN-γ at 100ng/ml; prior to flexing at 0.8Hz for 12hours.
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### Figure 1

#### a

| miR                  | Total Context Score | Regulation in psoriasis                                      |
|----------------------|---------------------|--------------------------------------------------------------|
| miR-10abc/10a-5p     | -0.57               | miR-10b 1.32-fold up-regulated in PN                        |
| miR-10a              | 0.67-fold down-regulated in PP |
| miR-490-3p           | -0.29               | n/a                                                         |
| miR-146ac/146b-5p    | -0.26               | miR-146a 3.10-fold up-regulated in PP, miR-146b-59 up-regulated 2.73-fold (Zibert et al. J Derm Sci 2010) |
| miR-146b             | 3.30-fold up-regulated in PP |
| miR-203              | -0.19               | 2.02-fold up-regulated in PP and 5.86-fold up-regulated in PP |
| miR-210              | -0.15               | n/a                                                         |
| miR-24/24ab/24-3p    | -0.15               | 1.51-fold and 1.41 up-regulated in PP and PN respectively (Sonkoly et al. J PLOS One 2007) |
| miR-122/122a/1352    | -0.13               | n/a                                                         |
| miR-103a/107/107ab   | -0.13               | 1.04-fold up-regulated in PP (Sonkoly et al. J PLOS One 2007) |
| miR-145              | -0.10               | n/a                                                         |
| miR-375              | -0.09               | n/a                                                         |

*PP – psoriatic lesion (plaque) PN – normal (clinically unaffected) psoriatic skin

#### b

- **16 miRs (broadly conserved in vertebrates) predicted to bind to the ACKR2 3'-UTR.**
- **47 miR differentially expressed in PP versus NN (Zibert et al. J Derm Sci 2010)**
- **29 miR differentially expressed in PP versus NN (Sonkoly et al. J PLOS One 2007)**
microRNA regulation of ACKR2 expression

Figure 2

a) IRAK1 and TRAF6 levels in control and miR-146b treated cells

b) ACKR2 levels in control and miR-146b, miR-10b, and miR-203 treated cells

c) IRAK1 and TRAF6 levels in control and miR-146b treated cells

d) ACKR2 levels in control and miR-10b, miR-146b, and miR-203 treated cells
microRNA regulation of ACKR2 expression

Figure 3

(a) Bar graph showing the number of copies of IRAK1/105 copies of TBP for control miR and miR-146b, with a p-value of <0.005.

(b) Bar graph showing the bioluminescence index (luciferase/renilla) for control miR, miR-10, miR-146, and miR-10/146, with significant differences indicated by ***.
microRNA regulation of ACKR2 expression

Figure 4
microRNA regulation of ACKR2 expression

Figure 5

(a) [Image: grayscale cell culture]

(b) [Image: fluorescently stained cells]

(c) scrambled control miR

(d) miR-10b

GREEN – ACKR2
BLUE – DAPI
microRNA regulation of ACKR2 expression

Figure 6

(a) Fold change of ACKR2 expression in static versus flexed T-cells, STIC T-cells, STATIC IFN-γ, FLEXED T-cells+a anti-IFN-γ, STATIC IFN-γ, FLEXED

(b) Fold change of ACKR2 expression in resting T-cells pre-treated with T-cell supernatant or IFN-γ

(c) Number of copies of ACKR2/10^5 copies TBP

(d) Fold change of miR-146a, miR-146b, miR-146a, miR-146b in static versus flexed T-cells

(e) Fold change of miR-146a, miR-146b, miR-146a, miR-146b in resting versus IFN-γ

P-values are indicated for statistical significance: P<0.05, P<0.01, P<0.005, P<0.001
MicroRNA-146 and cell trauma downregulate expression of the psoriasis-associated atypical chemokine receptor ACKR2
Kave Shams, Mariola Kurowska-Stolarska, Fabian Schütte, A. David Burden, Clive S. McKimmie and Gerard J. Graham

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