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Galectin-7 downregulation in lesional keratinocytes contributes to enhanced IL-17A signaling and skin pathology in psoriasis

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Psoriasis is a chronic inflammatory skin disease characterized by inflammatory cell infiltration, as well as hyperproliferation of keratinocytes in skin lesions, and is considered a metabolic syndrome. We found that the expression of galectin-7 is reduced in the skin lesions of patients with psoriasis. IL-17A and TNF-α, two cytokines intimately involved in the development of psoriatic lesions, suppressed galectin-7 expression in human primary keratinocytes (HEKn cells) and the immortalized human keratinocyte cell line HaCaT. A galectin-7 knockdown in these cells elevated the production of IL-6 and IL-8 and enhanced ERK signaling when the cells were stimulated with IL-17A. Galectin-7 attenuated IL-17A–induced production of inflammatory mediators by keratinocytes via the miR-146a–ERK pathway. Moreover, galectin-7–deficient mice showed enhanced epidermal hyperplasia and skin inflammation in response to intradermal IL-23 injection. We identified fluvastatin as an inducer of galectin-7 expression by connectivity map (cMAP) analysis, confirmed this effect in keratinocytes, and demonstrated that fluvastatin attenuated IL-6 and IL-8 production induced by IL-17A. Thus, we validate a role of galectin-7 in the pathogenesis of psoriasis, in both epidermal hyperplasia and keratinocyte-mediated inflammatory responses, and formulated a rationale for the use of statins in the treatment of psoriasis.
Introduction

Psoriasis is a complex inflammatory skin disease where both genetic and environmental factors participate in its pathogenesis. Important characteristics of this disease include keratinocyte hyperproliferation and leukocyte infiltration. Although the etiology is not clear, hyperactive keratinocytes and immune cells are key players in the pathogenesis of psoriasis, and the cross-talk between these cells contributes to the pathological phenotype. Moreover, psoriasis is considered as a metabolic syndrome, associated with dyslipidemia and an increased risk of cardiovascular disease.

In psoriasis, IL-17 cytokines secreted by activated T cells are the key mediators that coordinate local tissue inflammation. IL-17A induces keratinocyte hyperproliferation and can cause keratinocytes and other cell types to produce pro-inflammatory cytokines and neutrophil-mobilizing chemokines (including IL-6, CXCL8/IL-8, and CXCL2). The expression of these cytokines and chemokines is dependent on the IκB kinase (IKK)-α–NF-κB and ERK signaling pathways. Therefore, factors modulating the sensitivity of keratinocytes to IL-17 through the above signaling pathways may play a crucial role in the pathogenesis of psoriasis.

Galectin-7, a 15 kDa β-galactoside–binding protein, is highly expressed in epidermal keratinocytes and is induced by the tumor suppressor p53 (1). Galectin-7 is primarily an intracellular protein and can be located both in the nucleus and cytoplasm of keratinocytes (2, 3), although it also exists.
outside the cell and is capable of binding to various glycoconjuggates in the extracellular space.

Downregulation and nuclear translocation of galectin-7 in transformed and ultraviolet-light-irradiated keratinocytes have been reported, suggesting that galectin-7 may participate in cellular homeostasis (2, 4). In mice, galectin-7 was reported to regulate skin homeostasis during injury, but the detailed mechanism is not clear (5). Previously, we have investigated the role of galectin-7 in the regulation of keratinocyte proliferation (6), but how galectin-7 functions in keratinocytes in the inflammatory response occurring in human inflammatory skin diseases, such as psoriasis, is not clear.

A number of microRNAs (miRNAs) were found to be differentially expressed in psoriatic lesions (7). According to a number of mechanistic studies, miRNAs can drive various pathways in psoriasis pathogenesis, including regulation of T-cell activation and functions, IFN-α and TNF-α expression, keratinocyte hyperproliferation, cytokine or chemokine expression, and NF-κB signaling (8-11). We have previously identified two microRNAs that are regulated by galectin-7 (i.e., miR-146a and miR-203) (6). There, we reported that galectin-7 protects JNK1 from ubiquitination and degradation, which in turn upregulates expression of miR-203. We also demonstrated that miR-203 upregulates the expression of p63, which is known to suppress keratinocyte proliferation. Thus, galectin-7 suppresses keratinocyte proliferation through the JNK-miR-203-p63 pathway.

In the data obtained in our previous transcriptomic study (12), we found that galectin-7 mRNA is
downregulated in the skin lesions of patients with psoriasis (GSE153007). In the present study, we first explored the protein levels of galectin-7 in psoriasis and then provided evidence that its downregulation in keratinocytes, as noted in psoriatic lesions, contributes to the inflammatory response and epidermal hyperplasia associated with psoriasis. We then dissected the underlying mechanism and established that galectin-7 suppresses the inflammatory response associated with psoriasis by suppressing the miRNA-146a-ERK pathway in keratinocytes. Furthermore, we identified a mechanistic basis for the use of statins, commonly used in the treatment of dyslipidemia, as therapeutic agents in psoriasis attributable to their potent induction of galectin-7 expression.
Results

Galectin-7 expression is downregulated in human psoriatic lesions and mouse psoriasiform dermatitic lesions induced by IL-23

Consistent with the data from our previous transcriptomic study (12) (GSE153007), meta-analysis of the microarray and RNA sequencing datasets (GSE68937 and PRJNA421744) from other publications (13, 14) revealed that galectin-7 mRNA is downregulated in the epidermis of skin lesions of patients with psoriasis (Supplementary Table 1 and 2). To confirm this finding at the protein level, we examined galectin-7 protein expression in the skin lesions from a cohort of patients with psoriasis (n=27) and normal skin from healthy controls (n=75) by immunohistochemistry. As shown by the representative images (Figure 1A), galectin-7 was strongly expressed in all epidermal layers of normal skin (Figure 1A), but significantly downregulated in psoriatic skin (Figure 1A). The reduction in the galectin-7 protein levels in psoriatic epidermis was verified by computer-assisted quantitative analysis of immunohistochemical staining (Figure 1B). In addition, we confirmed galectin-7 protein expression is reduced in lesional skin compared with non-lesional skin from the same patients (n=10) (Figure 1C), and the quantification results are shown in Figure 1D. These immunohistochemical analyses (Figure 1A and 1C) showed galectin-7 is expressed mainly in the epidermis and is undetectable in the dermis. Moreover, we performed immunoblot analysis with samples from lesional and non-lesional skin (epidermal plus dermis) of three psoriasis patients. There, we confirmed galectin-7 protein levels were significantly
lower in the lesional skin compared with the non-lesional skin (Supplementary Figure 1). It is to be noted that an earlier study (15) did not observe reduced expression of galectin-7 in psoriasis lesions, which could be due to a number of possibilities, including the population of the patients (thus the severity of the disease), antibodies used, and tissue preservation methods.

We next addressed whether galectin-7 expression was also reduced in a mouse model of psoriasis, that involves intradermal IL-23 injections (16, 17). Injection of IL-23 into the ears of wild-type (WT) mice induced marked ear swelling, as expected. This response was associated with epidermal hyperplasia and leukocyte infiltration, which were not seen with PBS injection (controls; Figure 1C). IL-23–injected epidermis also showed drastically reduced galectin-7 expression (Figure 1E), as quantified in Figure 1F.

We then examined the effects of cytokines (e.g., IL-17A, IL-23, and TNF-α, which are known to promote psoriatic inflammation) (17-19) on galectin-7 expression in immortalized human keratinocytes (HaCaT cells) and primary neonatal epidermal keratinocytes (HEKn cells). We found that IL-17A alone reduced galectin-7 protein expression in both (Figure 1G). TNF-α also significantly reduced galectin-7 expression in HaCaT cells, while interferon (IFN)-γ, lipopolysaccharide (LPS), and IL-23 only had modest effects (Figure 1G).

Suppression of galectin-7 expression promotes production of pro-inflammatory cytokines and chemokines

Microarray analysis of galectin-7 knockdown HaCaT cells revealed upregulation of several chemokine
genes (CCL3, CCL4, CXCL2, CXCL3, and others) and psoriasis-related genes (including serine protease inhibitors, SERPINA3 and SERPINB4; S100 calcium-binding proteins, S100A7 and S100A7A; and defensin β4A, DEFB4A) in these cells, as compared with control cells (our unpublished observations). This finding suggested that reduced galectin-7 expression might promote inflammation, thereby contributing to the pathogenesis of psoriasis. We examined the expression of cell surface receptors, IL-17RA, TLR4, and IFN-γR by flow cytometry, and found no significant changes in galectin-7 knockdown cells compared with controls (our unpublished observations). Accordingly, we hypothesized that galectin-7 has a suppressive effect on the cell signaling pathways of inflammatory responses, i.e., galectin-7 downregulation favors pro-inflammatory cytokine production during inflammation. We therefore subjected both HaCaT and HEKn cells to different immune stimuli, including IL-17A, IL-21, IL-22, IL-23, TNF-α, LPS, or IFN-γ, and measured cytokine secretion by enzyme-linked immunosorbent assays (ELISAs). Among these stimuli, only IL-17A (Figure 2), TNF-α, and LPS (our unpublished observations) induced production of IL-6 and IL-8, two neutrophil-mobilizing chemokines highly implicated in the pathogenesis of psoriasis. Galectin-7 knockdown cells secreted greater amounts of IL-6 and IL-8 in response to stimulation with IL-17A, as compared to control cells (Figure 2). We also observed stronger constitutive secretion of IL-6 and IL-8 in galectin-7 knockdown cells without stimulation (Figure 2). In addition, an inhibitor of galectin-7 (lactose) had no observed effect on the production of IL-6 and IL-8 (supplementary Figure 3). Since lactose is not cell-permeable, this result suggests that galectin-7 functions in the intracellular region.
MiR-146a expression in keratinocytes is suppressed by galectin-7 and induced by IL-17A

By microarray analysis, deep sequencing, and real-time PCR analyses, we have previously found that miR-146a was upregulated in galectin-7 knockdown HaCaT cells (6). Here, we showed miR-146a was similarly significantly overexpressed in HEKn cells with a transient galectin-7 knockdown (Supplementary Figure 4A). We performed in situ hybridization using locked nucleic acid (LNA)-modified nucleotide probes and confirmed that miR-146a was also expressed in the epidermis of normal human skin, but only rather weakly (Figure 3A). We found that expression of this miR was greatly increased in all epidermal layers of psoriatic lesions compared to healthy human skin (Figure 3A). Tissues hybridized with LNA-modified U6 small nuclear RNA (snRNA)-targeting probes or scrambled probes served as positive and negative controls, respectively (Figure 3A). We treated HaCaT and HEKn cells with IL-17A and found that miR-146a expression was induced (Figure 3B). In addition, we observed that IL-17A (Figure 3B), but not IL-22 or IL-23 (our unpublished observations), induced miR-146a expression.

To examine the effects of miR-146a under inflammatory conditions, we generated HaCaT cells stably overexpressing miR-146a (Supplementary Figure 4B). These cells exhibited markedly enhanced production of IL-6 and IL-8 after IL-17A stimulation, as compared with control cells (Figure 3C and 3D), although their galectin-7 expression remained unaffected (Supplementary Figure 4C).

Galectin-7 knockdown enhances the IL-17–induced MAPK ERK signaling pathway

In response to IL-17A exposure, both the MAPK and NF-κB pathways participate in the production of
pro-inflammatory cytokines in keratinocytes (20, 21). To further dissect the mechanism underlying the involvement of galectin-7 in psoriasis pathogenesis, we focused on the IL-17A signaling pathway. To distinguish whether galectin-7 affects MAPK or NF-kB activation, we starved HaCaT cells overnight in serum-free medium and then incubated the cells in the presence or absence of IL-17A. By immunoblotting analysis, we found that resting cells contained small amounts of phosphorylated extracellular signal-related kinase 1 and extracellular signal-related kinase 2 (phospho-ERK1 and phospho-ERK2, respectively; Figure 4A). In contrast, exposure of cells to IL-17A induced strong phosphorylation of ERK1 and ERK2 in four galectin-7 knockdown cell lines (sh-1, sh-2, sh-3, and sh-4), with 2.0- to 6.6-fold increases compared to control cells (Figure 4A). The total ERK1 and ERK2 protein levels were comparable in all cells treated or not treated with IL-17A (Figure 4A and Supplementary Figure 5).

In contrast, the upstream components of the NF-kB pathway (including phospho-NF-kB, total NF-kB, phospho-IκBα, and total IκBα) remained unchanged in response to IL-17A (Figure 4A), suggesting that galectin-7 does not mediate activation of the NF-kB pathway. We then used specific chemical inhibitors, including MAP kinase p38 inhibitor (SB203580), JNK inhibitor (SP600125), NF-κB inhibitor (PDTC), phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), and MAPK/ERK inhibitor (PD98059), to target their corresponding pathways in galectin-7 knockdown keratinocytes after stimulation with IL-17A. Among these compounds, the MAPK/ERK inhibitor PD98059 significantly blocked IL-6 and IL-8
production induced by IL-17A (Supplementary Figure 6A and 6B). IL-17A–induced IL-6 and IL-8 secretion was not blocked by inhibitors of MAP kinases p38 (SB203580), JNK (SP600125), NF-κB (PDTC), and by the inhibitor of phosphatidylinositol 3-kinase (PI3K; LY294002; our unpublished observations). Our data suggest that the regulatory role of galectin-7 in IL-17A–induced cytokine expression is primarily mediated by its effect on the MAPK-ERK signaling pathway via suppression of ERK phosphorylation.

MiR-146a induces inflammatory mediators in keratinocytes through the MAPK-ERK pathway

To identify the intracellular pathways via which miR-146a increases the expression of pro-inflammatory cytokines after stimulation by IL-17A, we examined activation of the MAPK and NF-κB pathways in miR-146–overexpressing keratinocytes. As shown in Figure 4B, the MAPK-ERK pathway was highly activated by IL-17A in these cells, compared with control cells. We also found that ERK phosphorylation was significantly enhanced (more than four-fold, P < 0.01; Figure 4B). In line with the data from galectin-7 knockdown cells, there were no obvious differences in the phosphorylation of NF-κB and IκBα or their total protein levels between miR-146a–overexpressing cells and control cells (Figure 4B). Collectively, these results suggest that miR-146a promotes IL-17A–induced production of IL-6 and IL-8 through the MAPK-ERK pathway.

Galectin-7–deficient mice exhibit heightened IL-23–induced epidermal hyperplasia and skin inflammation

To further assess the participation of galectin-7 in the regulation of inflammatory responses in vivo, we
studied galectin-7–deficient mice to determine the role of galectin-7 in IL-23–induced skin inflammation.

In response to IL-23 stimulation, WT mice showed ear swelling resulting from hyperplasia of epidermal keratinocytes and leukocytes infiltration, as revealed by H&E staining (Figure 5A). Skin sections from galectin-7–deficient mice showed a greater increase in ear and epidermal thickness compared with their WT control littermates 14 days after IL-23 injection (Figure 5B and 5C). In addition, the numbers of infiltrating leukocytes were significantly higher in galectin-7–deficient mice (Figure 5D). IL-19 is known as a robust marker reflecting the magnitude of IL-23/IL-17 signaling in chronic inflammation (22). In microarray analysis, we also found that the amounts of pro-inflammatory-cytokine mRNAs (IL-17A, CXCL5, and IL-19) were elevated in galectin-7–deficient mice, compared to WT mice (Supplementary Table 3). Taken together, these results indicate that galectin-7 has a suppressive effect on keratinocyte-mediated inflammatory responses.

Live deep-tissue visualization of galectin-7 expression in EGFP-galectin-7 transgenic mice by two-photon microscopy

To examine the expression of galectin-7 in the skin during IL-23-induced inflammatory response in vivo, we generated a galectin-7-EGFP fusion transgenic mouse model. This involved CRSIPR/CAS9-mediated homologous recombination, which allowed insertion of an EGFP gene to fuse with the endogenous galectin-7 gene, where the expression of the fusion protein is under control of the native galectin-7 promoter. EGFP-galectin-7 mice exhibited an intense green fluorescence signal on the entire body
surface (Figure 6A). In the three-dimensional images obtained by two-photon microscopy (Figure 6B),
the green fluorescence signal was found to be confined to keratinocytes and hair follicles (Figure 6C)
(the projected green signals from hair follicles is the reflected light from the hair). Immunoblot analysis
indicated that EGFP-galectin-7 protein is expressed in the ear skin, dorsal skin, and esophagus, but not
in the lung, kidney, liver, intestine and colon (Figure 6D and our unpublished observations). In addition,
we found no green fluorescence signals in bone marrow cells from these transgenic mice (Figure 6E).
We further performed affinity purification by using lactose-Sepharose beads and demonstrated that
EGFP-galectin-7 from the skin lysates retained lactose-binding activity (Figure 6F).
We performed daily intradermal IL-23 injections on the ears of these transgenic mice for 14 days, with
the contralateral ear being injected with PBS as control. Compared to the control, the ear that received
IL-23 had a profound thickening of the epidermis. We also injected Evan’s blue dye through the tail vein
for visualization of the blood vessels (red fluorescence), and found that galectin-7 was not expressed in
endothelial cells, because there were no green fluorescence signals in the blood vessel areas, as well as
other areas in the dermis, in IL-23-treated skin (Supplementary Figure 7A). We further performed
analysis of three-dimensional images obtained on days 0, 7, 14 and 21, and showed an increase in the
epidermal thickness in the IL-23 injected ear skin along with reduced green fluorescence signals on days
14 and 21 (Supplementary Figure 7B).

Fluvastatin increases galectin-7 levels and suppresses production of pro-inflammatory cytokines in
keratinocytes, and attenuates IL-23-induced epidermal thickness in vivo

To utilize our observations on the anti-inflammatory and antiproliferative effects of galectin-7, we scrutinized microarray databases for approved drugs that can induce galectin-7 expression. Four compounds were identified by connectivity map data (cMAP) analysis: methylprednisolone, tobramycin, fluvastatin, and pempidine. In our microarray and proteomic data (our unpublished observations), we found that mRNA and protein levels of galectin-7 were negatively correlated with S100A7, which was reported to be upregulated in psoriatic keratinocytes (23). Among the predicted drugs, only fluvastatin induced galectin-7 mRNA and protein expression (Figure 7A, Supplementary Figure 8A) and reduced S100A7 mRNA expression (Figure 7B) in our in vitro experiments using HaCaT cells. In addition, we found that fluvastatin can induce p21 overexpression as revealed by immunoblot analysis (Supplementary Figure 8B). We next examined the influence of other statin drugs and found that atorvastatin, cerivastatin, pitavastatin, lovastatin, mevastatin, and simvastatin all induced galectin-7 expression in both HaCaT and HEKn cells (Supplementary Figure 8C and 8D), while pravastatin was the least able to enhance galectin-7 expression (Supplementary Figure 8C and 8D).

We found that fluvastatin attenuated IL-17A–induced IL-6 and IL-8 secretion in HaCaT cells (Figure 7C and 7D). We also observed that fluvastatin enhanced galectin-7 expression above basal levels, even in the presence of cytokines (TNF-α and IL-17A) that suppress galectin-7 expression (Figure 7E). In vivo, fluvastatin attenuated IL-23-induced ear skin thickening and epidermal hyperplasia (Figure 7F and
Supplementary Figure 9A and 9B). Consistent with our finding that pravastatin was barely able to enhance galectin-7 expression, it also had little effect on suppressing skin thickening in the same model (Figure 7F and Supplementary Figure 9C). We further tested whether the suppression of ear thickening by fluvastatin was dependent on galectin-7 by using galectin-7-deficient mice. We found that fluvastatin failed to suppress ear thickening in galectin-7-deficient mice (Supplementary Figure 10), suggesting that fluvastatin amelioration of skin thickening was dependent on the expression of galectin-7. Thus, fluvastatin is a potent galectin-7 inducer that can suppress IL-17A- and IL-23-induced cytokine production and epidermal hyperplasia.

Discussion

We conclude that galectin-7 is downregulated in the keratinocytes of psoriatic human skin. This phenomenon was also confirmed in a mouse model of psoriasis. Mechanistically, we linked this to the action of IL-17A and TNF-α on keratinocytes, as demonstrated with the HaCaT and HEKn cell lines. By using both cell lines and a mouse psoriasis model, we were able to link the downregulated expression of galectin-7 to two cardinal histopathological features of psoriasis: keratinocyte hyperproliferation and skin inflammation. The former is consistent with our previous demonstration of the anti-proliferative function of galectin-7 in keratinocytes (6). The latter is associated with the ability of endogenous galectin-7 to suppress IL-6 and IL-8 production in keratinocytes treated with IL-17A, as shown in this study. Moreover, we were able to link the anti-inflammatory function of
galectin-7 to its ability to suppress the MAPK pathway. In the skin, galectin-7 is expressed exclusively in keratinocytes. We thus believe downregulated expression of galectin-7 in psoriasis contributes to the pathogenesis solely through the dysregulated functions of lesional keratinocytes resulting from the deficiency of this protein.

Galectin-7, like other galectins, are synthesized without a classical leader peptide for protein secretion through the classical pathway. The protein is abundantly expressed in the cytosol and enriched in the nuclei in some cell types under certain conditions. Nevertheless, the protein can be released by cells into the extracellular space through unconventional protein secretory pathways.

Our previous work (6) and present study demonstrated that intracellularly in keratinocytes, this protein functions by regulating intracellular signaling mediators, including JNK1, ERK, and miRNA, independently of the carbohydrate-binding activity of the protein. Thus, the finding in an earlier study (15) that there was no alteration in the levels of glycoligands in psoriasis lesions does not contradict with our conclusions.

We previously found that galectin-7 regulates JNK1 stability, thereby controlling miR-203 expression. In the present study, we demonstrated that galectin-7 suppresses miR-146a expression, which in turn, promotes ERK signaling during stimulation with IL-17A. We believe that there are two independent pathways regulating the expression of miR-203 and miR-146a, respectively. One piece of evidence we have is that we did not observe cross-talk between miR-203 knockdown and miR-146a overexpression
in cells. Indeed, when we knocked down or overexpressed miR-203, we did not observe any effect on cytokine production, nor feedback regulation on the miR-146a level. On the other hand, in miR-146a-overexpressing cells, we did not observe a change in their proliferation ability, but only cytokine production was elevated. Taken together, we believe that although galectin-7 regulates these two microRNAs simultaneously, their effects are independent. The overall effect of galectin-7 deficiency through these two miRNAs is enhanced cell proliferation and cytokine production in keratinocytes, both of which lead to the psoriasis phenotype.

The single-nucleotide polymorphism rs2910164 in miR-146a was found to be associated with psoriasis through the regulation of EGFR expression (24). Overexpression of miR-146a in skin lesions was reported to correlate with clinical psoriasis scores (11). It is known that miR-146a is induced by zymosan through the NF-κB signaling pathway in keratinocytes. MiR-146a levels then soon decrease to a basal level because of negative feedback signaling (25). In this case, miR-146a was reported to exert an anti-inflammatory action in keratinocytes through its known targets, including IRAK1 and TRAF6 (which are also required for miR-146a expression), and subsequent suppression of IL-8 and CCL20 expression (25). In addition, upregulation of miR-146a in the skin of patients with psoriasis was believed to contribute to lymphocyte infiltration (26). In contrast, here we found that IL-17A induced miR-146a expression, and HaCaT cells overexpressing miR-146a produced larger amounts of the pro-inflammatory cytokines IL-6 and IL-8. Therefore, our demonstration of the effect of IL-
17A–miR‐146a signaling on the inflammatory response is opposite to that of TLR2–miR‐146a signaling reported previously (25). Another study identified miR‐146a in the suppression of IL‐17–mediated skin inflammation in mice induced by topical application of imiquimod (27). In this model, the effect of miR‐146a on skin inflammation may influence both immune cells and keratinocytes. Thus, our results and those of other researchers suggest that miRNAs may yield different responses in cells, depending on the experimental conditions, including culture conditions (e.g., growth factors), cell type, and activation status. Mechanistically, miRNA functions are dependent on their target mRNA levels. Thus, the overexpression of miR‐146a in psoriatic skin lesions might be related to its inability to inhibit target genes (11). In this case, transcriptomic analysis should provide further information, and this topic has yet to be explored.

Other studies revealed the participation of miRNA‐203 in the modulation of the inflammatory response. For example, miR‐203 can suppress the levels of IL‐8 mRNA in keratinocytes treated with TNF‐α (28, 29), including levels of the suppressors of cytokine signaling (SOCS3 and SOCS6) in gingival epithelial cells (30). We tested whether galectin‐7 affects the levels of SOCS1, SOCS3, and SOCS6 in keratinocytes by immunoblot analysis, but did not observe a consistent downregulation of these proteins in galectin‐7 knockdown cells (our unpublished observations). However, we cannot entirely exclude the possibility that galectin‐7 also regulates the inflammatory response through this miRNA. Psoriasis is considered as a metabolic syndrome associated with an increased risk of cardiovascular
diseases, including acute coronary syndrome, arterial hypertension, and myocardial infarction (31, 32). Patients with this condition are often found to also have higher blood levels of cholesterol and triglycerides. Accordingly, patients with psoriasis may benefit from statin drugs, a group of 3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase inhibitors commonly taken by patients with hypercholesterolemia resulting from abnormal lipid metabolism. Interestingly, the beneficial effects of statins on skin lesions in patients with psoriasis have already been documented and are possibly mediated by suppressed inflammation and improved skin regeneration (33-35). Our findings suggest that the beneficial effects of statins may be related to their upregulation of galectin-7 expression. Different statins may have different effects on psoriasis and those that are stronger inducers of galectin-7 (such as fluvastatin and simvastatin) may be preferable over weaker inducers (such as pravastatin). Indeed, it has been reported that the former are more effective at inhibiting CCL20 production by human keratinocytes induced by IL-1β, TNF-α, or IL-17A, than the latter (34). It is to be noted that a reduction in CCL20 expression can decrease the number of CCR6+ T helper 17 (Th17) cells, a cell type crucial for the development of psoriatic skin lesions. While we are yet to test whether galectin-7 can also suppress CCL20 production, our findings suggest that galectin-7 may be responsible for the specificity of statins in terms of regulating the production of pro-inflammatory cytokines in human skin, and thus contribute to their therapeutic effects on psoriasis. In summary, galectin-7 expression in keratinocytes is downregulated by cytokines responsible for
the development of psoriasis. This resulted in the hyperproliferation of keratinocytes and skin inflammation. Approaches to induce galectin-7 in keratinocytes may be useful for the treatment of psoriasis. We have identified one such approach: the use of selected kinds of statins, which is intriguing, as psoriasis is considered a metabolic syndrome associated with dyslipidemia, and thus patients would benefit from the use of statins. Additional investigations along this approach have the potential to identify statins that can be selected on the basis of their potency in the induction of galectin-7 and will be more effective in the treatment of psoriasis.
Methods

Human skin tissue samples

Human skin tissues from patients with psoriasis ($n=27$) and healthy volunteers ($n=75$) were acquired with informed consent in accordance with a protocol approved by the Chung Shan Medical School Institutional Review Board. Lesional and non-lesional skin section pairs were acquired in accordance with protocols approved by the Chung Shan Medical School ($n=5$ cases) and Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation ($n=5$ cases) Institutional Review Board, respectively. Patients with psoriasis vulgaris received a diagnosis based on clinical and histopathological criteria and patients receiving systemic therapies were excluded. All patients were not treated with topical medications for at least one month before the biopsies were taken.

Mice

Galectin-7$^{+/−}$ mice with a C57BL/6 background were generated by the European Conditional Mouse Mutagenesis Program (EUCOMM) from the embryonic stem cell line EPD0327_3_B05 and were acquired from the Wellcome Trust Sanger Institute (Cambridge, UK). We implemented a breeding scheme of heterozygotes crossed with heterozygotes, to generate WT, heterozygous, and homozygous mice. All the mice in this study were genotyped, and control WT mice were littermates of the homozygous mice. Both male and female mice of 6 to 8 weeks old were used in the experiments.

Keratinocyte culture and preparation of galectin-7 knockdown cells
The human keratinocyte cell line HaCaT were acquired as described in our previous publication(6), and the cells was maintained at 37°C in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin (Sigma, USA) and 100 μg/ml streptomycin (Gibco, CA, USA). Short hairpin RNA (shRNA) reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology and Genomic Research Center, Academia Sinica, supported by the National Core Facility Program for Biotechnology Grants of NSC (NSC 100-2319-B-001-002). Four shRNAs with clone IDs of TRCN0000057393, TRCN0000057394, TRCN0000057395, and TRCN0000057396 were used to generate stable galectin-7 knockdown HaCaT clones. HaCaT cells were infected with one of the four shRNA-expressing lentiviruses and puromycin was employed to select stable cell clones after 3 days of infection. After puromycin selection, four individual pooled clones were cultured in puromycin-free medium for two weeks before further analysis.

Primary neonatal human epithelial keratinocytes (HEKn cells) were purchased from Gibco (cat. No. C-001-5C). HEKn cells were cultured at 37°C in keratinocyte serum-free medium (K-SFM) supplemented with 30 μg/ml bovine pituitary extract and 5 ng/ml recombinant human epidermal growth factor obtained from the same manufacturer. Compared with HaCaT cells, HEKn cells have a limited proliferation ability and are not suitable for establishing shRNA-based stable knockdown cells; therefore, we used a small interfering RNA (siRNA) oligo to transiently knock down the expression of galectin-7.

The Silencer® Select siRNAs were purchased from Invitrogen, including negative control #1 and a pre-
designed siRNA targeting human galectin-7 (cat. No. s230574-75-76). HEKn cells were treated with thirty picomoles of siRNAs in a 6-well plate with $1 \times 10^6$ cells per well using Lipofectamine 2000 (Invitrogen, USA) as a transfection agent.

Measurement of cytokine production

To measure the concentrations of pro-inflammatory cytokines secreted by keratinocytes, we seeded 2 $\times 10^4$ cells per well in 96-well plates. Cells were incubated for 24 h and then treated with 50 µg/ml LPS (Sigma, cat. No. L4391), 100 ng/ml human TNF-α (Peprotech, cat. No. 300-01), 100 or 200 ng/ml human IL-17A (Peprotech, cat. No. 200-17), 200 ng/ml human IL-22 (Peprotech, cat. No. 200-22), 100 ng/ml human IL-23 (Peprotech, cat. No. 200-23), or 50 ng/ml IFN-γ (eBioscience, cat. No. 39-8319-65) for 48 h at 37°C. In addition, the MEK inhibitor PD98059 (Merck Millipore Calbiochem) was added along with stimulation by LPS or IL-17A (48 h). The supernatants were then collected and analyzed by ELISAs, which were performed using specific combinations of paired antibodies (capture and detection antibodies).

Immunoblot analysis

Cells were harvested and lysed in RIPA lysis buffer containing 1% Triton X-100 and a protease inhibitor cocktail (Sigma-Aldrich), and the total protein concentrations were measured with a Bradford Protein Assay Kit (Bio-Rad). Proteins in the samples of the lysates with the same amounts of total protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting. Primary antibodies against phospho-Erk1 (pT202)/Erk2 (pT185) (E337, Epitomics, cat.
No. 1481-1), Erk1 (EP4967, Epitomics, cat. No. 3739-1), Erk2 (E460, Epitomics, cat. No. 1586-1), NF-κB
p65 (E379, Abcam, cat. No. ab32536), phospho-NF-κB p65 (pS536) (EP2294Y, Abcam, cat. No. ab76302),
IκBα (E130, Abcam, cat. No. ab32518), phospho-IκBαpS32 (EPR3148, Abcam, cat. No. ab92700), β-actin
(AC-15, Sigma, cat. No. 5441), or GAPDH (EPR1977Y, Epitomics, cat. No. 2251-1) were applied to detect
the corresponding proteins. Goat anti-galectin-7 antibodies were used as described previously (3).
Horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse, rabbit, or goat IgG were
then incubated with the membranes, and the proteins were visualized by chemiluminescence according
to the manufacturer’s instructions (EMD Millipore, Advanta). Protein quantification was performed
using the Soft MaX Pro 6.2.1 software.

**Quantitative RT-PCR (RT-qPCR)**

Total RNAs (including mRNA and miRNA) were extracted from cultured cells or mouse ears using the
TRizol Reagent (Invitrogen). For measurement of human galectin-7, IL-6, IL-8, IL-17A, and IFN-γ mRNA
levels, mRNAs were reverse-transcribed into cDNA by means of the iScript cDNA Synthesis Kit (Bio-Rad),
and real-time PCR was carried out, using specific Universal Probe Library probes (Roche) accompanied
with primers targeting these gene products, according to the manufacturer’s instructions.

For miR-146a quantification, total RNA samples were converted to cDNA, and real-time PCR was
conducted with specific primers targeting miR-146a using the Mir-X miRNA First-Strand Synthesis and
SYBR qRT-PCR Kit (Clontech). Human GAPDH and U6 snRNA were selected as internal controls for
normalization of the levels of mRNAs and miRNAs, respectively. Relative levels of mRNAs and miRNAs were calculated, and fold changes were obtained using the ΔΔCt method and compared with vector-only control cells.

IL-23–induced mouse model of psoriasis

The IL-23–induced mouse psoriasis-like model was established as previously described (17). Intradermal injection of recombinant mouse IL-23 (1 μg; eBioscience, cat. No. 34-8231-85) (or PBS as control) into the right ear of anesthetized mice was performed using an insulin syringe every other day for 14 days. Ear thickness was measured before the intradermal injection and measurements were taken at the center of the ears using a pocket thickness gauge (Mitutoyo Corp.) Mice were euthanized on day 15; the ears were collected, and a half of the ears were embedded in paraffin for H&E, immunohistochemical, and in situ hybridization staining, while RNAs from the other half of the ears were extracted for analysis of the cytokine and miRNA profiles by RT-qPCR.

To study the effect of statins in this mouse psoriasis model, 30 mg/kg/day fluvastatin (Cayman, #10010337) and pravastatin (Cayman, #10010343) were administrated by oral gavage to the mice once daily for 14 days. Saline was used as a control. The experimental groups were designed and arranged as follows: PBS (n=2), IL-23 (n=3), PBS + Saline (n=5), IL-23 + Saline (n=5), PBS + fluvastatin (n=3), IL-23 + fluvastatin (n=4), PBS + pravastatin (n=4), IL-23 + pravastatin (n=4). Ear thickness was measured as described above. On day 15, the mice were euthanized, their ears were collected, and processed as
Histology and immunohistochemistry

Sections of paraffin-embedded mouse ears were prepared and stained with H&E by the Pathology Core Laboratory (IBMS, Academia Sinica). For immunohistochemical staining, 5-μm-thick sections of paraformaldehyde-fixed mouse ears or human skin were deparaffinized and hydrated with distilled water. Heat-induced epitope retrieval was performed by incubation in citrate buffer (Thermo) at 98°C for 10 min, and endogenous peroxidase was quenched by treatment with 3% H2O2 in PBS for 5 min. Next, 2.5% horse serum (Vector Lab) was applied for 1 h to block nonspecific binding on the tissue sections. Galectin-7 in both mouse and human skin was detected by incubation with a goat anti-galectin-7 antibody (3) (primary antibody) for 1 h. After washing with PBS, the histological slides were incubated with a polymer-HRP-conjugated horse anti-goat IgG antibody (Vector Lab) (secondary antibody) for 30 min. The staining reaction for galectin-7 was visualized by means of the ImmPACT™ DAB Peroxidase Substrate Kit (Vector Lab), and positive signals of 3,3′-diaminobenzidine (DAB) chromogen were developed as red-brown precipitates. The slides were counterstained with hematoxylin (Vector Lab) to detect nuclei. Quantification of immunohistochemical staining was performed by computer-assisted methods. The 100× magnified visual fields of the tissue sections (total area of 1.5 mm²) were examined. The epidermal region of each visual field was selected, and the intensity of galectin-7 staining from each pixel in the epidermis region was quantified using the ImageJ.
software (NIH, Bethesda, MD, USA). To calculate the mean intensity of galectin-7 staining in each skin section, the intensity of galectin-7 staining was divided by the size of the epidermal area observed.

In situ hybridization of miRNA

LNA-modified, digoxigenin (DIG)-labeled DNA probes complementary to human miR-146a (Exiqon) were designed for in situ hybridization of mouse and human skin sections according to the manufacturer’s instructions. A scrambled miRNA probe was a negative control, and the probe complementary to U6 snRNA served as a positive control. All the sections were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions. For reagent penetration, the sections were subjected to proteinase K digestion (5–10 μg/ml) for 5 min at 37°C, followed by treatment with 4% paraformaldehyde in PBS for 15 min, and with a prehybridization solution (Biochain) at 50°C for 3–4 h. The tissue samples were hybridized with specific probes overnight at 53°C. After the sections were washed with an SSC buffer (Biochain) dilution series, immunological detection with an alkaline phosphatase-conjugated anti-DIG antibody (Biochain) was carried out. Detection of signals was based on a reaction with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate (Biochain), followed by counterstaining of nuclei with nuclear fast red (Merck Millipore).

Generation of EGFP-galectin-7 transgenic mice

To generate mice expressing EGFP fused to the N-terminus of galectin-7, we designed a sgRNA targeted to the start codon of the galectin-7 gene, and used an EGFP-galectin-7 sequence plasmid (pcDNA3.1
with a 500 bp upstream and downstream galectin-7 sequence near the start codon and a linker GGGSGG

added to the C-terminus of the EGFP gene) as a homologous recombination repair template. The galectin-7 gene locus of CL57BL6 is located on chromosome 7: 28,863,853-28,866,284. The sgRNA target sequence of ENSMUSG00000053522 is GCCATGTCTGTAAGTATACT. The founder mice (F0), expressing a high green fluorescence signal on the whole-body surface, were selected and genotyped by PCR. The F1 of the founders were then crossed to acquire homo-transgenic mice (F2). Both male and female mice of 6 to 8 weeks old were used in the experiments.

**Two photon microscopy**

To visualize fluorescence signals from the deep tissue of the ear in live mice, we used an FVMPE-RS multiphoton laser cannning microscope (Olympus), equipped with Chameleon Vision II laser (Coherent). We used the excitation wavelength of 850 nm and green filter (495–540 nm) for GFP, and a red filter (575–645 nm) for red fluorescence signal. The 25× objective (XLPLN25xWMP2) was used for this experiment. For live animal imaging experiment, we anesthetized mice with isoflurane anesthesia and located the mice on a stage heated to 37 °C. The image data were further analyzed by using Image J software.

**Statistics**

All quantitative data are presented as mean ± SEM, SE, or SD unless indicated otherwise. We performed 2-tailed unpaired or paired Student’s t test. Either 1-way ANOVA or 2-way ANOVA with Tukey’s multiple
comparisons test was performed as described in figure legend, to compare the groups of samples, and
differences with P values less than 0.05 were considered statistically significant. For in vivo studies, the
statistical analyses were performed according to the description in the figure legends.

Study approval

The analysis of human tissue samples was approved by the Institutional Review Board of Academia
Sinica, Taiwan (AS-IRB01-16076 (N)). All animal experimental procedures were complied with the
guidelines approved by the Animal Care and Use Committee of the Institute of Biomedical Sciences
(IACUC Protocol ID: 10-11-090, Academia Sinica, Taipei, Taiwan).

Author contributions

FTL, CHL and HLC designed the research studies. HLC, CCH, CHL, MPL, PYH, CSC and DYC conducted
experiments, acquired, and analyzed data. DKH, YHL and PC provided valuable suggestions on the
projects and manuscript. YHL, YPH and TNL provided crucial reagents and materials. FTL, HLC, DKH and
CHL wrote the manuscript.

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Figure 1. The levels of galectin-7 are low in the epidermal keratinocytes of patients with psoriasis. (A) Representative Immunohistochemical (IHC) staining pictures of galectin-7 in skin sections from a healthy control and a psoriatic lesion. Scale bar: 100 μm. (B) Quantitation of IHC staining of galectin-7 in sections of normal skin from healthy controls (n = 75) and sections of lesional skin from patients with psoriasis (n = 27). Quantification of staining was performed by computer-assisted methods as described in the Methods. The y-axis indicates arbitrary numbers representing mean intensity across an area of the epidermis (InteDen/Area). (C) IHC staining of galectin-7 in lesional skin and non-lesional skin pair (D) Quantification of galectin-7 staining of paired lesional and non-lesional skin from the same patients (n=10 pairs). (E) IHC staining of galectin-7 in sections from intradermally IL-23–injected and PBS-injected mouse skin. Scale bar: 100 μm. (F) Quantification of galectin-7 staining of PBS–injected (n = 6) and IL-23–injected mouse skin (n = 13). (G) Immunoblot analysis of galectin-7 levels in HaCaT and HEKn cells stimulated with the indicated cytokines (e.g., IFN-γ, LPS, TNF-α, IL-23, or IL-17A). The concentrations of IFN-γ and LPS used were 100 ng/ml and 50 μg/ml, respectively. The concentrations of TNF-α, IL-23, and IL-17A used were as indicated in the figure. Cells were treated with the cytokines for two days, and cell lysates were prepared for immunoblot analysis. GAPDH served as a loading control. The intensity of the galectin-7 band was quantified by densitometry and normalized to GAPDH and control samples. The number below each band represents the relative galectin-7 amount. Statistical analyses were performed by unpaired (B and F) and paired (D) Student’s t test.*P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 2. Downregulation of galectin-7 in keratinocytes causes an elevated production of the pro-inflammatory cytokines IL-6 and IL-8 in response to IL-17A stimulation.

(A, B) Galectin-7 knockdown HaCaT cell lines (sh-1, sh-2, sh-3, and sh-4) and controls were incubated with or without IL-17A for two days, and IL-6 and IL-8 concentrations in the supernatants were measured by ELISA. All the experiments included three biological replicates. (C, D) HEKn cells were transfected with siRNA to knock down galectin-7 and then incubated with or without IL-17A for two days. The IL-6 and IL-8 concentrations in the supernatants were measured by ELISA. Three independent biological replicates were performed for the ELISA analysis. The results were presented as mean ± SD. For statistical analysis, 2-way ANOVA with Tukey’s multiple comparisons test were performed. Each shRNA- or siRNA-treated cell line was compared with its corresponding control (V or si-NC) for both untreated and IL-17–treated groups. The shRNAs and siRNAs for the knockdown of galectin-7 expression are as described in the Methods. ns: not significant, *P < 0.05, **P < 0.01.
MicroRNA-146a is upregulated in the epidermis of psoriatic lesions; this miRNA promotes production of the pro-inflammatory cytokines IL-6 and IL-8.

(A) MiR-146a in the epidermis of normal and psoriatic skin was detected by RNA in situ hybridization assay (ISH). Scale bar: 100 μm. (B) The miR-146a level in HaCaT cells treated with IL-17A was quantified by real-time PCR. (C, D) An miR-146a-overexpressing vector (pmiR-146a) and a scrambled control hairpin in pCDH-CMV-MCS-EF1-copGFP (pmiR) from SBI (System Biosciences) were used to generate stable miR-146a-overexpressing and control cell clones, respectively. The secretion of cytokines (IL-6 and IL-8) by HaCaT cells stably transfected with pmiR or pmiR-146a vectors was measured two days after stimulation with 25 or 100 ng/ml IL-17A. Three independent biological replicates were performed for the real-time PCR analysis. All results were presented as mean ± SD. For statistical analysis, unpaired Student’s t test (B) or 2-way ANOVA with Tukey’s multiple comparisons test (C and D) were performed. ns: not significant, **P < 0.01, ***P<0.001.
Figure 4. Reduced galectin-7 expression and miR-146a overexpression promote ERK1 and ERK2 signaling pathways triggered by IL-17A.

(A) Galectin-7 knockdown HaCaT cells and control cells were treated with IL-17A for 5 min, and cell lysates were analyzed by immunoblotting. Total ERK1, ERK2, NF-κB, and IκBα and their phosphorylated forms were detected with the corresponding antibodies. (B) HaCaT cells stably transfected with pmiR (control vector) or pmiR-146a were treated with IL-17A for 5 min. Immunoblotting was performed as described in (A). Protein quantification data on phospho-ERK1 (pERK1) and phospho-ERK2 (pERK2) were normalized to the control group in (A) and miR group in (B). Data on total protein levels and levels of phosphorylated NF-κB and IκBα and the total protein levels of ERK1 and ERK2 were normalized to the control (0 min).
Figure 5. Galectin-7–deficient mice exhibit hyperproliferative keratinocytes and increased leukocyte infiltration at the intradermally IL-23–injected sites, as compared with their littermate controls.

(A) Hematoxylin and eosin (H&E) staining of ear sections from WT or galectin-7–deficient (knockout; KO) mice injected intradermally with PBS or IL-23 every other day for 15 days. Scale bar: 50 μm. (B) Ear thickness of WT and galectin-7–deficient (KO) mice was measured before each intradermal injection and measurements were taken at the center of the ears (WT/PBS, n=5; WT/IL-23, n=18, KO/PBS, n=5, KO/IL-23, n=19). For statistical analysis, ear thickness of KO-IL-23 was compared with that in the corresponding WT-IL-23 group at each time point. (C) Epidermal thicknesses of WT and KO mice from H&E-stained sections as described in (A) obtained on day 15 from the same mice as described in (B). For each tissue section, three measurements were taken. (D) Leukocytes were counted in 400× magnified visual fields of tissue sections from IL-23–injected mice using the cell counting module in ImageJ software (WT, n=7; KO, n=5). All results were presented as mean ± SD. For statistical analysis, 2-way ANOVA with Tukey’s multiple comparisons test (B and C) or unpaired Student’s t test (D) were performed. ns: not significant, **P < 0.01, ***P < 0.001.
Figure 6. Two-photon deep tissue visualization of EGFP-galectin-7 expression in transgenic mice. (A) Detection of green fluorescent signal on the whole body surface. (B) Schematic representation of the two-photon image analysis of deep-tissue in the ear. (C) Three-dimensional images of the view of X-Y axis and Z-axis planes. (D) Immunoblot analysis of galectin-7-GFP expression from tissues of EGFP-galectin-7 transgenic mouse. (E) Flow cytometry analysis of bone marrow cells purified from wild type and EGFP-galectin-7 transgenic mice. (F) Affinity purification of EGFP-galectin-7 fusion protein from the lysates of skin samples from EGFP-galectin-7 transgenic mice, followed by immunoblot analysis.
Figure 7. Fluvastatin increases galectin-7 levels in keratinocytes, suppresses production of pro-inflammatory cytokines, and attenuates IL-23 induced epidermal thickness.

(A, B) Real-time PCR analysis of the mRNA expression of galectin-7 and S100A7 in HaCaT cells treated with methylprednisolone (10.6 μM), tobramycin (8.6 μM), fluvastatin (9.2 μM), pempidine (13 μM), or vehicle control (dimethyl sulfoxide; DMSO) for 24 h. The relative fold changes were calculated using the ΔΔCt method; data from all the samples were normalized to the control sample, and GAPDH served as an endogenous control.

(C, D) Production of IL-6 and IL-8 by HaCaT cells treated for two days with fluvastatin with or without IL-17A (200 ng/ml) was measured by ELISA. For statistical analysis, each group was compared with control group (first bar: without IL-17A, DMSO, and fluvastatin group).

(E) Immunoblotting analysis of galectin-7 in keratinocytes treated with TNF-α or IL-17A with or without fluvastatin or DMSO.

(F) Ear thicknesses of different groups (PBS (n=3), IL-23 (n=3), PBS + vehicle (n=5), IL-23 + vehicle (n=5), PBS + fluvastatin (n=3), IL-23 + fluvastatin (n=5), PBS + pravastatin (n=4), IL-23 + pravastatin (n=5)) of mice subjected to intradermal injections of IL-23 or PBS, as described in Figure 5, and treated with fluvastatin (blue line), pravastatin (green line), or saline. For statistical analysis, 1-way (A and B) or 2-way (C, D and F) ANOVA with Tukey’s multiple comparisons test were used. Three independent biological replicates were performed for real-time PCR and ELISA analyses. The results (adjusted p values) of group pairs are annotated on the graph (IL-23 + fluvastatin group vs. corresponding IL-23 + vehicle group (colored in gray); IL-23 + fluvastatin group vs. corresponding IL-23 + pravastatin group (colored in green)). ns: not significant, *P < 0.05, **P < 0.01, ***P < 0.001.