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Sensitivity of cervical carcinoma cells to vesicular stomatitis virus-induced oncolysis: potential role of human papilloma virus infection

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High-risk carcinogenic subtypes of human papilloma virus (HPV) are associated with the development of squamous cell carcinomas of the cervix (CC) and a subset of head and neck (HNSCC). Recurrent metastatic diseases of these sites display a dismal prognosis. Therefore, there is an urgent need to uncover innovative therapeutic strategies in this clinical setting. Oncolytic viruses, including vesicular stomatitis virus (VSV), were identified due to their ability to specifically target tumor cells that generally display defects in interferon (IFN) signaling. HPV expressed proteins can inhibit IFN signaling; therefore, HPV-infected cells may be particularly sensitive to VSV oncolysis. In this study, we evaluated the sensitivity of four CC (HPV+) and four HNSCC (HPV−) derived cell lines to VSV oncolysis. Interestingly, the CC cell lines were consistently more sensitive to VSV cytotoxicity than the HNSCC cell lines tested. Exogenous IFN addition or infection with two attenuated VSV variants that are more susceptible to IFN inhibition failed to attenuate VSV oncolysis in hypersensitive CC cell lines. Furthermore, the expression of HPV-E6, that inhibits IFN receptor signaling, in the VSV-resistant HNSCC cell line SCC25 attenuated VSV-induced IFN response and significantly enhanced VSV cytotoxicity. Finally, differential VSV infection and replication was confirmed in xenograft murine tumor models and explant tumor tissues from two patients with CC. Taken together, these results demonstrate that HPV-infected cells are susceptible to oncolytic virus therapy and that this approach may represent a novel therapeutic approach in HPV positive CC and HNSCC patients.

Human papilloma viruses (HPV) are small double-stranded DNA tumor viruses that replicate in differentiating keratinocytes of the skin and anogenital tract.[1–3] Three oncogenes, E5, E6 and E7, modulate the transformation process, two regulatory proteins, E1 and E2, modulate transcription and replication and two structural proteins, L1 and L2, form the viral capsid.[4] E6 and E7 proteins delay keratinocyte differentiation, trigger host DNA synthesis and enhance cell cycle progression, hijacking host DNA synthetic enzymes to replicate its genome. HPVs induce benign warts and papillomas; however, infection with high-risk types (HPV-16, −18, −31 and −45) is a major risk factor for the development of 100% of cervical carcinomas (CC), 50% of other anogenital carcinomas, 20% of head and neck squamous cell carcinomas (HNSCC) and may also play a role in oropharyngeal cancer.[5]

The E6 and E7 genes are of particular importance as they are retained and expressed in most CC and their continued expression is required to maintain the malignant phenotype.[6] An important target for E6 is E6-associated protein, a protein-ligase of the ubiquitin pathway that targets the tumor suppressor protein p53 for degradation. Loss of p53 leads to genetic instability and rapid malignant progression.[5,7] The E7 protein binds to the retinoblastoma protein and members of the retinoblastoma family enhancing cell cycle progression and DNA synthesis.[6] E6 and E7 exert overlapping effects on cell cycle control, and in combination, they efficiently immortalize human keratinocytes.[8] More recently, E6 has been shown to inhibit the cellular anti-viral interferon (IFN) response as its binding to Tyk2 inhibits its interaction with

Key words: cervical cancer, human papilloma virus (HPV), vesicular stomatitis virus (VSV), interferon, oncolytics
Abbreviations: CC: carcinomas of the cervix; HNSCC: squamous cell carcinomas of the head and neck; HPV: human papilloma virus; IFN: interferon; IRF: interferon regulatory factor; MOI: multiplicity of infection; VSV: vesicular stomatitis virus
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the Type I interferon receptor (IFNAR). Through this mechanism, IFN-induced activation of IFNAR and its downstream signaling cascades is inhibited by E6.[9] Recurrent and metastatic CC and HNSCC present with a very poor prognosis with limited treatment options.[10] Novel therapeutic approaches are required for effective treatment of these aggressive cancers.

Oncolytic viruses (OV) hold great promise to improve cancer patient outcomes through their tumor selective replication and multi-modality attack against cancers.[11–13] To date, clinical trials have demonstrated that OV-based therapeutics are well tolerated compared to conventional chemo- and radiation therapies.[14] However, limitations to systemic delivery and reduced efficacy against various resistant tumors have limited their use in a clinical setting.[14] Nevertheless, the reporting of clinical activity of a number of OVs has spurred further clinical evaluations.[15–19] Yet there remain challenges to the widespread use of replicating biological therapeutics; two important challenges are to overcome the innate and adaptive immune responses of the patient and to identify a patient population that would likely respond to this form of therapy.[14]

Vesicular stomatitis virus (VSV) is a small-envelope negative strand RNA virus that has proven effective in targeting tumors in several murine models.[20–22] While effectively replicating in cancer cells, wild type VSV does not infect normal mammalian cells efficiently as it is particularly sensitive to Type I IFN-induced cellular immunity.[21] The IFN system is critically important in preventing the unrestrained replication of a wide variety of viruses, including VSV.[23] There are two main families of IFNs referred to as Type I (α,β) and Type II (γ). Type I IFNs can be induced by a number of stimuli, including viruses, bacteria, dsRNA, growth factors and cytokines.[23] The IFN pathway is often defective in transformed cells lines as this signaling cascade is important as well in the regulation of cell growth.[20,24] Other antiviral regulatory mechanisms are similarly deregulated in cancer cells and play a role facilitating VSV replication as well.[25] The concomitant loss of antiviral host defense is an important mechanism that drives the susceptibility of tumor cells to VSV infection and oncolysis.

The selectivity of OV for tumor cells defective in their IFN response is enhanced by strategies that activate antiviral responses in normal cells.[26–28] VSV induces relatively little IFN production in infected cells due to the inhibition of host gene expression by the viral matrix (M) protein that blocks nuclear-cytoplasmic export of cellular mRNAs inhibiting the IFN driven expression.[29,30] A number of different mutations render the M protein defective in its ability to inhibit host gene expression without compromising its ability to function in virus assembly.[21] Viruses containing these M protein mutations are attenuated (VSV-AV); however, they can induce an IFN response in infected cells specifically protecting normal cells enhancing the therapeutic index of this OV.[21]

Therefore, VSV is an oncolytic agent that has specificity for tumor cells that have acquired defects in IFN signaling.[21] However, these defects are not well characterized with a variety of potential targets that can regulate IFN signaling and response and as yet there is no reliable marker of VSV oncolytic susceptibility. Due to its lack of inhibition of the IFN response in normal cells, VSV-AV represents an ideal anticancer therapeutic agent. In CC and HNSCC, HPV infection plays a role in their pathogenesis and represents an ideal clinical setting to evaluate VSV efficacy. HPV infection itself targets IFN response through E6 expression that is required to maintain cellular malignancy.[31,32] Inhibition of E6 expression induces apoptosis in CC cells, thus, tumor acquired resistance to VSV may be difficult to achieve. In this study, we examined the sensitivity of CC- and HNSCC-derived cell lines, xenografts and explant tumor tissue to VSV and VSV-AV infection, replication and oncolysis. We also evaluated the ability of VSV to infect human CC tissues *ex-vivo*. The results obtained suggest the utility of VSV as a therapeutic agent specifically in HPV-infected tumors, notably CC and a subset of HNSCC.

Material and Methods

Tissue culture

The SCC9, SCC25, FADU and Cal27 HNSCC were obtained from the ATCC (Rockville, MD). The CaSki, SiHa, HeLa and ME180 CC cell lines were provided by Dr. D. Hedley (University Health Network, Toronto). All cell lines were maintained in Dulbecco's-MEM (Media Services, Ottawa Regional Cancer Centre) supplemented with 10% fetal bovine serum (Medicorp, Montreal). The HPV status of each of these cell lines was confirmed by Dr. Patti E. Gravitt (John Hopkins School of Public Health, Baltimore, MD), as previously described.[33] The Indiana serotype of VSV was used throughout this study and was propagated in Vero cells. M mutant attenuated viruses AV1 and AV2 as well as the AV-green fluorescent protein (GFP) derived from the Indiana serotype of VSV were rescued and propagated as previously described.[21] SCC25-E6 expressing cells were retrovirally transduced with the pLPCX expression vector (provided by Dr. Bruce McKay, Ottawa Hospital Research Institute, Ottawa) containing E6 (HPV 18) and neomycin resistance genes. G418 treatment of 500 μg/ml for 2 weeks was used to select an E6 expressing pool of SCC25 cells. Real-time polymerase chain reaction (PCR) for HPV 18-E6 was done as previously described[34] with the following modifications; total RNA was isolated from HeLa, SCC25 and SCC25-E6 cells, with TRIZOL (Invitrogen Life Technologies, Carlsbad, CA) and the reverse transcription (RT) reaction was preformed using High Capacity cDNA Reverse Transcription kit (Invitrogen). RT-PCR conditions for amplifying cDNAs of E6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH); 95′ for 2 min, 35 cycles of 95′ for 30 sec, 60′ for 30 sec and 72′ for 30 sec, followed by 1 min of 72′. cDNA samples were run on 1.5% agarose gel. Following primers sequences were used for RT-PCR reactions: HPV 18E6 GenBank number AY_262282 forward 5′-CCAGAAACCGTTGAATCCAG-3′ reverse 5′-GGAGTCTTT CCTGTCGTGCT-3′; GAPDH GenBank number NM_002046 forward-5′-GAAGGTGAAGGTCGGAGTC-3′ reverse-5′-GAA

GATGGTGATGGGATTTC-3′. IFNα levels in media were determined following VSV infection (multiplicity of infection, MOI = 1) employing the Human ELISA IFNα detection kit (PBL Interferon Source, Piscataway, NJ). Two independent experiments were performed each with quadruplicate readings. The average of the eight readings is presented with the error as the SD of the mean.

MTT assay

In each experiment, the test cell line was seeded into 96-well plates at 5×10^4 cells/well in growth medium. Following overnight incubation, media were removed by aspiration and to each well was added 20 μl of virus-containing media (no serum) ranging in 10-fold increments or negative control media containing no virus (six replicates per dose). After a 60-min incubation to allow virus attachment, 80 μl of growth medium was added to each well, and the plates were incubated for another 48 hr. Following treatment, 50 μl of a 5 mg/ml solution in phosphate buffered saline of the MTT tetrazolium substrate (Sigma, St. Louis, MO) was added and incubated for up to 6 hr at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 100 μl of a 0.01M HCl/10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS) (Sigma) solution shaking overnight at 37°C. The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies at 570 nm to determine the optical density of the samples. To assay for IFN affects, cell lines were pretreated with 100-units/ml recombinant IFN-α (Sigma-Aldrich, St. Louis, MO) for 12 hr and then challenged with a range of doses of VSV.

Western blot analysis

Total cellular protein was extracted using a buffer that consisted of 1% Igepal CA-630 (Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (Sigma), 0.2 mM sodium orthovanadate (Sigma-Aldrich) and 0.2 mM phenyl methyl sulphonyl fluoride (Sigma-Aldrich) in 2× phosphate buffered saline (PBS). Approximately 200 μl of extraction buffer was used to treat 10^6 cells. Total protein was quantified with the Biorad Protein Assay using bovine serum albumin (Sigma-Aldrich) as standard. Protein extracts representing 50 μg total protein were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham, Toronto). Membranes were blocked in 5% skim milk powder in PBS 1 hr at room temperature. Primary antibodies specific for IRF7 (Santa Cruz Biotechnology, Santa Cruz, CA), p-IRF3 (Cell Signaling, Danvers, MA), IRF3 (Santa Cruz), MX2 (Santa Cruz) and actin (Sigma-Aldrich) were diluted in 5% skim milk powder in PBS and incubated with the membrane over night at 4°C. The secondary antibodies (Bio-Rad, Mississauga, Canada) were applied at a 1:5,000 dilution in 3% bovine serum albumin (BSA), 10% fetal bovine serum (FBS) in PBS and incubated for 1 hr at room temperature (washes following antibody incubations are 3× 5 min in PBS/0.05% Tween 80 (Sigma-Aldrich) then processed for chemiluminescent detection (Amersham, Oakville, Canada). After the desired exposure was obtained the membrane was stained with Coomassie Blue (Sigma-Aldrich) to ensure equal loading of the samples.

Xenograft and tumor slice evaluations

Mice were obtained from Charles River Laboratories. HeLa (3×10^6 cells) and SCC25 (1×10^7 cells) tumors were established subcutaneously in 6-week-old CD1 female nude mice ($N = 5$). Palpable tumors were formed within approximately 10 days after seeding. VSV-AV1-RFP was administered IV (1×10^7 pfu/mouse). Mice were sacrificed 3 days after injections and RFP associated to the virus was detected by microscope (Leica M205FA). For Immunohistochemistry, formalin-fixed SCC25 and HeLa-treated xenografts were paraffin embedded and cut into 5 μm sections. Sections were deparaffinized, treated with 3% H_2O_2 in tris buffered saline (TBS) for 10 min, rinsed in TBS for 5 min, blocked with universal blocking agent Background Sniper (Biocare Medical; Brampton, ON, Canada) for 20 min at room temperature, incubated with polyclonal antibodies specific to either VSV[35] (1:5,000 dilution) or caspase 3 (BD Bioscience, Mississauga, ON, CA, dilution 1/500) in DaVinci universal diluent (Biocare Medical) for 1 h at room temperature, incubated with Rabbit HRP Polymere (Biocare Medical) for 10 min at room temperature and developed for 5 min with the 3,3-Diaminobenzidine (DAB) chromatogen kit (Biocare Medical, Concord, CA). Slides were counterstained in hematoxylin for 1 min and mounted on cover slips with permount. All experiments were performed in accordance with institutional guidelines review board for animal care (University of Ottawa).

Explant preparation, culture, infection and titration

Primary CC tissue specimens were obtained from consenting patients who underwent tumor resection. Tissue specimens were processed within 48 hr post-surgical excision. Samples were sliced to a thickness of about 0.5 mm using a sterile vibratome, placed in 12-well plates, cultured with αMEM containing 10% fetal bovine serum and incubated at 37°C. The following day, slices are incubated with VSV-AV1 (10^8 pfu) that express GFP. Samples were monitored microscopically 24 hr later. After the indicated treatment condition, samples were weighed and homogenized in 0.5 ml of PBS using a homogenizer (Kinematica AG-PCU-11). Serial dilutions of tissue preparations were prepared in serum-free media and viral titers were quantified by standard VSV plaque assay.

VSV titration from in vitro cancer cells, in vivo mouse tumor and ex vivo patient tissue samples

Supernatants from each treatment condition were collected at the specified time point. A serial dilution was then performed in DMEM and 200 μl of each dilution was applied to a confluent monolayer of Vero cells for 45 min. Subsequently, the plates were overlaid with 0.5% agarose in DMEM-10% FBS and the plaques were grown for 24 hr. Conroy fixative

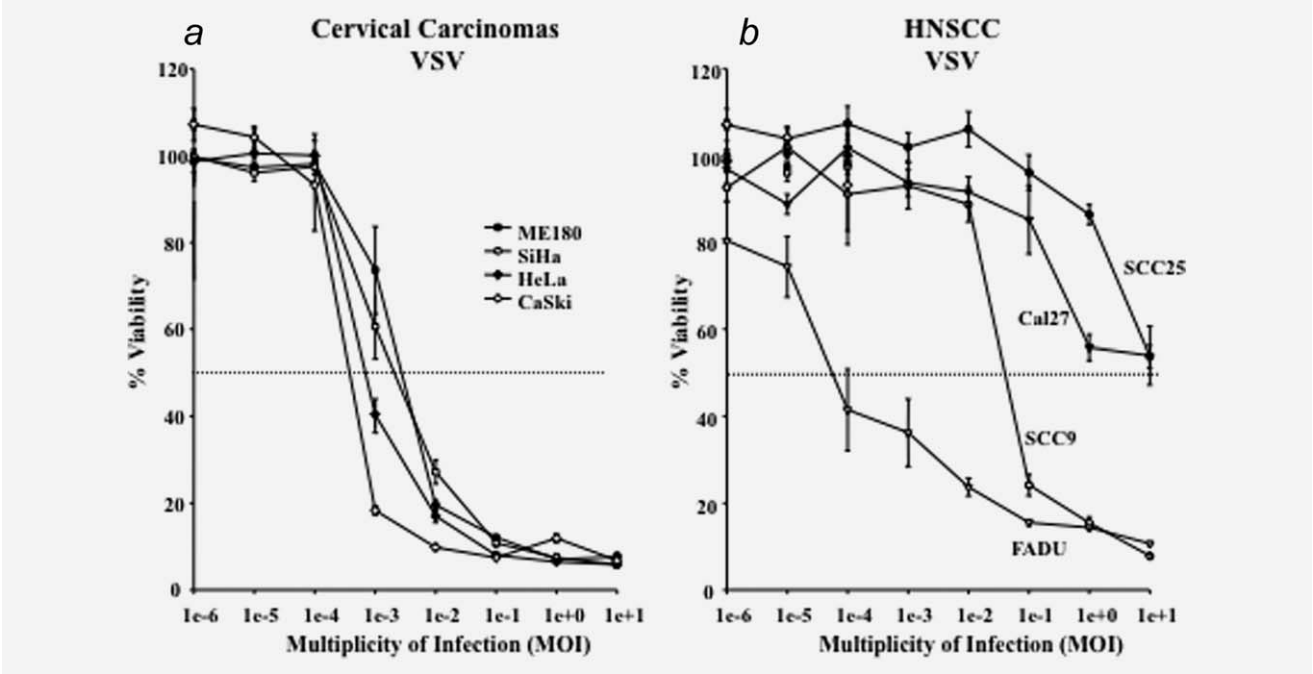

Figure 1. CC and HNSCC cell lines display differential sensitivity to VSV. (*a*) CaSki, SiHa, HeLa and ME180 CC cell line were evaluated for VSV-induced cytotoxicity as determined by the MTT cell viability assay. After overnight incubation, cells were exposed to VSVHR-containing media ranging in 10-fold increments or negative control media containing no virus was added for 48 hr. The plates were then analyzed reading at 570 nm to determine the optical density of the samples. Each virus dose tested was done in replicates of six with three independent experiments performed yielding similar results. (*b*) The SCC9, SCC25, FADU and Cal27 HNSCC cell lines were processed as in (*a*) and similarly evaluated using the MTT assay.

(methanol:acetic acid in a 3:1 ratio) was then applied directly on top of the overlay for 5 min. The overlay was gently lifted off using a spatula and the fixed monolayer was stained with 0.5% crystal violet for 5 min, after which the plaques were counted.

This study was approved by the Institutional Review Board of the Ottawa Hospital Research Ethics Board (Protocol for Obtaining Tumor Tissue for Research from Patients with Solid Organ Malignancies, Dr. John Bell). All patients provided written informed consent for the collection of samples and subsequent analysis. The animal work was approved by the appropriate committee (University of Ottawa, Animal Care Committee, ME-220 protocol, Dr. John Bell).

Results

Cervical carcinoma cells are sensitive to VSV oncolysis

In order to evaluate the potential of prior HPV infection as a mechanism of enhancing the oncolytic properties of VSV, we evaluated VSV-induced cytotoxicity in a panel of four CC- and four HNSCC-derived cell lines. Cells were infected at various multiplicity of infection (MOI) and the percentage of cell survival was determined by the MTT cell viability assay. These cell lines were tested for their susceptibility to infection and oncolysis by the heat-resistant Indiana strain of VSV.[20] Interestingly, the results show differential sensitivity for VSV oncolysis between these sets of tumor cells. The CC cells (ME180, SiHa, HeLa, CaSki) were exquisitely sensitive to VSV-induced cytotoxicity with a 50% lethal MOI between 1×10^{-2} (0.01) and 1×10^{-3} (0.001) classifying this group as permissive to VSV infection (Fig. 1*a*). On the contrary, the HNSCC group showed differential responses to infection of the heat-resistant VSV with only the FADU cell line showing sensitivity while SCC9, Cal27 and SCC25 were comparatively resistant to VSV oncolysis (Fig. 1*b*). For example, Cal27 and SCC25 were strongly resistant to VSV cytotoxicity as even high MOI treatment of 10 virus particles per cell failed to induce 50% cell death demonstrating greater than 1,000× resistance compared to the CC cells tested. These results highlight a potential role for prior HPV infection in VSV sensitivity; however, other determinants as evident in the HNSCC cell line FADU also contribute to VSV sensitivity. A number of studies have demonstrated that VSV sensitivity is multi-factorial and manifested in a wide variety of tumor cell types.[20–22]

This interesting difference in terms of sensitivity to VSV cytotoxicity motivated us to further investigate CC and HNSCC cells sensitivity to the VSV attenuated strains VSV-AV1 and VSV-AV2 that have been previously identified and characterized.[21] Cell viability for both groups was assayed at various MOI (1×10^{-6} to 1×10^{1}) of VSV, AV1 and AV2 (Fig. 2). The four CC cell lines evaluated segregated into two groups, the ME180 and SiHa cells showed attenuation of cytotoxicity with both the AV1 and AV2 strains of VSV

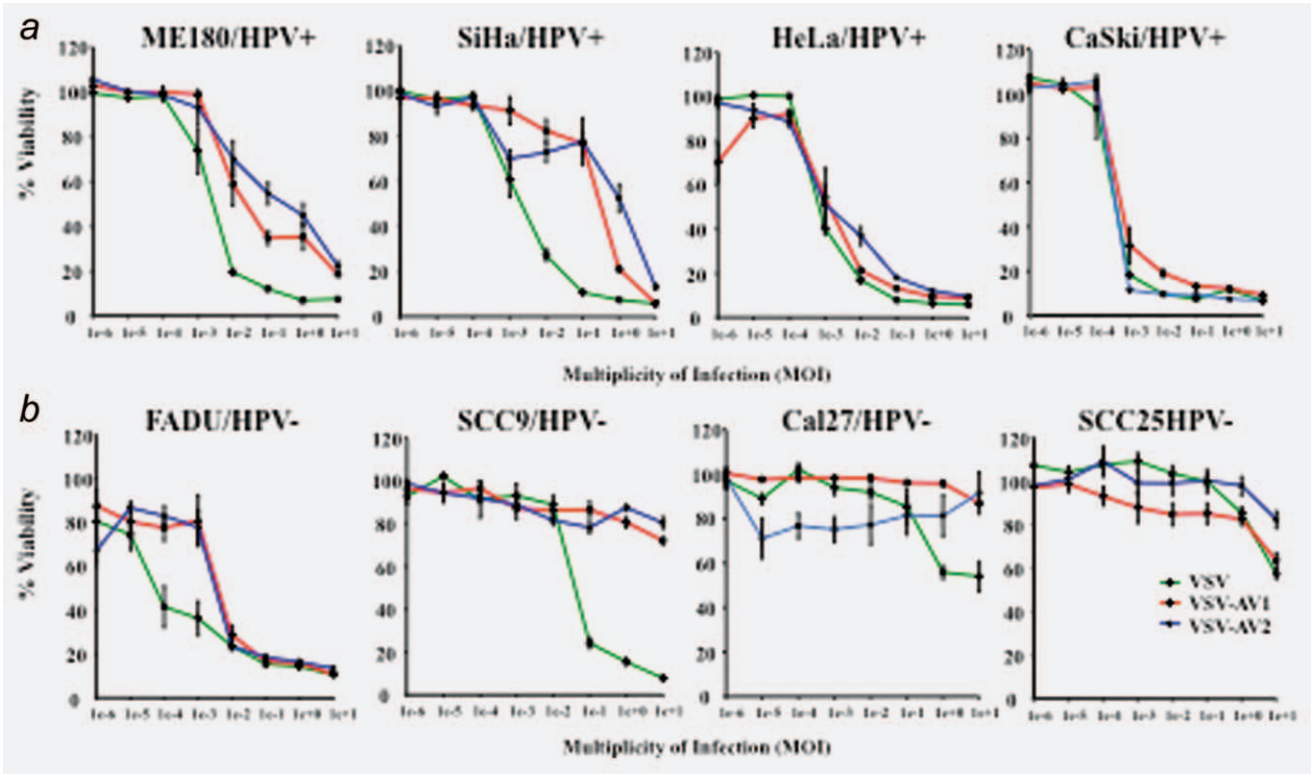

Figure 2. CC and HNSCC cell lines display differential sensitivity to attenuated VSV variants. (*a*) CaSki, SiHa, HeLa and ME180 CC cell line were evaluated for VSV-HR, VSV-AV1 and VSV-AV2-induced cytotoxicity as determined by the MTT cell viability assay. Each virus dose tested was done in replicates of six with three independent experiments performed yielding similar results employing the MTT cell viability assay after 48 hr treatment. (*b*) SCC9, SCC25, FADU and Cal27 HNSCC cell lines with associated HPV status were processed as above with VSV-HR, VSV-AV1 and VSV-AV2. Green line: VSV; red line VSV-AV1; blue line: VSV-AV2.

(approximately 100-fold) while the HeLa and CaSki cell lines were equally sensitive to all three strains tested (Fig. 2*a*). In the HNSCC cell lines, differential VSV strain sensitivity was observed as FADU cells also showed attenuation of cytotoxicity with both the AV1 and AV2 strains of VSV (approximately 100-fold), however, the three other HNSCC lines were resistant to the cytotoxic effects of both VSV attenuated strains (Fig. 2*b*). These results were summarized in the Supporting Information Table 1 displaying the LD_{50} (the MOI of VSV with a lethal dose of 50%) for each cell line and viral strain evaluated. The cell lines segregated into three groups based on their sensitivity to the strains of VSV tested: resistant, sensitive or hypersensitive to VSV cytotoxicity. While all of the CC cells fell into the hypersensitive and sensitive groups, 3/4 of the HNSCC cell lines were resistant. Only one HNSCC cell line was sensitive to VSV cytotoxicity (FADU). Since 100% of CC and 25% of HNSCC carcinomas are HPV positive, we next determined whether HPV infection status played a role in VSV sensitivity (Supporting Information Table 1). As expected, all four of the CC were shown to be HPV positive and, all of the HNSCC cell lines, including FADU, were HPV negative. This does not rule out the potential for HPV to sensitize CC cells to VSV infection but does suggest that VSV sensitivity can result from a number of mechanisms as has been documented in numerous previous studies.[21,24,25]

To confirm the various cell line sensitivities to VSV and its attenuated variants, a sensitive CC cell line (HeLa) and a VSV-resistant HNSCC cell line (SCC25) were infected with these VSV strains and observed microscopically at three different time points for cytopathic effects including 6-, 24- and 72-hr post-infection with an MOI of 1.0 (Fig. 3*a*). Similar to the result shown in Figures 1 and 2, HeLa cells infected by VSV and the attenuated VSV forms AV1 and AV2 showed significant cytotoxicity as cell rounding and floating cells were readily evident. SCC25 cells were comparatively resistant to VSV and its AV1 and AV2 variants (Fig. 3*a*). Furthermore, using methylene blue staining of cell monolayers in 100-mm plates treated with VSV and its variants for 96 hr at an MOI of 1.0, the differential sensitivity of SCC25 and HeLa cells to VSV oncolysis is clearly evident (Fig. 3*b*). Virus quantification by a standard titration assay in CC and HNSCC cells groups also confirmed differential infectivity and replication of VSV on the cell lines tested (Fig. 3*c*). VSV replication is the highest in CC group (SiHa, HeLa) and is at least 10-fold higher than the SCC9 HNSCC cell line at the

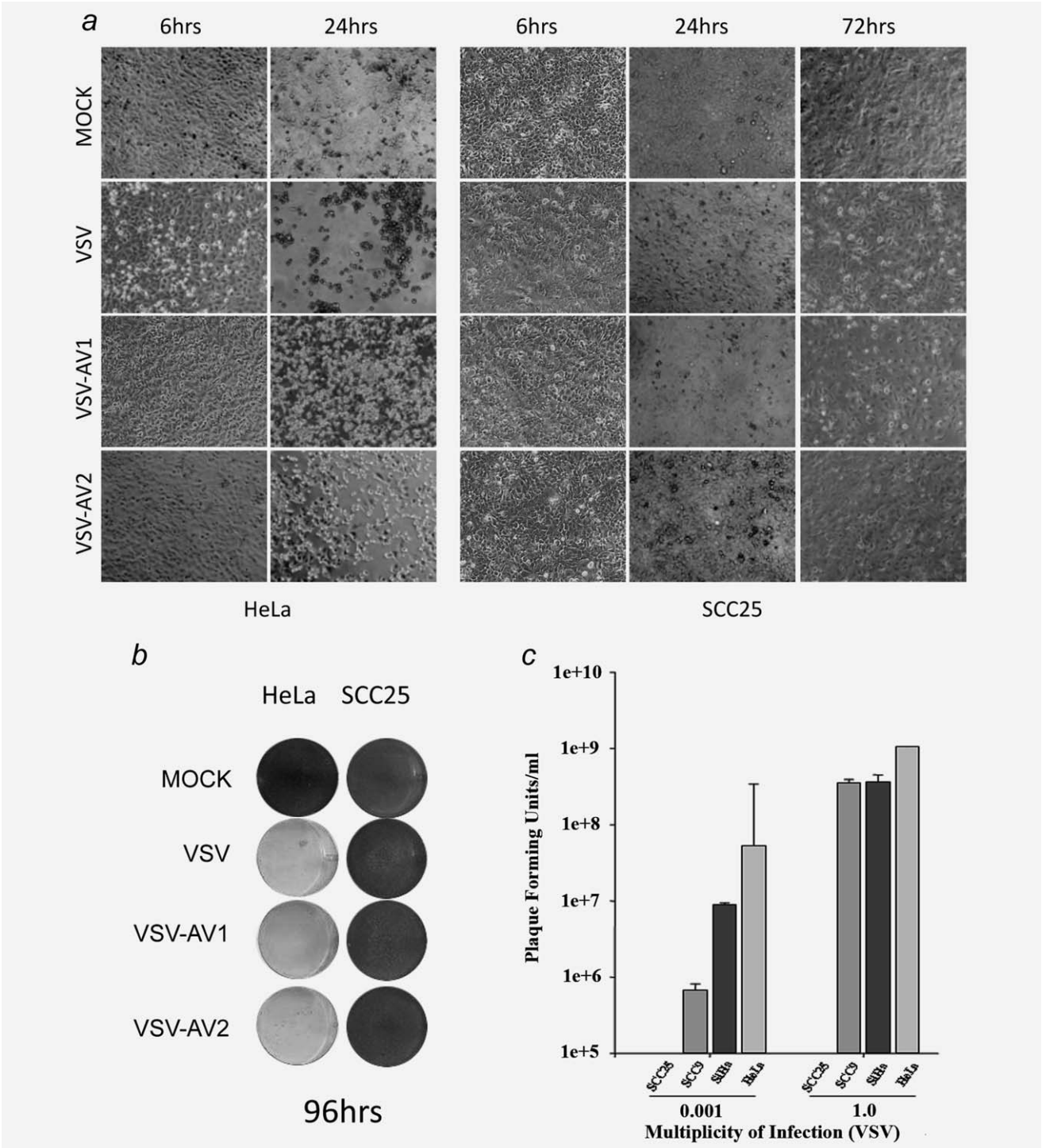

Figure 3. Cytopathic effects of VSV and its attenuated strains. (*a*) HeLa and SCC25 cell lines were left untreated or treated with VSV, VSV-AV1 or VSV-AV2 at MOI = 1.0 for 6 and 24 hr of infection. Observing changes in cell morphology as assessed by bright-field microscopy assessed viral oncolytic effect. Representative pictures of each treatment were obtained and presented. Each virus dose tested was done in triplicate. (*b*) Methylene blue stained HeLa and SCC25 monolayers treated with mock infection, VSV, VSV-AV1 and VSV-AV2 at an MOI = 1.0. Cells (10^6) plated in 100-mm dishes were treated with the viruses for 96 hr and stained. (*c*) SCC25, SCC9, SiHa and HeLa were treated with VSV at MOI 0.001 or 1.0 for 24 hr following VSV infection; VSV titers were determined by standard plaque assay. Mean ± SEM from replicates of six.

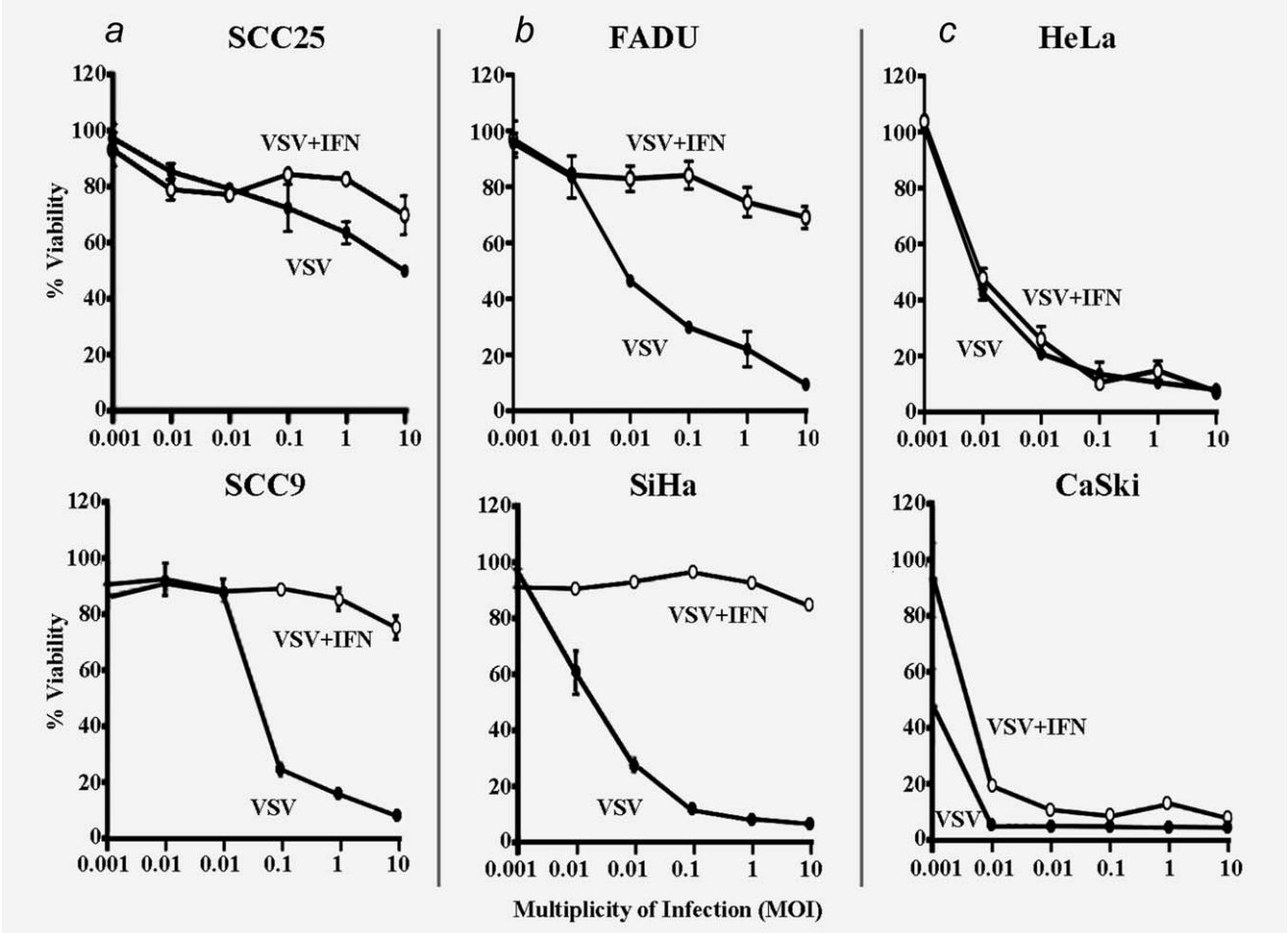

Figure 4. Role of IFN in VSV sensitivity. (*a*) The resistant cell lines SCC25 and SCC9; (*b*) the sensitive cell lines FADU and SiHa; (*c*) the hypersensitive cell lines HeLa and CaSki were evaluated by MTT assay either with VSV alone (closed circle) or with pretreatment with 100 units/ml recombinant IFN-α for 12 hr (open circles) and then challenged with same range of doses of VSV. Each virus dose tested was done in replicates of six and repeated with similar results.

low MOI of 0.001. Interestingly, the resistant HNSCC SCC25 cell line failed to demonstrate significant viral titers even at the high MOI of 1 clearly demonstrating the resistance of this line to VSV infection (Fig. 3*c*). Taken together, these results demonstrate that CC cells are sensitive to VSV-induced cytotoxicity while HNSCC are comparatively more resistant to the oncolytic effects of VSV.

Role of IFN pathway inhibition in VSV sensitivity

Due to the potential of HPV E6 protein to inhibit IFN activity by interfering with IFN receptor activation,[9] we evaluated whether the HPV status with respect to IFN activity had an effect on VSV efficacy. First, employing a cell line representative of each VSV sensitivity groups; SCC25 and SCC9 (resistant), FADU and SiHa (sensitive) and HeLa and CaSki (hypersensitive), we compared their sensitivity to VSV with or without addition of exogenous IFNα (100 units/ml). Cells were infected with various VSV MOI following 12 hr of IFN treatment. In the resistant and the sensitive cell lines, IFN treatment significantly repressed VSV-induced cytotoxicity (Figs. 4*a* and 4*b*). In contrast, there was no effect of IFN treatment on VSV-induced cytotoxicity in the hypersensitive cell lines tested (Fig. 4*c*).

These results suggest that HPV can block IFN induction potentially through HPV-E6 protein activity and in some tumor cells this effect can be rescued by the exogenous addition of IFNα. Therefore, we evaluated two important regulators of IFN anti-viral activity whose expression is induced or activated, in part, by the activation of IFNAR: Interferon Regulatory Factor-7 and −3 (IRF7 and IRF3), respectively.[21] We evaluated the expression of IRF7 and IRF3 together with its activated phosphorylation form following VSV, VSV-AV1 and VSV-AV2 infection (MOI = 1) at an early 6 and later 24 hr time-points in our resistant (SCC9 and SCC25), sensitive (FADU and SiHa) and hypersensitive (HeLa and CaSki) panel of cell lines. The IFN MX2 GTPase is part of the cell-autonomous innate immune response against viruses that importantly is induced by and can also inhibit VSV

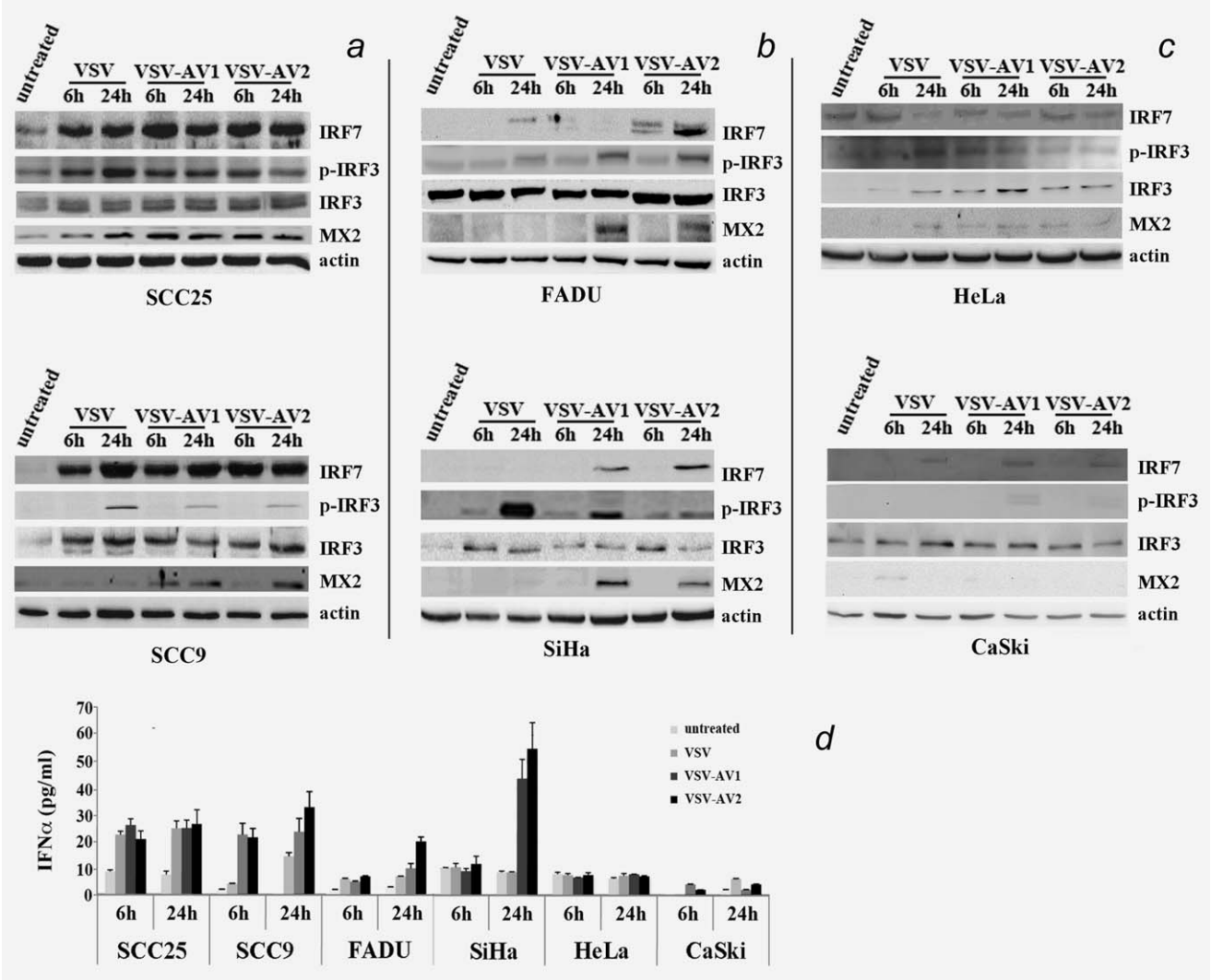

Figure 5. Western blot analysis of IRF7, IRF3 and MX2 expression and ELISA determination of IFNα secretion in response to VSV infection. (*a*) The resistant cell lines SCC25 and SCC9; (*b*) the sensitive cell lines FADU and SiHa; (*c*) the hypersensitive cell lines HeLa and CaSki were evaluated by Western blot analysis in untreated or cells infected with VSV, VSV-AV1 or VSV-AV2 for 6 or 24 hr of infection with an MOI = 1.0. Total cellular protein was extracted and quantified and evaluated for expression of IRF7, p-IRF3, total IRF3, MX2 and actin. (*d*) ELISA determination of IFNα production in mock infection, VSV, VSV-AV1 and VSV-AV2 in these cell lines under identical conditions as above. Media was assayed at 6 and 24 hr post-viral infection.

infection[36] and its expression level was similarly evaluated in this study. In the SCC25 VSV-resistant cell line, VSV, VSV-AV1 and VSV-AV2 all significantly induced expression of IRF7, p-IRF3 and MX2 at both the early and late time-points tested (Fig. 5*a*). SCC9 cells that demonstrate sensitivity to VSV but are resistant to the cytotoxic effects of VSV-AV1 and VSV-AV2 showed virus induction of IRF7 similar to SCC25; however, p-IRF3 was induced only at the late time-points by all three variants while MX2 induction was only observed with VSV-AV1 and VSV-AV2 infections (Fig. 5*a*). In the sensitive FADU and SiHa cell lines that are sensitive to VSV but display an attenuated response to VSV-AV1 and VSV-AV2, significant induction of IRF7, p-IRF3 and MX2 was generally observed in the late infection time-points in the two attenuated strains (Fig. 5*b*). In the hypersensitive cell lines HeLa and CaSki, all three VSV strains tested failed to significantly induce either IRF7, p-IRF3 or MX2 at the time-points tested (Fig. 5*c*).

We next determined whether the IFN response detailed in the Western blot analysis was associated with their potential to induce IFNα production. In SCC25 and SCC9 cells, release of IFNα into culture media was induced at both the 6- and 24-hr post VSV treatment (MOI = 1.0) as determined using an IFNα ELISA kit (Fig. 5*d*). In the sensitive FADU and SiHa cells, IFNα release was significantly induced only in the two VSV-AV strains at the 24 hr time-point assayed (Fig. 5*d*). In contrast, the hypersensitive cell lines HeLa and CaSki, VSV infection with either strain failed to induce IFNα production and release. These

results demonstrate that VSV sensitivity of these cell lines was associated with their inability to induce an IFN response.

To determine the ability of HPV to sensitize tumor cells to VSV, we exogenously expressed the HPV E6 protein in the SCC25 cells, the most resistant to VSV that lacks detectable HPV infection. A pool of E6 expressing SCC25 cells were significantly more sensitive to VSV cytotoxicity compared to empty vector selected cells (Fig. 6*a*). For example, the SCC25 cells expressing E6 protein have a 7% survival at MOI 1 compared to 75% for parental cells and were at least 100× more sensitive than their parental counterparts. The expression of E6 (HPV18) mRNA in SCC25-E6 cells was confirmed by RT-PCR with HeLa cells as a positive control (Fig. 6*b*). E6 expression in SCC25 cells also inhibited VSV-induced IFN response as induction of IRF7, and p-IRF3 and MX2 following VSV treatment was significantly attenuated (Fig. 6*c*) compared to parental SCC25 cells (see Fig. 5*a*). Together, these data strongly suggest that HPV E6 plays a role in regulating VSV sensitivity in HPV infected cells. In the case of FADU and the wide array of tumor-derived cell lines previously shown to be sensitive to VSV oncolysis without prior HPV infection,[20,21,24,25] it is clear that this is one of a variety of mechanisms targeting IFN response that infer sensitivity to this important viral agent.

In vivo and *ex vivo* sensitivity of cervical carcinomas to VSV infection

In order to establish that our *in vitro* evidence has potential therapeutic implications, we tested the efficacy of VSV in relevant tumor xenograft nude mouse models and in primary human samples. In this study, we injected/infected five nude mice with established HeLa or SCC25 subcutaneous tumors, two CC tissues and a normal uterine tissue sample obtained after surgery with either VSV-AV1-RFP (nude mice) or eGFP (patient samples). In the xenografted nude mice, HeLa-derived flank tumors showed VSV-induced RFP (red fluorescent protein) expression demonstrating permissiveness to intravenous injected VSV-AV1. In contrast, SCC25-derived flank tumors failed to express detectable levels of RFP indicating resistance to VSV infection (Fig. 6*d*). Furthermore, through immunohistochemical analyses, we confirmed the differential infectivity of these xenografts as VSV protein expression was readily detected in the HeLa but not in SCC25 xenografts. The presence of VSV in the HeLa xenografted tumors was also associated with the presence of cleaved caspase 3, a marker of the induction of apoptosis (Fig. 6*d*). The human tumor tissue explants employed in this study were obtained from patients undergoing surgical resection at our institute. Subsequent to processing, tissue slices were incubated with VSV-AV1-GFP (green fluorescent protein). As with our xenograft studies, viral infection was visualized by fluorescent microscopy after 48 hr. Interestingly, we observed significant eGFP expression with VSV after 48 hr of infection of the CC tumor tissue but not in the normal tissue analyzed (Fig. 6*e*). Differential VSV infectivity was also evident through assessing the production of infective virus particles in the SCC25 and HeLa xenograft tumor tissue and patient tissue samples by determining the plaque forming units per gram of tissue. HeLa xenografts and infected CC tumors produced greater than 1,000× higher VSV titers than the normal tissue analyzed (Figs. 6*d* and 6*e*). These results suggest the potential for VSV as a therapeutic option where HPV infection plays a role in tumor pathogenesis.

Discussion

Replicating OV, like VSV, have been designed or selected to specifically infect cancer cells with limited activity in normal cells.[21,37–39] A major cell defense pathway that inhibits virus infection and spread is the activation of the IFN transcription pathway followed by the release of this cytokine.[21,25,27] IFN activated host defense mechanisms in surrounding cells and tissues can limit and clear viral infections. Transformed cells are generally defective in IFN signaling as inhibition of this pathway fosters cell proliferation.[21,25,27] Defects in IFN

Figure 6. HPV-E6 protein's role in VSV sensitivity and *in-vivo*, *ex vivo* sensitivity of CC to VSV infection. (*a*) SCC25 cells expressing E6 or empty vector control were assayed for by MTT for cell viability following 48 hr VSV treatments. Each virus dose tested was done in replicates of six with three independent experiments performed yielding similar results. (*b*) RT-PCR analysis of SCC25, SCC25-E6 and HeLa cells for E6 expression with GAPDH expression analyzed to ensure fidelity of the cDNAs evaluated. These results confirmed E6 expression in the SCC25 E6 generated cell line. (*c*) SCC25 E6 cells were evaluated by Western blot analysis in untreated or cells infected with VSV for 6- or 24 hr of infection with an MOI = 1.0. Total cellular protein was extracted and quantified and evaluated for expression of IRF7, p-IRF3, total IRF3, MX2 and actin. (*d*) HeLa or SCC25 subcutaneous xenograft tumor models were established in nude mice. Each group received VSV-AV1 (1.10×10^7 pfu) injected IV and after 3 days of VSV injection, mice were sacrificed and viral replication at tumor site was imaged for red fluorescent protein (RFP). Two representative mice per group are presented. All xenografted tumor samples from (*b*) were weighed, homogenized, and virus quantification was performed by standard VSV plaque assay. $N = 5$ per group. Bars correspond to SEM. *T*-test between SCC25 and HeLa VSV-AV1 treated tumors. $^{**}p < 0.00001$. Immunohistochemical analysis of VSV and cleaved caspase 3 in SCC25 and HeLa xenografts are also presented clearly demonstrating enhanced infection and activity of intravenously delivered VSV in HeLa xenografted tumors. (*e*) Primary CC tissue was sliced and cultured with αMEM containing serum. The following day, slices were incubated with and without VSV-AV1 (10^8 pfu) expressing eGFP. Samples were monitored by microscopy 24 hr later with bright-field and green fluorescent protein (GFP) expression. Normal and cancer tissue of one patient is presented. Following the indicated treatment conditions, samples were weighed, homogenized and virus quantified by standard VSV plaque assay.

signaling are pleiotropic with a wide variety of target inactivated either through genetic mutations or epigenetic changes.[40–43] These targets may be re-engaged and confer resistance to these oncolytic viral agents.[40–43] The rhabdovirus VSV is an RNA-based virus that has a simple genome and has evolved a strategy of extremely rapid replication and spread to by-pass innate anti-viral immune responses.[44] Attenuated mutant strains of VSV are very sensitive to IFN and are unable to overcome endogenous IFN signaling within normal infected cells.[26–28] These properties make the naturally occurring attenuated variants of VSV-AV ideal oncolytic agents that can be readily evaluated in the clinic. However,

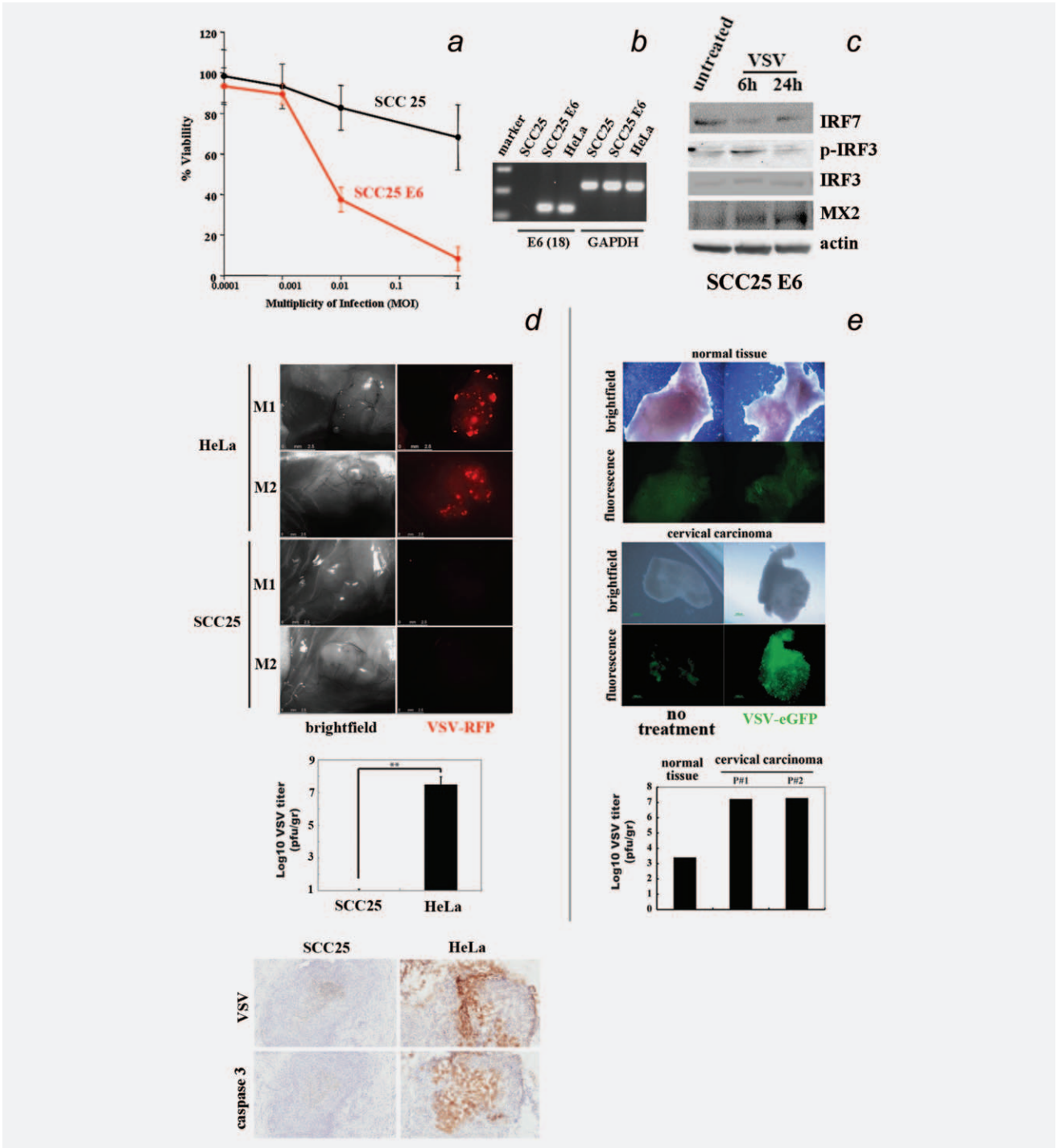

Figure 6.

residual activity of IFN-signaling pathway as well as acquired resistance can be insurmountable barriers for their clinical efficacy.[21,25,27]

In this study, we hypothesized that HPV infection, through the expression of its E6 protein, inhibits IFN receptor signaling as well as the requirement of E6 expression to initiate and maintain malignant transformation,[5,8] VSV would be an active agent in this setting. The cell lines evaluated in this study, segregated into three response groups based on their sensitivities to VSV and its two attenuated strains VSV-AV1 and VSV-AV2 to tumor cell oncolysis. The resistant cell lines readily induced the IFN pathway and IFNα secretion. The sensitive cells lines were sensitive to VSV-induced oncolysis but showed an attenuated response to VSV-AV1 and VSV-AV2. The induction of the IFN response was generally limited to the attenuated VSV variants as well. The hypersensitive group showed significant cytotoxicity with all three VSV variants and failed to induce an IFN response with either of these agents. These results confirm previous work highlighting the importance of IFN signaling in regulating response to VSV oncolysis.[21,25,27] With the exception of the FADU HNSCC cell line, sensitivity to VSV in this panel was associated with prior HPV infection. Other targets inhibiting IFN response also play a role especially in the FADU cell line as well as the hypersensitive HeLa and CaSki cell lines where more than one IFN target may be inhibited resulting in their enhanced sensitivity to VSV oncolysis. Expression of HPV-E6 in the VSV-resistant HNSCC cell line SCC25 inhibited IFN signaling and significantly enhanced the cytotoxicity of VSV compared to parental controls. Xenografted HeLa-derived tumors as well as explant tumor tissues from two CC patients demonstrated the ability of VSV to infect CC cells in a more clinical setting. Taken together, these results suggest that HPV-infected cells are susceptible to oncolytic virus therapy and that this approach may represent a novel therapeutic tool in HPV positive CC and HNSCC.

Of interest to our previous study, this natural event mimics the ability of combinations of two different viruses to induce co-operative oncolytic activity that we recently described.[45] In that study, we demonstrated synergistic tumor cell cytotoxicity between a poxvirus (vaccinia virus) and VSV. Infection by vaccinia virus inhibited IFN secretion that was beneficial for VSV oncolysis thus enhancing the efficacy of both viruses.[45] This strategy requires the administration of two independent viruses, however, due to the presence of a pre-existing viral infection in HPV positive tumors only one agent will be administered enhancing the safety of this approach as only tumor cells will be "exposed" to the second HPV virus. Clearly, in the case of CC where 100% and HNSCC where 25% of patients have tumors that are HPV positive,[4,5] we believe that VSV represents a viable therapeutic strategy. A clinical evaluation of CC and other tumor types, like HNSCC, with a high prevalence of HPV infection that pre-screens for HPV, status is warranted.

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