Human, Pig, and Mouse Interferon-Induced Transmembrane Proteins Partially Restrict Pseudotyped Lentiviral Vectors

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Lentiviral vectors are increasingly used in clinical trials to treat genetic diseases. Our research has focused on strategies to improve lentiviral gene transfer efficiency in the airways. Previously we demonstrated that a feline immunodeficiency virus (FIV)-based lentiviral vector pseudotyped with the baculovirus envelope glycoprotein GP64 (GP64-FIV) efficiently transduced mouse nasal epithelia *in vivo* but transduced mouse intrapulmonary airways with 10-fold less efficiency. Here, we demonstrate that members of a family of proteins with antiviral activity, *interferon-induced transmembrane proteins* (IFITMs), are more highly expressed in mouse intrapulmonary airways as compared with mouse nasal airways. Using GP64- and VSV-G (vesicular stomatitis virus G glycoprotein)-pseudotyped FIV, we show that expression of mouse IFITM1, IFITM2, and IFITM3 restricts gene transfer. Further, we show that both the nasal and intrapulmonary airways of IFITM locus knockout mice are more efficiently transduced with GP64-FIV than their heterozygous littermates. In anticipation of transitioning our studies into pig models of airway disease and clinical trials in humans, we investigated the ability of pig and human IFITMs to restrict lentiviral gene transfer. We observed that both human and pig IFITMs partially restricted both VSV-G-FIV and GP64-FIV transduction *in vitro*. Previous studies have focused on IFITM-mediated restriction of replication-competent wild-type viruses; however, these results implicate the IFITM proteins as restriction factors that can limit lentivirus-based vector gene transfer to airway epithelia. The findings are relevant to future preclinical and clinical airway gene therapy trials using lentivirus-based vectors.

INTRODUCTION

CURRENT LENTIVIRAL VECTOR TECHNOLOGY has progressed considerably toward the goal of creating a vehicle with the ability to efficiently, safely, and persistently express a transgene in the appropriate cell types.^{1,2} Several features of lentiviral vectors make them attractive vehicles for delivering therapeutic genes, including their large transgene-packaging capacity, efficient gene transfer capabilities, persistent expression, the capacity to transduce mitotically quiescent cells, and lack of virus-encoded proteins that could elicit undesirable immune responses.^{3–5} For our studies, we developed gene transfer vectors derived from a non-primate lentivirus, feline immunodeficiency virus (FIV).^{6,7} Tissue and cellular tropism of lentiviral vectors can be modified by substituting the envelope glycoprotein with other glycoproteins from many

different viruses. We previously demonstrated that FIV pseudotyped with the envelope glycoprotein from baculovirus *Autographa californica* multi-nucleocapsid nucleopolyhedrosis virus (GP64) confers apical entry into well-differentiated primary cultures of human, mouse, and pig airway epithelial cells.^{4,8–10} We further demonstrated that GP64-FIV efficiently transduced the airways of mice or pigs *in vivo*, was repeatedly administered without the generation of blocking immune responses, and expressed a reporter gene for the life span of mice.^{9,11} Thus, GP64-FIV is a candidate gene therapy reagent for genetic pulmonary diseases such as cystic fibrosis.

Multiple host factors have been identified that limit lentiviral gene transfer efficiency.^{12–16} The *interferon-induced transmembrane* family of proteins (IFITMs) are robustly stimulated by both type

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I and type II interferons.¹⁷ IFITM1, IFITM2, and IFITM3 are all expressed ubiquitously, whereas IFITM5 expression is unique to osteoblasts.¹⁸ Mice and pigs express IFITM orthologs of their human counterparts, and are induced by interferon similarly.¹⁹ The IFITM1, IFITM2, and IFITM3 proteins exhibit antiviral activity, including restricting influenza A, vesicular stomatitis virus (VSV), and severe acute respiratory syndrome (SARS) virus infection.^{20–22} IFITMs are the only known host restriction factors to block a prefusion step in virus replication.²³ This event occurs in the late endosome or lysosome; however, the exact molecular mechanism of restriction is still unclear. Studies suggest that the prefusion restriction is a result of IFITM presence in the endosomal membrane that renders it unable to achieve the conformational changes in membrane curvature required for hemifusion, an intermediate in the viral envelope fusion process.²³ One report suggests that IFITM proteins will also restrict wild-type, replication-competent HIV-1²⁴; in addition, IFITM proteins restrict *in vitro* entry of VSV G glycoprotein (VSV-G)-pseudotyped HIV-1-based vectors.^{24–26}

We previously reported that GP64-FIV transduces mouse nasal airway epithelia with ~10-fold greater efficiency than mouse intrapulmonary epithelia.¹⁰ This difference was not the result of surface area, immune responses, or clearance because the difference was recapitulated in well-differentiated primary cultures derived from mouse septa and trachea. In addition, the transduction efficiency difference was also observed with VSV-G- and Ebola-pseudotyped FIV.¹⁰ These data suggested that a cellular restriction factor was responsible for the difference in transduction efficiency. Here, we investigate whether the IFITM family of proteins contributes to the differential transduction efficiency observed between the nasal and tracheal airways.

MATERIALS AND METHODS

Viral vector production

Lentiviral vectors were produced and titered by the University of Iowa Viral Vector Core (Iowa City, IA; www.medicine.uiowa.edu/vectorcore). The FIV vector system used in this study^{27,28} expressed firefly luciferase or green fluorescent protein (GFP) from the FIV3.3RSV backbone.²⁹ VSV-G, Machupo virus (MACV), and baculovirus (GP64)-pseudotyped FIV vector particles were generated by transient transfection, concentrated 250-fold by centrifugation, and titered using real-time PCR as previously described.²⁹

Stable cell lines

The T-REx system (K1030-01; Invitrogen/Thermo Fisher Scientific, Waltham, MA) was used to generate doxycycline-inducible stable cell lines expressing IFITMs, according to the manufacturer's protocol. Briefly, T-REx HeLa cells (R714-07; Invitrogen/Thermo Fisher Scientific) that stably express the Tet repressor were transfected with pcDNA4/TO/*myc*-His (V1030-20; Invitrogen/Thermo Fisher Scientific) plasmids encoding individual IFITM genes. These transfected cells were then selected in Eagle's Minimum Essential Medium (EMEM) supplemented with blasticidin at 5 µg/ml (R210-01; Invitrogen/Thermo Fisher Scientific) and Zeocin at 50 µg/ml (R250-01; Invitrogen/Thermo Fisher Scientific). Monoclonal cell lines were isolated by means of cloning cylinders (CLS31668; Sigma-Aldrich, St. Louis, MO) and expanded. To verify IFITM expression, cells were treated with doxycycline (1 µg/ml) for 24 hr, cell lysates were collected, and Western blots were performed using standard techniques. Briefly, total protein was sonicated, loaded onto 15% Tris-HCl gels (4569035; Bio-Rad, Hercules, CA), and run at 100 V in Tris-tricine/SDS buffer (1610734; Bio-Rad) diluted to 1×. Protein was transferred to polyvinylidene difluoride (PVDF) membranes (1620177; Bio-Rad) at 0.3 A for 90 min at 4°C in Tris-glycine transfer buffer (LC3675; Thermo Fisher Scientific). Membranes were blocked overnight in 5% milk and incubated with mouse monoclonal primary antibody against C9 tag (diluted 1:1000) (ab5417; Abcam, Cambridge, UK) or c-Myc tag (diluted 1:1000) (R950-25; Thermo Fisher Scientific) for 3 hr at 4°C. Membranes were washed and then incubated for 45 min at room temperature with a goat anti-mouse secondary antibody (diluted 1:5000) (31437; Thermo Fisher Scientific) conjugated to horseradish peroxidase. Membranes were then developed with SuperSignal West Pico chemiluminescent substrate (34080; Thermo Fisher Scientific).

Restriction assays

Restriction assays were performed in 24-well plates coated with poly-L-lysine (P4707; Sigma-Aldrich). For each vector pseudotype and vector production lot, a vector dilution was chosen that conferred ~20% GFP⁺ cells in the T-REx parental cell line. The T-REx stable cell lines were maintained in EMEM (11965-084; Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (10082139; Thermo Fisher Scientific) and 1% penicillin–streptomycin. Cells were treated with either doxycycline (1 µg/ml) or vehicle (buffered culture medium) for 24 hr before transduction with

FIV expressing GFP. Cells were incubated with viral vectors for 4 hr in serum-free medium. After the transduction period, the serum-free transfection medium was replaced with maintenance medium. Forty-eight hours after transduction, cells were fixed with 2% formaldehyde, harvested, and suspended, using Accumax proteolytic cell detachment solution (SCR006; Millipore, Billerica, MA), and GFP expression was quantified with a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA).

IFITM locus knockout mice

IFITM^{del/+} mice were kindly provided by M. Farzan.²¹ Because of the inclusion of tyrosinase marker *Tyr* at the telomeric end of the deletion, the genotype segregates exclusively with coat color. Wild-type littermates are albino, hemizygous littermates are light tan, and knockout mice are light brown.³⁰ All genotypes were verified by PCR of genomic DNA. This study was approved by the University of Iowa Institutional Animal Care and Use Committee. Mice were first anesthetized by intraperitoneal injection of ketamine–xylazine (87.5 and 2.5 mg/kg, respectively). Approximately 1×10^7 transducing units (TU) of FIV vector formulated with 1% methylcellulose (1:1) in a total volume of 50 μ l was delivered to the nasal epithelia via direct instillation, or to the trachea epithelia via a 24-gauge Teflon catheter. Methylcellulose increases viral vector gene transfer efficiency in nasal and intrapulmonary airways without disrupting trans-epithelial resistance.⁴

Bioluminescence imaging

Mice were injected intraperitoneally with 200 μ l of D-luciferin (15 mg/ml in 1 $\times$ phosphate-buffered saline [PBS]; Xenogen, Alameda, CA), using a 25-gauge needle. After 5 min, mice were imaged with the Xenogen IVIS charge-coupled device camera while under 1–3% isoflurane anesthetization. Imaging data were analyzed and signal intensity was quantified with Xenogen Living Image software. The negative controls in all *in vivo* bioluminescence assays were naive strain matched mice. We included negative controls in every assay and report only background-subtracted levels of experimental groups.

Expression plasmids

For each species (mouse, pig, and human) the IFITM1, IFITM2, and IFITM3 expression vectors were generated for overexpression studies. Human IFITM1 (Cat. No. DC00426), IFITM2 (Cat. No. DC02727), and IFITM3 (Cat. No. DC02019) expression vectors in a pcDNA4.0 backbone with an

N-terminal c-Myc tag were purchased from Clontech (Mountain View, CA). Pig IFITM and mouse IFITM expression vectors were generated by designing sequence specific primers (Integrated DNA Technologies [IDT], Coralville, IA) and amplifying IFITM cDNA from porcine and murine cDNA libraries, respectively. Primer sequences were as follows: mouse IFITM1 forward 5'-CCG AGA GAT GCC TAA GGA GCA GCA AGA GGTG-3' and reverse 5'-GCA AGA CAT CTC ACA TCA TCT AAT GGC-3'; mouse IFITM2 forward 5'-CCA TGA GCC ACA ATT CTC AAG CCT TCTTG TCC-3' and reverse 5'-GCA AGA CAT CTC ACA TCA TCT AAT GGC-3'; mouse IFITM3 forward 5'-CCG CAC CAT GAA CCA CAC TTC TCA AGC C-3' and reverse 5'-CCT CTA TTA AGT GTG AAG GTT TTG AGC G-3'; pig IFITM1 forward 5'-GGA TCC ATG ATC AAG AGC CAG CAC GA-3' and reverse 5'-CTC GAG GTA GCC TCT GTT ACT CTT TGC-3'; pig IFITM2 forward 5'-GGA TCC ATG AAC TGC GCT TCC CAG CC-3' and reverse 5'-CTC GAG GTA GCC TCT GTT ACT CTT TGC-3'; pig IFITM3 forward 5'-GGA TCC ATGAAT TGC GCT TCC CAG CCC-3' and reverse 5'-CTC GAG GTA GCC TCT GTA ATC CTT TAT-3'. The amplified sequences were then gel purified and subcloned into pcDNA3.1/V5-His TOPO TA expression vector (K4800-01; Thermo Fisher Scientific). The murine Ifitm sequences were subsequently cloned into a pcDNA4.0 backbone. Sequences were verified with published Ifitm cDNA sequences from the National Center for Biotechnology Information (NCBI, Bethesda, MD). The mouse and pig sequences were cloned with an N-terminal nine-amino acid epitope from bovine rhodopsin (the C9 epitope) for detection via immunocytochemistry. Notably, we report here a pig IFITM1 sequence (KT873487) containing a single-nucleotide difference (alanine to guanine) from the NCBI sequence (JQ315414) that results in a histidine-to-glutamine change at amino acid position 113. In addition, our sequencing of pig IFITM3 (KT873488) differed from the NCBI sequence (NM_001201382) by two nucleotides: a synonymous thymine-to-cytosine change at nucleotide position 57 and a thymine-to-cytosine change resulting in an isoleucine-to-threonine change at amino acid position 117. These sequences were confirmed in cDNA isolated from eight pigs of similar breeds.

Real-time PCR for IFITM expression

Total cellular RNA was harvested from eight donor cultures of both nasal turbinate and tracheal cells using TRIzol–chloroform extraction. Reverse transcriptase PCR was performed with a high-capacity reverse transcriptase kit (4368813; Thermo Fisher

Scientific). Primers were designed with software available through the IDT website, and primers were subsequently ordered from IDT. Primer sequences were as follows: pig IFITM1 forward 5'-CTT TCG CCT ACT CCG TGA AG-3', reverse 5'-AGA TGT TCA GGC ACT TGG C-3'; pig IFITM2 forward 5'-CTG AAC ATC TGGGCT CTG ATC-3', reverse 5'-GCT CTA ACA TCT GGT AGG CTG-3'; pig IFITM3 forward 5'-CTG AAC ATC TGG GCT CTG-3', reverse 5'-AAA TTA CCA GGG AGC CAG TG-3'. Real-time quantitative PCR used SYBR green and was performed on a 7900HT fast real-time PCR machine (Applied Biosystems/Thermo Fisher Scientific), and analysis was performed with Applied Biosystems SDS 2.3 software, using the $2\Delta C_t$ analysis method to determine fold change relative to nasal turbinate.

Statistics

All numerical data are presented as means $\pm$ standard error. Statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA). Two-tailed, unpaired Student *t* tests were used to compare the experimental treatment group with the no-doxycycline control for the majority of the studies reported here. *F* tests were performed in parallel to determine that variance was similar between groups. For nonparametric data, the Mann-Whitney *U* test was used. To generate the error bars for the control samples, we first calculated the standard error for the raw data. The normalized error bars are calculated on the basis of the ratio to the mean expression, using the formula: standard error/mean expression $\times$ normalized expression = normalized standard error.

RESULTS

Expression of lentiviral restriction factors in mouse airways

In a previous study, we observed that GP64-FIV transduced mouse nasal airways with greater efficiency than the intrapulmonary airways.¹⁰ This observation was true both *in vivo* and in well-differentiated primary cultures derived from mouse nasal septa or trachea. We hypothesized that the differential expression pattern was due to a cell-specific factor. Using real-time PCR we assessed the mRNA transcript abundance of seven previously described lentiviral restriction factors^{12–16,22} from mouse nasal septa- or trachea-derived tissues. We observed no differences in the expression levels of CPSF6, SAMHD1, APOBEC3, or TRIM28. However, the basal mRNA expression levels of IFITM1,

Figure 1. Quantitative real-time RT-PCR was used to compare basal mRNA levels of known host restriction factors in the nasal septa and trachea of mice. Results are plotted as average fold expression in tracheal samples compared with nasal septum samples (mean $\pm$ standard error). n.d., not detected. *n* = 8; **p* < 0.01.

IFITM2, and IFITM3 were all elevated in mouse tracheal tissue as compared with the nasal septa (Fig. 1). *Zfhx4* expression is specific to nasal tissues and was used as a control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is expressed at similar levels in both nasal septa and trachea and was used as a control. On the basis of these data, the IFITM family members were identified as proteins of interest for further study.

Mouse IFITMs inhibit transduction of GP64-FIV

To investigate the role of the IFITM proteins in restricting GP64-FIV entry, we used IFITM over-expression assays. Doxycycline-inducible stable lines expressing C9-tagged mouse IFITM1, IFITM2, or IFITM3 were established. As demonstrated by Western blot, the levels of IFITM were detectable only in the presence of doxycycline (Fig. 2A). For the restriction assay, cells were treated with doxycycline or vehicle for 24 hr. The cells were then transduced with FIV expressing GFP and pseudotyped with VSV-G, GP64, or Machupo virus (MACV) envelope glycoprotein. MACV is a New World arenavirus that is reported not to be subject to IFITM restriction²² and serves as a negative control. The native VSV “G” envelope glycoprotein has reproducibly been shown to be restricted²² and thus serves as a positive control. The cells were transduced at multiplicities of infection (MOIs) sufficient to result in $\sim 20\%$ positive cells in the vehicle-treated groups. Three days after vector transduction, the percentage of GFP-positive cells was quantified by flow cytometry. In the presence of doxycycline, we observed that VSV-G-FIV was re-

Figure 2. Stable cell lines expressing C9-tagged mouse IFITM1 (mIFITM1), mIFITM2, and mIFITM3 under the control of a doxycycline-inducible promoter were established. **(A)** Western blots were performed on cells incubated in the presence or absence of doxycycline. α -Tubulin was used as a loading control. **(B)** Cells stably expressing mIFITM1 were incubated without (solid columns) or with (shaded columns) doxycycline for 24 hr, and then challenged with VSV-G-FIV-GFP, GP64-FIV-GFP, or MACV-FIV-GFP for 4 hr. Cells were washed and 4 days later the percentage of GFP-positive cells was quantified by fluorescence-activated cell-sorting (FACS) analysis. Results are plotted as average relative transduction compared with the no-doxycycline group $\pm$ standard error. The procedure was repeated for cells stably expressing **(C)** mIFITM2 and **(D)** mIFITM3. Dox, doxycycline; GP64, baculovirus envelope glycoprotein; IFITM, interferon-induced transmembrane protein; MACV, Machupo virus; VSV-G, vesicular stomatitis virus G glycoprotein. $n=8$; * $p<0.01$, ** $p<0.001$.

stricted by mIFITM1 (Fig. 2B), mIFITM2 (Fig. 2C), and mIFITM3 (Fig. 2D). No significant restriction was observed for MACV-FIV. Similar to VSV-G-FIV, GP64-FIV was also restricted by each of the IFITM family members tested. These results were the first indication that IFITM family members restrict viral vector pseudotyped with baculovirus GP64 and may help to explain why GP64-FIV transduces mouse nasal epithelia with greater efficacy than mouse intrapulmonary airways.

We used quantitative real-time RT-PCR to measure *Ifitm* mRNA expression in the stable cell lines (+Dox) and endogenous *Ifitm* mRNA baseline expression in mouse trachea (data not shown). These levels were normalized to the reference gene encoding hypoxanthine phosphoribosyltransferase 1 (*HPRT* or *Hprt*, respectively). In all cases, *Ifitm1*, *Ifitm2*, and *Ifitm3* levels were ~ 2 - to 4-fold higher than that of the reference gene, suggesting that *Ifitm* is expressed (at least at the mRNA level) at similar levels in our stimulated stable cell lines *in vitro* and in mouse trachea *in vivo*.

Improved GP64-FIV transduction in IFITM locus knockout mice

To determine whether IFITMs contribute to vector restriction in mouse airways *in vivo*, we

delivered GP64-FIV expressing firefly luciferase to *Ifitm* locus knockout ($-/-$) mice, hemizygous ($+/-$) littermates, or wild-type ($+/+$) littermates. The knockout mice, termed *IfitmDel*, were first described by Lange and colleagues.³⁰ These mice lack *Ifitm1*, *Ifitm2*, *Ifitm3*, *Ifitm5*, and *Ifitm6* genes. We observed significantly increased levels of gene transfer in both the nose and lung of knockout mice (Fig. 3). Because basal IFITM expression is higher in the intrapulmonary airways than in the nasal airways (Fig. 1), we hypothesized that in *Ifitm* locus knockout mice the level of transgene expression in the intrapulmonary airways would equilibrate to the levels of expression in the nasal airways. However, we observed that nasal luciferase expression after GP64-FIV transduction in knockout mice was enhanced to a greater extent than was observed in the lung. In addition, gene transfer to the lung of knockout mice was similar to gene transfer to the nose of hemizygous littermates. These data suggest that one or more of the IFITM family members are restriction factors to lentiviral gene transfer in both the nasal and intrapulmonary airways of mice. They may contribute to the differential transduction pattern observed between the lung and nose, but other variables are involved.

The airways of humans and pigs share many anatomical similarities; therefore, pigs are becoming a common preclinical model of gene therapy for pulmonary disease. For this reason, we turned our attention to human and pig IFITMs. IFITM protein alignments are shown in Fig. 4. Of note, pig and human IFITMs share the greatest homology, ranging in protein similarity from 73 to 81%. Pig versus mouse and human versus mouse protein

To determine whether human and pig IFITMs also restrict lentiviral vector transduction, we generated six additional doxycycline-inducible stable cell lines. Approximately 60 total monoclonal cell lines were screened for doxycycline-inducible IFITM expression by Western blot, and those cell lines with similar levels of expression were chosen for restriction assays (data not shown). Restriction assays were repeated in the human IFITM1, IFITM2, and IFITM3 and pig IFITM1, IFITM2, and IFITM3 cell lines. We observed that over-expressing IFITMs from pig (Fig. 6A) and human (Fig. 6B) significantly restricted GP64-FIV. VSVG-FIV was also significantly restricted by each IFITM. Conversely, MACV-FIV was not significantly restricted by any of the IFITMs.

Figure 5. Quantitative real-time RT-PCR was used to compare basal mRNA levels of known host restriction factors in the nasal septa and trachea of pigs. The results are plotted as average fold expression of tracheal samples compared with nasal samples $\pm$ standard error. $n=8$.

DISCUSSION

These studies began with the following question: Are IFITM1, IFITM2, and IFITM3 responsible for the less efficient lentiviral vector transduction in mouse intrapulmonary airways as compared with mouse nasal airway epithelia? We observed that

the IFITM proteins restrict GP64-FIV transduction of stable cell lines. In addition, *Ifitm* locus knockout mice were more permissive to GP64-FIV transduction than were hemizygous littermates. These results suggest IFITMs may contribute to the differential transduction of mouse nasal and lung airways. However, a greater increase in luciferase expression was observed in mouse nasal airways despite higher basal levels of *Ifitm1*, *Ifitm2*, or *Ifitm3*. Thus, additional factors must play a role.

Interestingly, we previously reported that lentivirus-mediated gene transfer to the nose and lung of interferon receptor α/β knockout mice was indistinguishable from that to strain-matched wild-type mice.¹⁰ Because IFITMs are induced by interferons, similar results might be expected between the IFITM knockout mice used in this study and the previously reported interferon receptor knockout mice. However, the mice used in the previous study were deficient only in type I interferon signaling and retained functional type II (IFN- γ) and type III (IFN- λ) interferon signaling. All three signaling pathways are thought to induce IFITM expression.¹⁷ These redundancies may explain why the interferon receptor α/β knockout mice were phenotypically identical to wild-type mice in terms of lentiviral gene transfer properties.

We next asked whether IFITM-mediated restriction of lentiviral gene transfer is species specific, and chose to compare pig and human IFITMs. This question is relevant to our goal of engineering a suitable gene transfer vector as a potential therapy for pulmonary diseases such as cystic fibrosis. Importantly, pig models of cystic fibrosis manifest airway disease phenotypes present in humans with cystic fibrosis.^{31,32} Advantages of the pig as a model include lung anatomy, physiology, histology, and biochemistry that are more similar to humans.³³ In addition, pigs share greater DNA sequence homology with humans than do mice, have a larger body size, and longer life spans. Similar to the mouse, we observed that pig and human IFITM1, IFITM2, and IFITM3 all restrict GP64-FIV transduction *in vitro*. However, unlike in mice, there was no difference in basal expression of *IFITM1*, *IFITM2*, or *IFITM3* in tracheal and nasal airway epithelial cells. This is consistent with our previous observation that GP64-FIV transduces pig nasal and tracheal epithelial cells with equal efficiency.¹⁰ We conclude that IFITMs partially restrict transduction of lentivirus-based vectors in both pig and human airway cells. We note that mouse, pig, and human IFITMs also restricted VSV-G-pseudotyped FIV, an envelope that

Figure 6. Stable cell lines expressing IFITM1, IFITM2, and IFITM3 under the control of a doxycycline-inducible promoter were established. (A) Cells stably expressing C9-tagged pig IFITM1, IFITM2, and IFITM3 were incubated without (solid columns) or with (shaded columns) doxycycline for 24 hr, and then challenged with VSV-G-FIV-GFP, GP64-FIV-GFP, or MACV-FIV-GFP for 4 hr. Cells were washed and 4 days later the percentage of GFP-positive cells was quantified by FACS analysis. Results are plotted as average relative transduction compared with the no-doxycycline group $\pm$ standard error. (B) The procedure was repeated for cells stably expressing Myc-tagged human IFITM1, IFITM2, and IFITM3. $n=11$; * $p<0.001$, ** $p<0.00001$.

is widely used in gene transfer applications.^{5,34} Because it is known that IFITMs restrict entry of other enveloped viruses, including influenza, Ebola, and others,^{20–22} our findings likely have broad relevance.

Previous studies investigating IFITM-mediated restriction have focused on replication-competent enveloped viruses.^{20–23} Traditionally, this class of restriction factor likely has evolved to prevent secondary rounds of viral spread. Gene expression is induced after the initial viral infection stimulates an interferon response. However, in this study we investigated the effect on replication-incompetent lentivirus-based vector. In this setting, it is likely that the basal levels of IFITM protein have one chance to prevent the trafficking and fusion steps necessary for endosomal fusion and entry. The overexpression of the IFITMs in the inducible cell lines may represent the “worst case scenario” for viral vector transduction. However, significantly greater levels of transduction were observed in IFITM locus knockout mice, indicating that even basal IFITM expression significantly alters transduction efficiency.

Given the sequence conservation between species, the observation that all IFITMs restricted vector entry similarly is perhaps not unexpected. In addition, neither IFITM1, IFITM2, nor IFITM3 was consistently most restrictive among the three species tested. We note that we first performed these studies using transient transfection of the IFITMs followed by vector delivery (data not shown). In those studies, results varied greatly from experiment to experiment. The generation of stable cell lines with inducible expression was necessary to achieve reproducible results.

Given the observed finding of IFITM-mediated restriction to lentiviral gene transfer, investigating potential methods to transiently knock down or inhibit IFITM function in airway epithelial cells

could provide a means for temporarily improving viral vector-mediated gene transfer. In fact, studies suggest that amphotericin B may provide such a reagent.^{35,36} Using technologies such as the Connectivity Map,³⁷ gene expression profiles from cells with and without IFITM expression could be used as queries to identify candidate drugs for screening in future work. Transient knockdown of IFITMs, using small interfering RNA (siRNA) technologies, is a less appealing strategy because of the resulting interferon stimulation that simultaneously augments antiviral defenses, including IFITMs.

In conclusion, our results identify the IFITM family of proteins as novel barriers to GP64-mediated lentiviral gene transfer. These results are consistent across species and have potential therapeutic implications for improving gene delivery to correct genetic diseases affecting the airway epithelium.

ACKNOWLEDGMENTS

The authors thank Anna Locke for technical assistance. The authors thank Kun Li and Ashley Cooney for critical comments on the manuscript. The authors also acknowledge the support of the University of Iowa Genomics Division, *In Vitro* Models and Cell Culture Core, Viral Vector Core, and Cell Morphology Core. This work was supported by National Institutes of Health R01 HL105821 (P.L.S.) and P01 HL051670 (P.B.M.). Core facilities at the University of Iowa were partially supported by the National Institutes of Health (P01 HL51670 and P01 HL091842) and by the Center for Gene Therapy for Cystic Fibrosis (P30 DK54759).

AUTHOR DISCLOSURE

No competing financial interests exist.

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Received for publication December 1, 2015;
accepted after revision March 19, 2016.

Published online: March 19, 2016.