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Reducing Influenza Virus Infection through shRNA-Targeting TMPRSS2 Protease

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Cleavage of influenza A virus hemagglutinin (HA) via host cell proteases is required for entry into the host cell. The serine protease, TMPRSS2, has been implicated as having an important role in HA cleavage and activation. To better understand the role of TMPRSS2 for avian, human and swine influenza virus infection, a lentivirus was constructed containing a doxycycline-inducible small hairpin ribonucleic acid (shRNA) to inhibit the TMPRSS2 gene via RNA interference (RNAi). This construct was used to transduce influenza-susceptible human cell lines with shRNA, and other necessary genes to facilitate development of a stable cell line. Transduced cells were isolated by cell sorting using flow cytometry, and puromycin selection was used to create cloned cell lines. TMPRSS2 gene silencing was validated by PCR. Despite blocking the production of TMPRSS2, human influenza virus infection was not substantially ($p > 0.05$) reduced, suggesting that other cellular proteases may compensate. Based on the insignificant reduction of infection, we can conclude that TMPRSS2 protease may not be the only protease used by influenza virus to cleave HA for infection. Other proteases may work alongside TMPRSS2 protease in the infection process.

INTRODUCTION

Influenza A virus causes seasonal outbreaks of mild to severe respiratory tract infection that can cause 3,000 to 49,000 deaths in the US alone, and between 250,000 to 500,000 deaths a year throughout the world (Mueller et al., 2010). Influenza virus is in the *Orthomyxoviridae* family, which contains three genera: influenza A, B, and C (Gangurde et al., 2011). Typically, humans are infected with influenza A and B viruses, while group C rarely causes illness (Potter, 2002). The most recent notable incidence of an influenza virus pandemic is the swine-origin H1N1 pandemic that emerged in Mexico in 2009 and took the lives of 7,000-13,000 individuals throughout the world (Secencan et al., 2011; Korteweg & Gu, 2010).

Influenza viruses are enveloped and contain a segmented, single-stranded, negative-sense ribonucleic acid (RNA) genome (Liu & Zhang, 2010). The virus acquires its envelope from the host cell membrane during budding, and has a morphology that can be pleomorphic or spherical with a diameter of 800-1200 angstroms (Potter, 2002). The genome of the virus consists of seven or eight segments of linear negative-sense RNA that code for 11 proteins (Chiu et al., 2003). The outer envelope consists of two major glycoproteins, which are hemagglutinin (HA) and neuraminidase (NA), and the less prominent matrix protein. The HA and NA are major antigenic determinants of the virus and their variation is responsible for the appearance of new epidemic and pandemic strains of influenza (Potter, 2002).

The influenza virus HA is the viral attachment protein. The HA also has an important role in the release of the viral RNA into the cell by causing fusion of viral and cellular membranes. HA must be cleaved by cellular proteases to be active as a fusion protein. The interaction between the virion and the sialic acid receptors on host cells leads to virus endocytosis, and the acidic pH in the endosome initiates a structural change in HA causing viral and endosomal membrane bonding (Garten & Klenk, 2008). During the fusion process, the precursor HA glycoprotein (HA0) is cleaved in two subunits: HA1 and HA2 (Potter, 2002; Garten & Klenk, 2008). These two subunits remain together at the surface of the virus particle after the cleavage process (Garten et al., 2004). The new N-terminal end of HA2 display a sequence of hydrophobic amino acids called fusion peptide, which is inserted into the endosomal membrane and causes fusion of the viral and cell membranes (Bosch et al., 1981). Consequently, the influenza viral RNAs can enter the human cell cytoplasm (Skehel & Wiley, 2000; Steinhuer, 1999; Garten et al., 2011). The cleavage sites present in HA vary between viral strains – a feature that contributes to virus spread, pathogenicity, and tissue tropism (Garten et al., 2011). Viral spread is restricted by tissue type because proteases that can cleavage HA are tissue specific (Bottcher-Friebertshauser et al., 2010). Most influenza viruses, including the H1, H2, and H3 subtypes typically infecting humans, require activation by trypsin-like proteases (Bosch et al., 1981). Trypsin-like proteases such as TMPRSS2 (transmembrane proteases serine S1 member 2) and HAT (Human airway trypsin-like protease) are known to be present in human airway epithelial cells and their full-length coding sequences are also known (Bottcher et al., 2006; Shulla et al., 2011). TMPRSS2 protease was evaluated in this study because TMPRSS2 has been shown to play an important role in

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facilitating influenza infections (Bottcher et al., 2006). Studies have not been conducted to test the role of HAT in influenza infections.

Based on prior studies, we hypothesized that stopping TMPRSS2 protease from being produced would reduce the infection of influenza since influenza requires this protease to cleave HA and gain entry into the host cell. In order to more thoroughly study the role of TMPRSS2 in influenza virus replication, a stable cell line expressing small hairpin RNA (shRNA) against TMPRSS2 protease was generated using a lentivirus construct. The lentivirus was used to transduce human alveolar basal epithelial (A549) cells using a cassette containing a shRNA against TMPRSS2, red florescent protein, puromycine selection gene, and doxycycline-inducible gene. If the results indicate a significant reduction of infection in cells with TMPRSS2 protease knocked out, it would open up the possibilities of a more permanent vaccine development rather than developing a new vaccine seasonally.

METHODS AND MATERIALS

Plasmid construction and purification:

A plasmid containing doxycycline selection, red fluorescent protein, puromycin selection, and a shRNA cassette expressing shRNA targeting the TMPRSS2 gene was used (OpenBioSystems, Lafayette, CO). The plasmid was transfected into competent *E. coli*, and the E. *coli.* was cultured in 10 mL of LB Broth containing 0.001 g carbenicillan added for selection of the gene of interest. The *E. coli* culture was placed on a shaker to facilitate bacterial growth at 37°C for 24 hours. An Accuprep Plasmid Extraction kit (Bioneer USA AccuPrep^{TM} SERIES, Alameda, CA) was utilized for the extraction of the plasmid from *E. coli.* A Nanodrop spectrophotometer (ThermoFisher Scientific, Pittsburgh, PA) was used to determine the deoxyribonucleic acid (DNA) plasmid concentration (ng/µL) by UV absorbance 260-280 µm.

Cell Culture (HEK-293 and A549):

Human embryonic kidney (HE293) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, Logan, UT) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, Logan UT), while human type II basal epithelial (A549) cells were grown in DMEM with 5% FBS. The cells were maintained at 37°C in 5% CO_2 environment and were sub-cultured with fresh media at 48-72 hour intervals.

Lentiviral Transduction:

One day prior to lentivirus transduction, two six-well flat-bottom tissue culture plates (Corning, St. Louis, MO) were seeded with HEK-293 at 5.5 x 10^5 cells/well. On the day of transduction, complexes were made where each plate consisted of DNA (30 µL packaging mix stock + 9 µg plasmid DNA) diluted into 1 mL serum-free media. This was subsequently diluted by transferring 187.5 µL Express-In into 1 mL serum-free media in a 15 mL conical tube, and the DNA and the media were then added to Express-In mixture (ThermoFisher Scientific, Pittsburgh, PA) and incubated for 20 min. at room temperature. Subsequently, 4 mL of serum-free media was added to the tube containing DNA/Express-In complex. After aspirating the media from HEK-293 cells in the six-well plates, 1 mL of the complex was added to each well. The plates were incubated at 37°C for 3-6 hours, after which the transfection media was aspirated and replaced with 2.5 mL of standard (10% FBS, DMEM) per well and the plate was returned back to the incubator. At 48, 72, 96, and 120 hours post-transfection, the media was collected from each plate and stored at -80°C. The efficiency of transduction was determined using a flow cytometer. No control was used during the procedure due to the known high efficiency of lentivirus in transduction.

Virus Purification:

A lentivirus purification step was performed using a Millipore Fast-Trap Lentivirus Purification and Concentration Kit (Billerica, MA). Briefly, the viral supernatants collected from transfection were filtered by passing through a series of Steriflip-HV units. After using the buffers provided in the kit, viral supernatant was passed through a charged filter unit to allow the virus to bind to the filter. The filter was washed to collect the purified and concentrated virus.

Transduction:

The purified lentiviral particles collected were used for transduction of the plasmid of interest into A549 cells. One day prior to transduction, T-25 flasks were prepared so that the cells were 50-60% confluent the next day. A mixture of purified virus and serum-free media (1 mL virus and 1 mL serumfree media) was added to these cells and incubated for 6-8 hours in standard media (5% FBS, DMEM).

Growth tube number	Extracted Plasmid (ng/uL)
1	170.8
2	212.2
3	151.4
4_2	180.3

Table 1: Plasmid Extraction. Plasmid of interest (transfer vector) containing the doxycycline selection, red fluorescent protein, puromycin selection, and shRNA cassette that expressed TMPRSS2 gene-targeting shRNA was delivered by OpenBio Systems and cultured in 4 growth tubes containing *E. coli*. A total of 714.7 ng/µL of plasmid was extracted and used to create the Lipofectamine complexes.

Induction:

The plasmid was induced by the addition of doxycycline (Fisher Scientific, Fair lawn, New Jersey). A stock solution of 0.001 g doxycycline and 10 mL of 5% media was prepared, and 400 µL of this solution was added to 10 mL of 5% media. Subsequently, 5 mL of this diluted solution was added to a T-25 flask

containing A549 cells. For selection of transfected A549 cells, puromycin (Fisher Scientific, Fair Lawn, New Jersey) was employed. Briefly, 13 µL of puromycin was added for every mL of media. Subsequently, a Becton Dicinson FACSCalibu flow cytometer and cell sorter (BD BioScience, Franklin Lakes, NJ) with green laser was utilized to sort the top 5% auto-fluorescing cells (No antibodies were used).

Polymerase Chain Reaction (PCR):

Cellular RNA was purified using the RNeasy kit (Qiagen, Valencia, CA), and complementary DNA (cDNA) was synthesized using the SuperScript First Strand cDNA synthesis kit (Invitrogen) or the Verso cDNA synthesis kit (Invitrogen). PCR was performed using the following primers: TMPRSS2_F: CAG GGT CAC CAC CAG CTA TT, TMPRSS2_R: CCG CTG TCA TCC ACT ATT CC, ACTB_F: GGC ATC CAC GAA ACT ACC TT, ACTB_R: AGC ACT GTG TTG GCG TAC AG. To assess the efficacy of siTMPRSS2 on influenza virus replication, quantitative real-time PCR (qPCR) was used to quantify copies of the influenza matrix protein (M) gene present in the cells. PCR was performed using the amplification cycle: 10 minutes at 95ºC followed by 40 cycles of 95ºC for 30 seconds, 60ºC for 1 minute, and 72ºC for 30 seconds. Gene copies were normalized to a standard curve.

Hemagglutination assay:

Induced and non-Induced Madin Darby canine kidney (MDCK) cells were plated at 5 x 10^5 cells/well in a 96-well plate. Cell monolayers were infected with A/New Caledonia influenza virus or A/WSN/33 influenza virus using 10-fold higher MOI of 0.5 MEM/0.3% BSA/2 µg/mL TPCK-trypsin. Hemagglutination assay was performed using the MDCK cell supernatant with 0.5% chicken red blood cells (cRBCs).

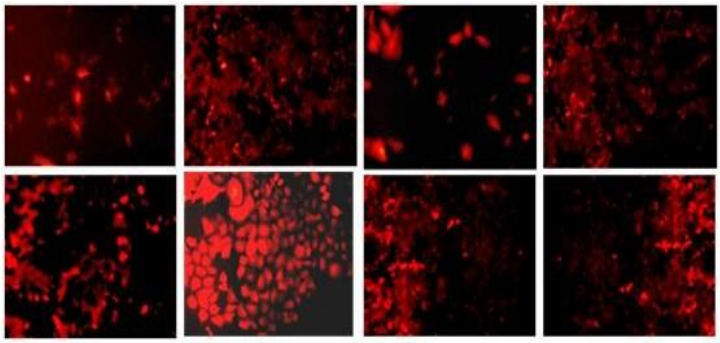

Figure 1: Florescence microscopy images showing successful plasmid transduction. The separated sub-cloned cell lines were created from the transfected A549 cell lines by puromycin selection and cell sorting. Successful expression of plasmid of interest is demonstrated by the red florescence when induced by doxycycline.

Cellomics Arrayscan:

Infected cells were fixed in ice cold methanol: acetone 20:80 solution for 10 min. and stained with a 5 µg/mL anti-NP (nucleoprotein) monoclonal antibody H16-L10-4R5 (Biovest International, Tampa, Florida). This is then followed by antibody staining, which is detected using AlexaFluor 488-labeled goat anti-mouse IgG (1 µg/mL; Invitrogen, Carlsbad, CA). Cells were counterstained with DAPI (2 µg/mL) (Invitrogen, Carlsbad, CA) and visualized by immunofluorescent microscopy (EVOS digital inverted fluorescent microscope, Advanced Microscopy Group, Bothell, WA).

RESULTS

Transfection of HEK-293 cells and transduction of A549 cells:

Approximately 714.7 ng/µL plasmid was extracted from *E. coli* (Table 1). During transfection, 120 mL of media containing the lentivirus construct was collected from the supernatant of HEK-293 cells. After concentration of the lentivirus using a standard centrifugation enrichment method, the lentivirus was used to insert the plasmid into A549 cell line. The transfected A549 cells were split into eight different sub-cultured cell lines which were cultured separately. The transduction of eight clone cell lines was successful as demonstrated by the images taken with a florescence microscope (Figure 1). The red florescence shows that plasmid transduction was successful and that the plasmid is inducible using doxycycline.

Infected wells per 8 replicates at 1 MOI		
New Caledonia	Induced	0/8
	Uninduced	5/8
WSN	Induced	8/8
	Uninduced	8/8

Table 2: Lower influenza titer found in TMPRSS2 suppression. Hemagglutination assay results were determined following 48 hours of infection. During the Hemagglutination assay, virus was found only at MOI of 1.0. The results indicate a decrease in infection for New Caledonia influenza virus in the induced (TMPRSS2 suppression) compared to the non-induced clones. Infection by WSN influenza virus, acting as our control, shows no differentiation between induced and non-induced clones.

PCR validation:

PCR was carried out on the eight cell lines created to test the difference in the production of TMPRSS2 RNA between the doxycycline-induced and non-induced clones PCR results indicated that the doxycycline-induced cells produced less TMPRSS2 RNA than the non-induced clones (Figure 2).

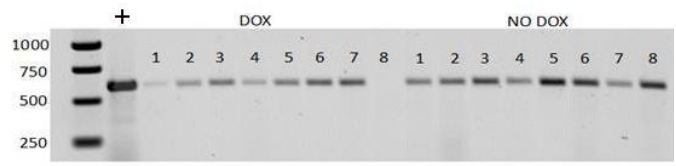

Figure 2: Doxycycline-induced cells produce less TMPRSS2 RNA compared to non-induced cells. PCR was carried out on eight A549 cell lines to test the difference in TMPRSS2 RNA expression between doxycycline induced (DOX) and non-induced (NO DOX) clones. The gel visually illustrates that doxycycline-induced cells produce less TMPRSS2 RNA than the non-induced clones. Samples were run on a 1% agarose/TAE gel for 2 hours at 100 V. Positive control (+) contains TMPRSS2 over expressing plasmid.

shRNA targeting TMPRSS2 reduces A/New Caledonia influenza virus infection:

To better understand the results, a quantitative analysis was carried out by looking at the band density. Doxycycline induction of the cloned cell lines was found to mediate a higher level of expression of TMPRSS2 shRNA (Figure 5). To evaluate the activity of the transfected cells, testing was carried out with subclone number 6 because of the high level of TMPRSS2 shRNA expression that was revealed by PCR compared to the other subclones. The induced and non-induced versions of clone 6 were infected with two different strains of influenza virus, either A/New Caledonia or A/WSN influenza virus. WSN influenza virus was utilized as a positive control because it does not require trypsin for infection. After 48 hours of infection at a multiplicity of infection (MOI) of 1.0, the influenza virus titer was determined by hemagglutination assay (Table 2) and by immunohistochemistry staining of the influenza N protein using a Cellomics Arrayscan (Figure 4). Results from these studies suggest the notion that TMPRSS2 has an important role in facilitating influenza virus replication, a feature shown by lower levels of New Caledonia influenza virus replication during TMPRSS2 shRNA induction.The positive control showed no reduction in infection, which was expected for WSN. The results from the Cellomics Arrayscan supported the findings from the hemagglutination assay shown in Table 2.

DISCUSSION

Previously studies have suggested that TMPRSS2 (transmembrane proteases serine S1 member 2) is involved in the infection and replication of influenza virus. TMPRSS has been shown to cleave the HA0 glycoprotein found on the viral envelope into HA1 and HA2 to facilitate the conformational change necessary for the virus to gain entry into the host cell by endocytosis (Potter, 2002; Garten & Klenk, 2008; Skehel & Wiley, 2000; Garten et al., 2004). Therefore, to better understand how the tempo of TMPRSS2 expression affects the influenza virus infection and replication, an inducible lentivirus construct expressing shRNA targeting the TMPRSS2 gene was constructed. The PCR results showed a reduction in TMPRSS RNA for the doxycycline-induced clones and this feature was linked to reduced virus replication. After PCR (Figures 4 & 5), a hemagglutination assay (Table 2) and Cellomics Arrayscan analysis (Figure 4) were used to show that there was indeed a reduction of infection between induced and non-induced cell clones.

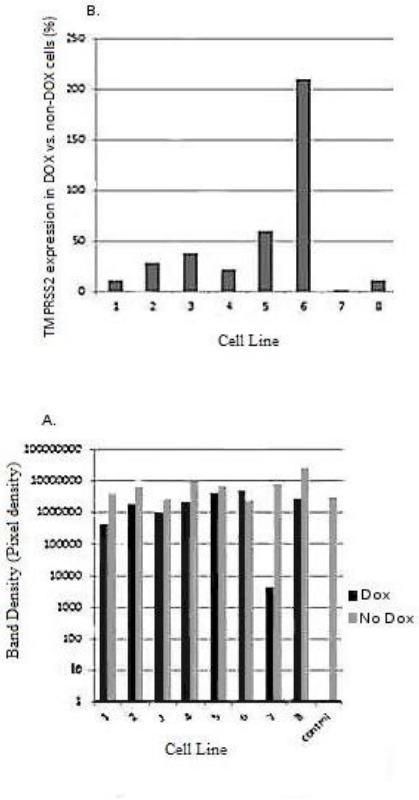

Figure 3: Quantitative analysis of PCR gel results using density analysis. There is a consistent trend of lower band density for doxycycline-induced clones compared to non-doxycycline induced clones (Figure 5a). This difference is better visualized in Figure 5b, where the density analysis is displayed in percent values ((doxycycline induced/non-induced) x 100%).

Even though our results indicate a reduction of influenza virus, the reduction is not as substantial as would be expected if TMPRSS2 protease was the only protease involved in cleaving HA0 and initiating the infection of influenza virus (Chiu et al., 2003). If TMPRSS were the only protease to be involved with virus entry, the reduction of infection by influenza virus would have been much more drastic (Shulla et al., 2011). This smaller

reduction implies that other proteases may be facilitating or synergizing with TMPRSS activities to achieve the conformational change needed in HA for the virus gain cell entry and thereby compensating for the loss of TMPRSS protease. More information may be gained by carrying out the activity testing with one of the other eight cell lines to see if the reduction of infection is consistent. This would also allow us to see whether there is direct correlation between the amount of TMPRSS2 silencing demonstrated by PCR and the reduction of infection seen. Since our results indicate that there may be other proteases working alongside TMPRSS2, we can carry out similar studies with other likely candidates to more precisely determine the roles of other proteases in the process of influenza infection.

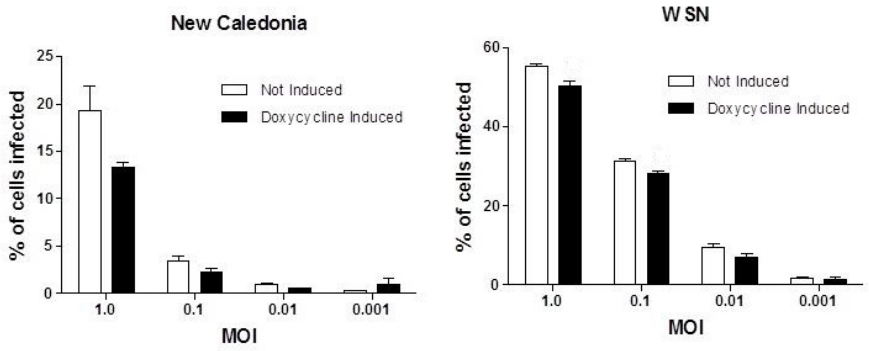

Figure 4: Comparison of Cellomics Arrayscan for detection of N protein in New Caledonia and WSN-infected cells. The results indicate a decreased rate of influenza infection for doxycycline-induced clones at various MOIs between 1.0 and 0.01. The most drastic decrease was found at 1.0 MOI.

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