UNIVERSITY OF CALIFORNIA SAN DIEGO

The Long Non-coding RNA *FLG-AS1* Regulates Keratinocyte Proliferation in Psoriasis

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Allison Delehoy is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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Psoriasis is a skin condition characterized by skin thickening, inflammation, and dysfunction in the epidermal skin barrier. At the molecular level, psoriasis occurs as keratinocytes experience impaired differentiation and excessive proliferation. Long non-coding RNAs (lncRNAs) are found within the non-coding region of the genome, and while they are known to be more cell and tissue-specific than other RNAs, their role in diseases is

not yet fully understood. Using a CRISPR interference (CRISPRi) screen and genome-wide association study (GWAS), lncRNAs involved in keratinocyte proliferation were mapped to psoriasis loci, and our laboratory identified *Filaggrin antisense RNA 1* (*FLG-AS1*) as a top candidate. The single nucleotide polymorphism (SNP) within *FLG-AS1*, rs12130219 (A>G), is associated with an increase the risk of psoriasis, but the specific mechanism is still unclear. It was first determined through an enhancer reporter assay that this SNP does not act through transactivation. Experiments perturbing expression of *FLG-AS1* RNA found that *FLG-AS1* promotes keratinocyte proliferation in both cell culture and organotypic skin culture. We proposed that rs12130219 may be linked to psoriasis through its effect on *FLG-AS1* RNA expression.

INTRODUCTION

About 2% of the human population is afflicted with psoriasis.[1] Psoriasis is characterized by dry, irregularly shaped red papules and plaques with silver scales on the skin, mostly found on extremities.[1] In the affected areas, patients experience skin thickening, inflammation, and dysfunction in the epidermal skin barrier. This is due to keratinocytes undergoing impaired differentiation and excessive proliferation while immune cells infiltrate the epidermis.[1,2]

During the differentiation process, keratinocytes migrate upwards towards the surface of the epidermis while a change in their function and morphology occurs. Differentiated keratinocytes form the cornified envelope of the epidermis so the skin can prevent water loss and provide protection from the environment.[3] Molecularly, keratin intermediate filaments are linked together to form the cytoskeleton of the cornified envelope, which is then completed by the joining of other proteins and lipids to form the insoluble protein-lipid structure.[3] Any disruptions of this process can result in the dysfunction of the epidermal barrier, which is one of the hallmarks of psoriasis.

The hyperproliferation of keratinocytes in psoriasis occurs due to the secretion of antimicrobial peptides by stressed keratinocytes in order to activate immune cells.[2] In turn, these immune cells produce cytokines that activate more keratinocytes, and the keratinocytes produce chemokines that recruit more immune cells.[2] This amplification leads to the excessive proliferation of keratinocytes, resulting in psoriasis patients experiencing inflammation and thickening of the epidermis.

While common biologic treatments of psoriasis target the cytokines and chemokines released from immune cells, other studies have shown that keratinocytes may act as the true trigger of inflammation in psoriasis.[1,4] Psoriasis is also estimated to be heritable at 60% to 90%,

and there have been over 40 susceptibility loci discovered that are associated with psoriasis.[5] Thus, for future therapeutics, targeting genes expressed in keratinocytes may be the key to more effective treatments of the disease.

About 98% of the human genome consists of non-protein coding regions.[6] Long non-coding RNAs (lncRNAs), which are a major component of the non-coding region, are RNAs longer than 200 nucleotides that do not code for protein.[7] They are known to play a role in gene expression, as they have been found to be more cell and tissue specific than other RNAs[7]; however, there is a gap in knowledge regarding the true function of these RNAs within diseases.

Our laboratory identified a lncRNA candidate *Filaggrin antisense RNA 1* (*FLG-AS1*) that is linked to psoriasis loci and is expressed in keratinocytes. The single nucleotide polymorphism (SNP), rs12130219, within exon 1 of this RNA substitutes an adenine for a guanine (A>G). A study has shown that this SNP was found to increase the risk of psoriasis[8], yet how this occurs mechanistically remains unknown.

It is hypothesized that rs12130219 is related to psoriasis through its association with *FLG-AS1*. In the context of psoriasis, we are investigating if *FLG-AS1* is a negative regulator of keratinocyte differentiation and a positive regulator of cell proliferation; if so, a decrease in *FLG-AS1* expression is expected to promote the keratinocyte differentiation process and impede the proliferation process, which would protect against the epidermal dysfunction and thickening that occurs in psoriasis. Through this study, we hope to better understand the role of rs12130219 and lncRNAs within psoriasis. Because the molecular causes of psoriasis are not yet fully understood, this investigation could prove to be helpful by illuminating lncRNAs as potential targets of future therapeutic endeavors.

MATERIALS AND METHODS

Primary Keratinocyte Culture

Epidermal keratinocytes isolated from discarded foreskin of neonatal circumcisions were propagated in EpiLife media with corresponding supplements and antibiotic-antimycotic (Thermo Fisher Scientific) added. Cells were incubated at 37°C and 5% CO_2.

Luciferase Assay

The enhancer-like region within *FLG-AS1* was cloned into the pGL4.24 vector (Promega) (Table 1). InFusion mutagenesis was performed to create the rs12130219 SNP (A>G) (Table 1). pGL4.24 FLG-AS1, pGL4.24 FLG-AS1 SNP, and an empty pGL4.24 vector as a control were transfected into 3×10^4 293T cells using Lipofectamine 3000 and P3000 Reagent (Invitrogen). Cells were incubated for 72 hours after transfection. DualGlo Luciferase Reagent (Promega) was added into the culture media per the manufacturer's instructions, followed by a ten-minute incubation at room temperature. Firefly and *Renilla* luciferase were measured on a SpectraMax iD3 microplate reader (Molecular Devices). Firefly luminescence was normalized to *Renilla* control luminescence.

Table 1: *FLG-AS1* enhancer-like regions cloned into pGL4.24.

Vector	Sequence
pGL4.24 FLG-AS1	TTCTTACTAATAATAGTTTAC**A**CTGTTT TACCGCTTCACTGCTTCACGTAAGTAA AGTAATTATTTCAAAAGGAAATGTTGG TCATTTATAAAATGGTGGTAAAGATGG TACAGCGGATTAATGTGAGGATTAAAT GAGATGATACATGAAAGTGCTTAAAA CAGTACAGGG
pGL4.24 FLG-AS1 SNP	TTCTTACTAATAATAGTTTAC**G**CTGTTT TACCGCTTCACTGCTTCACGTAAGTAA AGTAATTATTTCAAAAGGAAATGTTGG TCATTTATAAAATGGTGGTAAAGATGG TACAGCGGATTAATGTGAGGATTAAAT GAGATGATACATGAAAGTGCTTAAAA CAGTACAGGG

shRNA Design

Four independent non-overlapping short hairpin RNAs (shRNAs) targeting *FLG-AS1* and one scrambled non-targeting control (SCR) shRNA were designed (Table 2).

Table 2: shRNA sequences targeting *FLG-AS1*.

shRNA	Sequence
FLG-AS1 shA (sh1) Fwd	CCGGCTGGTGTGATCCGAATGTAACCTCG AGGTTACATTCGGATCACACCAGTTTTTG
FLG-AS1 shA (sh1) Rev	AATTCAAAAACTGGTGTGATCCGAATGTA ACCTCGAGGTTACATTCGGATCACACCAG
FLG-AS1 shB Fwd	CCGGTTTCCTCTTAGGATTTATTTCCTCG AGGAAATAAATCCTAAGAGGAAATTTTTG
FLG-AS1 shB Rev	AATTCAAAAATTTCCTCTTAGGATTTATTT CCTCGAGGAAATAAATCCTAAGAGGAAA
FLG-AS1 shC Fwd	CCGGCTGCCTAATCTCCTTTCTAAACTCGA GTTTAGAAAGGAGATTAGGCAGTTTTTG
FLG-AS1 shC Rev	AATTCAAAAACTGCCTAATCTCCTTTCTA AACTCGAGTTTAGAAAGGAGATTAGGCAG
FLG-AS1 shD (sh2) Fwd	CCGGCTGGAGACCTCATGAAGAATGCTC GAGCATTCTTCATGAGGTCTCCAGTTTTTG
FLG-AS1 shD (sh2) Rev	AATTCAAAAACTGGAGACCTCATGAAGAA TGCTCGAGCATTCTTCATGAGGTCTCCAG
SCR (non-targeting scrambled control)	CCTAAGGTTAAGTCGCCCTCG

RNAi-mediated Gene Knockdown

The four *FLG-AS1*-targeting shRNAs and one SCR shRNA were cloned into the pLKO.1 puro vector (Addgene #8453). 293T cells were transfected with packaging, transfer, and envelope vectors to produce lentivirus. The supernatants containing the lentivirus were collected after 48 hours and were concentrated using Lenti-X Concentrator (Takara). The lentivirus was then used to infect 5×10^5 primary keratinocytes, using media containing 3 μg/mL polybrene. The infected cells were selected with 1 μg/mL puromycin the following day.

Seeding Keratinocytes for Differentiation

2.5×10^5 of lentivirus-infected keratinocytes were seeded for differentiation, while the remaining undifferentiated "Day 0" keratinocytes were collected in RLT Buffer (Qiagen). Keratinocytes were induced to differentiate by plating to confluence in the presence of 1.2 mM calcium for 4 days. "Day 4" differentiated keratinocytes were then collected in RLT Buffer.

RNA Isolation and RT-qPCR

RNA of "Day 0" and "Day 4" keratinocytes in RLT Buffer were isolated using a RNeasy Plus Mini Kit (Qiagen). The RNA was reverse transcribed into cDNA using iScript Reverse Transcription Supermix (Bio-Rad). To measure the change in gene expression of differentiation markers (*KRT 1, KRT 10, FLG, LOR*) in "Day 4" keratinocytes, RT-qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with primers targeting the differentiation markers (Table 3). To test the knockdown of *FLG-AS1*, RT-qPCR was performed on "Day 0" cDNA using primers targeting *FLG-AS1* (Table 3).

Table 3: qPCR primer sequences targeting keratinocyte differentiation markers and *FLG-AS1*.

qPCR Primer	Sequence
KRT 1 Fwd	GAAGTCTCGAGAAAGGGAGCA
KRT 1 Rev	ATGGGTTCTAGTGGAGGTATCTA
KRT 10 Fwd	GCAAATTGAGAGCCTGACTG
KRT 10 Rev	CAGTGGACACATTTCGAAGG
FLG Fwd	AAAGAGCTGAAGGAACTTCTGG
FLG Rev	AACCATATCTGGGTCATCTGG
LOR Fwd	AGAAGCCATTGAGCTCTCCG
LOR Rev	ACTGGGGTTGGGAGGTAGTT
L32 Fwd (housekeeping gene)	AGGCATTGACAACAGGGTTC
L32 Rev (housekeeping gene)	GTTGCACATCAGCAGCACTT
FLG-AS1 Fwd	ATGTAACGGAGCGGACTTTG
FLG-AS1 Rev	AAGCCAAGCACAACCTCAGT

Cell Proliferation Assay

Cell growth was measured by plating 5,000 lentivirus-infected keratinocytes for each condition and time point (Day 0, 3, 6). At each time point, alamarBlue Cell Viability Reagent (Thermo Fisher Scientific) was added to the respective wells per the manufacturer's instructions, followed by a two-hour incubation at 37ºC. Aliquots of culture media were transferred to a separate plate to measure fluorescence using a SpectraMax iD3 microplate reader. Fluorescent signal at "Day 0" was set to 1 in order to measure the fluorescence at "Day 3" and "Day 6" relative to "Day 0."

Organotypic Skin Culture

5×10^5 of lentivirus-infected keratinocytes were seeded onto the basement membrane of air-dried 1.5 × 1.5 cm sections of devitalized human dermis. The cells were propagated for 8 days in KGM media, which was replaced every other day to induce epidermal stratification. Half of the tissue was collected in RLT Buffer for RNA isolation and RT-qPCR to confirm *FLG-AS1* knockdown, while the other half was placed in O.C.T. Compound (Fisher HealthCare) to be sectioned at 7 μm on a cryostat.

Hematoxylin/Eosin (H&E) Staining

Slides of the tissue sections were submerged in 100% methanol for 1 minute, washed with tap water, submerged in Hematoxylin (Richard-Allan Scientific) for 7 minutes, washed with tap water, submerged in Eosin (Richard-Allan Scientific) for 10 seconds, washed with 100% ethanol, and were then air-dried. The slides were mounted using ProLong Gold Antifade Mountant, and the tissues were imaged.

Immunofluorescence (IF) Staining

Slides of the tissue sections were fixed for 10 minutes using 4% paraformaldehyde (for KRT10) or 100% acetone (for Ki67). They were then washed with 0.1% PBS-Tween, followed by applying 0.2% Triton-X to permeabilize. A blocking reagent of PBS-Tween + 2% goat serum was applied for 1 hour at room temperature. Primary antibodies (Table 4) were added in a buffer of PBS + 2% goat serum, and the slides incubated overnight at 4ºC. Slides were washed with 0.1% PBS-T and were incubated with the secondary antibody (Table 4) in PBS + 2% goat serum for 45 minutes at room temperature. Slides were washed with 0.1% PBS-T and stained with 1x Hoechst stain (Invitrogen) for 2 minutes at room temperature. Slides were washed with PBS and Milli-Q water and were air-dried. The slides were mounted using ProLong Gold Antifade Mountant, and the tissues were imaged.

Table 4: Antibodies used for IF staining of organotypic skin.

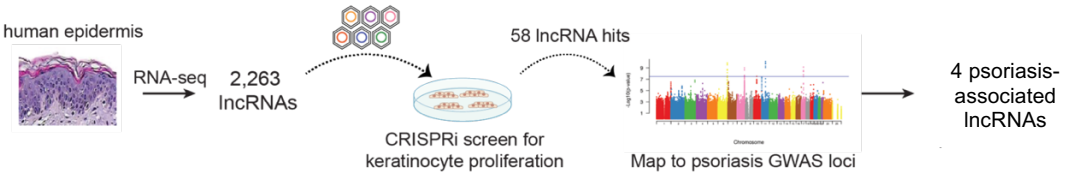
Antibody	Type	Dilution
KRT 10 (rabbit)	Primary (Cell Signaling Technology)	1:1000
Ki67 (rabbit)	Primary (Invitrogen)	1:100
Goat anti-rabbit 555	Secondary (Invitrogen)	1:500

RESULTS

GWAS and CRISPRi screen identifies *FLG-AS1* as a psoriasis-associated lncRNA

2,263 lncRNAs are known to be expressed in the human epidermis (Figure 1A). Based on a genome-wide association study (GWAS) and CRISPR interference (CRISPRi) screen, several psoriasis-associated loci were found to map to lncRNAs[9] (Figure 1A). Four lncRNAs were identified as top candidates for involvement in psoriasis, with one of these being *FLG-AS1* (Figure 1B). It has been previously discovered in microarrays that *FLG-AS1* expression is increased in psoriasis skin compared to control skin[10], as well in lesional psoriasis compared to non-lesional psoriasis[9] (Figure 1C). Therefore, it was decided to proceed with *FLG-AS1* in downstream experiments.

A



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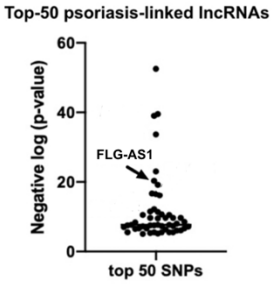


C

Psoriasis Microarray Group Comparison	Average log2FC of *FLG-AS1* Expression
Lesional vs. non-lesional psoriasis	2.405
Control vs. psoriasis skin	0.79

Figure 1: The lncRNA *FLG-AS1* was discovered to be associated with psoriasis. (*A*) RNA sequencing (RNA-seq) was performed on the human epidermis, and 2,263 lncRNAs were found to be expressed. A CRISPRi screen identified 58 of the lncRNAs to be involved in keratinocyte proliferation, a process evident in psoriasis in excess. These lncRNAs were mapped to psoriasis GWAS loci that are known to not be linked to protein-coding genes, and the top 4 lncRNA candidates (including *FLG-AS1*) were chosen. (*B*) The top 50 psoriasis-linked lncRNAs are graphed by their corresponding negative p-value that measures their association to psoriasis. Each dot represents one susceptibility locus. *(C)* Average log fold change of *FLG-AS1* expression was determined from microarrays performed on keratinocytes derived from the skin of psoriasis and control patients (n = 2).

FLG-AS1 in human keratinocytes

A SNP in *FLG-AS1*, rs12130219, has been found to be associated with an increased risk of psoriasis[11], but the specific mechanism still remains unclear. Seen in a map of the gene locus, the SNP lies within an enhancer-like region (Figure 2A). This begs the question if the rs12130219 SNP plays a role within psoriasis by differential enhancer activity.

FLG-AS1 is transcribed alongside and acts as an antisense RNA to *Filaggrin* (*FLG*), a known marker of keratinocyte differentiation (Figure 2B). Antisense lncRNAs are known to control neighboring gene regulation by acting as positive and negative modulators of protein-coding genes.[12] An antisense relationship to *FLG* may indicate the involvement of *FLG-AS1* in the keratinocyte differentiation process.

We hypothesized two possible mechanisms for how rs12130219 acts within psoriasis — either by altering a regulatory DNA element, such as an enhancer, or through affecting RNA transcript levels or activity. Because rs12130219 is found within an enhancer-like region, the SNP could alter the expression of a gene related to the pathogenesis of psoriasis. Additionally, since *FLG-AS1* is upregulated in lesional psoriasis (Figure 1C), this points towards the RNA possibly playing a role within the cellular processes of psoriasis: differentiation and proliferation. Therefore, the SNP could change the activity of *FLG-AS1* transcripts, which could in turn dysregulate keratinocyte differentiation and proliferation. It was decided to first explore if rs12130219 affects enhancer activity.

The SNP rs12130219 has no significant effect on the enhancer-like element in *FLG-AS1*

A pGL4.24 vector containing the enhancer sequence with the rs12130219 SNP (G) or reference allele (A) was transfected into 293T cells. A DualGlo Luciferase Assay was performed by measuring Firefly and *Renilla* luminescence. A luciferase assay utilizes Firefly and *Renilla*

luciferases as co-reporters to quantify gene expression.[13] *Renilla* acts as transfection control and is expressed constitutively from a separate vector.[14] Therefore, a ratio of Firefly to *Renilla* luminescence experimentally normalizes the signal.

It was found that there was no significant difference in luciferase luminescence between the cells transfected with the vector containing the *FLG-AS1* enhancer-like sequence with the reference allele and with the SNP (Figure 2C). This shows that rs12130219 has no major effect on the activity of the enhancer element in *FLG-AS1*. In fact, the empty pGL4.24 vector acting as a control had substantially more luciferase signal than both vectors containing the *FLG-AS1* enhancer-like sequences. This reveals that what was labeled as an enhancer element could possibly act as a repressor. Regardless, we observed that the rs12130219 SNP did not affect the transactivation activity of this DNA element.

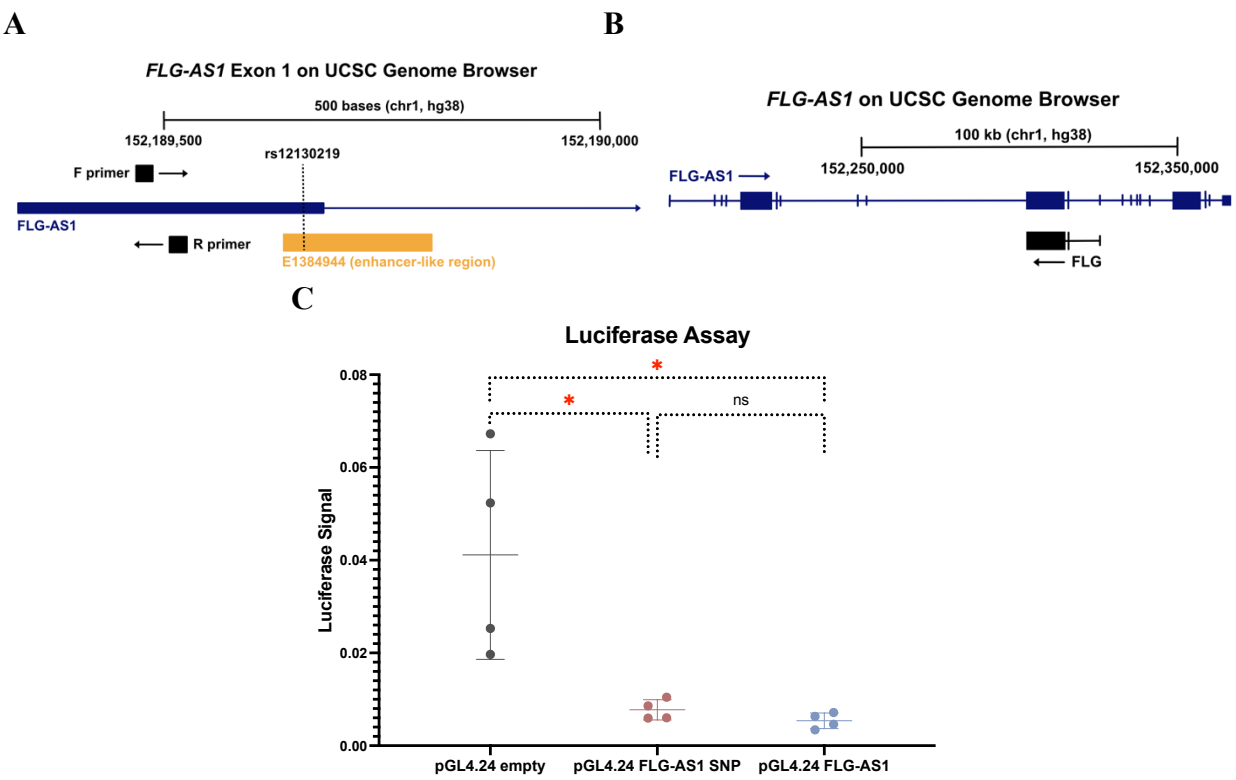


Figure 2: *FLG-AS1* locus and luciferase assay in human keratinocytes. *(A)* Schematic of exon 1 in *FLG-AS1* and the rs12130219 SNP within the enhancer-like region. The location of qPCR forward (F) and reverse (R) primers are mapped, used to measure *FLG-AS1* expression (sourced from the UCSC Genome Browser). *(B)* Schematic of *FLG-AS1* and *FLG* loci. *(C)* Luciferase assay performed in 293T cells using recombinant pGL4.24 vectors containing *FLG-AS1* enhancer-like region with and without the rs12130219 SNP, alongside an empty pGL4.24 vector as a control. Error bars, mean with SD; n = 4. Comparisons were evaluated by Student's t-test, and astericks indicate statistical significance ($p < 0.05$).

FLG-AS1 knockdown by shRNAs

To investigate if *FLG-AS1* acts through RNA transcript levels or activity to affect the pathogenesis of psoriasis, a knockdown was performed using shRNAs to deplete *FLG-AS1* expression within keratinocytes. shRNAs are designed to suppress the mRNA levels of a targeted gene in mammalian cells through its hairpin-like double-stranded structure.[15] These homologous strands of sequence are cleaved into small interfering RNAs (siRNAs), in which one strand localizes to and degrades target RNA transcripts.[15] The four shRNAs (A-D) designed to target non-overlapping sequences within *FLG-AS1* were infected into keratinocytes via lentivirus, and

the expression of *FLG-AS1* was measured by RT-qPCR. With the expression of *FLG-AS1* after knockdown normalized to the non-targeting control SCR, FLG-AS1 shA and FLG-AS1 shD, now designated FLG-AS1 sh1 and FLG-AS1 sh2 (Figure 3A), resulted in the greatest knockdowns of over 50% *FLG-AS1* depletion (Figure 3B) and were used for downstream experiments.

***FLG-AS1* knockdown may inhibit keratinocyte differentiation**

We examined the effect of *FLG-AS1* knockdown on keratinocyte differentiation, a key process in psoriasis. Additional lentivirus containing FLG-AS1 sh1 and sh2 were produced, and the degree of *FLG-AS1* knockdown was measured again in undifferentiated "Day 0" keratinocytes to confirm knockdown prior to further analysis. FLG-AS1 sh1 resulted in about 52% and FLG-AS1 sh2 resulted in about 64% FLG-AS1 depletion (Figure 3C).

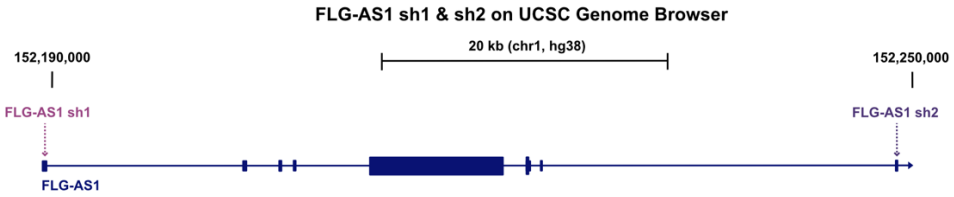
By performing RT-qPCR on RNA from "Day 4" differentiated keratinocytes, the expression of differentiation markers (*KRT 1, KRT 10, FLG, LOR*) was measured in cells with *FLG-AS1* knockdown and was normalized to the non-targeting SCR control. By multiple t-tests, all markers were found to be significantly downregulated following *FLG-AS1* knockdown by FLG-AS1 sh1 ($p < 0.01$) (Figure 3D). Yet, *FLG-AS1* knockdown by FLG-AS1 sh2 did not result in any significant changes in expression of the differentiation marker expression (Figure 3D). A possible explanation for this is an off-target effect due to the independent targeting locations of the two shRNAs (Figure 3A). Because of the different results from the shRNAs, we cannot confidently conclude if there is a definite effect of *FLG-AS1* on keratinocyte differentiation. However, the trend does loosely demonstrate that *FLG-AS1* knockdown may impede the keratinocyte differentiation process.

FLG-AS1 expression is required for keratinocyte proliferation

The cell proliferative rate, another hallmark of psoriasis, was then investigated using a proliferation assay on keratinocytes infected with FLG-AS1 sh1, FLG-AS1 sh2, and SCR lentivirus. alamarBlue was added to the culture media for each condition at Day 0, Day 3, and Day 6 timepoints, and fluorescence was read in order to quantify cell growth. alamarBlue measures actively dividing cells by acting as a substrate that can be reduced to a fluorescent product by cellular metabolism.[16] The fluorescent signal can then be measured, which is proportional to the number of viable cells.[16]

With "Day 0" set to 1 in order to normalize proliferation to the initial day of growth, keratinocytes with *FLG-AS1* knockdown displayed inhibited proliferation when compared to the non-targeting SCR control (Figure 3E). In the FLG-AS1 sh1 and FLG-AS1 sh2 conditions, there are more stable levels relative proliferation across Day 0, Day 3, and Day 6 (Figure 3E). In the SCR condition, the keratinocytes grew at an expected rate for the given timepoints (Figure 3E). The difference in relative proliferation at Day 6 between the *FLG-AS1* knockdown conditions and SCR were found to be significant by a two-sided t-test ($p < 0.05$) (Figure 3E).

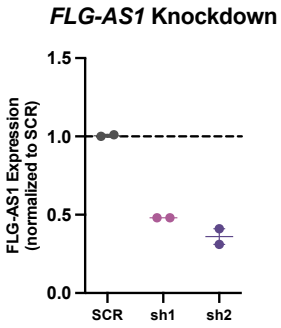
A



B

shRNA	*FLG-AS1* expression after knockdown
FLG-AS1 shA*	0.1591
FLG-AS1 shB	0.8224
FLG-AS1 shC	0.9092
FLG-AS1 shD*	0.4939

C



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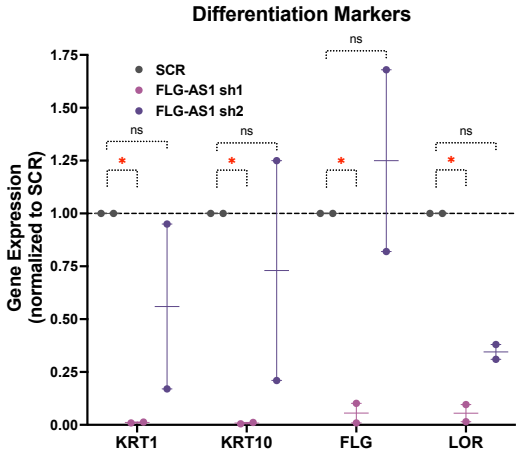

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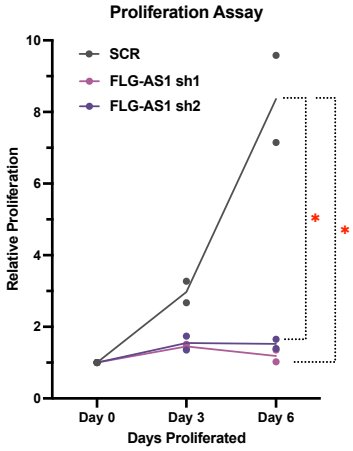

Figure 3: *FLG-AS1* transcript levels affect psoriasis-related cell processes of proliferation and differentiation. *(A)* Map of FLG-AS1 sh1 and sh2 designed to target *FLG-AS1* and induce knockdown. *(B)* Initial test of four shRNAs on inducing *FLG-AS1* knockdown in one independent keratinocyte cell line. *FLG-AS1* expression was normalized to SCR, a non-targeting control. FLG-AS1 shA and shD were designated "FLG-AS1 sh1" and "FLG-AS1 sh2". *(C)* *FLG-AS1* knockdown by FLG-AS1 sh1 and sh2, prior to differentiation. Error bars, mean with SEM; n = 2 independent keratinocyte cell lines. *(D)* Expression of differentiation markers *KRT1*, *KRT10*, *FLG*, and *LOR* in "Day 4" differentiated keratinocytes with *FLG-AS1* knockdown. Expression was measured by RT-qPCR and normalized to SCR, a non-targeting control. Error bars, mean with SEM; n = 2 independent keratinocyte cell lines. Comparisons were evaluated by Student's t-test, and astericks indicate statistical significance ($p < 0.01$). *(E)* Keratinocyte proliferation assay performed on keratinocytes with and without *FLG-AS1* knockdown, measured by fluorescence for quantification. Plotted values represent an increase in keratinocyte proliferation relative to Day 0. Line represents mean of n = 2 independent keratinocyte cell lines at each time point. Comparisons between *FLG-AS1* knockdown and non-targeting control on Day 6 were evaluated by Student's t-test, and astericks indicate statistical significance ($p < 0.05$).

FLG-AS1 is essential for organotypic epidermis formation

With *FLG-AS1* knockdown showing results in affecting the keratinocyte differentiation and proliferation processes, it was decided to model the same results three-dimensionally by the formation of organotypic skin.

Keratinocytes infected by SCR and FLG-AS1 sh1 lentivirus were seeded onto sections of devitalized human dermis and grew for 8 days into a stratified epidermal layer. The tissues were then sectioned and stained using Hematoxylin and Eosin (H&E) staining, allowing for the visualization of the multiple layers of the epidermis; Hematoxylin stains the cell nuclei purple, and Eosin stains the cytoplasm and extracellular matrix pink. Stratification in the epidermis is evident in the tissue seeded with keratinocytes infected with the non-targeting SCR control (Figure 4A). In the tissue with *FLG-AS1* knockdown, stratification is present but is less prominent (Figure 4A).

The tissue sections also underwent immunofluorescence (IF) staining, which resulted in images with KRT10/Ki67 stained red and cell nuclei stained blue (Figure 4A). In the SCR tissue, it is seen how cell nuclei become sparser towards the surface of the epidermis due to differentiation, along with an intense signal of the differentiation marker KRT 10 (Figure 4A). More nuclei also exhibited a signal from proliferation marker Ki67, indicating that cell proliferation was taking place (Figure 4A). In the FLG-AS1 sh1 tissue, a similar structure and effect can be seen but with less intense KRT10 signal and less cell nuclei with Ki67 signal, demonstrating a dysregulation in the differentiation and proliferation processes (Figure 4A).

KRT10 was quantitated by measuring the average intensity of the red signal across multiple tissue sections (Figure 4B), and Ki67 was quantitated by the average number of nuclei with visible expression across multiple tissue sections (Figure 4C). Two-tailed t-tests showed

that there is a significant difference in both KRT10 ($p < 0.005$) and Ki67 ($p < 0.05$) signals between the *FLG-AS1* knockdown and SCR conditions (Figure 4B, 4C). Thus, *FLG-AS1* knockdown in organotypic epidermis results in impeded keratinocyte differentiation and proliferation.

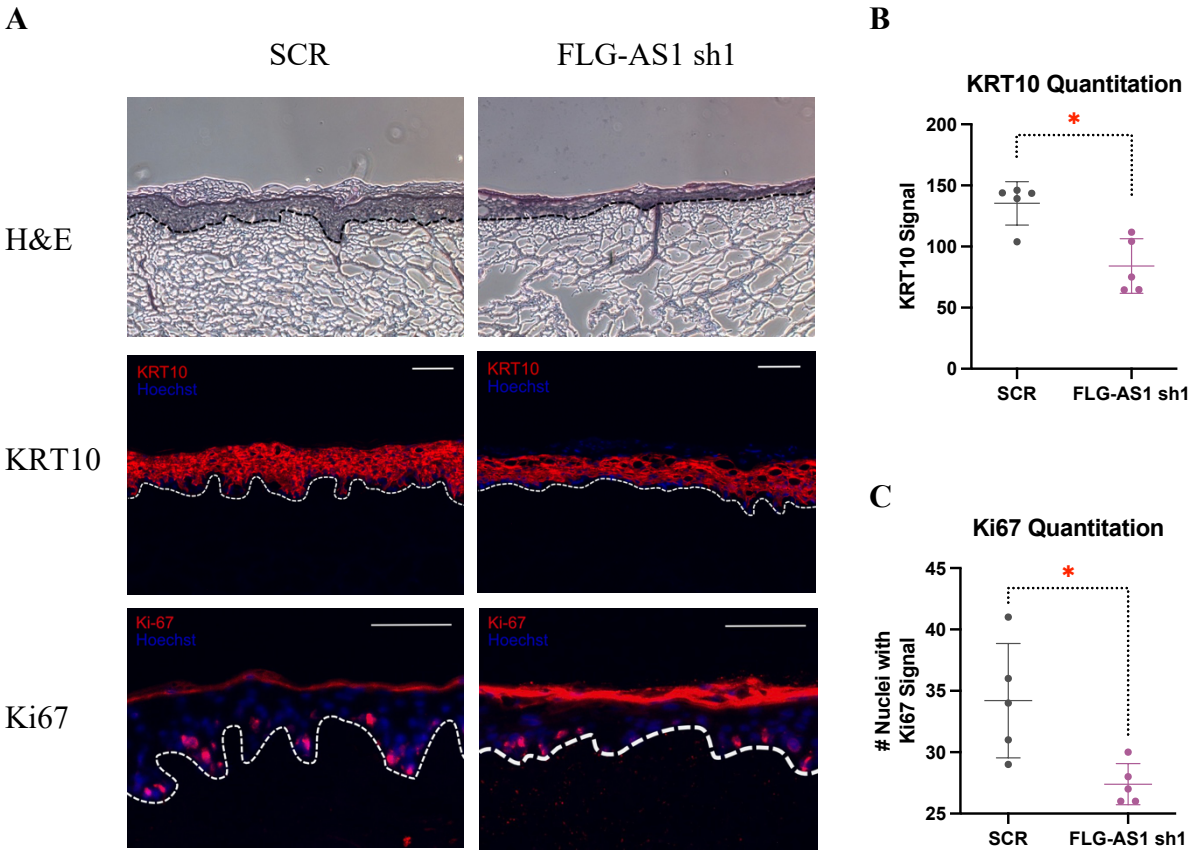

Figure 4: *FLG-AS1* expression is necessary for keratinocyte differentiation and proliferation within organotypic epidermis. *(A)* Hematoxylin and eosin staining (top), immunofluorescence staining of differentiation marker KRT10 (middle), and immunofluorescence staining of proliferation marker Ki67 (bottom) in control organotypic epidermis (SCR) and organotypic epidermis with *FLG-AS1* knockdown (FLG-AS1 sh1). Nuclei are stained in Hoechst (blue). Scale bars, 100 μM. *(B)* KRT10 quantitation measured by signal intensity. Error bars, mean with SD; n = 5 images of each tissue. Comparisons were evaluated by Student's t-test, and astericks indicate statistical significance ($p < 0.005$). *(C)* Ki67 quantitation measured by number of nuclei with Ki67 signal in each tissue. Error bars, mean with SD; n = 5 images of each tissue. Comparisons were evaluated by Student's t-test, and astericks indicate statistical significance ($p < 0.05$).

DISCUSSION

The lncRNA *FLG-AS1* has been identified to be linked to psoriasis, a skin disease characterized by dysfunction and autoinflammation within the epidermal layer of the skin. In this study, it was first found that *FLG-AS1* and the SNP rs12130219 (A>G) do not play a role within psoriasis through transactivation of DNA elements. *FLG-AS1* RNA transcripts were then investigated accordingly, and in line with the psoriasis phenotype, *FLG-AS1* was found to be a positive regulator of keratinocyte proliferation within cell culture and organotypic epidermis formation. This, in turn, suggests a potential mechanism for the involvement of the SNP rs12130219 within the pathogenesis of psoriasis.

In psoriasis, keratinocytes experience impaired differentiation and excessive proliferation.[1] It was found from microarrays performed on the skin of patients that *FLG-AS1* expression is increased in psoriasis and even moreso increased in lesional psoriasis (Figure 1C). In this study, when *FLG-AS1* expression was depleted, there was a significant downregulation of all keratinocyte differentiation gene markers using one shRNA (Figure 3D). Additionally, there was a significant decrease in KRT10 signal in organotypic skin with *FLG-AS1* knockdown (Figure 4B). These results denote a possible impairment in the differentiation process when FLG-AS1 expression is depleted, which contradicts the phenotype of psoriasis.

However, *FLG-AS1* depletion also curbs the growth of keratinocytes, demonstrated when keratinocytes with *FLG-AS1* knockdown exhibited significantly inhibited growth in a cell proliferation assay (Figure 3E). The same findings occurred three-dimenionally in organotypic epidermis formation, where there was significantly less nuclei exhibiting Ki67 expression in tissues with *FLG-AS1* knockdown (Figure 4C). Therefore, a depletion in *FLG-AS1* expression

leads to an impediment of the keratinocyte proliferation process, which aligns with the psoriasis phenotype.

These results raise the possibility that *FLG-AS1* transcripts may be involved in psoriasis through the aspect of proliferation, rather than differentiation. This can be further reinforced by the initial CRISPRi screen and GWAS that linked *FLG-AS1* to both keratinocyte proliferation and psoriasis (Figure 1A). Because of the association of the SNP rs12130219 with *FLG-AS1*, it can then be inferred that rs12130219 may play a role in the pathogenesis of psoriasis through regulating the keratinocyte proliferation process.

As researching the non-coding region of the human genome has been on the rise in recent years, lncRNAs have been categorized into four functions: epigenetic regulators, transcription factor regulators, post-transcription regulators, and protein scaffolds.[17] In terms of involvement in cell proliferation, there is evidence that some lncRNAs interact with transcription factors responsible for regulation of the cell cycle.[18] Specifically, lncRNAs have been shown to regulate cyclin-dependent kinases (CDKs), which bind to cyclins during different phases of the cell cycle and enable progression through the process.[17] Through further experimentation, it could be possible that *FLG-AS1* may behave by a similar mechanism in the context of psoriasis; with an increase of *FLG-AS1* expression, the transcripts could promote the expression of CDKs and cyclins or suppress CDK inhibitors, leading to the hyperproliferation of keratinocytes and the appearance of psoriasis lesions.

There are other investigations that have claimed the involvement of *FLG-AS1* in human diseases other than psoriasis, including multiple cancers. *FLG-AS1* was found to be downregulated in oral[19] and esophageal[20] squamous cell carcinomas. The downregulation of *FLG-AS1* is also linked to cervical[21] and gastric[22] cancer. High expression of *FLG-AS1* is

associated with higher overall survival of low-grade gliomas[23,24]. While *FLG-AS1* seems to play a protective role within these cancers, no specific functions have been unveiled, which begs the question of what the true function of *FLG-AS1* may be; however, since cancers are characterized by the hyperproliferation of cells, these studies can nonetheless uphold the possible mechanism of *FLG-AS1* functioning in psoriasis through regulating keratinocyte proliferation.

The potential involvement of *FLG-AS1* in psoriasis could prove to be helpful in the development of therapeutics for the skin disease. In recent times, RNA-based technologies have been widely researched and utilized across the world, with the Sars-CoV-2 mRNA vaccine being the most notable for having shown high efficacy in mitigating its respective virus.[25] This newfound focus on RNA as a target for treatment and prevention could be important in developing new RNA technologies for all types of diseases, including psoriasis. RNAs have also grown in popularity in usage as biomarkers for diseases, specifically in cancers where lncRNAs can be found within biological fluids.[26] As a lncRNA, *FLG-AS1* could serve as a biomarker for psoriasis or as a potential target for therapeutics in the future.

Nevertheless, there remains a possibility that *FLG-AS1* contributes to the pathogenesis of psoriasis due to its involvement in keratinocyte proliferation. Other aspects of *FLG-AS1* regarding psoriasis are planned to be explored, such as the phenotype following *FLG-AS1* overexpression. Since *FLG-AS1* is known to be upregulated in psoriasis patients, the psoriasis model would be better reflected by an overexpression of *FLG-AS1* rather than a knockdown (Figure 1C). Additional follow-up experiments will include assessing the effects on keratinocyte proliferation when *FLG-AS1* is overexpressed in cell culture and in organotypic skin. It is also planned to investigate if *FLG-AS1* expression regulates psoriasis-specific genes, which would associate *FLG-AS1* more directly with the disease. These include small molecules that are

expressed in keratinocytes and are involved in the immune response of psoriasis, such as the chemokine CCL20 that recruits helper T cells[27] and the cytokine IL-23 that activates memory T cells.[28] Ultimately, the *FLG-AS1* SNP rs12130219 will be incorporated into keratinocytes through CRISPR-based genome editing, and previous experiments will be repeated in order to determine if the SNP affects the cellular processes of psoriasis in similar ways. This data will lead to further insight about the role of *FLG-AS1* and its respective SNP in psoriasis, as well as insight about the role of lncRNAs in diseases overall.

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