MicroRNA Modification of Coxsackievirus B3 Decreases Its Toxicity, while Retaining Oncolytic Potency against Lung Cancer

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We recently discovered that coxsackievirus B3 (CVB3) is a potent oncolytic virus against KRAS mutant lung adenocarcinoma. Nevertheless, the evident toxicity restricts the use of wild-type (WT)-CVB3 for cancer therapy. The current study aims to engineer the CVB3 to decrease its toxicity and to extend our previous research to determine its safety and efficacy in treating TP53/RB1 mutant small-cell lung cancer (SCLC). A microRNA-modified CVB3 (miR-CVB3) was generated via inserting multiple copies of tumor-suppressive miR-143/miR-145 target sequences into the viral genome. In vitro experiments revealed that miR-CVB3 retained the ability to infect and lyse KRAS mutant lung adenocarcinoma and TP53/RB1-mutant SCLC cells, but with a markedly reduced cytotoxicity toward cardiomyocytes. In vivo study using a TP53/RB1-mutant SCLC xenograft model demonstrated that a single dose of miR-CVB3 via systemic administration resulted in a significant tumor regression. Most strikingly, mice treated with miR-CVB3 exhibited greatly attenuated cardiotoxicities and decreased viral titers compared to WT-CVB3-treated mice. Collectively, we generated a recombinant CVB3 that is powerful in destroying both KRAS mutant lung adenocarcinoma and TP53/RB1-mutant SCLC, with a negligible toxicity toward normal tissues. Future investigation is needed to address the issue of genome instability of miR-CVB3, which was observed in ~40% of mice after a prolonged treatment.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death among both men and women worldwide.1 There are two major forms of lung cancer, non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), with the former constituting ~85% of all lung cancer cases. Among them, adenocarcinoma is the most common type of lung cancer, responsible for almost half of all lung cancers, and is associated with both smokers and non-smokers.2 Genetic mutations play a critical role in the development of lung adenocarcinoma. The well-identified oncogenic driver mutations in lung adenocarcinoma include those in epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and Kirsten rat sarcoma viral oncogene homolog (KRAS), which occur in ~15%, ~7%, and ~30% of lung adenocarcinoma, respectively.3 SCLC accounts for ~15% of all lung cancers and is almost exclusive to smokers. Between 60% and 90% of SCLC cases feature mutations in gene encoding tumor protein p53 (TP53mut) and/or retinoblastoma protein (RB1mut).4 Although lung adenocarcinoma associated with EGFR mutations (EGFRmut) or ALK translocations can be clinically treated using tyrosine kinase inhibitors,2 lung adenocarcinoma with KRAS mutations (KRASmut) and SCLC are currently undruggable and associated with a poor prognosis.4,5 Highly effective and innovative treatment modalities for these subsets of advanced lung cancer are therefore urgently needed.

Recent advances in oncolytic virotherapy provide a promising new treatment approach.6,7 Oncolytic viruses are a group of viruses that are genetically engineered or naturally occurring to specifically destroy cancer cells while sparing normal tissues. Their unique tumor-destructive mechanism lies in their ability of lytic replication, resulting in the lysis of cancer cells and the release of viral progeny to infect neighboring cells. Moreover, oncolytic viruses can overcome the immunosuppressive effects of tumors and have the ability to initiate anti-tumor immunity.6,7 In October 2015, the US Food and Drug Administration (FDA) approved the first genetically modified herpes simplex virus 1 (talimogene laherparepvec [T-VEC]) for the treatment of melanoma.8 During the past decades, several oncolytic viruses (both RNA and DNA viruses), including coxsackievirus A21,9 reovirus,10 vaccinia virus,11 adenovirus,12 measles virus,13 New- castle disease virus,14 and vesicular stomatitis virus,15 have been...
Coxsackievirus B3 (CVB3) is an enterovirus in the family of Picornaviridae. It is a small, non-enveloped virus that contains a positive RNA genome encoding a single open reading frame flanked by 5' and 3' untranslated regions (UTRs). Although CVB3 infection can be severe in children and immunocompromised individuals, causing myocarditis, pancreatitis, and meningitis, infection in adults is generally asymptomatic or causes mild flu-like symptoms. Our recent study has demonstrated that CVB3 is an extremely potent oncolytic virus against KRASmut lung adenocarcinoma, while sparing normal lung epithelial cells, and EGFRmut lung adenocarcinoma. Despite this promising discovery, we found that wild-type (WT)-CVB3 causes damage to multiple organs, particularly to the heart, in immunodeficient mice.

In the current study, we aimed to use microRNA (miRNA) targeting to modify the CVB3 genome to lessen its toxicity to normal tissues while maintaining oncolytic properties specifically in cancer cells. miRNAs are a class of endogenous small non-coding RNAs that are evolutionarily conserved and act as key regulators in a wide range of fundamental cellular functions, including cell proliferation, differentiation, and apoptosis, by binding to the mRNAs with complementary sequences. Subsequently, they promote either mRNA degradation or suppression of translation. Recent evidence suggests that miRNAs also play a key role in tumorigenesis and progression of cancers. miRNAs are commonly downregulated in different types of cancer tissues in comparison with normal tissues. This unique feature of cancer cells can be exploited to develop miRNA-sensitive, tumor-specific oncolytic viruses. In this study, we showed that inclusion of tumor-suppressive miRNA complementary target sequences into the CVB3 genome markedly reduces its virulence to normal tissues without compromising its anti-tumor potency. Moreover, we demonstrated that, in addition to KRASmut lung adenocarcinoma, CVB3 also acts as a potent oncolytic virus against TP53mut/RB1mut SCLC.

RESULTS

miR-145 and miR-143 Are Significantly Downregulated in Lung Cancer Cells Compared with Normal Lung Epithelial Cells and Cardiomyocytes

As alluded to above, our recent in vitro and in vivo studies discovered that WT-CVB3 effectively destroys KRASmut lung adenocarcinoma. Nonetheless, it was observed that the efficient tumor suppression is accompanied by damage to normal tissues, particularly the heart in immunocompromised mice. In this study, we aimed to genetically engineer the CVB3 genome to decrease its toxicity to normal tissues.

The miRNAs miR-145 and miR-143 have been reported to be tumor suppressive and significantly downregulated in lung cancer tissues. To confirm their relative abundance in lung cancer versus normal tissues, quantitative PCR (qPCR) was conducted to measure the levels of miR-145 and miR-143 in various lung cancer and normal cells. As shown in Figures 1A and 1B, the expression of both miR-145 and miR-143 was significantly downregulated in lung cancer cells, including KRASmut lung adenocarcinoma cells (H2030, H23, and A549) and TP53mut/RB1mut SCLC cells (H524, H526, and H528), and HeLa cells were measured by qRT-PCR and calculated using the equation RQ (relative quantity) = 2^-DDCt. The results are presented as mean ± SD (n = 3). One-way ANOVA was used to assess the differences among different cell types. *p < 0.05, **p < 0.01, ***p < 0.001. (C) Construction of miRNA-modified CVB3 (miR-CVB3). Four copies of miR-145 (5'-AAGGGATTCCTGGGAAAACTGGAC-3') and two copies of miR-143 (5'-GAAGCTACAGTGCTTCATCTCA-3') target sequences were inserted between the 5' UTR and VP4 of the CVB3 genome. A Kozak sequence was added before the start codon (ATG) of VP4 to facilitate the translation of viral protein in cancer cells.

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H526), than in normal lung epithelial cells (BEAS2B and primary lung epithelial cells) and cardiomyocytes (mouse HL-1 cardiomyocytes and human induced pluripotent stem cell [iPSC]-derived cardiomyocytes [iCMs]). Also note that the levels of miR-145 and miR-143 in HeLa cells, in which WT-CVB3 and miR-CVB3 were grown and titered, were also very low. Our data suggest that miR-145 and miR-143 serve as candidate targets for restricting oncolytic CVB3 replication to tumor cells.

Construction of miRNA-Modified CVB3

We then engineered several miRNA-modified CVB3s, in which multiple copies of miR-145 target sequences alone (either in its forward or reverse orientation) or in combination with miR-143 target sequences (the core sequences of miR-145/miR-143 between mice and humans are 100% identical) were inserted into the 5′ UTR or 3′ UTR of the CVB3 genome to promote tumor-targeted viral replication. Among them, we found that miRNA-regulated CVB3s, in which four copies of miR-145 and two copies of miR-143 target sequences were inserted into either the 5′ UTR or 3′ UTR of the CVB3 genome (Figure 1C), displayed the least cardiotoxicity and the highest anti-tumor potency in vitro (Figure S1). In this study, we used miRNA (5′ UTR)-modified CVB3 (denoted hereinafter by miR-CVB3) to generate virus stocks for subsequent experiments.

miR-CVB3 Shows a Significantly Reduced Cytotoxicity toward Cardiomyocytes

To test the safety of the newly generated recombinant CVB3, we assessed the cytotoxicity of miR-CVB3 toward mouse HL-1 cardiomyocytes, as this cell line has been extensively used to study CVB3-induced cardiac damage, and it highly expresses miR-145 and miR-143 (Figures 1A and 1B). After a 72-h viral infection over a range of multiplicity of infection (MOI) of 0.01 to 100, we demonstrated a significant reduction of cytotoxicity in miR-CVB3-treated cells in comparison with WT-CVB3-treated cells, as assessed through morphological observation (Figure 2A), crystal violet staining (Figure 2B), and a cell viability assay (Figure 2C). The decreased cardiotoxicity of miR-CVB3 was further verified in human cardiomyocytes (iCMs) by the cell viability assay (Figure 2D).

miR-CVB3 Retains Its Lytic Potency against KRASmut Lung Adenocarcinoma Cells

We next sought to determine whether inclusion of miRNA targets to the CVB3 genome affects its lytic ability against lung cancer cells. Consistent with our previous observation that KRASmut lung adenocarcinoma cells are acutely permissive to WT-CVB3, we found that WT-CVB3 efficiently killed KRASmut lung adenocarcinoma cells (H2030, H23, A549) in a dose-dependent manner (Figure 3). Moreover, we found that miR-CVB3 retained its ability and potency to lyse KRASmut cells, although at a slightly reduced level compared to WT-CVB3.

Both WT-CVB3 and miR-CVB3 Effectively Induce SCLC Cell Death

As discussed earlier, SCLC is the most aggressive subtype of lung cancer and is associated with poor prognosis.\(^4\) Most patients with SCLC are diagnosed late when the cancer is already advanced and there is no
targeted therapy for SCLC. In the current study, we extended our previous work on NSCLC to investigate the ability of both WT-CVB3 and miR-CVB3 in killing SCLC. H524 and H526, two SCLC cell lines carrying TP53/RB1 mutations characteristic of this disease, were utilized in this study. Following a 72-h viral infection at an MOI of 0.1, 1, and 10, both WT-CVB3 and miR-CVB3 caused severe cytopathic effects at all viral doses (Figure 4A). Furthermore, cell viability assays revealed that miR-CVB3 dose-dependently destroyed these cells in a comparable manner to WT-CVB3 (Figure 4B).

Lastly, BEAS2B, a normal human lung epithelial cell line that expresses elevated levels of miR-145 and miR-143 (Figures 1A and 1B), was used to examine the possible cytotoxicity of miR-CVB3 toward normal lung cells. In agreement with our earlier report,17 infection with WT-CVB3 caused a marginal cytopathic effect and cell death at the higher MOIs examined (Figures 4C and 4D). However, as anticipated, we observed that miRNA modification of CVB3 significantly reduced the toxicity induced by WT-CVB3 to normal lung epithelial cells (Figure 4D).

We further determined whether reduced cytotoxicity in cardiomyocytes and normal lung epithelial cells is a consequence of decreased viral infectivity in these cells. Median tissue culture infection dose (TCID50) assay was performed on supernatant collected from various cell types treated with WT-CVB3 or miR-CVB3 at an MOI of 0.1 for 36 h as indicated to measure infectious viral titers. As shown in Figure 5A, viral titers were strikingly decreased in miR-CVB3-treated HL-1 cardiomyocytes in comparison with WT-CVB3-treated cells. Although a significant decrease in miR-CVB3 titers in H526 TP53mut/RB1mut cells was also observed, the ratio of reduction was much less as compared to that in cardiomyocytes.

We also examined the kinetics of viral RNA replication (Figure 5B) and titers (Figure 5C) in different cell lines incubated with WT-CVB3 or miR-CVB3 at an MOI of 10 for indicated times by qPCR and a TCID50 assay, respectively. We showed that starting at 5–7 h post-infection, viral RNA copy numbers and titers were considerably lower in miR-CVB3-treated as compared to WT-CVB3-treated HL-1 cardiomyocytes (Figures 5B and 5C, left panels). It was also found that viral RNA copies and titers were decreased in H2030 KRASmut and H526 TP53mut/RB1mut cells upon miR-CVB3 treatment, but at a much lesser extent (Figures 5B and 5C, middle and right panels). Taken together, these data suggest that the miR-modified version of CVB3 has an increased therapeutic window between cancer and normal cells than does the WT counterpart.

Intraperitoneal Injection of miR-CVB3 Leads to a Significant Reduction of Tumor Volume with Markedly Decreased Cardiotoxicity in Mice

After verification of tumor specificity and efficacy of the miR-CVB3 in vitro, we next characterized its safety and effectiveness in vivo using a non-obese diabetic (NOD)-severe combined immunodeficiency
SCID xenograft mouse model. Since CVB3 infection triggers more severe inflammation in males than in females, only male mice were used in this study to determine the oncolytic efficacy and toxicity of miR-CVB3.

We first tested the possible systemic toxicity of miR-CVB3 in NOD-SCID mice without a prior tumor implantation. Mice were intraperitoneally inoculated with either WT-CVB3 (n = 4) or miR-CVB3 (n = 5) at a single dose of $1 \times 10^8$ plaque-forming units (PFU) for 14 days, which represents the peak time of CVB3-induced tissue injuries. Various mouse organs were then harvested for the analysis of tissue damage and viral infection. Consistent with our early report, NOD-SCID mice treated with WT-CVB3 showed severe toxicity, and only one mouse survived (25% survival rate) throughout the time course, while all mice treated with miR-CVB3 survived (100% survival rate) to the end of the experiment (Figure 6A). Hematoxylin and eosin (H&E) staining (Figure 6B) and pathological quantitation (Figure 6C) revealed a massive inflammatory infiltration and necrosis in the heart of mice treated with WT-CVB3. Remarkably, we observed no apparent cardiac pathology in mice treated with miR-CVB3. There were also no evident damages to other organs, including the lung, liver, and spleen, in both WT-CVB3- and miR-CVB3-treated mice. Modest pancreatic pathology was observed in both groups (Figures 6B and 6C). Viral quantitation by VP1 immunostaining (Figures 6D and 6E) and plaque assay (Figure 6F) demonstrated a significant reduction in viral protein VP1 expression (almost undetectable) and viral titers (i.e., ~1 million-fold lower) in the heart of miR-CVB3 mice as compared to WT-CVB3 mice, indicating that decreased cardiovirulence in miR-CVB3 mice is mainly due to reduced viral replication. It was also observed that VP1 expression (nearly undetectable) and viral titers were significantly decreased in the pancreas of miR-CVB3 mice compared to WT-CVB3 mice (Figures 6D–6F).

Finally, a different cohort of NOD-SCID mice was used to generate the H526-derived TP53mut/RB1mut SCLC xenograft model to determine the anti-tumor potency and the long-term toxicity of miR-CVB3. The mouse model was established through subcutaneous injection of H526 cells ($1 \times 10^7$ cells) into the left and right flank of the mice. After ~10 days, the implanted tumor reached a palpable size. WT-CVB3 or miR-CVB3 was then given via intraperitoneal injection as described above. Mice treated with PBS were used as controls. Similar to the observations made in cultured cells (Figures 1A and 1B), we showed that the levels of miR-145 and miR-143 were significantly lower in implanted SCLC as compared to normal mouse tissues, including heart, pancreas, lung, liver, spleen, kidney, intestine,
and brain (Figures 7A and 7B). Kaplan-Meier survival analysis revealed that all mice treated with WT-CVB3 succumbed to death or had to be euthanized due to severe morbidity associated with viral infection at or prior to day 15 post-injection, whereas 100% of miR-CVB3-treated mice survived until day 35 post-infection, and the overall survival rate in the miR-CVB3 group at day 56 post-infection was 57.14% (Figure 7C). In the sham group, six out of eight PBS-treated mice continued to grow until the end of experiment, while upon WT-CVB3 or miR-CVB3 treatment the tumor volumes were marked reduced in a comparable manner between the two groups (Figure 7D). Tumor weight measurement on day 25 also showed a substantial reduction in mice treated with miR-CVB3 compared with PBS treatment (Figure 7E). Tumor volumes remained very small or undetectable in miR-CVB3-treated mice until the experimental endpoint (i.e., day 56 post-infection, data not shown). Viral titers were extremely low in the heart and lung, but moderately high in the pancreas and tumor, of mice injected with miR-CVB3 on day 25 (Figure 7F). There was no evident tissue damage in mice treated with miR-CVB3 or PBS on day 25 post-treatment (Figure 7G, pathological scores were not shown due to undetectable pathology). For long-term toxicity analysis (i.e., after day 35 post-infection), we found that mice that survived the entire experimental period (i.e., day 56) displayed no tissue damage and VP1 positive staining, similar to the data shown in Figure 7C. However, evident cardiotoxicity and positive VP1 staining was observed in the heart and pancreas of mice that died between day 35 and 56 post-treatment (Figure S2A). To determine whether the recurrence of cardiotoxicity is due to genome instability of the miR-CVB3, sequencing was conducted, confirming the loss of inserted miRNA target sequences (Figure S2B). Future investigation is needed to address this issue. Taken together, our data suggest that miR-CVB3 retains the oncolytic effectiveness against TP53mut/RB1mut SCLC, but with substantially decreased tissue toxicity.

**DISCUSSION**

In this study, we genetically modified the CVB3 genome through a miRNA-targeting approach. Using both in vitro cell culture and in vivo mouse xenograft models, we demonstrated that miR-CVB3 infection is specific to KRASmut lung adenocarcinoma and TP53mut/RB1mut SCLC cells with little to no observable damage to normal lung or heart cells, leading to significant tumor regression and improved overall survival. The oncolytic effect of miR-CVB3 is very powerful, as evidenced by the observation that one dose of viral infection through systemic administration results in more than 90% reduction of tumor volume. The advantage of systemic delivery of oncolytic virus is apparent. In addition to being clinically feasible, it also has potential to kill metastatic tumors.

The selection of miR-145/miR-143 for the present study is based on our prior experience using their target sequences to augment tumor selectivity of oncolytic herpes simplex virus-1,26,27 and on our current evidence that expression levels of miR-145/miR-143 are particularly low in various types of lung cancer cells in comparison to normal lung epithelial cells and cardiomyocytes. This differential expression pattern enables selective viral replication in lung cancer cells. miR-145/miR-143, together with many other miRNAs, have been revealed to function as tumor suppressors by negatively regulating the expression of multiple oncoprogens.28 Oncogenic RAS has been shown to transcriptionally suppresses miR-145/miR-143,29 whereas activation of tumor suppressor protein p53 causes upregulation of this miRNA cluster.30

As a RNA virus, the CVB3 genome is less stable than that of DNA viruses. To reduce the potential loss of miRNA target function due to viral RNA mutation, we used different miRNA targets and incorporated multiple copies of individual miRNA target sequences. In addition to the recombinant CVB3 generated by inserting the miRNA
target sequences in their forward orientation (targeting the positive-strand of CVB3) as described in the current study, we also tested the virus modified with reverse-oriented miRNA targets (targeting the negative-strand of CVB3). We found that the cardiotoxicity caused by the former virus at MOIs of 1 and 10 is much less than that of the latter (Figure S1), suggesting that targeting the positive-strand of CVB3 is more efficient in reducing viral proliferation and consequent cytotoxicity in normal tissues.

Miyamoto et al.31 previously reported that intratumoral injection of WT-CVB3 substantially suppresses NSCLC tumor growth in nude mice without apparent treatment-related toxicity and death. The same research group has since revised their earlier report regarding safety, having shown significant tissue toxicity following WT-CVB3 treatment.32 They further demonstrate that modification of CVB3 by inserting miR-34 target sequences into the 5’ UTR and 3’ UTR of the viral genome greatly decreases WT virus-induced organ damage and improves mouse survival.32 The differences between our present work and their research include selection of different miRNA target sequences (miR145-miR143 versus miR-34), different route (systemic intraperitoneal versus local intratumoral injection), and frequency (single injection versus multiple injections for up to five times) of viral administration. Despite these differences, both studies provide strong evidence that miRNA-regulated oncolytic CVB3 serves as a valuable tool to be further developed for lung cancer therapy.

We have previously demonstrated that aberrant activation of the ERK1/2 signaling pathway and compromised type I interferon immune response in KRASmut lung adenocarcinoma cells are key factors contributing to the sensitivity to CVB3-induced cell death.33 This then opens up the question of what mechanism underlies CVB3 specificity in TP53mut/RB1mut SCLC compared to normal cells. TP53 and RB1 are the most important and frequently mutated tumor-suppressor genes. Mutations and inactivation of these genes play a key role in the development of various human malignancies. In addition to its best characterized functions in cell cycle arrest and cell death, p53 has also been uncovered to have an antiviral activity through interferon-dependent antiviral immunity and by stimulating apoptosis. It was shown that TP53 can be transcriptionally activated by type I interferon, a key antiviral mediator, in response to viral infection.34 Studies have further revealed that p53 contributes to innate immune response through promoting interferon-mediated antiviral activity.35 Transgenic mice carrying an extra copy of the Tp53 gene were found to exhibit increased resistance to viral infection.36 Additionally, p53 has also been reported to
directly inhibit viral transcription. For example, p53 regulates HIV-1 gene expression by suppressing transcriptional activation of the long terminal repeat.\textsuperscript{17} It was also found that p53 binds to simian virus large T antigen and blocks its function in mediating viral replication.\textsuperscript{36} Of note, we have previously found that CVB3 infection facilitates p53 degradation, and overexpression of WT-p53 suppresses viral replication.\textsuperscript{39} Thus, it is conceivable that loss of p53 activity in SCLC cells benefits CVB3 by providing a favorable cellular environment for viral propagation and consequent oncolysis. Similar to TP53, the tumor-suppressor gene RB1 has also been implicated in host immune responses and inflammatory diseases.\textsuperscript{36} Although the role of RB1 in viral infection remained to be determined, our early findings that CVB3 infection results in a marked reduction of RB1 protein expression associated with virus-induced cell growth arrest\textsuperscript{40} point to a viral strategy to counteract the possible antiviral function of RB1.

One possible limitation of the miRNA strategy is attenuation of the virulence of modified virus in lung carcinoma cells due to the expression of miRNAs in these cells, although at a considerably lower level as compared to that in normal cells. However, given the wide window of the viral dosages that cause tissue toxicity and kill tumor cells, we expect that a slight increase of viral concentration will overcome this drawback. Another potential issue is the genome instability of the miRNA-modified virus. The loss of the incorporated miRNA target sequences observed in the current study is likely due to the formation of loop structures of the insertion. For cloning purpose, we designed two 14-nt-long sequences containing the PacI and BamHI cut sites, which are reverse complementary to each other, flanking the miR-145/miR-143 modification region in the miR-CVB3. As a consequence, the secondary structure could generate a stem loop covering the region of miRNA modification in both strands of the virus, and subsequently the genetic reversal might happen during genome replication by skipping the region of miRNA modification. Despite the fact that the loss of insertion and consequent recurrence of viral pathogenicity were observed after day 35 of treatment in NOD-SCID mice, it is expected that genetic reversal of miR-CVB3 might be less harmful in immunocompetent mice due to more effective immune clearance of virus as compared to immunocompromised mice. Nonetheless, future research is required to address this problem to minimize the potential risk.
As discussed earlier, both direct oncolysis and anti-tumor immunity triggered by virus infection are thought to contribute to the efficacy of cancer virotherapy.

The present research focuses mainly on the oncolytic mechanism of miR-CVB3. It is fully recognized there is a lack of data on assessing the role of the host immune system in virotherapy, although NOD-SCID mice lacking functional T and B lymphocytes preserve some natural killer cell and macrophage activity. Future research will focus on using immunocompetent transgenic mouse models to evaluate the therapeutic efficacy and safety of the engineered CVB3. We expect that miR-CVB3 will exert an anti-tumor effect by triggering direct tumor lysis and eliciting tumor-specific immunity.

In conclusion, we have generated a recombinant CVB3 with value to be further exploited for the treatment of KRASmut lung adenocarcinoma and TP53mut/RB1mut SCLC, the two most devastating subtypes of lung cancer.

**MATERIALS AND METHODS**

**Generation of miRNA-Modified CVB3**

The miRNA-modified CVB3 (denoted hereinafter by miR-CVB3) was created by insertion of four copies of miR-145 and two copies of miR-143 target sequences into the 5′ UTR of the CVB3 genome. The plasmid pCVB3/T7 containing the intact genome of CVB3 (Kandolf strain) was used as the backbone to generate miR-CVB3. Briefly, pCVB3/T7 was digested by Xbal to remove the BamHI sites while sparing the 5′ UTR-VP4 region. The resulting plasmid was then mutagenized with a primer (5′-GGT GAT ACT TCT GAA ATC ATG CCC ATT TGG CTC TAT GGA TCC TTT GCT GTA TTA AAC TTA ACA ATG-3′) harboring a BamHI site and a Kozak consensus sequence between the 5′ UTR and the start codon of VP4. The mutant backbone was further modified by inserting a BamHI-digested PCR product that includes four-copy miR-145 target sequences and a ClaI site amplified using a primer pair (5′-AAT GGA TCC TTA ATT AAC GAA GGG ATT CCT GG-3′ and 5′-AAT GGA TCC TTA ATT AAA TCG ATA GCG TCC AGT TTG C-3′) from the plasmid pCMV-ICP27-145T. The CVB3 genome in the resultant plasmid was then repaired by replacing the BglII-SalI fragment with the corresponding fragment in pCVB3/T7 to construct pCVB3-miR-145. Finally, the plasmid pCVB3-miR-145/miR-143 was generated by a ClaI site insertion of an annealed oligonucleotide pair (5′-cgT GAG CTA CAG TGC TCC TTC GCC AGC AGT GCT TCA TCT ATc cta gaa t-3′ and 5′-cga ttc tag aTG AGA TGA AGC ACT GTA GCT CAA TCG TGA TGA GAA GCA CTG TAG CTC A-3′), including two copies of miR-143 target sequences. All restriction enzymes used were from Thermo Fisher Scientific.

To produce miR-CVB3 and WT-CVB3 (Kandolf strain) stock, viral genome was synthesized from pCVB3-miR-145/miR-143 and pCVB3/T7 linearized by SalI digestion, respectively, using a HiScribe T7 quick high yield RNA synthesis kit (#E2050S, New England Biolabs). Subsequently, viral RNA was transfected into HeLa cells and the supernatant was collected at ~72 h post-transfection when cytopathic effects were most prominent. The virus-containing supernatant was further propagated in HeLa cells until viral titers reached desirable levels (i.e., ~5 × 10⁹ PFU/mL) for storage.

**Cell Lines**

The KRASmut lung adenocarcinoma cell lines of epithelial origin (i.e., A549 cells [CCL-185], H230 cells [CRL-5914], and H23 cells [CRL-5800]), the TP53mut/RB1mut SCLC epithelial cell lines (i.e., H524 cells [#CRL-5831] and HS26 cells [#CRL-5811]), and the BEAS2B human normal lung epithelial cell line (#CRL-9609) were all obtained from the American Type Culture Collection. All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (#11875093, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Human primary airway epithelial cells isolated from normal donors were generously provided by Dr. Tillie Hackett at the University of British Columbia. The HL-1 cardiomyocytes (#SCC065, Sigma-Aldrich) were cultured in Claycomb medium (#51800C, Sigma-Aldrich) as previously described. Human cardiomyocytes (iCMs) were generated by differentiation of the human induced pluripotent stem cell line, iPS (IMR90)-I, using the G4i protocol, which involved timed inhibition of GSK3 and Wnt signaling. Upon differentiation, the iCMs were maintained in RPMI/B27/insulin media (RPMI 1640 with B-27 supplement complete with insulin [Life Technologies, #17504-044]) through the infection studies.

**Animal Studies**

The immunocompromised NOD-SCID mice (NOD.CB17-Prkdcscid/J, #001303, The Jackson Laboratory) were bred at the Animal Resource Centre of BC Cancer Research Centre. All mouse experiments were conducted at the Centre for Heart Lung Innovation Animal Facility of the University of British Columbia in strict accordance with the recommendation in the *Guide for the Care and Use of Laboratory Animals* (Canadian Council on Animal Care, Ottawa, ON, Canada). The protocol was approved by the University Animal Care Committee (A18-0275).

Two different cohorts of male NOD-SCID mice at the age of ~6–8 weeks were used for this research. The first cohort was conducted to determine the potential systemic toxicity of the viruses. NOD-SCID mice were injected intraperitoneally with a single dose of WT-CVB3 (n = 4 mice) or miR-CVB3 (n = 5 mice) at 1 × 10⁸ PFU in a volume of 100 µL for 14 days (peak time of virus-induced tissue damage). For the second cohort of the study, H526 cells (1 × 10⁷ cells) were injected subcutaneously into the left and right flank of each mouse to generate the TP53mut/RB1mut SCLC xenograft mouse model. Once the tumor volume reached a palpable size (~100 mm³ on each side), mice were inoculated with WT-CVB3 (n = 5 mice) or miR-CVB3 (n = 15 mice; 7 mice were used for long-term survival analysis, and the other 8 mice were euthanized on day 25 post-infection for the measurement of tumor weight, viral titers, and toxicity) as described above. Mouse treated with an equal volume of PBS (n = 8 mice) were used as controls. The animals were then monitored daily and body weight was measured every 2 days. Tumor size was measured twice weekly until the experimental endpoint, and...
tumor volume on each side was calculated as length $\times$ width$^2 \times 0.52$ and presented as a total volume of both sides. If the tumor exceeded 20 mm in diameter or mice presented severe symptoms linked to viral infection, the mice were euthanized prior to the experimental endpoint as per the approved animal protocol. Mouse organs, including heart, lung, liver, kidney, spleen, and pancreas, were collected for subsequent analysis.

### Quantitative Reverse Transcriptase PCR (qRT-PCR)

Total cellular RNA was extracted using the Monarch total RNA mini-prep kit (#XT2010, New England Biolabs). To measure the relative levels of viral RNA, qRT-PCR targeting CVB3 2A, mouse and human GAPDH was performed using the Luna universal one-step qRT-PCR kit (#E3005, New England Biolabs) on a ViiA 7 real-time PCR system (Applied Biosystems). The primer pairs used for viral RNA measurement are as follows: CVB3 2A (forward, 5’-GCT TGG CAG ACA TTC GTG ATC-3’; reverse, 5’-CAA GCT GTG TTC CAC ATA GTC CTT CA-3’), mouse GAPDH (forward, 5’-GGC AAA TTC AAC GGC ACA GT-3’; reverse, 5’-AGA TGG TGA TGG GCT TCC C-3’), and human GAPDH (forward, 5’-AAT CCC ATC ACC ATC TTC CA-3’; reverse, 5’-TGG ACT CCA CGA GTT ACT CA-3’). The CVB3 2A gene level was first normalized to GAPDH mRNA, and then to cell numbers.

To determine the relative expression level of miR-145 and miR-143, three stem-loop primers (miR-145, 5’-CTC AAC TGG TGT CGT GGA TGC GTC AAT TCA GTT GAG AGG GAT TC-3’; miR-143, 5’-GTC GTA TCC AGT GCT GTT CGT G-3’; reverse, 5’-CTG GAT ACG ACT GAC TCA-3’), and miR-93 [which has been identified as a reference gene for qPCR analysis of miRNA levels$^{46,51}$], 5’-CTC AAC GGT GTC GTG GAG TCG GCA ATT CAG TTT AGG TAC CTG C-3’) were used for RT with an iScript cDNA synthesis kit (#1708890, Bio-Rad) according to the manufacturer’s instructions. qPCR was then conducted using three primer pairs targeting miR-145 (forward, 5’-CGG CGG GTC GAG TGC TTT TCC CAG G-3’; reverse, 5’-CTG GTG TCG TGG AGT CGG CAA TTC-3’), miR-143 (forward, 5’-CCT GGC CTG AGA TGA AGC AC-3’; reverse, 5’-CAG TGG TGC GTC GAG CTA A-3’), and miR-93 (forward, 5’-CGG CGG CAA AGT GCT GTT CTG G-3’; reverse, 5’-CTG GTG TCG TGG AGT CGG CAA TTC-3’). Samples were run in triplicate and analyzed using the comparative CT (2$^{-\Delta\Delta CT}$) method with control samples and presented as relative quantitation.

### Crystal Violet Assay

Crystal violet staining was performed for assessment of cell death. Briefly, after removal of culture medium and washing with PBS, adherent cells were fixed and stained with 0.4% crystal violet solution for 30 min.

### Cell Viability Assay

Cell viability was evaluated using the alamarBlue assay according to the manufacturer’s protocol (#BUF012A, Bio-Rad). Briefly, the alamarBlue solution was added to the 48-well plate to a final concentration of 10%. After incubation at 37°C for 4 h, the absorbance was measured at 570 nm and 600 nm on a microplate reader. Percentage survival of CVB3-infected cells is expressed relative to that of sham controls, which is arbitrarily set as 100% survival.

### Viral Titer Quantification

A plaque assay was conducted on CVB3-infected tissue homogenates to assess viral titers as previously reported.$^{48}$ The viral titers were calculated and presented as PFU/g.

The TCID$_{50}$ assay was performed on supernatant collected from CVB3-infected cells for measuring viral titers as described previously.$^{49}$

### Histological Examination

Potential tissue toxicity was evaluated by histological analysis following H&E staining. The pathological score was graded based on inflammation, necrosis, calcification, lesion area, and cellular vacuolization as previously described.$^{20}$

### Immunohistochemical Staining

Immunohistochemical staining was performed using the primary antibody of monoclonal anti-CVB3 capsid protein VP1 (1:1,200, Cox mAb 31A2, Medagnost, Germany) as described previously.$^{51}$ VP1 staining was quantified by ImageJ (version 1.0) and presented as relative optical density normalized to sham infection as previously reported.$^{52}$

### Statistical Analysis

All results are expressed as mean ± standard deviation (SD). Statistical analysis was conducted using one-way ANOVA or an unpaired Student’s t test as indicated in the figure legends. A value of $p < 0.05$ was considered statistically significant. All results presented are representative of at least three independent experiments.

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.01.002.

### AUTHOR CONTRIBUTIONS

H. Liu, W.W.G.J., W.W.L., and H. Luo designed the studies. H. Liu, Y.C.X., H.D., Y.M., and C.S.N. performed the experiments. A.C. and C.J.L. generated the iCMs. H. Liu and H. Luo wrote and revised the manuscript.

### CONFLICTS OF INTEREST

W.W.G.J. is the Chief Scientific Officer at Virogin Biotech Ltd. H. Liu and C.S.N. are partially sponsored by Virogin Biotech Ltd. through the MITACS Accelerate Program. The remaining authors declare no competing interests.

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