In a rare example, Kelly *et al.* showed that oncolytic coxsackie virus A21 with inserted muscle-specific miRNA target sequences escaped from the cellular miRNA system by mutation of the target inserts.[26] We did not find any indication of escaped mutants from LC16m8Δ-B5R$_{let7a}$/LG, as shown in the bioluminescence images of the BxPC-3 model (**Figure 6e**). Furthermore, sequence analysis did not show any mutations in the target inserts of LC16m8Δ-B5R$_{let7a}$/LG or LC16m8Δ-B5Rgfp$_{let7a}$ during cell culture passages. As reported previously,[20] B5R^{+} revertants spontaneously emerged from LC16m8 by frameshift mutation resulting from a single nucleotide insertion at site just upstream of the deletion site in the open reading frame of the *B5R* gene. Since the MRVV has four copies of miRNA complementary target sequences for let-7a in the 3′UTR (and not the open reading frame) of the *B5R* gene, it is unlikely that a slight mutation would result in a significantly different phenotype, even if a mutation in these target inserts occurred during viral replication.

On the other hand, the spread of LC16m8Δ-B5R$_{let7a}$/LG was much less than that of the unregulated LC16mO/LG or the control LC16m8Δ-B5R$_{let7a\text{-}mut}$/LG in SCID mice and therefore did not cause any pock lesions in normal mouse tissues where let-7a is abundant (**Figure 5a–c**). However, quantitation of the bioluminescence signal from the luciferase-expressing vaccinia revealed that the LC16m8Δ-B5R$_{let7a}$/LG signal was still higher by 1–2 log orders than the B5R-deleted LC16m8Δ/LG signal (**Figure 5c**). These results suggest that miRNA-mediated inhibition of B5R expression may be overcome by miRNA saturation, which has been observed by Kelly *et al.* previously.[44] The possibility is also supported by another data that no B5R-EGFP expression was observed in HeLa cells infected with LC16m8Δ-B5Rgfp$_{let7a}$ at an multiplicity of infection (MOI) of 0.1 (**Figure 3d**); however, 100-fold higher input multiplicities of LC16m8Δ-B5Rgfp$_{let7a}$ allowed B5R-EGFP expression in HeLa cells (data not shown). Thus, more attention should be paid to miRNA saturation rather than to mutation of miRNA target inserts in designing vaccinia viruses for future use. In this regard, incorporation of different miRNA target sequences of more than one miRNA species might be one strategy to address the question of miRNA saturation.

In conclusion, we developed a highly attenuated MRVV with let-7a miRNA complementary target sequences in the 3′UTR of the *B5R* gene. This MRVV could selectively replicate and induce oncolysis in tumor cells without affecting normal cells, depending on the miRNA expression level. More generally, this study shows that control of viral replication and oncolytic activity by miRNA-based gene regulation provides a potentially novel and versatile platform for engineering vaccinia viruses for cancer virotherapy.

MATERIALS AND METHODS

Plasmid construction. The construction of all plasmids used in this study is described in the **Supplementary Materials and Methods**.

Cell culture. Human carcinoma cell lines [lung A549 (Ham's F12K); pancreatic BxPC-3, PANC-1 and neuroblastoma SK-N-AS (RPMI-1640); colorectal Caco-2, epidermoid HEp-2 (E-MEM); cervical HeLa and breast MDA-MB-231 (D-MEM)] and rabbit kidney-derived RK13 cells (E-MEM) were obtained from the American Type Culture Collection (Manassas, VA) and grown in their respective mediums (Wako, Osaka, Japan) with 10% fetal bovine serum (Hyclone, Waltham, MA) at 37 °C in a humidified atmosphere with 5% CO_2. NHLF cells was purchased from TaKaRa Biomedicals (Otsu, Japan) and cultured according to the manufacturer's protocol. HeLa-let7aKD or HeLa-NC cells were generated by infecting HeLa cells with lentivirus expressing tough decoy (TuD) RNA against let-7a or a negative control, respectively, with the human 7SK RNA polymerase III promoter,[45] as described previously.[46] Single HeLa-let7aKD or HeLa-NC cell isolates were expanded and selected in media containing 5 μg/ml puromycin (Sigma, St Louis, MO).

Virus construction. To construct viruses with B5R, namely, LC16m8Δ-B5R and LC16m8Δ-B5Rgfp, RK13 cells were infected with B5R-deleted LC16m8Δ viruses[20] at a MOI of 0.02, and then transfected with pB5R or pTN-B5Rgfp. After harvesting the progeny viruses 2–5 days later, LC16m8Δ-B5R and LC16m8Δ-B5Rgfp were selected on the basis of larger plaque size and/or enhanced EGFP expression, by three serial plaque purifications. Finally, the insertion of *B5R* was verified by sequencing the modified region.

Similarly, miRNA-regulated viruses, namely, LC16m8Δ-B5R$_{let7a}$, LC16m8Δ-B5R$_{let7a\text{-}mut}$, LC16m8Δ-B5Rgfp$_{let7a}$, and LC16m8Δ-B5Rgfp$_{let7a\text{-}mut}$ were constructed by infecting RK13 cells with LC16m8Δ viruses, as described above, and then transfecting them with pTN-B5R$_{let7a}$ × 4, pTN-B5R$_{let7a\text{-}mut}$ × 4, pTN-B5Rgfp$_{let7a}$ × 4, or pTN-B5Rgfp$_{let7a\text{-}mut}$ × 4, respectively.

Likewise, the viruses expressing luciferase and EGFP, namely, LC16mO/LG, LC16m8Δ/LG, LC16m8Δ-B5R/LG, LC16m8Δ-B5R$_{let7a}$/LG, and LC16m8Δ-B5R$_{let7a\text{-}mut}$/LG, were constructed by infecting RK13 cells with LC16mO, LC16m8Δ, LC16m8Δ-B5R, LC16m8Δ-B5R$_{let7a}$, or LC16m8Δ-B5R$_{let7a\text{-}mut}$ viruses, respectively, as described above, and then transfecting them with pSFJvnc110-LucIRESgfp. All viruses were propagated and titrated in RK13 cells and stored at −80 °C.

Quantification of let-7a miRNA. First, total RNA was isolated from A549, BxPC-3, HeLa, PANC-1, and NHLF cells and also normal brain, heart, kidney, liver, lung, ovary, spleen, and tail of 6-week-old female athymic nude mice (Charles River Laboratories, Yokohama, Japan) using the mirVana microRNA isolation kit (Ambion, Carlsbad, CA). Then, expression of mature let-7a miRNA and the endogenous control were quantified by real-time PCR using the TaqMan microRNA assay kit (Applied Biosystems, Carlsbad, CA) for has-let-7a miRNA and U6 small nuclear RNA (snRNA), respectively. The relative expression of let-7a was calculated by using the comparative threshold method (Applied Biosystems User Bulletin No. 2).

Luciferase reporter assay. Cells in 96-well optical-bottom white plates (Nunc, Rochester, NY) were transfected with 0.1 μg of pMirGlo$_{let7a}$ or pMirGlo$_{let7a\text{-}mut}$ plasmid containing two expression units that encode Renilla luciferase (*RLuc*) acting as a transfection control and *FLuc* with four copies of let-7a target sequences or the disrupted sequences in the 3′UTR respectively, using Fugene HD (Roche, Basel, Switzerland). At 24 hours after transfection, the cells were analyzed for luciferase activities using the Dual-Glo Luciferase Assay System (Promega, Madison, WI).

Viral infection. Each cell line was infected with a vaccinia virus at an MOI of 0.1 or 0.5 plaque-forming unit (pfu)/cell, respectively, in Opti-MEM medium (Invitrogen, Carlsbad, CA) for 1 hour at 37 °C in 24-well or 96-well plates. Seventy-two hours after infection, the cells in the 24-well plate were photographed under phase-contrast or fluorescence microscopy. Subsequently, the infected cells were harvested into 1 ml of growth medium and sonicated to release the replicated viruses for titration in RK13 cells. One hundred twenty hours after infection, the viability of the cells in the 96-well plate was determined by using the CellTiter 96 AQueous cell proliferation assay kit (Promega).

In vivo *experiments.* The protocols for the following animal experiments were approved by the Animal Experiment Committee of the Institute of Medical Science, University of Tokyo, Japan.

In the first *in vivo* experiment, BxPC-3 cells stably expressing luciferase (5×10^6 cells in 100 µl of phosphate-buffered saline, pH 7.4) were intraperitoneally injected into 6-week-old female SCID mice (Charles River Laboratories) on day 0. Seven days later, the mice were administered a single intraperitoneal injection of LC16mO or LC16m8Δ (1×10^7 pfu in 100 µl of Opti-MEM per mouse). Control animals (mock therapy) were injected with 100 µl of Opti-MEM without any virus. To monitor *in vivo* tumor growth noninvasively, 150 µl of D-luciferin (15 mg/ml) was administered to the treated mice on days 4, 18, and 29. The mice were anesthetized with isoflurane before imaging the tumors with the IVIS 100 bioluminescence imaging system (Xenogen, Hopkinton, MA). The bioluminescence signals were quantified according to the manufacturer's protocol.

In the second *in vivo* experiment, 6-week-old female SCID mice were intraperitoneally injected with a single dose of each vaccinia virus expressing luciferase (1×10^7 pfu in 100 µl of Opti-MEM per mouse) on day 0. To monitor the *in vivo* viral growth, D-luciferin was injected into the mice on days 3, 9, or 16, and then they were examined by bioluminescence imaging, as described above.

In the third *in vivo* experiment, subcutaneous tumors were established by injecting A549 or BxPC-3 cells (5×10^6 cells in 100 µl of phosphate-buffered saline, pH 7.4) into the right flank of 6-week-old female athymic nude mice (Charles River Laboratories). When the tumors reached 5–8 mm in diameter, the mice received three intratumoral injections of each vaccinia virus (1×10^7 pfu in 100 µl of Opti-MEM per mouse) on days 0, 3, and 6. Control animals (mock therapy) were injected with 100 µl of Opti-MEM without any virus. The mice were euthanized at the end of the experiment or when any of the following occurred: tumor burden exceeded 2,500 mm^3, tumor ulceration occurred, or symptoms of severe viral toxicity, such as pock lesions on body surfaces and weight loss of >30%, manifested. The diameter of tumors was measured three times per week, and the volume of a tumor was calculated according to the formula: volume = $0.5 \times \text{length} \times \text{width}^2$. The virus biodistribution was determined by injecting D-luciferin into the mice on day 27 or 52, followed by bioluminescence imaging, as described above.

Statistical analysis. The differences in cytolytic activity, *in vivo* viral replication, and tumor burden between treatment groups were analyzed for statistical significance by one-way or two-way ANOVA and the Bonferroni test when ANOVA showed overall significance. P values <0.05 were considered to be statistically significant. Survival curves were constructed using the Kaplan–Meier method. Survival times were statistically analyzed by using the log-rank test. Data were analyzed using GraphPad Prism Ver 5 (GraphPad Software).

SUPPLEMENTARY MATERIAL

Figure S1. Inhibitory effects of TuD RNA on endogenous let-7a activity.

Figure S2. B5R expression of miRNA-regulated vaccinia virus in normal tissues.

Figure S3. Representative images of the biodistribution of MRVV/LG, determined by noninvasive imaging after intraperitoneal injection of D-luciferin into the mice that are shown in (**Figure 6c,d**) on days 3, 10, and 20.

Figure S4. B5R expression of miRNA-regulated vaccinia virus in subcutaneous mouse xenografts that expressed low levels of let-7a.

Materials and Methods.

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