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Assessment of oncolytic HSV efficacy following increased entry-receptor expression in malignant peripheral nerve sheath tumor cell lines

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Abstract

Limited expression and distribution of nectin-1, the major herpes simplex virus (HSV) type-1 entry-receptor, within tumors has been proposed as an impediment to oncolytic HSV (oHSV) therapy. To determine whether resistance to oHSVs in malignant peripheral nerve sheath tumors (MPNSTs) was explained by this hypothesis, nectin-1 expression and oHSV viral yields were assessed in a panel of MPNST cell lines using $\gamma_1$34.5-attenuated ($\Delta\gamma_1$34.5) oHSVs and a $\gamma_1$34.5 wild-type (wt) virus for comparison. Although there was a correlation between nectin-1 levels and viral yields with the wt virus ($R = 0.75$, $P = 0.03$), there was no correlation for $\Delta\gamma_1$34.5 viruses (G207, R7020 or C101) and a modest trend for the second-generation oHSV C134 ($R = 0.62$, $P = 0.10$). Nectin-1 overexpression in resistant MPNST cell lines did not improve $\Delta\gamma_1$34.5 oHSV output. While multistep replication assays showed that nectin-1 overexpression improved $\Delta\gamma_1$34.5 oHSV cell-to-cell spread, it did not confer a sensitive phenotype to resistant cells. Finally, oHSV yields were not improved with increased nectin-1 *in vivo*. We conclude that nectin-1 expression is not the primary obstacle of productive infection for $\Delta\gamma_1$34.5 oHSVs in MPNST cell lines. In contrast, viruses that are competent in their ability to counter the antiviral response may derive benefit with higher nectin-1 expression.

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CONFLICT OF INTEREST

JMM, GYG, and RJW are co-founders, stockholders and consultants for Catherex, Inc., which holds intellectual property related to oncolytic HSV.

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INTRODUCTION

Malignant peripheral nerve sheath tumors (MPNSTs) are a highly aggressive cancer of the peripheral nervous tissue believed to originate within the Schwann cell lineage[1] and are most commonly associated with the genetic condition neurofibromatosis type-1. Treatment options for MPNSTs beyond surgery are inadequate, resulting in a median survival of only 26 months.[2] Oncolytic virotherapy by attenuated herpes simplex type-1 viruses (oHSVs) has been proposed as an alternative to chemotherapy and radiotherapy for the treatment of MPNSTs.[3–7] HSVs with $\gamma_1$34.5 neurovirulence gene deletions are safe in humans and have been shown to selectively replicate in tumor cells.[8] These attenuated $\Delta\gamma_1$34.5 oHSVs have a clinically verified safety profile in patients with malignant glioma and have been associated with measurable antitumor responses.[9–13] However, these patient responses have varied widely, likely due to tumor susceptibility. Therefore, we have sought to further elucidate the mechanisms of oHSV resistance.

In our initial investigation into potential oHSV resistance mechanisms within MPNSTs, we have tested the hypothesis that oHSV resistance is attributable to the insufficient expression of HSV-1 entry receptors by tumor cells.[14–18] Four viral glycoproteins (gD, gB and gH/gL) and a cellular glycoprotein D (gD)-interacting receptor have been demonstrated as necessary and sufficient to trigger cellular entry.[19–23] Of the three cellular HSV-1 gD-interacting receptors, nectin-1, a cellular adhesion protein expressed in epithelial cells,[24] fibroblasts and neurons,[25] has been proposed as the major HSV-1 entry receptor.[26] Herpes virus entry mediator (HVEM) [27] and 3-*O*-sulfated heparan sulfate (3-OS-HS)[28] have also been demonstrated to facilitate HSV-1 entry. Additional cell-surface molecules that interact with other viral glycoproteins have been identified, though the broad necessity of these in permitting HSV-1 infection and spread remains to be determined and the lack of these molecules has not yet been implicated in limiting the oncolytic capacity of oHSV.

Here, we have investigated the hypothesis that HSV entry-receptor expression is a determinant of oHSV efficacy in MPNST cells and have identified whether an increase in entry-receptor expression improves the viral yield and spread of oHSVs. The influence of entry-receptor expression was examined in the context of an array of viral genotypes, including a representative wild-type (wt) $\gamma_1$34.5 HSV-1, a fully attenuated $\Delta\gamma_1$34.5 oHSV, and an attenuated second-generation oHSV capable of host antiviral evasion. We report the following conclusions: (1) correlation of nectin-1 expression with viral production capacity appears more important in viruses which are genetically competent to counter the intrinsic antiviral response, (2) increased expression of entry-receptor molecules modestly improves cell-cell spread of $\Delta\gamma_1$34.5 oHSVs, but yields little benefit to viral production and (3) increases in entry-receptor expression do not render resistant MPNST cell lines permissive to $\Delta\gamma_1$34.5 oHSV infection.

RESULTS AND DISCUSSION

MPNST cell lines have been previously identified as susceptible to oHSV infection and cytotoxicity.[3] To examine the correlation between viral production capacity and entry-receptor expression, human MPNST cell lines STS-26T, T265-2c, NMS2-PC, S462, YST-1,

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90-8, ST88-14 and 2XSB, or their luciferase-expressing derivatives (' − luc'), were first infected at a multiplicity of infection (MOI) of 10 (single-step replication assay) with a panel of genetically modified HSV-1 and cellular lysates collected 24 h post infection (h.p.i.) for viral recovery analysis (Supplementary Figure 1). The viral yields from this assay are presented in correlation with nectin-1 expression in Figures 1a–e.

HSV-1 entry receptors have not been previously identified in MPNSTs or cells of the Schwann cell lineage. Upon examination of nectin-1 and HVEM expression in our panel of MPNST cell lines, we found detectable levels of nectin-1 in all of the lines, with population-wide (>95%) expression of nectin-1 in five of eight cell lines (Figure 1f). Population-wide expression of HVEM in MPNST cell lines was observed in only one of eight lines (Supplementary Figure 2D); therefore, HVEM was excluded as a candidate for the major entry receptor in MPNSTs. The other established entry receptor 3-OS-HS was not examined in this study due to the lack of a commercially available antibody. However, HSV-1 infection of the resistant cell lines selected for further study (STS26T-luc and T265-luc) was found to be dependent on nectin-1 expression alone by nectin-1 neutralization assays (Supplementary Figure 2).

Pearson's correlation coefficients were calculated between viral recovery data and nectin-1 expression levels. Possible positive associations were found for the wt M2001 ($R = 0.75$; $P = 0.03$) and attenuated second-generation oHSV C134 ($R = 0.62$, $P = 0.10$) viruses (Figures 1a and b). While prior studies involving thyroid cancer[14] and head and neck squamous cell carcinoma[15] cell lines demonstrated correlation between viral yields of oHSV NV1023 (a derivative of R7020) and nectin-1 expression, no such associations were observed for G207, C101 or R7020 oHSVs suggesting that the specific viral genotype influences the outcome of infection.

The lack of a clear association of nectin-1 expression with attenuated $\Delta\gamma_1$34.5 oHSV viral yields led us to further evaluate the functional impact of increased entry-receptor expression on oHSV sensitivity. To assess this, the oHSV-resistant cell lines, T265-luc and STS26T-luc were transduced with full-length human nectin-1 (nectin-1α) using lentivirus LV2114CK. An mCherry expressing lentivirus was used as a control and confirmed that transduction alone did not alter viral production (data not shown).

If low entry-receptor expression diminishes the ability of oHSV to establish an initial infection, we would predict that increased nectin-1 expression would increase the initial opportunity for entry, resulting in replication within a greater number of cells and an increase in the total production of virus. To determine the impact of increased nectin-1 expression on viral yields in MPNSTs, single-step (MOI= 10, 24 h.p.i.) and multistep (MOI= 0.1, 24, 48, 72 h.p.i.; Supplementary Figure 3) viral recovery assays were performed using the parent and nectin-1 transduced cell lines. Because the reliable titering repeatability of HSV is within approximately 0.5 log, only changes in titer greater than 0.5 log are considered to be biologically relevant. The nectin-1 transduction of T265-luc and STS26T-luc resulted in abundant nectin-1 expression in T265-N1 and STS26T-N1 cell lines, respectively (Figures 2a and e). While increased entry-receptor expression improved the yields of a representative wt virus (Figures 2b and f), the increased expression did not

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improve the titers of a next-generation oHSV C154, an EGFP expressing variant of C134 (Figures 2c and g) or first-generation $\Delta\gamma_1$34.5 oHSV C101 (Figures 2d and h). To demonstrate that increased nectin-1 expression would be expected to improve viral production, the HSV receptor-deficient cell line CHO-K1 was transduced with nectin-1. A significant and greater than 5 log increase in wt HSV-1 titers was observed (Figures 2i and j).

Interaction with HSV entry receptors is essential for initial HSV entry as well as the subsequent cell-to-cell spread of HSV.[29] To assess the effect of increased nectin-1 expression on viral spread, we measured viral GFP expression in MPNST cells over the time in multistep assays following infection with GFP expressing C101 and C154 (Figure 3) or M2001 (Supplementary Figure 4). The results show that nectin-1 overexpression improved the ability of C101 to undergo cell-to-cell spread and increased the proportion of cells infected from 3 to 27% and from 1 to 7% of the cell population in T265-N1 and STS26T-N1 in multistep replication assays (MOI = 0.1, 24, 48 and 72 h.p.i.) respectively (Figures 3a and b). Despite this improved spread in resistant lines, the maximum spread was much less than that observed in the naturally permissive S462-luc and NMS-2PC MPNST cell lines, where C101 was capable of infecting >80% of the cells (Figure 3c). This suggests that endogenous levels of entry receptors are sufficient to permit infection and sustain $\Delta\gamma_1$34.5 oHSV spread in these lines and that increased entry-receptor expression is not sufficient to render resistant cell lines with a permissive phenotype. Of note, the overexpressed nectin-1 levels far exceeded the highest endogenous levels in the permissive lines (Supplementary Figure 5), suggesting that restricted entry is not an explanation for MPNST resistance to oHSVs. This conclusion is further supported by the fact that infection of the same cell lines with a second-generation oHSV (that is, C134 or C154) capable of evading the antiviral response[30] resulted in approximately 10- 100 fold increase in viral titers and notably greater cell-to-cell spread as compared with C101 (Figures 3d and e).

To determine the extent to which *in vivo* studies recapitulated these results, athymic nude mice were engrafted with either parent or nectin-1 expressing cell lines. Of the resistant cell lines, only STS26T-luc and the nectin-1 overexpressing variant established flank tumors. Tumors were injected with 1×10^7 plaque forming units of C101 or C154, and viral recovery was measured on days 3 and 5 post injection. Similar to the *in vitro* results (Figure 3g and h), the next-generation virus had a >10-fold viral production advantage over the $\Delta\gamma_1$34.5 oHSV C101, however neither virus demonstrated an increased viral titer between days 3 and 5 even with increased nectin-1 expression. Tumors were also collected for immunohistochemistry and staining for HSV-1 confirmed that increased nectin-1 expression did not benefit oHSV spread between days 3 and 5 (data not shown). The *in vivo* results therefore confirmed that neither the first-nor the second-generation oHSVs derived a benefit to viral output from increased entry-receptor expression.

In summary, the work presented here provides insight into one of the proposed determinants of oHSV therapeutic efficacy. We conclude that the primary mode of MPNST resistance to $\Delta\gamma_1$34.5 oHSVs is not due to limited expression of nectin-1. Despite the primary conclusions of previously published work that entry-receptor expression is predictive of a productive infection by oHSV, we suggest that the use of viruses in these previous studies

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which contained at least one functional copy of the $\gamma_1$34.5 gene (NV1023)[14,15,18] or $\gamma_1$34.5 under a nestin promoter (rQnestin34.5)[16] is in line with our conclusions that viruses which are genetically competent to counter the intrinsic antiviral response benefit the most from increased entry-receptor expression. Similarly in our work, the wt HSV-1 and C134 viruses derived greater benefit from higher entry-receptor expression than did the first-generation $\Delta\gamma_1$34.5 oHSVs. Furthermore, the work of Wang *et al*[16] showed that only the $\gamma_1$34.5 containing virus was able to substantially benefit from increased nectin-1 expression while the $\Delta\gamma_1$34.5 control virus did not. Future work should therefore include the characterization of the capacity for an intrinsic antiviral response as the major mechanism for oHSV resistance in MPNSTs.

MATERIALS AND METHODS

Cell lines

MPNST cell lines STS26T-luc, T265-luc, ST88-14-luc, S462-luc, 90-8- luc, NMS2-PC, YST-1 and 2XSB were provided by Dr Steve Carroll (University of Alabama at Birmingham). Cell lines STS26T-luc, T265- luc, and ST88-14-luc express firefly luciferase and have been previously described.[31] S462-luc and 90-8-luc were transduced *via* lentivirus to express Renilla luciferase. HSV-1 entry receptor-deficient cell line CHO-K1 was generously provided by Dr Yancey Gillespie (University of Alabama, Birmingham). All MPNST cell lines were maintained in DMEM, 10% FBS, and 1% P/S. CHO-K1 cells were maintained in Ham's F12, 10% FBS and 1% P/S. Vero cells were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in MEM and 5% BGS. All cell lines were confirmed to be free of Mycoplasma by DAPI staining and PCR detection.

Viruses

All viruses have been previously described. Briefly, M2001 was constructed by insertion of the gene encoding EGFP under the control of the CMV immediate early promoter into the U_L3-U_L4 intergenic region of the prototypical wt HSV-1 (F) strain.[32] C101 and C134 were derived from the $\Delta\gamma_1$34.5 mutant HSV-1 R3616 by insertion respectively of the *EGFP* or HCMV *IRS1* genes under the control of the CMV immediate early promoter in the U_L3-U_L4 intergenic region.[33] C154 is derived from C134 by insertion of EGFP into the deletion loci of $\gamma_1$34.5. G207 (Medigene, Inc., San Diego, CA, USA) is a clinical grade oHSV derived from R3616 with the additional insertion of *lacZ* in the U_L39 region.[34] R7020 (kindly provided by Bernard Roizman; University of Chicago, Chicago, IL, USA), is a clinical grade oHSV derived from HSV-1 (F) strain by insertion of a region of the HSV-2 genome encoding glycoproteins G, D, I and a portion of E into one of the internal repeat regions of HSV-1 (F) disrupting one copy of the neurovirulence gene $\gamma_1$34.5.[35]

Viral titering assays

Viral titers were determined by limiting dilution plaque formation assays as previously described.[33] MPNST cells were incubated for 2 h with virus diluted in 100 μl infection media (DMEM + 1% FBS) and replaced with growth media after infection. An equivalent volume of sterile milk was added and the plate subjected to three cycles of freeze-thaw at − 80 °C. Lysate was collected, sonicated, serially diluted in Vero infection media (MEM

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+ 1% BGS), and incubated on Vero monolayers. Infection media was replaced with growth media containing 0.01% human AB serum (Corning Cellgro, Corning, NY, USA). After 48 h, plaques were counted following May-Grunwald/methanol staining as previously described. All experiments were performed in triplicate and the average total plaque forming units reported with standard deviation.

Viral entry-receptor quantification

Expression of entry receptors was quantified by flow cytometry using either phycoerythrin-conjugated mouse monoclonal antibodies to nectin-1 (R1.302) (Biolegend, San Diego, CA, USA), HVEM (Biolegend), or isotype control (BD Biosciences, San Jose, CA, USA). Antibody concentrations used were confirmed to be saturating. Cells analyzed using a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Concurrently, Quantum Simply Cellular beads (Bangs Laboratories, Fisher, IN, USA) were used to determine the antibody binding capacity of each cell line. Mean fluorescence analysis was performed using FlowJo (v 7.6.1; Tree Star, Ashland, OR, USA) and the receptor-mean fluorescence intensity data converted to antibody binding capacity using a script provided with the Quantum Simply Cellular kit. All measurements were averaged from three independently seeded wells, and the final antibody binding capacity reported as the difference above the isotype control.

Correlation of nectin-1 and viral recovery

Pearson's correlation coefficients between nectin-1 expression and viral recovery were determined by analysis of the data in Prism 5 (GraphPad Software, La Jolla, CA, USA). Cutoff for statistical significance was set at $P<0.05$.

Nectin-1 overexpression

A self-inactivating lentiviral vector was used to overexpress nectin-1 or control mCherry in oHSV-resistant cell lines. Human nectin-1 clone (Clone ID: 8322523) was obtained from Open Biosytems (Thermo Scientific, Waltham, MA, USA). Nectin-1 cDNA was PCR amplified using primers 5′-CGGATCCCGGGTCGACCCGATGGCTC GGATGGGGCTT-3′ and 5′-CCGGGTCGAGCGGCCGCGCTACACGTAC CACTCCTTCTTGGAA-3′ (IDT, Coralville, IA, USA) in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The recipient vector has been previously described.[36] An intermediate lenti-vector was first constructed by insertion of an IRES-puromycin *N*-acetyl-transferase cassette into the *Not*I- and *Eco*RI-digested pLVmnd lentivirus. The intermediate construct was then digested with *Sal*I and *Not*I for subsequent insertion of the PCR-amplified Nectin-1 cDNA. The sequence of the coding region of the resulting lentiviral vector pCK2114 was verified. The control lentivirus (pLVmnd.CIP) was similarly constructed by insertion of mCherry upstream of the IRES-puromycin *N*-acetyl-transferase cassette in the intermediate virus. Lentiviruses were produced by co-transfection of pCK2114 or pLVmnd.CIP with pMD.G (VSVG pseudotype), and pCMV. deltaR8.91 (HIV packaging) in 293T cells using Lipofectamine 2000 and OptiMEM media (Gibco, Carlsbad, CA, USA). After 12 h, transfection media was replaced with DMEM/F12 with 10% FBS. Lentiviral-enriched supernatant was collected 48 h post transfection, filtered through a 0.22-micron filter, and mixed with polybrene (8 μgml $^{-1}$). STS26T-luc and T265-luc cells were

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subsequently transduced with the resulting lentiviruses enriched by puromycin selection (5 $\mu g ml^{-1}$), followed by fluorescence activated cell sorting to obtain pure populations (UAB Comprehensive Flow Cytometry Core, Birmingham, AL, USA).

HSV titers in nectin-1 overexpressing cell lines

The impact of increased nectin-1 expression on viral titers in resistant cell lines was determined as described above with the exception of replacing infection media with MPNST growth media and 0.01% human AB serum to minimize extracellular spread of the virus. Statistical significance was determined by two-tailed Student's *T*-test assuming equal variance. Star notation indicating significant differences is as follows: (*) for $P<0.05$, (**) for $P<0.01$ and (***) for $P<0.001$.

HSV spread as measured by GFP expression

Cells were incubated with virus or mock infected for 2 h at MOI = 0.1 in infection media. Infection media was replaced with growth media and 0.01% human AB serum following incubation. Cells were harvested at 12 h intervals and analyzed by flow cytometry. The percent GFP-positive measurement was assessed by defining the GFP (FL1) gate at 1% positive of the mocktreated cells. The percentage of the infected cell population expressing GFP was then recorded. All data points were performed in triplicate, averaged, and the standard deviation reported.

In vivo viral recovery

Six-week-old athymic nude (nu/nu) mice (NCI-Frederick, Frederick, VA, USA) were obtained and allowed to adjust for a period of 2 weeks. Bilateral, subcutaneous tumors were engrafted in the flank by injection of 5×10^6 cells suspended in 50:50 BD Matrigel (Becton Dickinson) and serum-free DMEM. Four tumors were used for each virus and timepoint. When tumors reached an average size of $300mm^3$, 1×10^7 plaque forming units of oHSV suspended in saline-buffered solution was injected intratumorally. At days 3 and 5 following infection, mice were euthanized and tumors recovered in DMEM and kept on ice. After tumors were mechanically dissociated, an equivalent volume of sterile milk was added to the homogenate and viral titering was performed as described above. Titers are plotted with standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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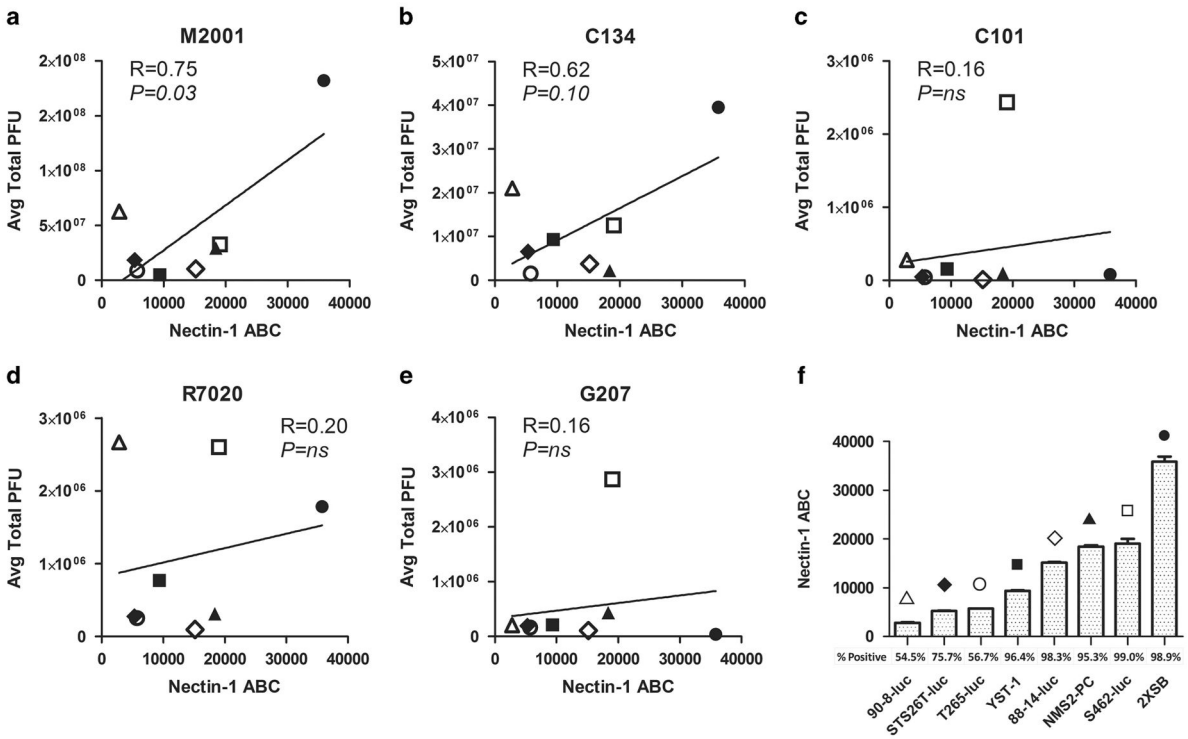

Figure 1.

Correlation of nectin-1 expression with viral titers. Pearson's correlation coefficients (**a–e**) were calculated between the viral titering data from M2001, C134, C101, R7020, G207 (Supplementary Figure 1) and the nectin-1 expression levels (**f**) from cell lines 90-8-luc (open triangle), STS26T-luc (closed diamond), T265-luc (open circle), YST-1 (closed square), 88-14-luc (open diamond), NMS2-PC (closed triangle), S462-luc (open square), and 2xSB (closed circle). A strong and significant correlation was noted for M2001. Cells were infected in triplicate by a single-step replication assay (MOI =10) and the lysates collected and titered at 24 h.p.i. Nectin-1 expression was quantified by flow cytometry after incubation with phycoerythrin (PE)-conjugated mouse monoclonal antibody with subsequent quantification using antibody quantification beads. The percentage of the cell population staining above the isotype control is also reported. Receptor quantification was performed in triplicate with the standard deviation reported.

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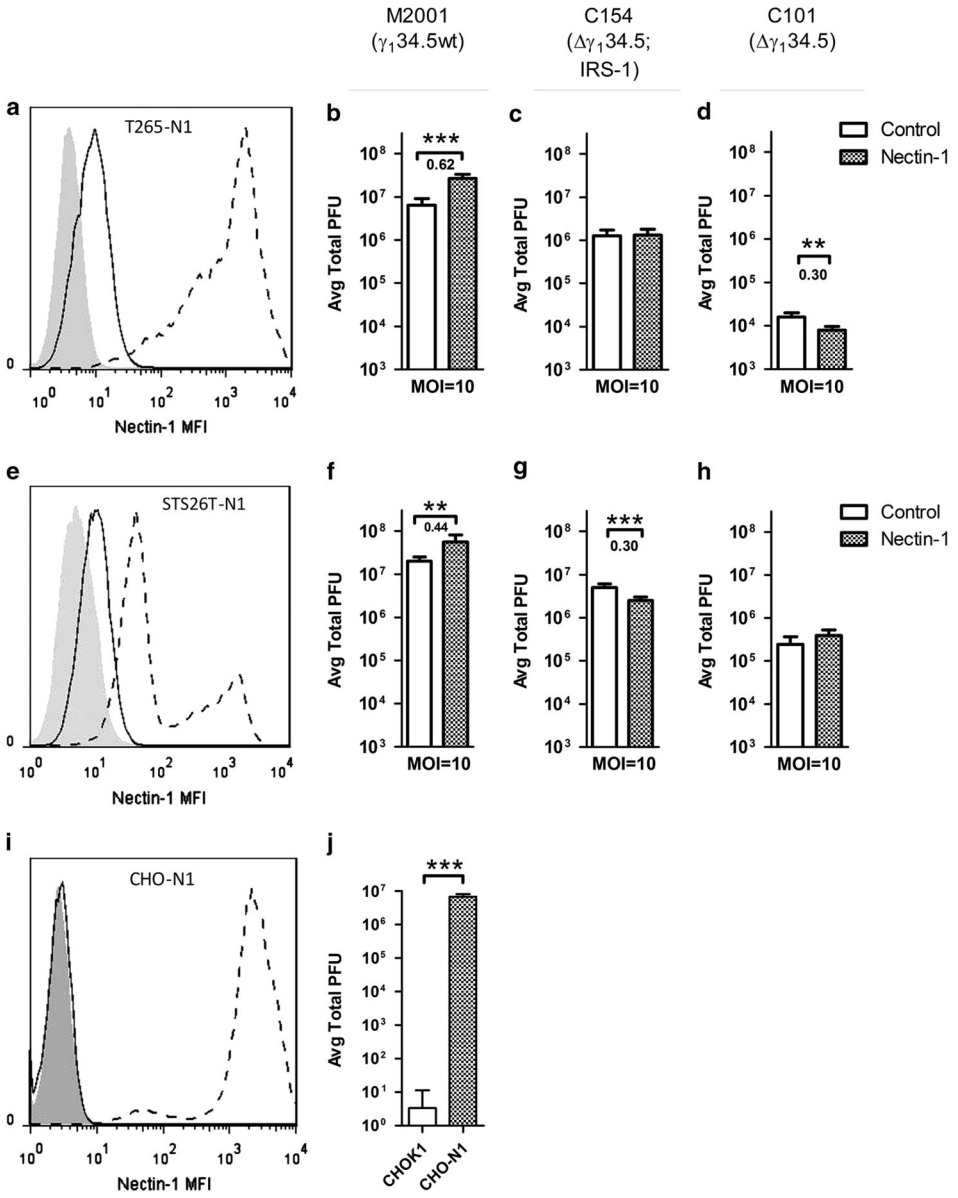

Figure 2.

Overexpression of nectin-1 in resistant cells and impact on single-step replication assays. Nectin-1 was transduced via lentivirus into oHSV-resistant cell lines T265-luc (**a**) and STS26T-luc (**e**) as well as control cell line CHO-K1. Isotype control (shaded), parent (solid line) and transduced (dashed line) cell lines are shown. Transduction of the nectin-1-deficient cell line (**i**) demonstrated function as an entry receptor as apparent by M2001 replication (**j**). The impact of nectin-1 overexpression in resistant cell lines was tested by single-step (MOI =10) replication by viruses M2001 (**b**, **f**), C154 (**c**, **g**) and C101 (**d**, **h**) and compared with control cell lines. Significance was determined by two-tailed Student's *t*-test with unequal variance. Significance was set at $P<0.05$. For cells with significant changes in titer, the logarithm of the absolute value of the increase was reported below the significance

marking. Changes in titer greater than 0.5 log are considered to be biologically relevant. ** P>0.01 and *** P>0.001.

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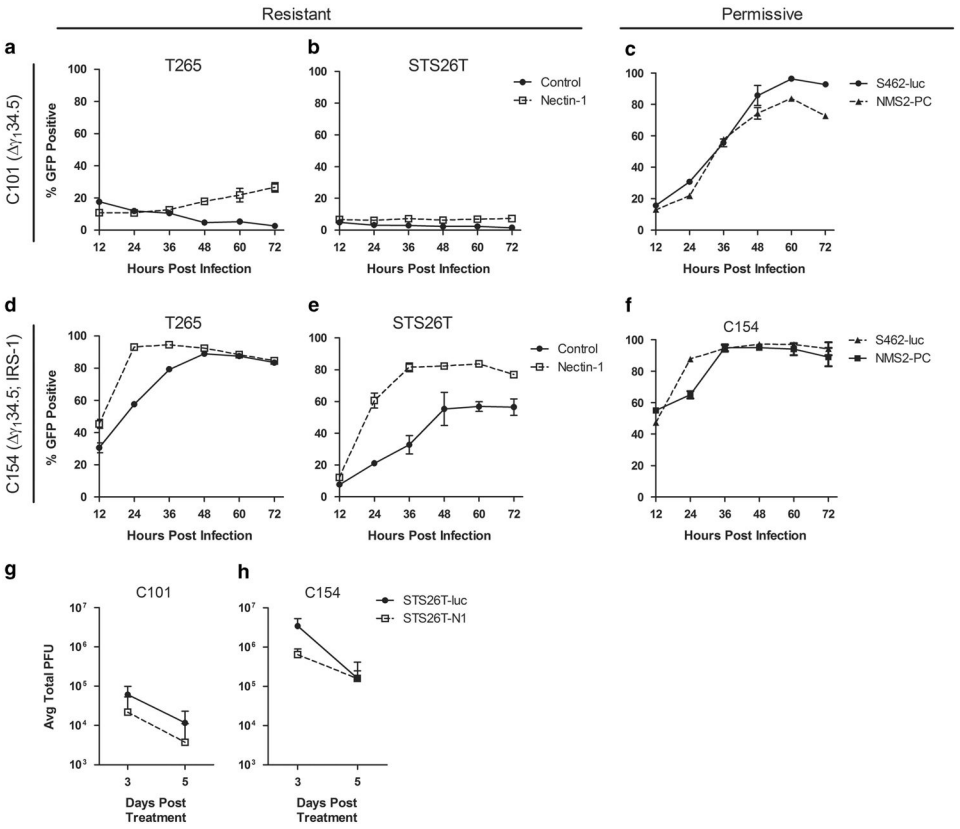

Figure 3.

Impact of increased nectin-1 expression on oHSV spread *in vitro* and viral recovery *in vivo*. Resistant cell lines STS26T-luc and T265-luc and their nectin-1 transduced variants, as well as permissive cell lines S462-luc and NMS2-PC, were infected in a multistep assay (MOI =0.1) with fully attenuated oHSV C101 (**a–c**) or second-generation C154 expressing HCMV IRS1 (**d–f**) and monitored by flow cytometry over time for viral infection as evident by expression of viral GFP. STS26T-luc and STS26T-N1 cells were engrafted in the flanks of nude mice and following tumor formation were injected with 1×10^7 plaque forming units (PFU) of C101 or C154. Tumors were harvested and viral titers determined at days 3 and 5 following infection (**g** and **h**). Data are representative of four tumors with standard deviation reported.