Molecular Therapy
Nucleic Acids
Brief Report

Multispecies-targeting siRNAs for the modulation of JAK1 in the skin

Qi Tang,[1,2,6] Katherine Y. Gross,[1,6] Hassan H. Fakih,[1,6] Samuel O. Jackson,[1] Mohammad Zain U.I. Abideen,[1] Kathryn R. Monopoli,[1,3] Carine Blanchard,[4] Claire Bouix-Peter,[4] Thibaud Portal,[4] John E. Harris,[2] Anastasia Khvorova,[1,5] and Julia F. Alterman[1]

[1]RNA Therapeutics Institute, University of Massachusetts Chan Medical School, Worcester, MA 01605, USA; [2]Department of Dermatology, University of Massachusetts Chan Medical School, Worcester, MA 01605, USA; [3]Bioinformatics and Computational Biology Program, Worcester Polytechnic Institute, Worcester, MA 01609, USA; [4]Aldena Therapeutics, London E1 6RA, United Kingdom; [5]Program in Molecular Medicine, University of Massachusetts Chan Medical School, Worcester, MA 01605, USA

Identifying therapeutic oligonucleotides that are cross-reactive to experimental animal species can dramatically accelerate the process of preclinical development and clinical translation. Here, we identify fully chemically-modified small interfering RNAs (siRNAs) that are cross-reactive to Janus kinase 1 (JAK1) in humans and a large variety of other species. We validated the identified siRNAs in silencing JAK1 in cell lines and skin tissues of multiple species. JAK1 is one of the four members of the JAK family of tyrosine kinases that mediate the signaling transduction of many inflammatory cytokine pathways. Dysregulation of these pathways is often involved in the pathogenesis of various immune disorders, and modulation of JAK family enzymes is an effective strategy in the clinic. Thus, this work may open up unprecedented opportunities for evaluating the modulation of JAK1 in many animal models of human inflammatory skin diseases. Further chemical engineering of the optimized JAK1 siRNAs may expand the utility of these compounds for treating immune disorders in additional tissues.

INTRODUCTION

Small interfering RNAs (siRNAs) are an emerging class of medicines that can reduce the expression of disease-related genes in a highly specific and sustained manner.[1,2] Following cellular internalization, the siRNA duplex (~20 bp) harnesses a natural search engine, called RNA interference (RNAi).[3] The siRNA guide strand is assembled into the Argonaute 2 protein to form an RNA-induced silencing complex capable of catalytically degrading complementary mRNAs, thus aborting the translation of target proteins.[4] With a straightforward sequence-based design and a well-defined mechanism of action, siRNAs have quickly garnered attention in pharmaceutical development.[5] Since 2018, multiple liver-delivering siRNA drugs have been approved by the US Food and Drug Administration, with many more under clinical validation for treating diseases of extrahepatic tissues.[6–10] More recently, we have shown that RNAi represents a highly programmable approach for targeted modulation of inflammatory signaling in the skin,[11] establishing a path toward siRNA treatments for inflammatory and autoimmune skin diseases.

An essential step toward the clinical development of siRNA therapeutics for skin diseases is to evaluate the safety and pharmacokinetics/pharmacodynamics (PK/PD) of this drug modality in animal models with tissue characteristics or pathological mechanisms resembling those of human skin. Rodents and nonhuman primates (NHPs) are commonly used models, and pig skin is recognized as the most accurate comparative model of human skin.[12,13] Identifying siRNAs with cross-species activity for testing in human, NHP, rodent, and pig skin would, therefore, accelerate the preclinical development of dermatological siRNA therapeutics. Here, we present strategies that support the identification and validation of siRNA with cross-species activity. We have identified and optimized several Janus kinase 1 (JAK1)–targeting siRNAs that have a broad cross-species targetability. These validated compounds may be used in various experimental and therapeutic applications.

RESULTS

Identification of a human and pig cross-reactive JAK1 siRNA

We have recently reported an siRNA (named si3033) that potently silences the human, NHP, and rodent JAK1 mRNAs.[14] si3033 demonstrates disease-modifying efficacy in a mouse model of vitiligo—a $CD8^+$ cytotoxic T cell–mediated autoimmune skin disease driven by the JAK1-dependent interferon-γ signaling pathway.[15] However, si3033 targets a site of human JAK1 mRNA with limited homology to pig JAK1, precluding efficacy and safety evaluations in pig models. Through bioinformatic analysis, we identified a previously validated human JAK1-targeting siRNA (referred to as si883; the modification pattern shown in Table S1) that the target site is fully conserved in the

https://doi.org/10.1016/j.omtn.2024.102117.

[6]These authors contributed equally

Correspondence: John E. Harris, Department of Dermatology, University of Massachusetts Chan Medical School, Worcester, MA 01605, USA.
E-mail: john.harris@umassmed.edu
Correspondence: Anastasia Khvorova, RNA Therapeutics Institute, University of Massachusetts Chan Medical School, Worcester, MA 01605, USA.
E-mail: anastasia.khvorova@umassmed.edu
Correspondence: Julia F. Alterman, RNA Therapeutics Institute, University of Massachusetts Chan Medical School, Worcester, MA 01605, USA.
E-mail: julia.alterman@umassmed.edu

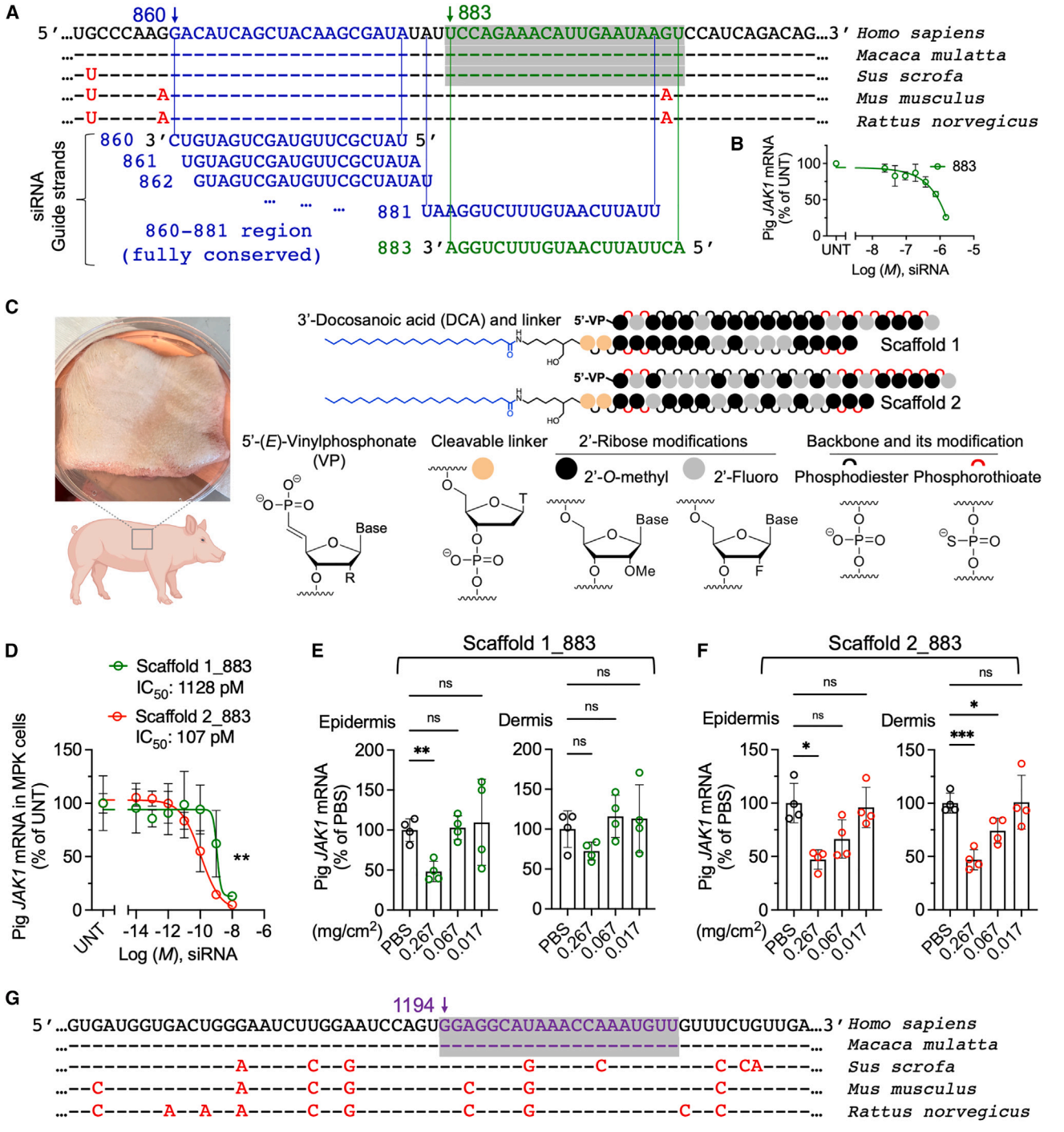

Figure 1. siRNA design for the modulation of JAK1 in multiple species

(A) Cross-species siRNA targeting different JAK1 mRNA transcript sites; shown guide sequence of siRNAs are complementary to target mRNA sequences. Red letters represent mismatched bases aligned to human sequence. (B) *In vitro* efficacy of siRNA 883 (cholesterol-conjugated) in minipig MPK cell line. Cells were treated with siRNA at 1.5 μM (highest dose, 2× serial dilutions were used in dose-response study) for 72 h; mRNA level was measured by QuantiGene 2.0 assay. UNT, untreated control. Data represented as percentage of UNT (n = 3 of independent biological replicates, mean ± SD). (C) Schematic of fully chemically-modified asymmetric siRNA scaffolds for skin delivery. (D) Minipig MPK cells were transfected with 2 variants of DCA-siRNA 883 for 72 h through lipofectamine-mediated uptake, and mRNA levels were measured using

(legend continued on next page)

pig JAK1 mRNA sequence (Figure 1A). We hypothesized that this human compound may also be able to silence pig JAK1 mRNA transcripts. We therefore tested the activity of si883 in a minipig MPK cell line *in vitro* and indeed observed an approximately 70%–80% downregulation of pig JAK1 mRNA (Figure 1B). This finding confirms that cross-species activity of siRNAs relies on target site homology between species and suggests that the strategy we used is a viable approach to validate the cross-species targetability of active human siRNAs.

We next evaluated the potency of si883 in pig skin explants. To optimize the chemistries of si883 for skin delivery, we covalently linked si883 to a hydrophobic moiety (i.e., docosanoic acid), which supports the delivery of siRNAs into all major skin cell types.[11,14,16] 5′-vinylphosphonate, 2′-*O*-methyl, 2′-fluoro, and phosphorothioate linkage modifications were also applied to si883 to improve metabolic stability.[17] Because the chemical modification pattern of siRNA scaffolds may affect their potency in certain sequence contexts,[10,11,14] we chemically engineered si883 in two different siRNA scaffolds (i.e., scaffold 1 and 2) and compared their potency in pig cells *in vitro* and skin explant *ex vivo* (Figure 1C). Scaffold 1 has higher 2′-*O*-methyl content (over 2′-fluoro), whereas scaffold 2 has a balanced 2′-*O*-methyl and 2′-fluoro configuration. In pig cells, scaffold 2 exhibited approximately 11-fold higher potency (half-maximal inhibitory concentration [IC_{50}]: 107 pM versus 1,128 pM) than scaffold 1 (Figure 1D). Scaffold 2 was also more potent than scaffold 1 in the epidermis and dermis of pig skin explant, following intradermal injection in a dose-response study (Figures 1E and 1F). Our findings identify si883 in scaffold 2 as an optimized siRNA candidate for evaluating the safety and PK/PD profile of JAK1 targeting siRNAs in pig models.

Identification of multispecies-targeting siRNAs that efficiently silence JAK1 across various species

Upon further bioinformatic analysis of the human JAK1 mRNA sequence, we discovered that a region upstream of the si883 target site (region 880–881: 22 bases) is evolutionarily fully conserved in a large number of animal species (with NCBI Blast algorithm). This region is not only homologous to pig JAK1 but also to NHP, mouse, and rat JAK1 transcripts (Figure 1A). This finding presented an opportunity to design JAK1-targeting siRNAs with broad cross-species targetability. To identify active siRNAs that target the 860–881 sites of JAK1 mRNA, we synthesized a panel of 22 siRNAs covering this entire region (Table S1). As a control, we used si1194, a previously validated siRNA for silencing human JAK1. si1194 is fully complementary to the site 1194 in human and NHP JAK1 mRNA, but possesses two mismatches to this site in pig, mouse, and rat JAK1 (Figure 1G; Table S1). si1194 should, therefore, efficiently silence human and NHP JAK1, but not pig, mouse, and rat JAK1. As a negative control, we used an siRNA with a chemical construct identical to the JAK1-targeting siRNAs but with a randomized nucleotide sequence, referred to as nontargeting control (NTC) siRNA. Test and control compounds were screened *in vitro* in cell lines of five species (i.e., human HeLa, NHP DBS-FRhL-2, minipig MPK, mouse Neuro-2a, and rat McA-RH7777).

As expected, si1194 showed high efficacy in human and NHP cells, but limited (up to 30% silencing) or no activity in mouse, rat, and pig cells (Figure 2A). This observation agrees with previous reports that high complementarity of siRNA to the target mRNA sequence, particularly in the seed region (i.e., 2^{nd}–7^{th} or 8^{th} nucleotide) of the guide strand, is essential for its silencing efficacy.[18] Our screen identified multiple hits that enabled more than 50% of JAK1 silencing in all tested species compared to untreated cells (Figure 2A). Bioinformatic analysis of the top two hits, si860 and si880 (showed more than 75% silencing efficacy in all five cell lines), validated that both siRNAs have no off-targeting predictions. si860 and si880 also exhibited comparable potency (IC_{50}) as determined by seven-point dose-response studies (Figures 2B and 2C). As si860 showed a slightly better potency in human cells, it was selected as a lead compound and was synthesized in scaffold 2 configurations for evaluation in skin tissues (Figure 1C).

We next validated the efficacy of si860 in the skin tissues of four species: human, pig, mouse, and rat. The compounds were intradermally injected into human and pig skin explants *ex vivo* and into mouse and rat skin *in vivo* (Figure 2D). We observed approximately 60%–70% silencing of JAK1 in the skin of all four tested species (Figure 2E). This result suggests that targeting conserved sites of mRNAs across multiple experimental species is a viable strategy for identifying cross-species reactive compounds for preclinical development. The bioinformatically predicted activity of si860 in other animal species remains to be validated in future studies, and this could be conducted in a relatively straightforward manner, as demonstrated in this work.

DISCUSSION

Oligonucleotide-based therapeutics are rationally-designed informational drugs based on the genetic sequences of disease targets. In this work, we identified and optimized therapeutic siRNAs with cross-species targetability against JAK1 by using RNA bioinformatics and chemical biology approaches. Similarly, the strategies could be applied to the design of other types of oligonucleotide therapeutics, such as antisense oligonucleotides.[19,20]

JAK family enzymes play crucial roles in mediating signaling transduction of a wide range of inflammatory cytokine pathways. JAK

QuantiGene 2.0 assay (n = 3 independent biological replicates, mean ± SD; 2-way ANOVA multiple comparison, **p < 0.01). (E and F) Dose response study (4-fold serial dilutions) of JAK1 silencing in epidermis and dermis. Skin biopsies (8-mm) were injected with siRNAs for 4 days; epidermis and dermis were separated by dispase. mRNA levels were quantified by QuantiGene 2.0 assay (n = 4 independent skin tissue samples, mean ± SD; 1-way ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001; and ns, not significant). (G) Target site of siRNA 1194 on JAK1 mRNA transcripts.

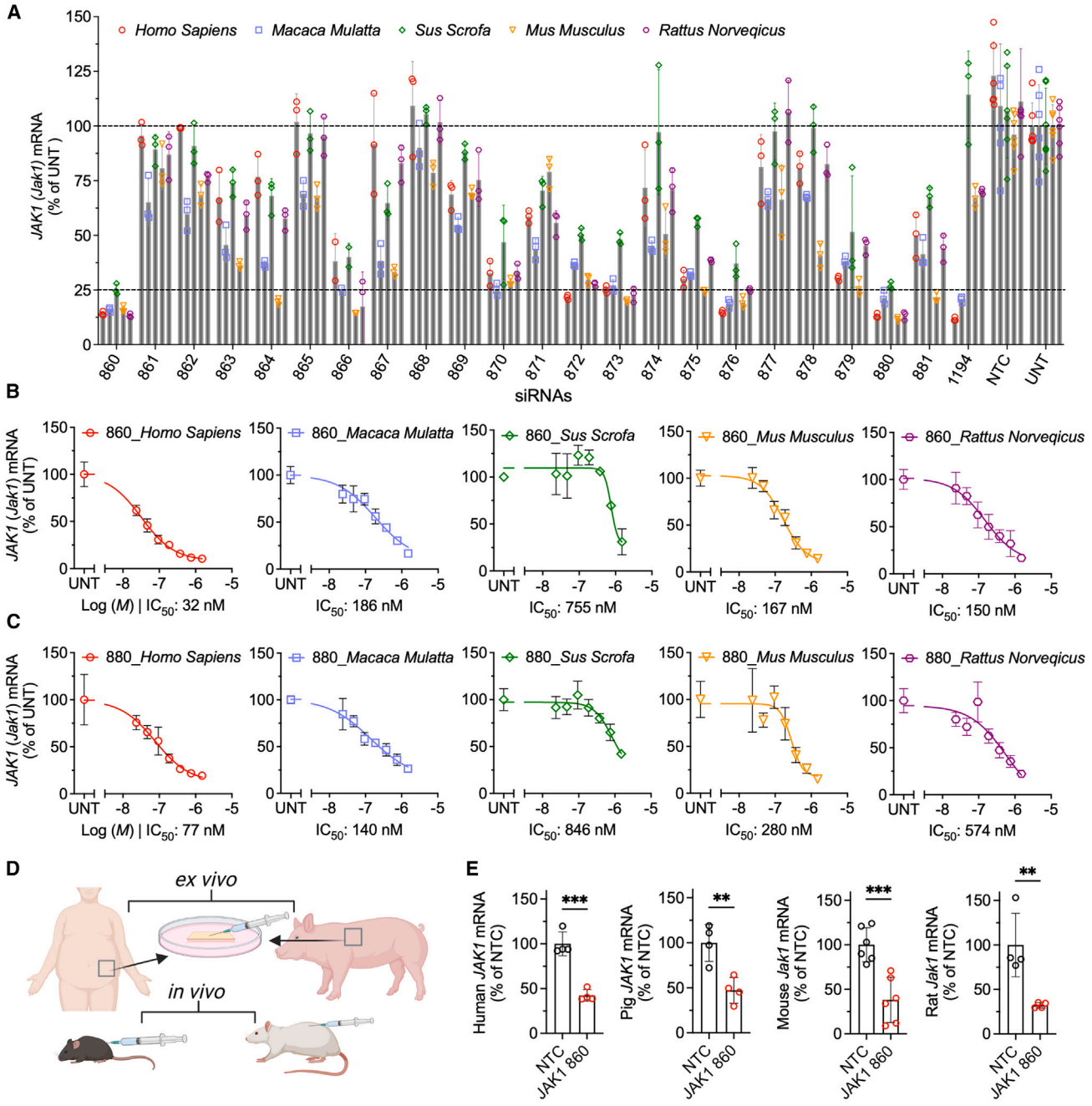

Figure 2. *In vitro* screen and *in vivo* efficacy validation of cross-species targeting JAK1 siRNAs

(A) *In vitro* screen of JAK1 siRNAs (cholesterol-conjugated) in human, NHP, pig, mouse, and rat cell lines. Cells were treated with compounds at 1.5 μM for 72 h; mRNA levels were measured by the QuantiGene 2.0 assay. NTC: siRNA with scrambled sequence that does not target known genes. Data represented as percentage of UNT (n = 3 independent biological replicates, mean ± SD). (B and C) Seven-point dose-response curves of lead siRNA 860 and 880 (cholesterol-conjugated; passive delivery); highest dose: 1.5 μM, 2× serial dilutions. (D and E) Intradermal injection of DCA-siRNA 860 in human and pig skin *ex vivo* (0.13 mg in 8-mm punch biopsy) as well as mouse (0.08 mg/injection) and rat (0.13 mg/injection) skin *in vivo*. JAK1 mRNA levels were quantified 4 days postinjection in human and pig skin and 7 days postinjection in mouse and rat skin by QuantiGene 2.0 assay (n = 4 independent skin tissue samples, mean ± SD; unpaired t test; **$p < 0.01$; ***$p < 0.001$). Alterman and colleagues design and identify multispecies-targeting siRNAs that potentiate the modulation of Janus kinase 1 (JAK1) across a wide range of animal species using a single molecular entity. JAK1 is a master regulator of many inflammatory signaling pathways, and dysregulation of these pathways is involved in various immune disorders.

inhibition has recently emerged as a popular strategy for the treatment of many inflammatory disorders,[21] such as rheumatoid arthritis[22] and autoimmune skin diseases.[23] Because the targeting site of si860 is evolutionarily conserved in a large number of species, the optimized si860 compound could be tested further in animal models of human inflammatory skin disease to evaluate the therapeutic potential of modulating JAK1. Future work can also apply the strategy outlined in this work to modulate additional disease targets in the skin.

The chemical configuration and modification patterns of an siRNA have a profound impact on its activity in silencing the target gene expression. In the present work, we have optimized the scaffold pattern of the multispecies-targeting JAK1 siRNAs. As the pharmacokinetic profile of a siRNA is primarily defined by the conjugate, further chemical engineering of the JAK1 compounds using proper conjugation strategies to deliver the siRNAs to tissues beyond the skin may open up additional opportunities.

MATERIALS AND METHODS

siRNA sequence design

siRNA sequences were designed based on 20-base target sequences of human JAK1 transcript (NCBI accession number: NM_001321852). Sequences with one of the following conditions were excluded: (1) >56% GC content, (2) $\geq$4 single nucleotide stretches, or (3) perfect homology to miRNA seeds at positions 2–7 of the guide sequence. To minimize transcriptomic off-target effects, siRNAs were excluded if positions 2–17 of the guide sequence had full complementarity to nontarget mRNAs. Targeting sequences (along with +10/−15 flanking nucleotides) were scored using a weight matrix,[24] and top-scoring sequences in each species were identified. Cross-species targeting was determined based on perfect homology of the 16-nt homology region within the target sequence (positions 2–17) to the target sequence in the other species. siRNAs were termed based on the position in the target transcript from which they were extracted (e.g., human si883 was extracted from positions 883–902 of NM_001321852); for cross-species targeting sequences, naming was based on the human transcript.

Oligonucleotide synthesis

Compounds for *in vitro* screening were synthesized using standard solid-phase phosphoramidite chemistry on a Dr. Oligo 48 high-throughput RNA synthesizer (Biolytic); compounds for *in vivo* injection were made using a MerMade 12 (BioAutomation) synthesizer. Standard RNA 2′-*O*-methyl and 2′-fluoro modifications were applied for improving siRNA stability (Chemgenes). The sense strands of *in vitro* compounds were synthesized at a 1-μmol scale on a cholesterol-functionalized controlled pore glass (CPG) solid support (Chemgenes), and the sense strands of *in vivo* compound were synthesized at a 5-μmol scale on an in-house synthesized docosanoic acid–functionalized CPG, as previously described.[16,25] Guide strands were synthesized on CPG functionalized with an Unylinker (Chemgenes), bis-cyanoethyl-*N*,*N*-diisopropyl (CED) phosphoramidite (Chemgenes) was used to introduce a 5′-monophosphate for *in vitro* experiments, and custom 5′ (*E*)-vinylphosphonate phosphoramidite (Chemgenes) was applied for *in vivo* studies. Cy3-phosphoramidites (Gene Pharma) were used for fluorescence labeling of the 5′ of sense strands.

Oligonucleotide deprotection

For postsynthesis deprotection, sense strands were cleaved from the CPG and deprotected using 40% aqeous methylamine and 30% NH_4OH (1:1, v/v) at room temperature for 2 h. Guide strands were cleaved and deprotected with 30% NH_4OH containing 3% diethylamine for 20 h at 35°C. The deprotected oligonucleotide solutions were filtered to remove CPG residues. The filtrates were immediately frozen in liquid nitrogen and dried down by a SpeedVac vacuum centrifuge in a chemical hood. The resulting pellets were reconstituted in 5% acetonitrile for subsequent purifications.

HPLC purification

Oligonucleotide purification was carried out on an Agilent 1290 Infinity II system. Sense strands were purified using a reverse-phase preparative column (Hamilton PRP-C18) under the following conditions: buffer A, 50 mM sodium acetate in 5% acetonitrile; buffer B, 100% acetonitrile; the gradient was 0%–20% for 3 min, 20%–70% for 23 min, then cleaned and recalibrated for 9 min; column temperature was 60°C; and the flow rate 40 mL/min. Guide strands were purified using an anion-exchange column (with SOURCETM 15Q) under the following conditions: buffer A, 10 mM sodium acetate in 20% acetonitrile; buffer B, 1 M sodium perchlorate in 20% acetonitrile; the gradient was 0%–20% for 3 min, 20%–70% for 23 min, then cleaned and recalibrated for 9 min; column temperature was 55°C; and the flow rate was 40 mL/min. Oligonucleotides were detected by measuring peaks with UV absorbance at 260 nm. Peak fractions were automatically collected for confirming identities. The oligonucleotide fractions were quality-controlled by liquid chromatography-mass spectrometry (LC-MS), and pure fractions were combined, frozen, and dried down in a Speed Vacuum centrifuge overnight. The dried oligonucleotides were resuspended into water and desalted by size-exclusion chromatography (with Sephadex G-25 Fine).

LC-MS characterization

The identity of all of the oligonucleotides was analyzed on an Agilent 6530 accurate mass Q-TOF LC-MS system using ion-pair reverse-phase chromatography (LC column: Agilent 2.1 × 50 mm AdvanceBio C18 oligonucleotide) under the following conditions: buffer A: 9 mM triethylamine/100 mM hexafluoroisopropanol in water; buffer B: 9 mM triethylamine/100 mM hexafluoroisopropanol in methanol; column temperature, 60°C; and flow rate, 0.5 mL/min. Peaks were detected by measuring UV at 260 nm. MS parameters were ion source, electrospray ionization; ion polarity, negative mode; mass scan range, 100–3,200 *m/z*; scan rate, 2 spectra per second; capillary voltage, 4,000 V; fragmentor voltage, 180 V.

Cell culture

Human HeLa cells (American Type Culture Collection [ATCC]; no. CCL-2), minipig MPK cells (ATCC; no. CLL-166), and rat

McA-RH7777 (ATCC; no. CRL-1601) were maintained in DMEM (Corning Cellgro; no. 10-013CV). NHP DBS-FRhL-2 (ATCC; no. CL-160) and mouse Neuro-2a cells (ATCC; no. CLL-131) were maintained in Eagle's minimal essential medium (ATCC; no. 30–2003). All media were supplemented with 10% fetal bovine serum (FBS; Gibco; no. 26140) with no antibiotics. All of the cells were grown at 37°C with 5% CO_2 supply and were split every 2–4 days and discarded after 15 passages.

In vitro screening

Cells were treated with cholesterol-conjugated siRNAs at 1.5 μM, which also served as the maximal dose for dose-response assays, for 72 h in 3% FBS media that was made from 50/50 volume of 6% FBS media/Opti-MEM media (Gibco; no. 31985–079). Cells were harvested in diluted lysis mixture consisting of a 1:2 ratio of lysis mixture (Invitrogen; no. 13228) to water and 0.2 mg/mL of proteinase K (Invitrogen; no. 25530–049), then lysed at 55°C for 30 min. Target mRNA levels were measured using Quantigene 2.0 assays (Affymetrix). All QuantiGene detection probe sets were ordered from Thermo Fisher: human JAK1 (no. SA-50455), NHP JAK1 (no. SF-4213912), pig JAK1 (no. SF-4295068), mouse Jak1 (no. SB-3029714), and rat Jak1 (no. SC-3062255). JAK1 mRNA expression was normalized to housekeeping genes and the probe sets used were human actin beta (ACTB) (no. SA-10008), NHP ACTB (no. SF-4205083), pig ACTB (no. SF-4295627), mouse Actb (no. SA-10003), and rat Actb (no. SC-16967).

Ex vivo human and pig skin studies

Deidentified surgically discarded fresh abdominal skin was obtained from the University of Massachusetts Chan Biospecimen, Tissue, and Tumor Bank. The collection of these samples from patient donors was approved by the institutional review board at the University of Massachusetts Chan Medical School. All of the participants gave written informed consent before surgical procedures. Fresh flank pig (*Sus scrofa*) skin samples were a gift from Dr. Kevin Donahue's laboratory at the University of Massachusetts Chan Medical School. Subcutaneous fat was fully removed and 8-mm skin biopsies were taken and injected intradermally with siRNAs (200 μM) in a volume of 50 μL. Skin biopsies were cultured in a 24-well plate with 2 mL/punch of Iscove's modified Dulbecco's media (Sigma-Aldrich; no. I3390) containing 10% FBS, 100 U/mL penicillin-streptomycin, and 50 μM 2-mercaptoethanol for 96 h at 37°C. The skin biopsies were treated with siRNAs within 24 h after the excision of specimens. To separate epidermis and dermis of pig skin, biopsies were incubated with 2 mL of 2.5 mg/mL dispase II (Roche; no. 04942078001) at 37°C for 1 h. Epidermis and dermis were manually separated. Both epidermis and dermis were mechanically homogenized in 500 μL of homogenizing solution (Invitrogen; no. QS0517) containing 0.2 mg/mL Proteinase K (Invitrogen; no. AM3546), followed by incubation at 55°C for 30 min. The processed skin homogenate was then centrifuged at 14,000 × *g* for 5 min, and the clear supernatant was collected for subsequent assays. Human and pig JAK1 mRNA expression levels were quantified using the QuantiGene Singleplex assay kit (Invitrogen; no. QS0016) with the following probe sets: human JAK1 (no. SA-50455), human ACTB (no. SA-10008), pig JAK1 (no. SF-4295068), and pig ACTB (no. SF-4295627).

In vivo mouse and rat skin studies

Female wild-type C57BL/6J mice were purchased from The Jackson Laboratory. Mice were 8–12 weeks of age at the time of the experiments. The female Lewis rats were purchased from Charles River Laboratory and were at 8–10 weeks of age at the time of the experiment. Animal studies were performed in accordance with the guidelines of University of Massachusetts Chan Medical School Institutional Animal Care and Use Committee. All of the procedures were approved under the protocol no. 202000010 (Khvorova laboratory) and no. 201900330 (Harris laboratory) and in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. The colonies were maintained and housed at pathogen-free animal facilities at the University of Massachusetta Chan Medical School with 12 h light/12 h dark cycle at controlled temperature (23°C ± 1°C) and humidity (50% ± 20%), with free access to food and water. Mouse and rat skin biopsies (8-mm punch) at the injection site were collected at the indicated time points and stored in RNA later solution (Sigma-Aldrich; no. R0901) at 4°C overnight. Skin biopsies were mechanically homogenized in 500 μL of homogenizing solution (Invitrogen; no. QS0517) containing 0.2 mg/mL Proteinase K (Invitrogen; no. AM3546), followed by incubation at 55°C for 30 min. The processed skin homogenate was then centrifuged at 14,000 × *g* for 5 min, and the clear supernatant was collected for subsequent assays. The mRNA levels of mouse and rat Jak1 expression were quantified using the QuantiGene Singleplex assay kit (Invitrogen; no. QS0016) with the following probe sets: mouse Jak1 (no. SB-3029714), mouse Actb (no. SB-10003), rat Jak1 (no. SC-3062255), and rat Actb (no. SC-16967).

Statistical analysis

Data were analyzed using GraphPad Prism 9 software. Sample size and statistical methods used for analyzing each experiment are detailed in the corresponding figure legends. Differences in all of the comparisons were considered significant at $p < 0.05$.

DATA AND CODE AVAILABILITY

All of the data from this work are included in the article. Requests for additional data or materials should be directed to the corresponding authors Dr. Julia Alterman, Dr. John E. Harris, or Dr. Anastasia Khvorova.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2024.102117.

ACKNOWLEDGMENTS

This project was supported by Aldena Therapeutics (sponsored research to J.F.A.) and the National Institutes of Health (grant nos. R35 GM131839 and R01 NS104022 to A.K.; grant no. R01 AR069114 to J.E.H.; and K99 AR082987 to Q.T.). We thank Dr. Kevin Donahue and Dr. Weilan Mo for supporting the pig skin explant

samples. We thank Dr. Emily Mohn Haberlin for helping revise and proofread this manuscript.

AUTHOR CONTRIBUTIONS

Q.T., C.B., C.B.-P., T.P., J.E.H., A.K., and J.F.A. conceived the project. K.R.M. and Q.T. carried out the computational design of the siRNAs. S.O.J. synthesized the *in vitro* compounds. H.H.F. synthesized the *ex vivo* and *in vivo* compounds. Q.T., K.Y.G., and M.Z.U.I.A. conducted the *in vitro* and *in vivo* studies. Q.T. and K.Y.G. prepared the manuscript draft. J.E.H., A.K., and J.F.A. reviewed and edited the manuscript. All of the authors have proofread the manuscript for publication.

DECLARATION OF INTERESTS

A.K. is a founder of Atalanta Therapeutics and Comanche Biopharma; serves on the Scientific Advisory Board of Aldena Therapeutics, Prime Medicine, Alltrna, and Evox Therapeutics; and owns equities in RXi Pharmaceuticals and Advirna. J.E.H. owns equities in Rheos Medicines, and is a founder of Villaris Therapeutics, Aldena Therapeutics, NIRA Biosciences, Vimela Therapeutics, and Klirna Therapeutics. Q.T., H.H.F., K.Y.G., M.Z.U.I.A., K.R.M., J.E.H., A.K., and J.F.A. are listed as inventors of RNAi technology patents and patent applications. C.B., C.B.-P., and T.P. are executives of Aldena Therapeutics.

REFERENCES

1. Khvorova, A. (2023). siRNAs—A New Class of Medicines. JAMA *329*, 2185–2186.
2. Egli, M., and Manoharan, M. (2023). Chemistry, structure and function of approved oligonucleotide therapeutics. Nucleic Acids Res. *51*, 2529–2573.
3. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806–811.
4. Matranga, C., Tomari, Y., Shin, C., Bartel, D.P., and Zamore, P.D. (2005). Passenger-Strand Cleavage Facilitates Assembly of siRNA into Ago2-Containing RNAi Enzyme Complexes. Cell *123*, 607–620.
5. Garber, K. (2018). Alnylam launches era of RNAi drugs. Nat. Biotechnol. *36*, 777–778.
6. Klein, D., Goldberg, S., Theile, C.S., Dambra, R., Haskell, K., Kuhar, E., Lin, T., Parmar, R., Manoharan, M., Richter, M., et al. (2021). Centyrin ligands for extrahepatic delivery of siRNA. Mol. Ther. *29*, 2053–2066.
7. (2022). Lipophilic siRNA conjugates yield durable silencing in extrahepatic tissues. Nat Biotechnol *40*, 1439–1440.
8. Brown, K.M., Nair, J.K., Janas, M.M., Anglero-Rodriguez, Y.I., Dang, L.T.H., Peng, H., Theile, C.S., Castellanos-Rizaldos, E., Brown, C., Foster, D., et al. (2022). Expanding RNAi therapeutics to extrahepatic tissues with lipophilic conjugates. Nat. Biotechnol. *40*, 1500–1508.
9. Malecova, B., Burke, R.S., Cochran, M., Hood, M.D., Johns, R., Kovach, P.R., Doppalapudi, V.R., Erdogan, G., Arias, J.D., Darimont, B., et al. (2023). Targeted tissue delivery of RNA therapeutics using antibody–oligonucleotide conjugates (AOCs). Nucleic Acids Res. *51*, 5901–5910.
10. Davis, S.M., Hariharan, V.N., Lo, A., Turanov, A.A., Echeverria, D., Sousa, J., McHugh, N., Biscans, A., Alterman, J.F., Karumanchi, S.A., et al. (2022). Chemical optimization of siRNA for safe and efficient silencing of placental sFLT1. Mol. Ther. Nucleic Acids *29*, 135–149.
11. Tang, Q., Sousa, J., Echeverria, D., Fan, X., Hsueh, Y.-C., Afshari, K., MeHugh, N., Cooper, D.A., Vangjeli, L., Monopoli, K., et al. (2022). RNAi-based modulation of IFN-γ signaling in skin. Mol. Ther. *30*, 2709–2721.
12. Abd, E., Yousef, S.A., Pastore, M.N., Telaprolu, K., Mohammed, Y.H., Namjoshi, S., Grice, J.E., and Roberts, M.S. (2016). Skin models for the testing of transdermal drugs. CPAA *8*, 163–176.
13. Dellambra, E., Odorisio, T., D'Arcangelo, D., Failla, C.M., and Facchiano, A. (2019). Non-animal models in dermatological research. ALTEX *36*, 177–202.
14. Tang, Q., Fakih, H.H., Zain Ui Abideen, M., Hildebrand, S.R., Afshari, K., Gross, K.Y., Sousa, J., Maebius, A.S., Bartholdy, C., Søgaard, P.P., et al. (2023). Rational design of a JAK1-selective siRNA inhibitor for the modulation of autoimmunity in the skin. Nat. Commun. *14*, 7099.
15. Harris, J.E., Harris, T.H., Weninger, W., Wherry, E.J., Hunter, C.A., and Turka, L.A. (2012). A mouse model of vitiligo with focused epidermal depigmentation requires IFN-γ for autoreactive $CD8^+$ T-cell accumulation in the skin. J. Invest. Dermatol. *132*, 1869–1876.
16. Biscans, A., Coles, A., Haraszti, R., Echeverria, D., Hassler, M., Osborn, M., and Khvorova, A. (2019). Diverse lipid conjugates for functional extra-hepatic siRNA delivery in vivo. Nucleic Acids Res. *47*, 1082–1096.
17. Khvorova, A., and Watts, J.K. (2017). The chemical evolution of oligonucleotide therapies of clinical utility. Nat. Biotechnol. *35*, 238–248.
18. Birmingham, A., Anderson, E., Sullivan, K., Reynolds, A., Boese, Q., Leake, D., Karpilow, J., and Khvorova, A. (2007). A protocol for designing siRNAs with high functionality and specificity. Nat. Protoc. *2*, 2068–2078.
19. Rinaldi, C., and Wood, M.J.A. (2018). Antisense oligonucleotides: the next frontier for treatment of neurological disorders. Nat. Rev. Neurol. *14*, 9–21.
20. Kulkarni, J.A., Witzigmann, D., Thomson, S.B., Chen, S., Leavitt, B.R., Cullis, P.R., and Van Der Meel, R. (2021). The current landscape of nucleic acid therapeutics. Nat. Nanotechnol. *16*, 630–643.
21. Schwartz, D.M., Kanno, Y., Villarino, A., Ward, M., Gadina, M., and O'Shea, J.J. (2017). JAK inhibition as a therapeutic strategy for immune and inflammatory diseases. Nat. Rev. Drug Discov. *16*, 843–862.
22. Tanaka, Y., Luo, Y., O'Shea, J.J., and Nakayamada, S. (2022). Janus kinase-targeting therapies in rheumatology: a mechanisms-based approach. Nat. Rev. Rheumatol. *18*, 133–145.
23. Damsky, W., and King, B.A. (2017). JAK inhibitors in dermatology: The promise of a new drug class. J. Am. Acad. Dermatol. *76*, 736–744.
24. Shmushkovich, T., Monopoli, K.R., Homsy, D., Leyfer, D., Betancur-Boissel, M., Khvorova, A., and Wolfson, A.D. (2018). Functional features defining the efficacy of cholesterol-conjugated, self-deliverable, chemically modified siRNAs. Nucleic Acids Res. *46*, 10905–10916.
25. Biscans, A., Coles, A., Echeverria, D., and Khvorova, A. (2019). The valency of fatty acid conjugates impacts siRNA pharmacokinetics, distribution, and efficacy in vivo. J. Control. Release *302*, 116–125.