Enhancers of Adeno-associated Virus AAV2 Transduction via High Throughput siRNA Screening

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Intracellular barriers to adeno-associated virus (AAV) transduction may limit gene delivery. We screened a short interfering RNA (siRNA) library targeting 5,520 genes to help identify pathways that modulate AAV transduction of human endothelium. In replicate screening, 50 pools (three siRNAs per gene) resulted in greater than eightfold reporter gene expression enhancement. Single siRNA confirmation tests demonstrated that at least one siRNA from each of the top 10 pools provided greater than twofold enhancement. Several siRNAs when used together resulted in additive effects and two of the most potent siRNA sequences were enhancers in cultured airway epithelium. However, enhanced transduction was not correlated with mRNA knockdown by quantitative real time PCR, indicating an off-target mechanism. In fact, four of the five most potent siRNAs contained a consensus hexamer region 5′-UGUUUC-3′ at positions 2–7 of the antisense strand. The point mutation U4A within this region (but not mutations at positions 1 or 14) disrupted transduction enhancement, indicating a microRNA (miRNA)-like mechanism. Transcription profiling indicated that the hexamer also resulted in perturbation of the interferon pathway via reduced interferon-induced protein 44-like (IFI44L), interferon-inducible myxovirus resistance 1 (MX1), and interferon-induced protein with tetratricopeptide repeats (IFIT5) mRNAs. Direct interferon (α, β, and ω) receptor 2 (IFNAR2) knockdown resulted in greater than twofold transduction enhancement. In addition to providing insight into AAV biology and enhanced transduction, the results demonstrate certain beneficial siRNA off-target effects.

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INTRODUCTION

Adeno-associated virus (AAV) is a promising vector for gene delivery. AAV vectors have several advantages including: low pathogenicity, low immune response, long term episomal expression in nondividing cell types, and specific organ targeting based on the serotype used.[1–4] However, insufficient transgene expression has limited the success of a number of human clinical trials that used AAV vectors.[5–8] Although AAV vectors result in higher transduction than nonviral methods, a major goal is to increase the efficiency of gene transfer. This cannot be simply overcome by continually increasing vector dosages, as higher doses are more likely to elicit an immune response and would present additional challenges to manufacturing capacity, cost of treatment, and/or treatment administration.

The biological pathway for AAV viral entry and gene expression contains numerous steps involving host proteins that control the level of transgene delivery and expression. AAV must bind to heparan sulfate proteoglycan cell surface receptors.[9,10] Following binding, AAV must be endocytosed in the presence of $\alpha_V\beta_5$ integrin and with activation of Rac-1[10]. Following endosome escape, the viral genome must gain entry to the nucleus, where viral DNA synthesis and transcription of the viral genome take place. Although the early steps of binding and endocytosis are well studied, many virus–host interactions remain unknown which may enhance or reduce viral transgene expression. The ability of knocking down individual targets makes short interfering RNA (siRNA) extremely useful for high throughput screening. A number of published studies have used this technique to examine virus/host cell interactions,[11–17] usually in the context of reducing viral infection (as opposed to enhancing transgene expression).

To help identify intracellular barriers to AAV transgene expression, we conducted a high throughput screen using siRNA to knock-down mRNA corresponding to 5,520 Applied Biosystems "druggable genome" targets. Despite optimization of siRNA sequence selection, both sequence-dependent and sequence-independent off-target effects may occur, causing unintended effects.[18–25] For example, an individual siRNA targeting a particular gene can show a different mRNA expression profile from another siRNA that successfully targets the same gene.[18] An additional type of sequence-dependent off-target effect arises from the hexamer seed region, located at positions 2–7 of the siRNA sequence. This region can bind to the 3′UTR of various mRNA species and can lead to a complex pattern of mRNA cleavage and translational silencing,[20,22] thereby functioning similar to microRNA (miRNA). In the present study, off-target effects caused by a common seed region sequence were observed in four of the top five screening hits. Also, mRNA profiling was used to investigate additional off-target effects where a complex phenotype emerged involving downregulation of genes of the interferon pathway.

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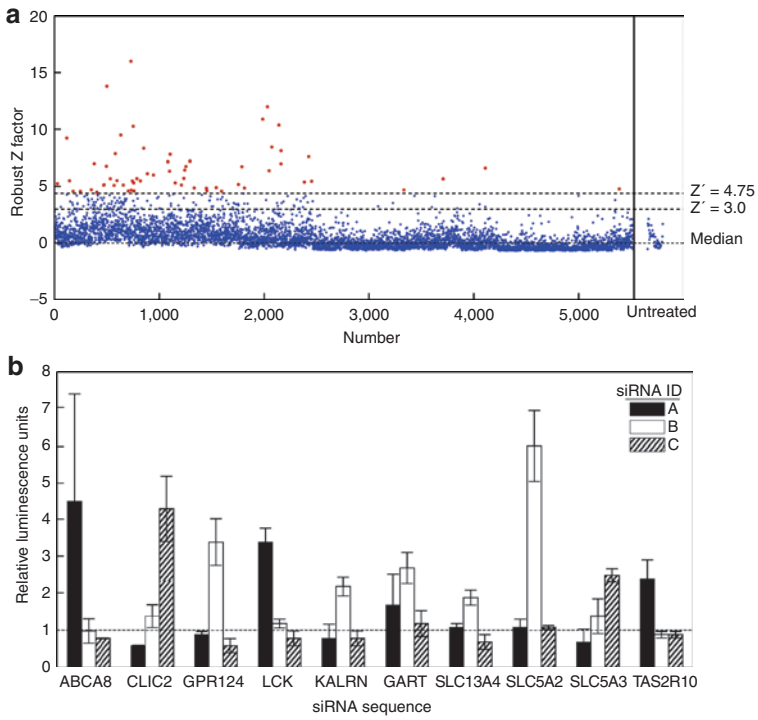

Figure 1 Primary and secondary screening for short interfering RNA (siRNA) enhancers of adeno-associated virus (AAV) transduction. (**a**) Robust Z-factor for 5,520 siRNA pools (average of three replicates) examined in primary screen for enhancement of AAV transduction as detected by enhancement of the luciferase transgene. A cutoff of Robust Z-factor > 4.75 defined 50 hits (red). Data from untreated wells (average of eight replicates) is shown on the right. Robust Z-factor = 0 (median) and Robust Z = 3.0 are marked as a reference. (**b**) A total of 10 of the top 50 pooled screening hits were confirmed as enhancers of AAV transduction when each siRNA (sequences A, B, C) of each pool was tested individually. At least one of the three sequences tested in each pool resulted in a significant enhancement of luciferase expression. ABCA8, ATP-binding cassette sub-family A member 8; CLIC2, chloride intracellular channel 2; GART, phosphoribosylglycinamide formly transformylase, phosphoglycinamide synthetase, phosphoribosylaminoimidazole synthetase; GPR124, G-protein coupled receptor 124; KALRN, kalirin; LCK, lymphocyte-specific protein tyrosine kinase; SLC5A2, solute carrier family 5 (sodium/glucose cotransporter), member 2; SLC5A3, soluble carrier family 5 (sodium/myoinositol cotransporter), member 3; TAS2R10, taste receptor type 2 member 10.

RESULTS

Primary and secondary screening

An siRNA library targeting 5,520 gene sequences was screened as pools (three siRNA pooled per targeted gene) to examine the effect of each targeted gene on adeno-associated virus serotype 2 (AAV2) transduction of cultured human aortic endothelial cells (HAEC). Three siRNAs at a concentration of 10 nmol/l per targeted gene were pooled (30 nmol/l total) and reverse transfected into HAEC in three replicate wells. At 1 day post-siRNA delivery, the HAEC were transduced with AAV2 coding for the firefly luciferase gene (AAV2-*Luc*) at 8.60×10^6 genome copies per well and the luciferase was then assayed 24 hour post-transduction (**Figure 1a**). The Robust Z-factor[26,27] provides a metric of the median absolute deviation by which an individual knockdown condition (averaged over three replicates) differs from the population median (median luminescence signal of 3.9×10^3 relative luminescence units). A total of 50 hits (~1 % hit rate) were scored as those siRNA pools with Robust Z-factor > 4.75, corresponding to replicate wells having >8.4 fold enhancement of luciferase expression as compared with negative control.

A total of 50 genes identified from the pooled primary screening were retested in a secondary screen by individually assaying each of the three siRNAs (data not shown). Due to a change in the normalization calculation following primary screening hit selection, the siRNAs tested do not exactly correspond to the 50 hits having Robust Z-factor >4.75. In this confirmation test, each individual siRNA was added such that the siRNA concentration prior to virus addition was 30 nmol/l (see **Supplementary Table S1** for siRNA sequences). A total of 10 targeted genes were confirmed that had at least one siRNA sequence providing significant improvement in transduction efficiency (**Figure 1b**). Three of the top ten gene hits (solute carrier family 13 (sodium/sulfate symporters), member 4 (*SLC13A4*), solute carrier family 5 (sodium/glucose cotransporter), member 2 (*SLC5A2*), solute carrier family 5 (sodium/myoinositol cotransporter), member 3 (*SLC5A3*)), came from solute carrier families, with sequence B against *SLC5A2* resulting in greater than sixfold enhancement of luciferase transgene expression. Sequence C against chloride intracellular channel 2 (*CLIC2*) resulted in greater than fourfold enhancement of transgene expression. In a separate experiment, cell viability and total cell protein following knockdown with *SLC5A2* sequence B and *CLIC2* sequence C were unchanged (**Supplementary Figure S1 and S3**), indicating that large enhancements in transgene expression (22 fold for *CLIC2* sequence C) were not likely due to toxicity of a particular siRNA sequence.

Off-target effect of siRNA sequences against CLIC2

Since only sequence C against *CLIC2* enhanced transgene expression, we used quantitative real time PCR to verify the extent of *CLIC2*

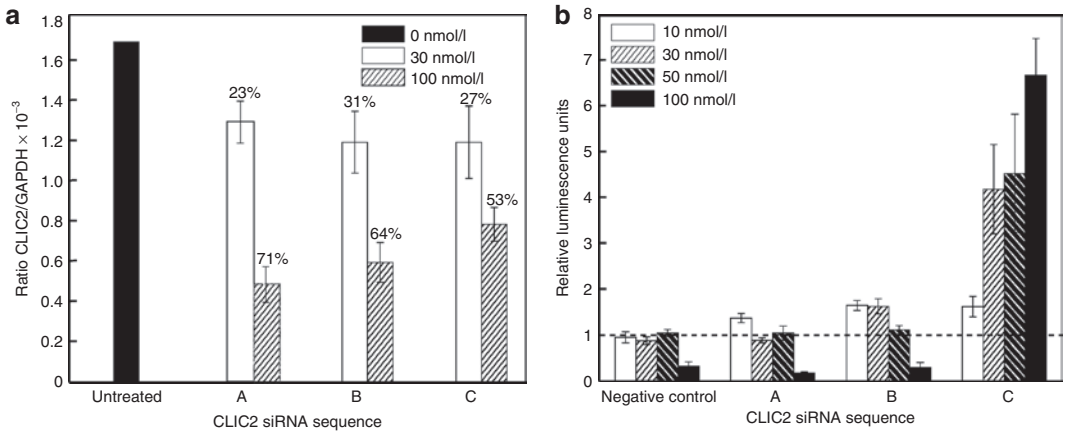

Figure 2 Demonstration of off-target mechanism of action of CLIC2 short interfering RNA (siRNA). (**a**) Quantitative real time PCR measurement of CLIC2 mRNA knockdown in human aortic endothelial cells at 24 hour following transfection with three different siRNA sequences at concentrations of 30 nmol/l and 100 nmol/l, normalized against GAPDH mRNA signal. (**b**) Luciferase luminescence relative to scrambled siRNA negative control for CLIC2 siRNA sequences A, B, and C used at four concentrations of 10, 30, 50, and 100 nmol/l. Adeno-associated virus (AAV) transgene expression was uncorrelated with CLIC2 mRNA knockdown. CLIC2, chloride intracellular channel 2.

Table 1 Short interfering RNA (siRNA) sequences providing the top hits in secondary screening of individual siRNAs, and point-mutated siRNA sequences based on chloride intracellular channel 2 (CLIC2) sequence C

Gene name	Applied Biosystems siRNA ID number	Letter code	Antisense sequence	Primary screen fold increase (pooled)	Secondary screen fold increase (single siRNA)
SLC5A2	41847	B	5′-ACAGUGCCUCUGUUGGUUCtg-3′	13.9	6.0
ABCA8	117435	A	5′-U*UGUUUC*AUAACAAUGAGCtg-3′	17.7	4.6
CLIC2	145736	C	5′-A*UGUUUC*UAAGGAGCAGGGtg-3′	16.9	4.3
GPR124	34695	B	5′-A*UGUUU*AGUCGGAGAAGCCtg-3′	7.0	3.4
LCK	668	A	5′-A*UGUUUC*ACCACCUCUCCCtg-3′	22.1	3.4
CLIC2(C)-A1U mutant			5′-**U***UGUUUC*UAAGGAGCAGGGtg-3′		
CLIC2(C)-U4A mutant			5′-A*UG**A**UUC*UAAGGAGCAGGGtg-3′		
CLIC2(C)-G14C mutant			5′-A*UGUUUC*UAAGGA**C**CAGGGtg-3′		

Abbreviations: ABCA8, ATP-binding cassette sub-family A member 8; CLIC2, chloride intracellular channel 2; GPR124, G-protein coupled receptor 124; LCK, lymphocyte-specific protein tyrosine kinase; SLC5A2, solute carrier family 5 (sodium/glucose cotransporter), member 2.
The bases shared in the seed region between four of the five sequences are italicized. The point mutations made to CLIC2 sequence C are in bold font and underlined.

mRNA knockdown. As shown in **Figure 2**, the amount of *CLIC2* mRNA knockdown was similar for each sequence at siRNA concentrations of 30 nmol/l or 100 nmol/l. We conclude that the mechanisms by which sequence C caused a substantial, dose-dependent increase in AAV2 transduction was not due to the reduction in *CLIC2* mRNA.

Seed region off-target effects

Analysis of the top siRNA sequence hits from single siRNA confirmation screening revealed that three of the top five shared an identical nucleotide sequence at positions 2–7 of the antisense strand and that a fourth siRNA shared positions 2–6 with those sequences (**Table 1**). To investigate if the observed off-target effect stemmed from this U_2GUUUC_7 seed region of the antisense strand, three siRNAs consisting of the *CLIC2* sequence C containing point mutations were examined (**Table 1**). These siRNAs were then transfected into HAEC at a range of concentrations from 10 to 100 nmol/l and AAV2-*Luc* was added 24 hours later (**Figure 3**). Where the point mutation was introduced into position 1or position 14 of the sequence, increases in transduction were comparable to the original *CLIC2* sequence C. However, when the U4A point mutation was introduced into the middle of the hexamer seed region, the siRNA sequence performance was similar to the negative control and did not display the increases in transduction efficiency observed with the other sequences. Comparison of knockdowns with the *CLIC2* sequence C and the mutated *CLIC2* sequence C in which the nucleotide at position four (U4A) indicates a miRNA-like mechanism for the off-target siRNA mediated enhancement of luciferase expression.

Transcription profiling following siRNA transfection implicate interferon pathways

Additional off-target effects of siRNA can arise through global phenotypic changes in the mRNA profile due to the siRNA. Differences in the mRNA expression profile between *CLIC2*(C) and *CLIC2*(C)-U4A mutant sequences due to off-target effects specific to the hexamer seed region were tested by mRNA profiling. In comparing the HAEC response to *CLIC2* sequence C versus U4A mutant siRNA sequence (no AAV2 added), a total of

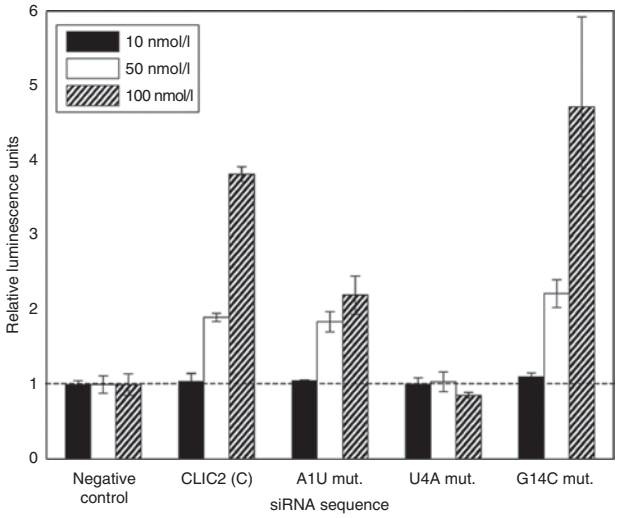

Figure 3 Hexamer region of CLIC(C) antisense strand mediates enhanced AAV2 transgene expression. Effect of CLIC2 short interfering RNA (siRNA) sequence C and CLIC2(C) siRNA mutants on AAV2 transduction of human aortic endothelial cells, normalized to scrambled siRNA negative control. AAV2, adeno-associated virus serotype 2; CLIC, chloride intracellular channel.

28 transcripts were enhanced, while 40 transcripts were decreased (**Supplementary Table S2**). Several transcripts related to the interferon pathway were downregulated: interferon-induced protein 44-like (*IFI44L*), interferon-inducible myxovirus resistance 1 (*MX1*), and interferon-induced protein with tetratricopeptide repeats 5 (*IFIT5*). The *IFI44L* and *MX1* transcripts were specifically among the top five transcripts identified, and the top two for which a known function or pathway could be assigned. Transfection with *CLIC2* sequence C siRNA resulted in a reduction of *IFI44L*, *MX1*, and *IFIT5* mRNAs relative to transfection with *CLIC2*-U4A mutant siRNA as confirmed by quantitative real time PCR (**Supplementary Figure S2**).

To further investigate the interferon pathway which is a known modulator of viral processes,[28–30] several knockdowns were conducted. *IFI44L*, *MX1*, and *IFIT5* knockdowns when tested individually did not result in an enhancement of AAV2-*Luc* transduction (data not shown), indicating that *CLIC2* sequence C siRNA creates a complex phenotype that results in enhanced transgene expression. In an additional test of interferon pathway processes, two unique siRNA sequences targeting the interferon (α, β, and ω) receptor 2 (*IFNAR2*) led to an increase in virus transduction (**Figure 4a**). In contrast, the addition of recombinant α interferon and β interferon directly into the cell culture at the time of transduction led to a decrease in transgene expression (**Figure 4b**) with no change in cell viability (data not shown).

Human airway culture

We evaluated if the enhancing effect of *CLIC* sequence C siRNA was cell-specific by testing enhancement of AAV2 transduction of a human bronchial epithelium. In order to test the effectiveness of these siRNA sequences in a primary cell line, both the *SLC5A2* sequence B and *CLIC2* sequence C were evaluated in human bronchial epithelium using AAV type 2 coding for enhanced green fluorescent protein, followed by evaluation using fluorescence

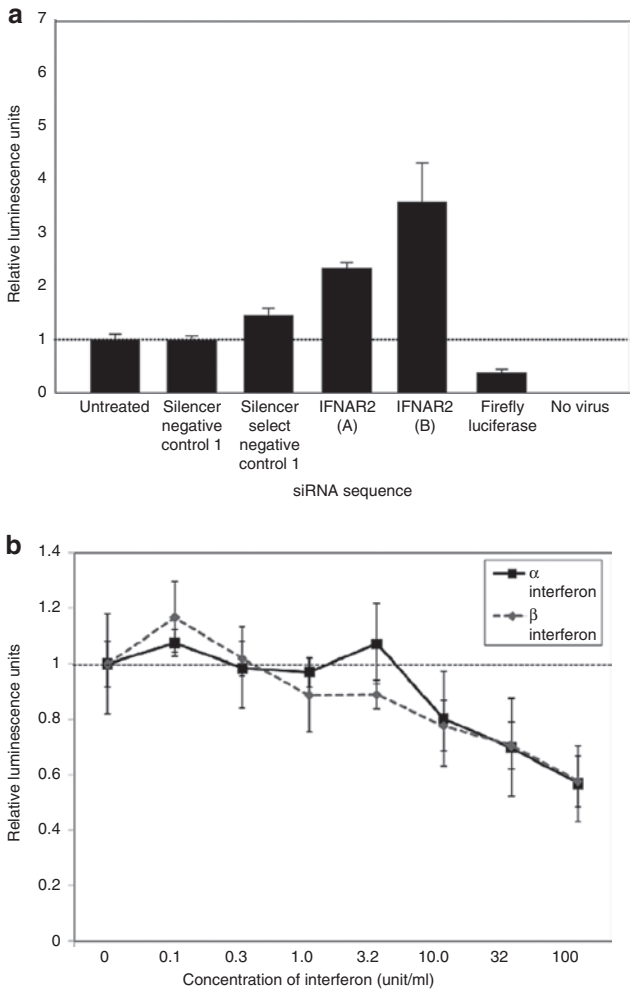

Figure 4 Effect of antagonism or agonism of interferon pathways on adeno-associated virus (AAV) transduction. **(a)** Effects of negative controls, interferon (α, β, and ω) receptor 2 (IFNAR2(A)) siRNA and IFNAR2(B) siRNA, and firefly luciferase siRNAs at 50 nmol/l on luminescence following transduction with AAV2-Luc. All results are normalized to the untreated condition. (**b**) Recombinant α and β interferons reduce transgene expression. siRNA; short interfering RNA.

microscopy and flow cytometry. At the highest siRNA concentration tested, an increase in fluorescence of 27% was observed for *SLC5A2* sequence B and an increase in fluorescence of 61% was observed for *CLIC2* sequence C (**Figure 5**). Although the siRNA sequences were identified using HAEC cells, the results were not specific to the endothelium and may be useful for gene therapy applications in respiratory diseases such as cystic fibrosis. These results also confirm that the enhancing effects were not unique for the firefly luciferase reporter gene product since *SLC5A2* sequence B and *CLIC2* sequence C siRNAs increased in fluorescence from enhanced green fluorescent protein as measured using flow cytometry and were consistent with the luciferase enhancements.

Combination effects

Two experiments were carried out in which the effect of pairwise combinations of siRNAs was examined. The total siRNA concentration used was constant at 50 nmol/l. In the first of these experiments, pairwise combinations of the top single siRNA sequence

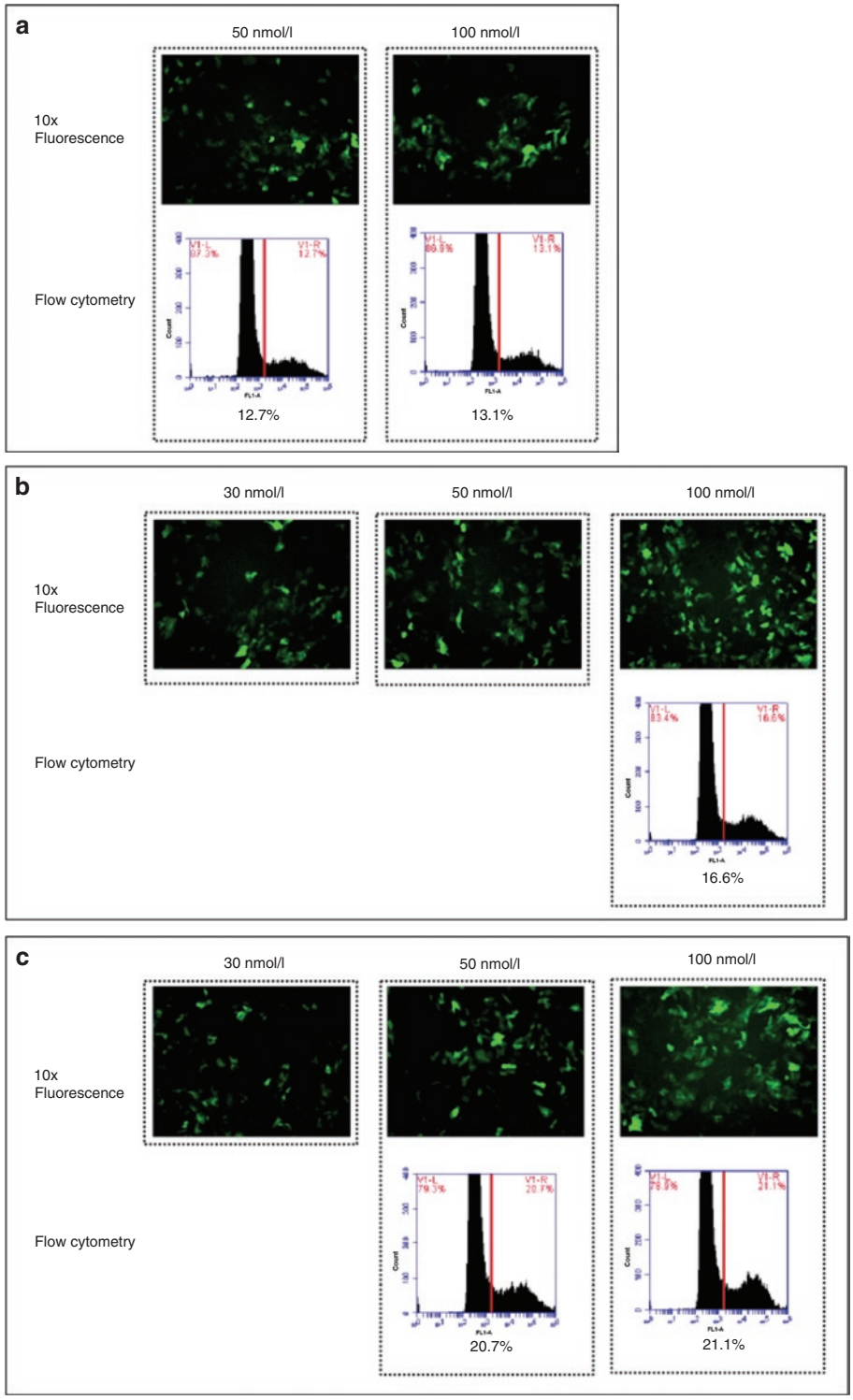

Figure 5 Short interfering RNA (siRNA) mediated enhancement of AAV2 transduction of human epithelial cells. Fluorescence microscopy images and flow cytometry data for human bronchial epithelium culture treated with **(a)** scrambled siRNA negative control, **(b)** SLC5A2 sequence B, and **(c)** CLIC2 siRNA sequence C and then transduced with AAV2 containing enhanced green fluorescent protein. AAV2, adeno-associated virus serotype 2; CLIC2, chloride intracellular channel 2; SLC5A2, solute carrier family 5 (sodium/glucose cotransporter), member 2.

for each of the top 10 genes were examined (Figure 6a). In the second, pairwise combinations of the three siRNA sequences for the top three genes (*CLIC2*, G-protein coupled receptor 124 (*GPR124*), and *SLC5A2*) were examined (Figure 6b). As expected, *SLC5A2* sequence B and *CLIC2* sequence C both provided high results both alone and in combination with other sequences. *GPR124* sequence B also provided a strong signal. In each of the two experiments, the highest signal came from a mixture of two

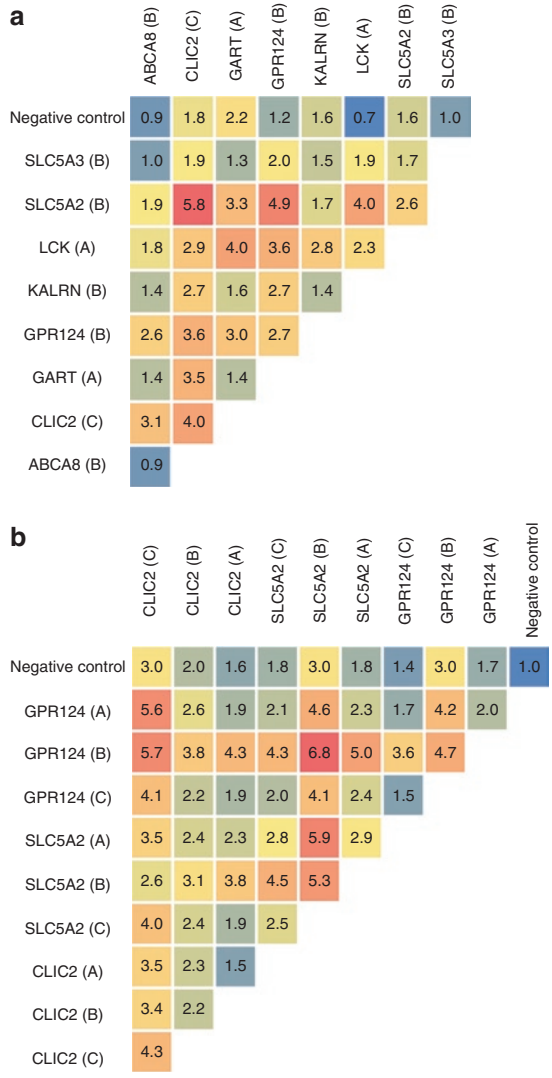

Figure 6 Pairwise interactions among confirmed short interfering RNA (siRNA) hits. Heat map of pairwise interactions between siRNA sequences (total siRNA concentration/well = 50 nmol/l) for the top 10 confirmed hits (**a**) or the three different sequences against the top three hits (**b**). ABCA8, ATP-binding cassette sub-family A member 8; CLIC2, chloride intracellular channel 2; GART, phosphoribosylglycinamide transformylase, phosphoriboglycinamide synthetase, phosphoribozy aminoimidazole synthetase; GPR124, G-protein coupled receptor 124; LCK, lymphocyte-specific protein tyrosine kinase; SLC5A2, solute carrier family 5 (sodium/glucose cotransporter), member 2; SLC5A3, solute carrier family 3 (sodium/myoinositol cotransporter), member 3; TAS2R10, taste receptor type 2 member 10.

siRNA sequences. For example, *GPR124* sequence A provided twofold improvement when used on its own, and CLIC2 sequence C provided 4.3-fold improvement on its own, but the combination gave 5.6-fold improvement. The pairwise tests generally resulted in additive enhancement but not synergistic enhancements.

Coadministration of siRNA with AAV

In order to examine the use of siRNA in combination with adeno-associated viral gene delivery, two experiments were performed in which the siRNA was coadministered with the viral vector. In the first, the virus was premixed with the siPort/siRNA mixture and then cells were added within thirty minutes (**Figure 7a**). In the second, the virus was added immediately following the addition of cells to the siPort/siRNA mixture (**Figure 7b**). The main difference between these two experiments was the exposure of virus to a higher concentration of siPort/siRNA for a short period of time in the premixed experiment. The experiments showed similar results to each other, and additionally followed the same general trend of results observed for the standard transduction protocol in which 24 hours elapsed between addition of siRNA and addition of virus. For the *SLC5A2* sequence B and *CLIC2* sequence C sequences, increases in viral transduction in the range of 50–150% were observed for the 50 nmol/l condition.

DISCUSSION

The use of siRNA high throughput screening targeting 5,520 genes allowed the identification of enhancers of adeno-associated viral gene delivery. By screening pools of three siRNAs per targeted gene in triplicate, a stringent Robust *Z*-score >4.75 provided for a ~1% hit rate. Overall, 60% of genes tested resulted in >1.0 fold enhancement. When individual siRNA sequences were retested, only 10 of the 50 targeted genes (20% confirmation rate) resulted in AAV-*Luc* enhancements (**Figure 1**). The observed improvements to AAV transduction were modest, generally sixfold in typical cases, but can rise to up to 22-fold improvements depending on delivery conditions. We note that a sixfold improvement could ultimately correspond to a sixfold reduction in: (i) bioreactor volume for production, (ii) number of injections to achieve efficacy, or (iii) overall cost of a therapy.

One of the strongest inducers was the *CLIC2*(C) sequence which had a beneficial action on transduction that was not correlated to *CLIC2* mRNA knockdown (**Figure 2**), indicating an off-target effect. Inspection of a number of the confirmed siRNA sequences that enhanced transgene expression led to the identification of a common hexamer seed region (5′-U_2GUUUC_7-3′) (**Table 1**). The U4A mutation in this hexamer seed region of the antisense strand destroyed the enhancing activity of the *CLIC2*(C) siRNA (**Figure 3**), indicating an important role for off-target miRNA-like silencing as a mechanism enhancing AAV2 transduction. An examination of all sequences screened shows that 14 pools contained an siRNA that included the common hexamer seed region (5′-U_2GUUUC_7-3′) (**Supplementary Table S5**) and that all of these pools resulted in an increase in transduction; this significant correlation has a *P* value of 0.0008. At present, no off-target silenced mRNAs have been identified that result in the enhanced transduction. A search through the miRBase (www.mirbase.org) does not reveal any wild-type human miRNAs containing the hexamer seed sequence identified in this study. The off-target effects of siRNAs were beneficial to AAV2 transduction of both human endothelium and human bronchial epithelium (**Figure 5**). Interestingly, siRNA sequences when used together provided additive benefits to AAV2 transduction (**Figure 6**) and never resulted in cross-antagonism (less than onefold enhancement).

Several of the top mRNA levels that are downregulated specifically by the *CLIC2*(C) siRNA sequence but not the *CLIC2*(C)-U4A mutant siRNA were interferon-inducible genes (*IFI44L*, *MX1*, and *IFIT5*). Due to this result, the interferon pathway was further explored and knockdown of the interferon (α, β, ω) receptor 2 was shown to improve transduction (**Figure 4a**). This receptor is

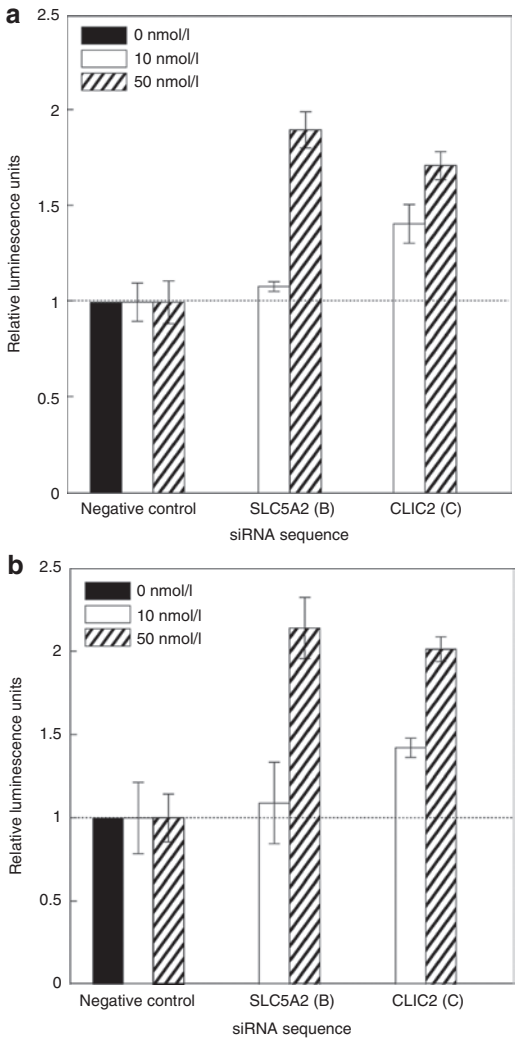

Figure 7 Coadministration of adeno-associated virus serotype 2 and short interfering RNA (siRNA) to human aortic endothelial cells enhances transgene expression. (**a**) Virus was premixed with siRNA lipoplexes and then added to cells. (**b**) Cells were reverse transfected and virus was immediately added after adding cells to siRNA formulation. CLIC2, chloride intracellular channel 2.

activated by type I interferons. The type I interferons serve as an early warning system in antiviral defense and previous work has shown them to be stimulated by AAV.[31] In response to a stimulus from a pathogen, type I interferons are synthesized and secreted.[30] The type I interferons then bind to receptors *IFNAR1* or *IFNAR2*, and the janus kinase, tyrosine kinases, and signal transducers and activators of transcription comprise the downstream pathways leading to production of interferon-induced proteins.[28,29,32,33] Many proteins are induced by the interferons, although a main pathway consists of the expression of protein kinase R. Protein kinase R then inhibits protein synthesis by inducing RNAse L to destroy RNA and by activating eukaryotic initiation factor 2 to lessen protein translation.[34,35] Other mechanisms additionally recruit the adaptive immune response.[28] Notably, a current literature search shows several studies in which AAV has been used to deliver interferon-β for cancer gene therapy.[36–38] These studies show promising results; however the results presented in this paper suggest that caution should be used when combining the AAV vector with interferon, due to the potential that the interferon could inhibit future readministration of the vector.

This work focused on AAV2. However, the population in general has a high rate of pre-existing immunity to AAV2, which is limiting for the use of this serotype in treating the largest possible base of patients.[39] In future work, it would be interesting to test these RNA sequences with other AAV serotypes. For comparison, an adenovirus primary screen was also conducted, with results shown in **Supplementary Table S4**. In general, there was little similarity between the primary screen results from adenovirus and AAV. Out of the top 50 hits, only three hits were common between the two screens: phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase, olfactory receptor, family 51, sub-family E, member 1, and G protein-coupled receptor kinase interactor 2. These few differences and similarities (6% overlap) will be the subject of future study.

Although the focus of this article has been on identifying enhancers to AAV2 transduction, future work could include examination of the inhibitors, which are important to furthering the understanding of cellular machinery used by the virus. Primary screen inhibitor hits (50 smallest luminescence values) (**Supplementary Table S6**) included ADP-ribosylation factor 1 and ADP-ribosylation factor 6, two members of the ADP-ribosylation family involved in vesicular transport. In fact, all six members of this family included in the screen showed a fold change of 0.8 or below. *RAB8A* and *RAB8B*, the two isoforms of the *RAB8* protein, play a role in protein transport and were also identified as a primary screen hit. However, prior to elucidating a specific mechanism, further work is required to confirm that the decreased transduction signal is a result of a change in viral transduction and not cytotoxicity.

With respect to therapeutic strategies to enhance transduction, we report that coadministration of siRNA lipoplexes with AAV2 results in enhanced transgene expression (**Figure 7**), suggesting that the enhancement is due to siRNA-modulated pathways distal of changes in receptor engagement, endocytosis, or endosome escape. We conclude that siRNA sequences containing the hexamer seed region (5′-U_2GUUUC_7-3′) result in a complex alteration of phenotype involving both translational silencing and multiple off-target mRNA knockdowns that together modulate the interferon pathway response to viral infection. However in regard to gene therapy, this alteration of phenotype can lead to enhancements in AAV transgene expression in human endothelium and epithelial cells.

MATERIALS AND METHODS

Cell culture. Human aortic endothelial cells (HAEC; Lonza, Walkersville, MD) were cultured in supplemented Clonetics EGM-2 (Lonza) at 37 °C and 5% CO_2. Prior to siRNA treatment, cells were rinsed with Dulbecco's phosphate-buffered saline and incubated with 0.05% Trypsin-EDTA (Invitrogen, Santa Clara, CA), then seeded onto 96 or 384 well flat bottom plates (BD Bioscience, Franklin Lakes, NJ). Human bronchial epithelial cells (Lonza) were grown in cell culture flasks containing supplemented bronchial epithelial growth media for 4–5 days at 37 °C and 5% CO_2. Human bronchial epithelial cells were then seeded at a density of 10^5 per well onto 96 well flat bottom plates (BD Bioscience) and cultured in an equal mixture of bronchial epithelial basal media and Dulbecco's modified Eagle's medium

(Invitrogen, Santa Clara, CA). Cells became confluent within 4 days and were allowed to grow for 2 weeks prior to forward transfection.

Druggable Genome Library. The Druggable Genome Library (Applied Biosystems, Foster City, CA) was used for screening. This library consists of 5,520 gene targets and three siRNAs per gene. The library was provided in 384 well plates, each well containing 0.25 nmol of lyophilized siRNA. The two columns on the right of the plate were left empty for controls. Sterile nuclease-free water (Applied Biosystems) was added to each well in order to resuspend the siRNA at a concentration of 3,125 nmol/l. Additional nuclease-free water was used to dilute the siRNA to a working concentration of 330 nmol/l. For the primary screen, the three siRNAs targeting the same gene were pooled together in equal quantities to create a pooled master. The pooled master was then used to create assay plates containing 2 µl of siRNA, with each individual siRNA at a concentration of 110 nmol/l, for a total pooled siRNA concentration of 330 nmol/l. For the confirmatory screen, the desired individual siRNAs were aliquoted from the diluted master, and used to create assay plates containing 2 µl of siRNA, with each individual siRNA at a concentration of 330 nmol/l. Seven columns on the right and left sides of each plate contained controls. Following the confirmatory screen, work was performed at larger scale (96 wells or less per plate), and in those cases the siRNA were ordered individually and resuspended using sterile nuclease-free water at a concentration of 3,125 nmol/l. For full screening results, see **Supplementary Table S3**. For comparison, an identical siRNA screening experiment against HAEC was conducted with adenovirus AdV5 at 10 genome copies per well (**Supplementary Table S4**).

Mutated siRNAs. For examination of off-target effects resulting from the seed region of the siRNA sequences, three mutated siRNA sequences were designed in which individual point mutations were introduced into positions 1, 4, and 14, respectively, of the siRNA strand corresponding to Applied Biosystems siRNA 145736 (*CLIC2* sequence C). In each case, the siRNA sense strand was complementary to its mutated antisense strand. The mutated siRNAs were chemically identical to the original Applied Biosystems Silencer siRNAs, with the exception of the point mutations, and sequences are given in **Table 1**.

Reverse transfection protocol. The protocol used for HAEC transfection was adapted from the method described by Barker and Diamond.[26] HAEC were cultured in Clonetics EGM-2 (Lonza). siRNA (Applied Biosystems) was added to a well plate. The well plate was either frozen overnight or held at room temperature for less than two hours. If the plate was frozen, it was thawed and allowed to equilibrate to room temperature prior to use. siPort NeoFX (Applied Biosystems) diluted in Opti-Mem (Lonza) was added to the siRNA plate and allowed to incubate at room temperature for 10 minutes. HAEC grown in a cell culture flask were then added to the plate at a seeding density of 4.5×10^4 cells per cm^2. The siRNA were allowed to transfect the cells for 24 hours.

Forward transfection protocol. siRNA (Applied Biosystems) was thawed at room temperature and then added to siPort NeoFX (Applied Biosystems) diluted in Opti-Mem (Lonza). The mixture was allowed to incubate at room temperature for 10 minutes prior to addition to 96 well plates. The siRNA were allowed to transfect the cells for 24 hours.

Interferon protocol. Frozen recombinant human αA-interferon and β-interferon (Calbiochem, San Diego, CA) were thawed on ice and diluted in Dulbecco's phosphate-buffered saline (Invitrogen, Santa Clara, CA) and serially diluted prior to addition to a 96 well plate. Virus addition followed within 20 minutes of addition of interferon to the well plate.

Luciferase transduction protocol. AAV2 containing a cytomegalovirus promoter and firefly luciferase sequence was added to the well plate. The virus was then allowed to transduce the cells for 24 hours. On the third day, cells were assayed for gene expression using the Bright-Glo assay kit (Promega, Madison, WI) following the vendor's protocol. A scrambled siRNA sequence was used as a negative control (Silencer Negative Control 1; Applied Biosystems).

Fluorescence transduction protocol. After 24 hours of exposure to siRNA, AAV2, containing a cytomegalovirus promoter and enhanced green fluorescent protein sequence was added to the plate. The virus was then allowed to transduce the cells for a minimum of 48 hours prior to imaging and flow cytometry analysis.

Flow Cytometry. An Accuri C6 Flow Cytometer (Accuri Cytometers, Ann Arbor, MI) was used for quantitative analysis of individual cell fluorescence. Cells were harvested into Dulbecco's phosphate-buffered saline (Invitrogen, Santa Clara, CA) and then held on ice until measurement. 20,000 counts per sample were recorded.

Cell viability assay. Cells were assayed for viability using the Cell Titer Glo assay kit (Promega) following the vendor's protocol.

Quantitative real-time PCR. Cells were treated with siRNA and were then harvested a day later for total RNA content using the Absolutely RNA microprep kit (Stratagene, La Jolla, CA). Superscript III reverse transcriptase and oligo(dT) (Invitrogen, Carlsbad, CA) was used to reverse transcribe the RNA. The resulting cDNA was then purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA). The *CLIC2* forward primer used was CACTACAAGCTAGACGGT and the reverse primer used was CCAGGAACGGAGGATT. The *MX1* forward primer used was CGCAGGGACCGCCTTGGACC and the reverse primer was GGGTGG GATGCAGCAGCTGGA. The *IFI44L* forward primer used was GGTGG GTCCAGTTGGGTCTGGA and the reverse primer was GCACAGTCC TGCTCCTTCTGCC. The *IFIT5* forward primer was AGGCTGTTACC CTGAACCCAGAT and the reverse primer was GGTCTGTTGTGT GTGGCCTTCT. The *GAPDH* transcript was used to normalize between samples. The *GAPDH* forward primer used was TGCACCACCAACTG CTTAGC and the reverse primer used was GGCATGGACTGTGGTCA TGAG. A Roche LightCycler (Roche, Indianapolis, IN) was used to generate a standard curve and optimize PCR conditions for each primer. The LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche) and Light Cycler melting curve analysis was used to perform quantitative real-time PCR.

Transcription profiling. Cells were grown in 24 well plates at a seeding density of 45,000 cells/cm^2. Cells were harvested using 0.05% Trypsin-EDTA (Invitrogen, Carlsbad, CA) 24 hours after siRNA transfection. Total RNA was purified from cell lysate using the Absolutely RNA kit (Agilent, Santa Clara, CA). For each sample, 0.2–0.3 µg of purified RNA was amplified, fragmented, and then hybridized to the Human Gene 1.0ST microarray (Affymetrix, Santa Clara, CA) according to the Affymetrix GeneChip Expression Analysis Technical Manual protocol. Following hybridization, washing, and staining, the microarray was imaged using a confocal scanner with fluorescence excitation at 570 nm. Two sequential scans were conducted and a mean fluorescence signal was calculated. The resulting signals were analyzed using the Affymetrix Microarray Suite 5.0 and default values provided by Affymetrix. Fold change, *P* value, and significance analysis of microarray[40] q-value were calculated. Transcripts whose significance analysis of microarray q-value were <25 and having a fold change difference >1.25 (indicating upregulation) or less than −1.25 (indicating downregulation) were identified.

SUPPLEMENTARY MATERIAL

Figure S1. Viability of HAEC 48 hours following knockdown with siRNA sequences indicated.

Figure S2. Real-time quantitative real time PCR results for (a) MX1, (b) IFIT5, and (c) IFI44L message expressed following treatment with siRNA CLIC2(C) and U4A mutant sequences.

Figure S3. siRNA knockdown at 50 nmol/l followed by (a) AAV2 luciferase transduction and luminescence assay, or (b) cell lysis and assay for total protein content.
Table S1. siRNA sequences investigated in detail during confirmation of secondary screen and microarray results.
Table S2. HAEC mRNA transcripts that were upregulated or downregulated following delivery of CLIC(C) siRNA relative to CLIC(C)-U4A mutant.
Table S3. Full siRNA screening results for AAV2 transduction (EXCEL file).
Table S4. Full siRNA screening results for AdV5 transduction (EXCEL file).
Table S5. siRNA sequences tested in primary screening containing hexamer seed region 5′-UGUUUC-3′ (EXCEL file).
Table S6. Annotated table of siRNA inhibitors of AAV2 transduction (EXCEL file).

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