Osteosarcoma (OS) is the most common primary bone tumor affecting children and young adults, and development of metastatic disease is associated with poor prognosis. The purpose of this study was to evaluate the antitumor efficacy of virotherapy with engineered measles virus (MV) vaccine strains in the treatment of OS. Cell lines derived from pediatric patients with OS (HOS, MG63, 143B, KHO5-312H, U2-OS and SJSA1) were infected with MV expressing green fluorescent protein (MV-GFP) and MV-expressing sodium iodide symporter (MV-NIS) strains. Viral gene expression and cytotoxicity as defined by syncytial formation, cell death and eradication of cell monolayers were demonstrated. Findings were correlated with in vivo efficacy in subcutaneous, orthotopic (tibial bone) and lung metastatic OS xenografts treated with the MV derivative MV-NIS via the intratumoral or intravenous route. Following treatment, we observed decrease in tumor growth of subcutaneous xenografts (P = 0.0374) and prolongation of survival in mice with orthotopic (P < 0.0001) and pulmonary metastatic OS tumors (P = 0.0207). Expression of the NIS transgene in MV-NIS infected tumors allowed for single photon emission computed tomography and positron emission tomography–computed tomography imaging of virus infected tumors in vivo. Our data support the translational potential of MV-based virotherapy approaches in the treatment of recurrent and metastatic OS.
In this study we demonstrated significant antitumor efficacy of MV derivatives (including MV-NIS and MV-GFP) against OS lines and xenografts. Expression of NIS in MV infected cells resulted in effective tumor cell uptake of 99mTc and F-18 tetrafluoroborate for in vivo monitoring of virus infection by SPECT and PET-CT. Moreover, we demonstrated that treatment of athymic nude xenografts with MV-NIS resulted in statistically significant decrease in the growth of orthotopic tumors, and conferred a significant survival advantage in mice bearing orthotopic tibial bone or metastatic pulmonary tumors.

**MATERIALS AND METHODS**

**Cell culture**

HOS, MG63, 143B, KHOS-312H, U2OS and SJSA1 OS cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). SJSA1 cells were grown in Roswell Park Memorial Institute medium and all other cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1 × penicillin-streptomycin. Cells were kept at 37 °C in a humidified atmosphere of 5% CO2.

**MV strains**

Construction of MV-GFP and the NIS protein (MV-NIS) has been previously described.\(^2\),\(^6\),\(^9\) The viruses were propagated in Vero cells and titrated as described.\(^2\),\(^6\),\(^9\) The viruses were released by two cycles of freeze/thawing, and the viral titer was determined by end point dilution assay and expressed as 50% tissue culture infectious dose (TCID\(_{50}\)) per ml on Vero cells.

**Assessment of viral replication in OS cell lines**

OS lines were plated in six-well plates at a density of 4 × 10\(^5\) per well. Cells were infected at an multiplicity of infection of 1 and 0.1, in 50 μl of opti-minal minimal medium. On days 1 through 4 after infection, cell viability was measured using the MTS cell proliferation assay (Promega, Madison, WI, USA), following the manufacturer recommendations.

**Cell viability assays**

HOS, MG63, 143B, KHOS-312H, U2OS and SJSA1 cells (10 000 cells per well) were seeded in a 96-well plate and infected with the indicated virus on the following day at a multiplicity of infection of 1 and 0.1, in 50 μl of opti-minimal essential medium. On days 1 through 4 after infection, cell viability was measured using the MTS cell proliferation assay (Promega, Madison, WI, USA), following the manufacturer recommendations.

**Western blot**

Cells were collected in RIPA buffer and samples loaded on 7.5% precast polyacrylamide gel (Bio-Rad, Hercules, CA, USA) for SDS-PAGE. Gel was transferred to polyvinylidene difluoride membrane. Membranes were blocked (5% nonfat milk in Tris-buffered saline–Tween) and incubated with anti-N protein antibody developed in our laboratory (publication pending, lanko lankov) overnight at 4 °C. Rabbit mouse-specific polyclonal immunoglobulin (G, A, M) HRP conjugate (diluted 1:2000 in 5% dry milk in phosphate buffered saline) was used as the secondary antibody (Pierce, Rockford, IL, USA). Anti-human β-actin was used as a control to ensure uniform loading. Antibody binding was visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

**Animal experiments**

143B OS cells were transduced with lentivirus-expressing firefly luciferase (143B-luc). 1 × 10\(^6\) cells were then injected subcutaneously into the right flank or into the right tibial bone of 5-week old nude mice, as described.
elsewhere. For the lung metastasis model, $1 \times 10^6$ cells were injected into the tail vein of mice and lung tumors allowed to engraft over 10 days. Mice were considered to have reached the euthanasia endpoint if $> 20\%$ weight loss, tumor exceeding $10\%$ of body weight or if their tumors developed ulcerations. All experimental protocols were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

**In vivo imaging**

Engraftment in tibial bone was verified 4 days post implantation and pulmonary engraftment was verified 10 days post implantation on a Xenogen bioluminescence Imaging System (Waltham, MA, USA). In order to assess viral replication *in vivo*, subcutaneous xenografts were treated intratumorally with MV-NIS every 4 days for 2 weeks. On day 14 mice were injected with $^{99}$Tc (100 $\mu$Ci) intraperitoneally, and tumor imaging was performed serially with a high-resolution micro single-photon emission computed tomography (SPECT) system (X-SPECT; Gamma Medica-Ideas, Northridge, CA, USA).

Intravenously treated ortothoxic xenografts on day 14 of MV-NIS therapy. Following intraperitoneal injection of 0.07 MBq Na $^{18}$F$^+$ per gr of weight, animal PET scans were acquired for 60 min followed by an X-ray scan using the GENISYS4 PET imaging system (Soiie Biosciences, Culver City, CA, USA). The images were analyzed for standardized uptake value in tumor, stomach, thyroid and bladder using AMIDE image analysis tool (Stanford University, Stanford, CA, USA) and fluoride separation on two Waters neutral alumina SPE cartridges (Milford, MA, USA). The product was then sterile filtered through a 0.2 $\mu$ filter and radiochemical purity was $> 99\%$ by silica gel thin-layer chromatography (MeOH). The specific activity was $2 – 5 \mu$Ci $\mu$g$^{-1}$.

**Statistical analyses**

Data were analyzed using GraphPad Prism (GraphPad software, San Diego, CA, USA).

**RESULTS**

MV-GFP and MV-NIS have potent anti-tumor activity against OS cell lines *in vitro*

We used two trackable oncolytic MV-Edm derivatives in our *in vitro* studies (Figure 1a): MV-GFP, expressing the green fluorescent protein, and MV-NIS. A panel of six OS cell lines were studied and all of them expressed high levels of the MV receptor, CD46 (Figure 1b). Cell lines were also analyzed for expression of Nectin-4, the epithelial receptor for MV and no significant expression was detected (data not shown).

Following infection with MV-GFP at an MOI of 1 there was efficient killing of four of six OS cell line monolayers (U2OS, HOS, KHOS-312H and SJSA1 cells) within 48–72 h (Figure 2a). MV-NIS had superior activity as compared with MV-GFP, with activity against all six lines, including the MG63 and 143B cells (Figure 2b). Because of the transcriptional gradient

**Figure 2.** Osteosarcoma (OS) cell lines are susceptible to measles virus (MV) infection. (a) Significant cytopathic effect observed against different OS cell lines following MV-expressing green fluorescent protein (MV-GFP) infection at an MOI of 1 ($n = 3$ independent experiments). (b) MV-expressing sodium iodide symporter (MV-NIS) infection at an MOI of 1 similarly led to significant cell death ($n = 3$ independent experiments). The MV-NIS cytopathic effect peaked earlier in the majority of cell lines, likely reflecting the impact of different transgene position on viral replication.

**Figure 3.** Measles virus-expressing green fluorescent protein (MV-GFP) infection kinetics in the moderately susceptible 143B sarcoma cell line. MV-GFP infection leads to (a) GFP expression is increased over time until monolayer obliteration. Images A1, 2 and 3 were taken on days 1, 2 and 3, respectively following infection (4× magnification). (b) Increased expression of measles N protein during the same time course.
Figure 4. Measles virus-expressing green fluorescent protein (MV-GFP) and MV-expressing sodium iodide symporter (MV-NIS) replicate efficiently in tumor lines, as demonstrated by one-step viral growth curves. Increased replication of MV-NIS as compared with MV-GFP was seen in the panel of six osteosarcoma cell lines.

Figure 5. Measles virus-expressing sodium iodide symporter (MV-NIS) treatment schema. 143B-luc osteosarcoma cells were implanted into the right flank, right tibial bone or via tail vein injection (lung metastasis model). On the day prior to the MV-NIS treatment initiation, engraftment was confirmed by Xenogen bioluminescence imaging. Mice were then randomized to receive intravenous MV-NIS treatment or heat-inactivated virus every 4 days for a total of 4 weeks. Two weeks after the initiation of therapy, mice were also imaged by single photon emission computed tomography or positron emission tomography–computed tomography to monitor in vivo viral activity.
during MV replication\textsuperscript{31} the different transgene positions within the MV genome (position one for MV-GFP vs position six for MV-NIS) could explain the difference in the cytopathic effect we observed between the two strains.

Though the pulmonary metastatic 143B cell line was immediately susceptible to MV-GFP oncolysis, infection with MV-GFP led to abundant GFP expression. Green fluorescence increased over the course of infection and syncytia grew in size and number ultimately leading to eradication of the monolayer. MV nucleoprotein (N-protein) expression was verified by western blot and N-protein expression increased over the first 3 days of infection (Figures 3a and b). Relative resistance to infection of 143B cells to MV-GFP could be overcome by increasing the multiplicity of infection (data not shown). In one step viral growth curves both strains resulted in replication, although higher titers of MV-NIS vs MV-GFP were obtained (Figure 4).

Characterization of 143B-luc flank, orthotopic and metastatic OS xenograft models

To investigate the therapeutic potential of MV-NIS treatment in the recurrent or pulmonary metastatic setting the aggressive and highly metastatic 143B cell line was chosen for xenograft development. 143B cells were transduced by a lentivirus-expressing firefly luciferase, to generate 143B-luc cells. We injected 100 μl of 143B-luc tumor cells (1 × 10\textsuperscript{6}) into the right flank, right tibial bone or intravenously into the tail vein of 5 week old mice to establish xenografts. Following verification of engraftment mice were then treated either with MV-NIS or heat-inactivated control virus every 4 days for a total of 4 weeks (Figure 5). Subcutaneous and orthotopic tibial tumors could be detected by luciferin bioluminescence within 4 days of implantation. Lung engraftment was similarly verified at 10 days post intravenous (IV) implantation (Supplementary Figure).

MV-NIS has potent antitumor activity in mouse xenografts

Flank tumors were generated, and were treated every four days with intratumoral (IT) injections of 1 × 10\textsuperscript{6} TCID\textsubscript{50} MV-GFP, MV-NIS or heat-inactivated control virus (n = 5 per group) (similar to the schema of Figure 5) and tumor size was measured with digital calipers. A decrease in tumor growth was observed (Figure 6) with MV-GFP (P = 0.0407) and MV-NIS treatment (P = 0.0374).
compression and hind limb paralysis secondary to metastatic involvement of the vertebral spine.

We also assessed survival following intravenous MV-treatment both in tibial orthotopic and pulmonary metastatic OS xenografts. Xenografts treated every 4 days with MV-NIS (n = 10) at a dose of $1 \times 10^6$ TCID$_{50}$ had a statistically significant prolongation of survival compared with mice treated with heat-inactivated virus (n = 10) both in the orthotopic tibial bone model (P < 0.0001, Figure 8a) and the pulmonary metastatic model (P = 0.0207, Figure 8b). In the orthotopic model, median survival of the treated mice was 60 days compared with 30 days for control animals. In fact, all MV-NIS-treated mice with orthotopic tibial xenografts were alive on day 40, as compared with none of the mice in the control group.

MV-NIS infects OS xenografts and NIS transgene expression can be monitored in vivo

In a separate study in order to assess viral replication in vivo, mice were treated with MV-NIS every 4 days for 2 weeks either directly into flank tumors or intravenously in established orthotopic xenografts. Monitoring of viral infection and replication in vivo was then performed using two different imaging modalities. In the subcutaneous flank tumor model MV-NIS-treated mice (IT injection) were administered Tc-99m into the peritoneum (IP) on day 14 and then imaged 30 min later by CT-SPECT. IT accumulation of Tc-99m in subcutaneous flank tumors was seen in mice treated with MV-NIS (Figure 9a), but not in animals treated with the inactive virus preparation (Figure 9a, lower panel). Cross-sectional imaging demonstrated MV-NIS gene expression in tumors; expression of NIS by infected tumor cells resulted in Tc-99m concentration, which we could detect by SPECT imaging.

We next evaluated the ability of IV MV-NIS therapy to result in the IT concentration of the radioactive tracer $[^{18}]$F tetrafluoroborate, through NIS mediated transport. PET-CT imaging performed 60 min after IP administration F-18 tetrafluoroborate resulted in significant uptake in tibial tumors (Figure 9b) and thereby convincingly demonstrated viral gene expression in OS tumors following systemic viral administration.

**DISCUSSION**

OS is an aggressive bone cancer with peak incidence in childhood, adolescence and early adulthood. Although surgical advancements and combination chemotherapy have led to a 65–70% cure rate, no additional improvement in survival...
has materialized during the last two decades; new therapeutic approaches are urgently needed for this young patient population.

Oncolytic measles virotherapy provides a novel and safe therapeutic strategy for treatment of recurrent and metastatic OS. The fact that viruses have adapted over millennia of evolution to efficiently invade cells and overtake their biosynthetic machinery, makes them attractive candidates for the development of novel antitumor approaches. Other groups have used replication-competent oncolytic viruses in the treatment of OS mostly focusing on in vitro efficacy studies and loco-regional in vivo delivery. Oncolytic Semliki forest virus was previously shown to have in vitro activity, and IT injections in an orthotopic K7M2 osteosarcoma model showed tumor regression and survival benefit. Polyomavirus targets the cell surface receptor CD155, and was found to induce apoptosis through induction of caspases 7 and 3 in bone and soft tissue sarcoma cells. Vesicular stomatitis virus delivered by isolated limb perfusion has similarly been shown to suppress osteosarcoma growth in an immune competent rat model. Our study represents the first report that demonstrates efficacy of an engineered MV strain in treatment of bone sarcomas, following both IT and systemic administration in challenging to treat orthotopic and lung metastatic models.

In this study, we have demonstrated that MV infects OS cell lines and that replication of MV is efficient, with evidence of viral replication to high titers in OS cells. Indeed, IV delivery of MV-NIS resulted in a decrease in growth of 143B orthotopic tumors and prolongation of survival of tumor-bearing mice. We have also shown that SPECT or PET-CT allow for efficient tracking of infected OS cells in vivo. We showed that IT and IV therapy can both be monitored in vivo, and that SPECT and PET-CT imaging are sensitive enough to differentiate infected tumor cell uptake. The ability to monitor viral replication and transgene expression in vivo also allows for pharmacodynamic measurements of viral distribution and dissemination, and various radioactive substrates can be used to quantify NIS gene expression including $^{125}$I, F-18 tetrafluoroborate and 99mTc. Recently reported clinical data support that SPECT-CT can be used to monitor viral replication in patients, for example, myeloma tumor deposits following systemic administration. NIS may also be used as a therapeutic transgene capable of further increasing the oncolytic potency of MV-NIS by allowing the intracellular concentration of radioisotopes, such as the beta particle emitter, $^{131}$I, as a mediator of radiovirotherapy.

Infection with oncolytic MV has also been shown to directly activate the immune system in immune competent preclinical models. The viral hemagglutinin has been shown to interact with and induce toll-like receptor-2 (TLR-2) signaling, and TLR-7 and TLR-9 have also been shown to be involved in viral nucleic acid detection and antiviral signaling. In preclinical studies MV vaccine-infected tumor cells cocultured with plasmacytoid dendritic cells induce maturation of dendritic cells into efficient antigen-presenting cells, with upregulation of costimulatory molecules CD40 and CD86. The antitumor activity of DCs dependent upon interferon-α (IFN-α)-mediated, autocrine stimulation and IFN-α can also directly stimulate apoptosis in tumor cells. IFN-α induction by 2-methoxyestradiol in OS cells has similarly been shown to have antiproliferative effects in vitro. MV-Edm derivatives have also been engineered to express immunostimulatory cytokines including granulocyte-macrophage colony stimulating factor (MV-GM-CSF) and IFNβ (MV-IFNβ), both important immune regulators that have been shown to induce antitumor immune responses in several tumor types. Work in our laboratory has also demonstrated that MV engineering to express the immunomodulatory Helicobacter pylori neutrophil-activating protein induces a brisk Th1 cytokine response in vivo, associated with high levels of TNF-α production as well as prolongation of survival in an aggressive model of lung metastatic breast cancer.

These engineered strains could also have excellent applicability in the treatment of OS as they could bridge oncolytic virotherapy with sarcoma immunotherapy, and they are currently in preclinical investigation.

MV has a proven record of safety in large-scale immunization campaigns; however, widespread immunization in the Western world can also pose a major challenge to oncolytic measles virotherapy approaches. Although delivery challenges are difficult to address in immunocompromised xenograft models, several techniques have been used to augment treatment efficacy. Rapid clearance of virus can take place shortly after the virus is introduced into an immunized host and represents a potential limitation for systemic administration approaches. Strategies for augmenting potency and ensuring safety of virotherapy include viral genetic manipulations, as already described, concurrent chemotherapy and immunomodulatory use of cyclophosphamide as has been demonstrated for MV-NIS in primate models. IV delivery of high doses of MV in patients lacking anti-MV-neutralizing antibodies has been successfully employed against refractory multiple myeloma. However, in the presence of neutralizing titers of anti-measles antibodies infected cell carriers have been used efficaciously to deliver MV to tumor cells; the infected cell carriers can circumvent and increase efficacy in the setting of pre-existing anti-measles humoral immunity. This concept has been demonstrated by employing dendritic cells in breast cancer xenografts and mesenchymal stem cell carriers in passively immunized mice bearing ovarian xenografts. More recently, human bone marrow-derived mesenchymal stromal cell carriers were similarly used in passively immunized mice to efficiently deliver MV in a systemic xenograft model of precursor B-lineage-acute lymphoblastic leukemia.

In summary, our results demonstrate that MV-NIS exhibits significant therapeutic effect against human OS cell lines in vitro and orthotopic and metastatic disease models in vivo. MV could provide a new therapeutic addition to multimodality treatment of patients with recurrent or metastatic OS, especially given the lack of cross-resistance with existing therapies and continuing improvements in the production of high titer therapeutic viral preparations. Promising emerging data in other tumor types such as ovarian cancer and multiple myeloma further highlight this potential. Based on these encouraging results, this approach has significant translational potential, and further preclinical and clinical studies are warranted.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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