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Coxsackievirus Type B3 Is a Potent Oncolytic Virus against *KRAS*-Mutant Lung Adenocarcinoma

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$KRAS$ mutant ($KRAS^{mut}$) lung adenocarcinoma is a refractory cancer without available targeted therapy. The current study explored the possibility to develop coxsackievirus type B3 (CVB3) as an oncolytic agent for the treatment of $KRAS^{mut}$ lung adenocarcinoma. In cultured cells, we discovered that CVB3 selectively infects and lyses $KRAS^{mut}$ lung adenocarcinoma cells (A549, H2030, and H23), while sparing normal lung epithelial cells (primary, BEAS2B, HPL1D, and 1HAEo) and $EGFR^{mut}$ lung adenocarcinoma cells (HCC4006, PC9, H3255, and H1975). Using stable cells expressing a single driver mutation of either $KRAS^{G12V}$ or $EGFR^{L858R}$ in normal lung epithelial cells (HPL1D), we further showed that CVB3 specifically kills HPL1D-$KRAS^{G12V}$ cells with minimal harm to HPL1D-$EGFR^{L858R}$ and control cells. Mechanistically, we demonstrated that aberrant activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and compromised type I interferon immune response in $KRAS^{mut}$ lung adenocarcinoma cells serve as key factors contributing to the sensitivity to CVB3-induced cytotoxicity. Lastly, we conducted *in vivo* xenograft studies using two immunocompromised mouse models. Our results revealed that intratumoral injection of CVB3 results in a marked tumor regression of $KRAS^{mut}$ lung adenocarcinoma in both non-obese diabetic (NOD) severe combined immunodeficiency (SCID) gamma (NSG) and NOD-SCID xenograft models. Together, our findings suggest that CVB3 is an excellent candidate to be further developed as a targeted therapy for $KRAS^{mut}$ lung adenocarcinoma.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in both males and females in North America and worldwide.[1,2] Currently, most patients with lung cancer are diagnosed at an advanced stage when potentially curative treatment is no longer possible. Histologically, adenocarcinoma is the most common type of lung cancer.[3] Further subcategorization has been achieved by molecular criteria, such as specific driver mutations in genes that encode signaling proteins crucial for cellular proliferation and survival.[4] Somatic mutations in epidermal growth factor receptor (*EGFR*) have been identified in ~15% of all patients with lung adenocarcinoma, with the proportion increasing to 50% in patients who have never smoked.[4] Although patients with *EGFR* mutant ($EGFR^{mut}$) lung adenocarcinoma have increased sensitivity to tyrosine kinase inhibitors, primary and acquired resistance toward these agents remains a major clinical obstacle.[5] Conversely, Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations are more common in patients who had a history of cigarette smoking and account for ~25% of lung adenocarcinomas.[6] However, these patients have a poor prognosis because of the lack of survival benefit from adjuvant chemotherapy and resistance to targeted kinase inhibitors.[7] Therefore, there is an urgent need for developing new therapeutics for this subgroup of the patients.

Oncolytic virus (OV) is clinically defined by its ability to induce lysis of malignant cells through a self-replication process without causing damage to normal tissues.[8,9] Over the past decades, a better understanding of tumor biology and molecular mechanisms of viral cytotoxicity has provided a scientific rationale to develop more efficient oncolytic viruses as potent, self-amplifying cancer therapeutics.[10] As a result, several viruses including adenovirus, herpes simplex virus 1 (HSV-1), coxsackievirus A21 (CVA21), measles virus, and reovirus have demonstrated varying degrees of success in clinical trials,[11–16] whereas a modified HSV-1 has been approved by the US Food and Drug Administration in October 2015 for the treatment of melanoma.[17] On the other hand, there are still several hurdles to overcome for oncolytic viruses to become clinically effective, which includes

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poor tropism for targeted organs and pre-existing immunity against oncolytic virus replication in adults.[10]

Coxsackievirus type B3 (CVB3), a non-enveloped, human pathogenic enterovirus of the *Picornaviridae* family, encompasses a 7.4-kb single-stranded, positive-sense RNA genome. Although CVB3 infection is associated with high incidence of myocarditis, pancreatitis, meningitis, and encephalitis in children and adolescents, infection in adults is generally asymptomatic or causes mild flu-like symptoms.[18–20] Recently, large-scale screening of 28 enterovirus strains has identified CVB3 as one of the most potent oncolytic viruses against a panel of different human cancer cells, including non-small-cell lung cancer (NSCLC).[21] In addition to its natural tropism for NSCLC cells, CVB3 also possesses two features that make it an excellent candidate for oncolytic virotherapy. First, CVB3 preferentially infects and lyses actively dividing cells over quiescent cells, thus activation of oncogenic signaling pathways within tumor cells creates a permissive microenvironment supporting virus replication.[22] Second, CVB3 infection is profoundly inhibited by type I interferon; as a result, normal cells with intact interferon signaling are more resistant to CVB3 infection than tumor cells that often display an impaired interferon signaling.[23–25]

In this study, we showed that wild-type (WT) CVB3 specifically targets $KRAS^{mut}$ lung adenocarcinoma cells with limited effects on normal lung epithelial cells and $EGFR^{mut}$ lung adenocarcinoma cells. Mechanistically, we demonstrated that enhanced extracellular signal-regulated kinase 1/2 (ERK1/2) activation and impaired type I interferon response contribute, at least in part, to the sensitivity of $KRAS^{mut}$ lung adenocarcinoma cells to CVB3-induced cytotoxicity. Xenograft models of lung adenocarcinoma demonstrated that treatment with WT-CVB3 results in a significant decrease in tumor size in immunocompromised mice bearing $KRAS^{mut}$ lung adenocarcinoma. Taken together, our findings suggest that CVB3 could be an excellent candidate for further development into a novel oncolytic virus for the treatment of $KRAS^{mut}$ lung adenocarcinoma.

RESULTS

CVB3 Specifically Infects and Decreases the Viability of $KRAS^{mut}$ Lung Adenocarcinoma Cells

The development of targeted therapies to driver oncogenes has led to a substantial benefit for NSCLC patients carrying *EGFR* and other specific mutations; however, *KRAS* mutations are currently undruggable. This evokes us to question whether CVB3-based virotherapy can be a novel approach targeting $KRAS^{mut}$ lung adenocarcinomas. In fact, previous studies have shown that tumor selectivity of several oncolytic viruses can be enhanced by gain-of-function mutations in given oncogenes of the Ras-signaling pathways.[26] To test our hypothesis, seven patient-derived lung adenocarcinoma cell lines, including three $KRAS^{mut}$ (H23, H2030, and A549) and four $EGFR^{mut}$ (H1975, H3255, PC-9, and H4006) cells were selected to examine their sensitivities to CVB3 infection. We also chose three normal lung epithelial cell lines (1HAEo, HPL1D, and BEAS2B) and primary airway epithelial cells isolated from normal donors to evaluate the specificity of CVB3 treatment *in vitro*. As shown in Figure 1A, CVB3 exhibited powerful cytotoxic activities against $KRAS^{mut}$ lung adenocarcinoma cells in a dose-dependent manner. However, $EGFR^{mut}$ lung adenocarcinoma cells and normal lung epithelial cells displayed only minimal cytopathic effects after 48-h infection with CVB3 even at the highest MOI tested. Cell viability assays further validated that CVB3 infection resulted in a profound reduction (~85%) of cell survival in $KRAS^{mut}$ lung adenocarcinoma cells (Figure 1B). No significant reduction in cell viability in $EGFR^{mut}$ lung adenocarcinoma cells or slight decrease of cell survival in normal lung epithelial cells was observed upon CVB3 infection, especially at the lower dose of CVB3 (Figure 1B). Moreover, we examined the replication ability of CVB3 in lung adenocarcinoma and normal lung epithelial cells by plaque assay. Figure 1C showed that the virus titers in the supernatant of CVB3-infected $KRAS^{mut}$ lung adenocarcinoma cells were significantly higher than those from $EGFR^{mut}$ lung adenocarcinoma and normal lung epithelial cells, suggesting that CVB3-mediated oncolytic effect is highly associated with its replicative capacity. As a positive control, we showed that WT-CVB3 infection of HeLa cells, a human cervical cancer cell line that has previously been shown to be extremely sensitive to CVB3 infection, caused substantial lysis at all concentrations tested (Figure 1D). Together, these results indicate that CVB3 specifically infects and kills $KRAS^{mut}$ lung adenocarcinoma to exert its oncolytic effects through self-replication.

KRAS Mutation Is a Determinant of Lung Adenocarcinoma Susceptibility to CVB3-Induced Cell Death

It has become evident that lung adenocarcinoma is a heterogeneous disease marked with a high rate of somatic mutations.[4,27] In addition to the driver oncogene, each lung adenocarcinoma cell line tested in this study has multiple somatic mutations that may produce a synergistic role in supporting viral replication. Therefore, to specifically determine the effect of *KRAS* or *EGFR* mutation on CVB3 tropism, we generated isogenic cells from the normal lung cell line HPL1D expressing a single driver mutation of either *KRAS* ($KRAS^{G12V}$) or *EGFR* ($EGFR^{L858R}$). HPL1D cells expressing GFP were used as a negative control. Western blot analysis verified overexpression of KRAS or EGFR in these cell lines (Figure 2A). We found that WT-CVB3 specifically targeted and lysed HPL1D-$KRAS^{G12V}$ cells with very minimal harm to HPL1D-$EGFR^{L858R}$ and normal cells (Figures 2B–2D), and UV-inactivated CVB3 (UV-CVB3) failed to cause apparent cell death (Figure 2E). Our results indicate that *KRAS* mutation is a determinant of viral sensitivity.

ERK1/2 Signaling in $KRAS^{mut}$ Adenocarcinoma Cells Enhances CVB3 Replication

We next investigated the potential mechanism by which CVB3 preferentially replicates in $KRAS^{mut}$ lung adenocarcinomas. Previous *in vitro* and *in vivo* evidence has demonstrated that CVB3 replication relies largely on the activation of oncogenic signaling pathways, among which the ERK1/2 signaling is the best characterized and proven to be the most important signaling pathway hijacked by CVB3 for effective replication.[28,29] To determine the potential contribution of ERK1/2 activation in permissiveness to CVB3-mediated cell

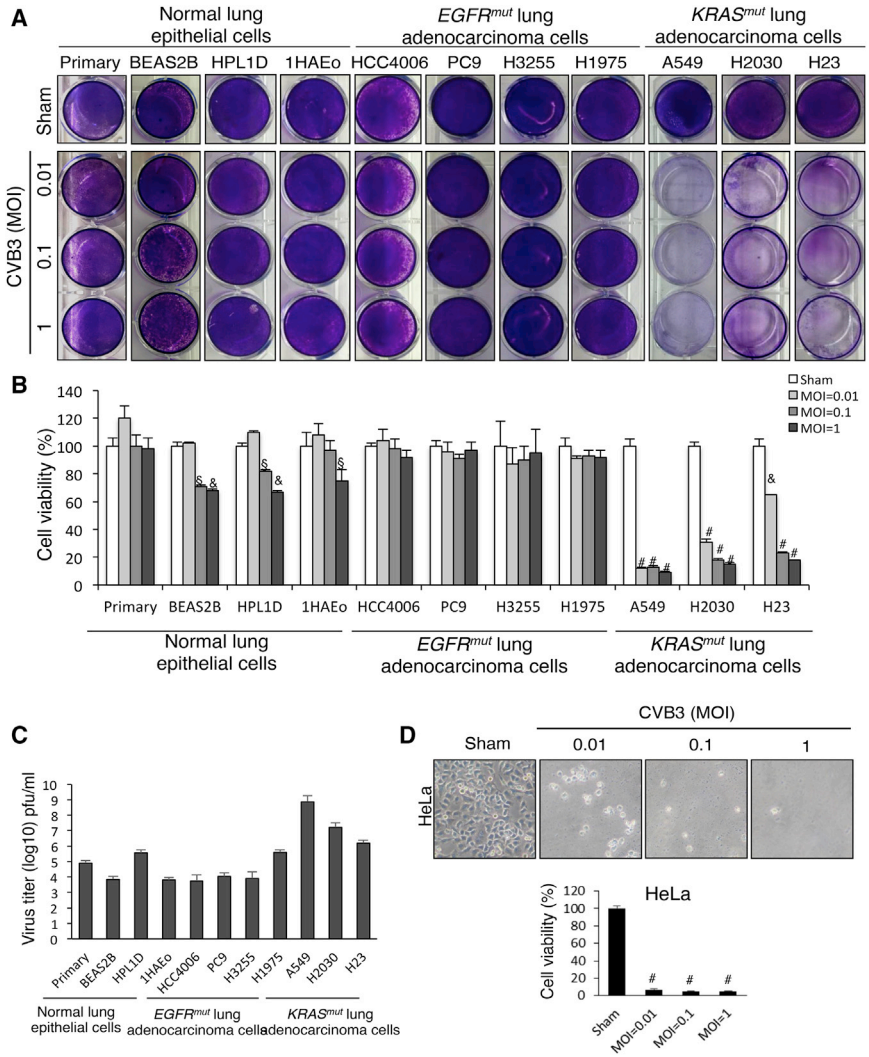

Figure 1. CVB3 Selectively Infects and Lyses *KRAS*^{mut} Lung Adenocarcinoma Cells

Various lung adenocarcinoma cells, including patient-derived *KRAS*^{mut} (H23, H2030, and A549), *EGFR*^{mut} (H1975, H3255, PC-9, and H4006), and normal lung epithelial cells (1HAEo, HPL1D, BEAS2B, and primary airway epithelial cells) were sham or CVB3 infected at different MOIs as indicated for 48 h. (A) Cytotoxicity was evaluated by crystal violet staining. (B) Cell viability was determined by the MTS assay. Each value of CVB3-infected cells was normalized to that of sham-infected cells (arbitrarily set at a value of 1) and expressed as mean ± SD (n = 3). $^{\#}p < 0.001$; $^{\&}p < 0.005$; $^{\S}p < 0.01$ compared with sham infection. (C) Virus titers in the supernatant of cells infected with CVB3 at an MOI of 0.1 for 24 h were measured by plaque assay. The results are presented as means ± SD (n = 3). (D) HeLa cells were infected with CVB3 at various MOIs as indicated for 48 h. Cytotoxicity and cell viability were determined as above. $^{\#}p < 0.001$ compared with sham infection.

death, we examined ERK1/2 activation or phosphorylation status in different lung adenocarcinoma cells and isogenic HPL1D cells expressing different mutant oncogenes. We found that the ERK1/2 was activated or phosphorylated in *KRAS*^{mut} cells to a greater degree as compared with *EGFR*^{mut} and normal lung epithelial cells (Figures 3A and 3B). We further showed that inhibition of ERK1/2 using a mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor (U0126) decreased viral protein levels and virus titers in a dose-dependent manner in both patient-derived *KRAS*^{mut} H2030 (Figures 3C and 3D) and HPL1D-*KRAS*^{G12V} cells (Figures 3E and 3F). Together, our data suggest that enhanced ERK1/2 activation contributes, at least in part, to the susceptibility of *KRAS*^{mut} lung adenocarcinoma cells to CVB3-induced cytotoxicity.

CVB3-Induced Type I Interferon Production Is Impaired in Cells Expressing *KRAS*^{mut}

Because type I interferon plays a key role in the innate immune response against CVB3 infection, we also questioned whether increased CVB3 susceptibility of *KRAS*^{mut} cells could be a result of compromised type-I interferon response. HPL1D cell lines stably expressing *GFP* (control), *KRAS*^{G12V}, or *EGFR*^{L858R} were used to determine *Ifnb1 (IFN-β* gene) expression upon sham or CVB3 infection at an MOI of 1.0 for different time points (Figure 4A) or at various MOIs for 7 h (Figure 4B). As expected, infection with CVB3 resulted in an upregulation of *Ifnb1* (*IFN-β*) gene in HPL1D-*GFP* control cells in a time- and dose-dependent manner (Figure 4). This induction of *Ifnb1* gene was significantly suppressed in HPL1D-*KRAS*^{G12V} cells, but further enhanced in HPL1D-*EGFR*^{L858R} cells (Figure 4). Our results suggest that impaired type I interferon production in *KRAS*^{G12V} cells may serve as an additional factor contributing to selective CVB3 replication and consequent oncolysis in these cells.

To further understand the mechanism by which *KRAS*^{G12V} inhibits CVB3-induced *Ifnb1* gene production, we examined the phosphorylation status of eukaryotic initiation factor 2α (eIF2α) as a marker of the activation of the double-stranded RNA (dsRNA)-activated protein kinase R (PKR). As a pattern recognition receptor for viral dsRNA, PKR has been shown to be significant for type I interferon production during viral infection.[30] As shown in Figure 4C, we found that the levels of phosphorylated eIF2α were markedly increased at 7 h after CVB3 infection in all three cell types, consisting with our early findings.[31] However, neither *KRAS*^{G12V} nor *EGFR*^{L858R} appeared to affect the phosphorylation status of eIF2α, indicating that suppression of *Ifnb1* production in cells expressing *KRAS*^{G12V} is not through inactivation of the PKR-eIF2α pathway.

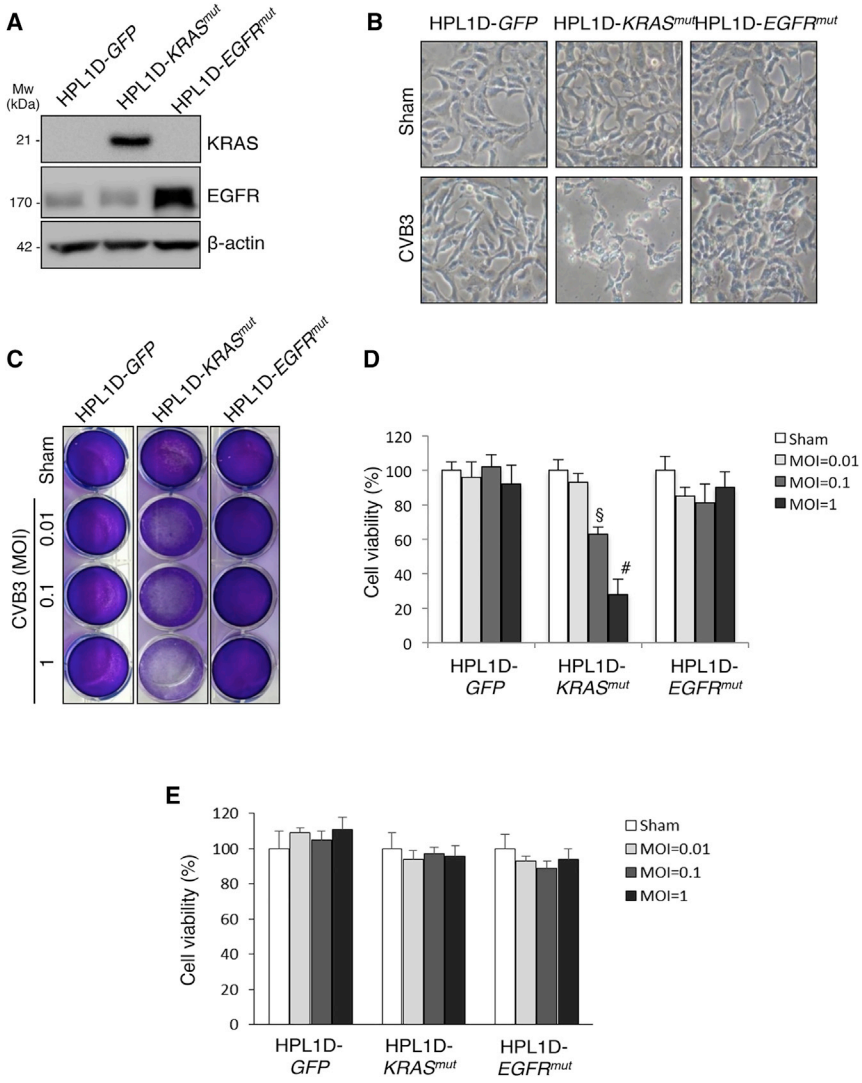

Figure 2. CVB3 Specifically Infects and Kills Lung Epithelial Cells Stably Expressing *KRAS*^{mut}

(A) HPL1D cell lines stably expressing *GFP* (control), *KRAS*^{G12V}, or *EGFR*^{L858R} were harvested, and protein expression of RAS and EGFR was validated by western blot analysis. (B) Various HPL1D stable cells were sham or CVB3 infected (MOI = 1) for 48 h. Cell morphology was examined by light microscopy (original magnification ×10). (C and D) Various HPL1D stable cells were sham or CVB3 infected at different MOIs as indicated for 48 h. Cytotoxicity was examined by crystal violet staining (C). Cell viability was measured by the MTS assay (D). Each value of CVB3-infected cells was normalized to that of sham-infected cells, which was arbitrarily set at a value of 1, and presented as the mean ± SD (n = 3). $^{§}p < 0.01$, $^{\#}p < 0.001$ compared with sham infection. (E) Different HPL1D stable cells were sham infected or infected with UV-CVB3 at various MOIs as indicated for 48 h. Cell viability was measured by MTS assay and normalized as above.

Protein Level of Viral Receptors Is Not a Major Determinant of Increased Susceptibility of *KRAS*^{mut} Lung Adenocarcinomas to CVB3

The coxsackievirus-adenovirus receptor (CAR) is the primary receptor responsible for CVB3 internalization.[32] We next sought to determine whether CAR expression is a determining factor for the sensitivities of lung adenocarcinoma to CVB3-induced cell death. Western blot results showed that protein levels of CAR were noticeably higher in H23 and H2030 (*KRAS*^{mut}) cells as compared with H1975, HCC4006, and H3255 (*EGFR*^{mut}) and normal lung epithelial cells, indicating a potential relationship between *KRAS* status and CAR expression (Figure 5A). To assess the effects of KRAS activation on CAR, we examined protein levels of CAR in tetracycline-inducible HPLID-*KRAS*^{mut} stable cells. Of interest, we found that addition of doxycycline resulted in decreased CAR levels (Figure 5B). This finding was further confirmed with the experiment of KRAS inhibition, which showed that treatment with ARS853, a KRAS^{G12C} inhibitor, increased protein levels of CAR in both H23 and H2030 *KRAS*^{mut} lung adenocarcinomas cells (Figure 5C). CAR expression has been previously reported to be associated with ERK1/2 signaling.[33] We further questioned whether ERK activation plays a role in *KRAS*-induced downregulation of CAR. Consistent with the early report,[33] we found that inhibition of ERK1/2 with U0126 caused an upregulation of CAR in both H23 and H2030 *KRAS*^{mut} cells (Figure 5D). Moreover, through the assessment of expression levels for the gene (*CXADR*) encoding CAR across a panel of 230 lung adenocarcinoma tumors profiled by The Cancer Genome Atlas (TCGA), we found that the transcriptional levels of *CXADR* between *KRAS*^{mut} and *EGFR*^{mut} (and *KRAS*^{mut} and *KRAS/EGFR*^{WT}) tumors were statistically insignificant (Figure 5E). Finally, we performed virus uptake assay using HPL1D stable cell lines expressing *GFP*, *KRAS*^{G12V}, or *EGFR*^{L858R} to determine whether increased viral particle uptake is a factor contributing to viral sensitivity of *KRAS*^{mut} cells. As shown in Figure 5F, we observed no significant differences in virus uptake between control and *KRAS*^{mut} or *EGFR*^{mut} cells. Taken together, our results suggest that KRAS negatively regulates CAR expression via ERK1/2 activation, and that enhanced protein levels of CAR detected in H23 and H2030 cells are independent of KRAS activation. Thus, CAR expression and/or altered virus entry are unlikely major determinants for the hypersensitivity of *KRAS*^{mut} lung adenocarcinomas to CVB3-induced cell death. We also examined the protein levels of decay-accelerating factor, the co-receptor for CVB3,[34] in various lung adenocarcinomas cells. However, no apparent changes were observed (data not shown).

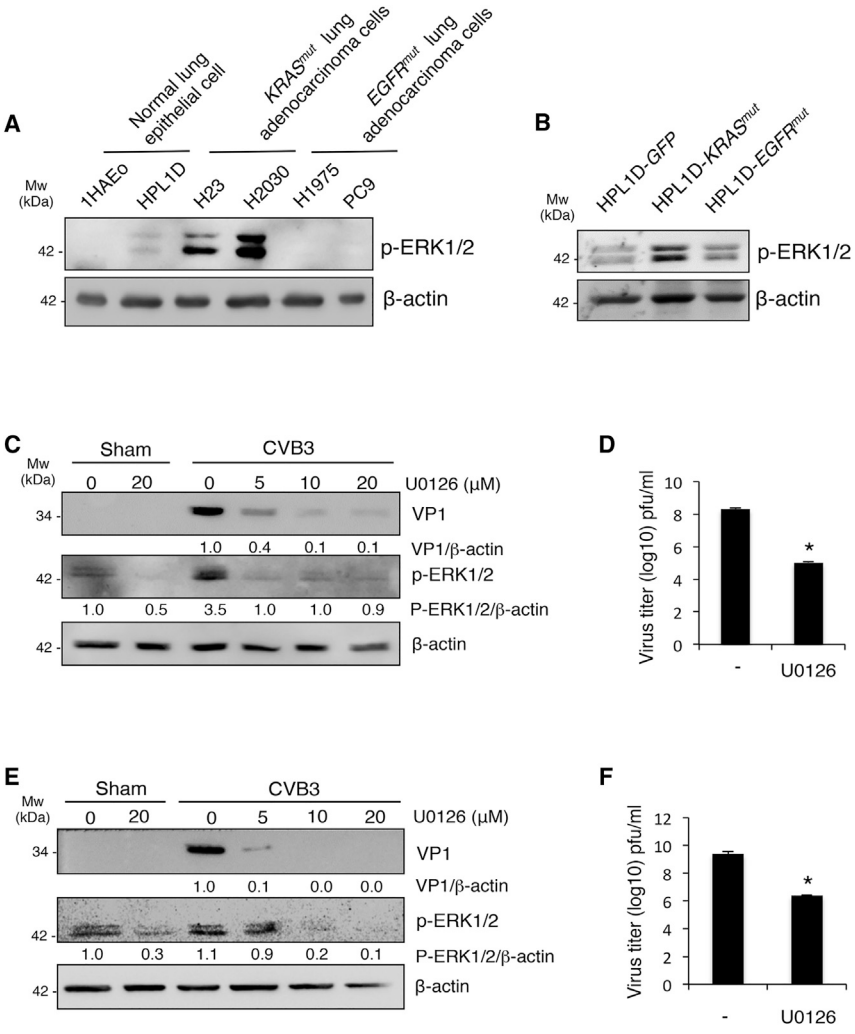

Figure 3. Aberrant Activation of ERK1/2 Signaling in *KRAS*^{mut} Lung Adenocarcinoma Cells Promotes CVB3 Replication

(A and B) Levels of p-ERK1/2 in various adenocarcinoma and normal lung epithelial cells (A) or in HPL1D cells stably expressing GFP (control), *KRAS*^{G12V}, or *EGFR*^{L858R} (B). (C–F) Inhibition of ERK1/2 activation blocks CVB3 replication in *KRAS*^{mut} cells. H2030 (C and D) and HPL1D-*KRAS*^{mut} cells (E and F) were infected with CVB3 (MOI = 10) in the presence or absence of different concentrations of MEK inhibitor U0126 as indicated for 7 h. Cells were harvested for western blot analysis of p-ERK1/2, VP1, and β-actin (C and E). Protein levels of VP1 and p-ERK1/2 were quantitated by densitometric analysis using NIH ImageJ, normalized to β-actin, and presented underneath of each blot as fold changes compared with the first lane (arbitrarily set at a value of 1) of sham or CVB3 infection as indicated. Culture medium was collected for plaque assay (D and F), and the results are presented as means ± SD (n = 3). *p < 0.05 as compared with vehicle-treated controls (–).

Intratumoral Injection of CVB3 Leads to a Significant Regression of *KRAS*^{mut} Xenograft Lung Tumors in an NSG Mouse Model

We next conducted xenograft animal experiments using *KRAS*^{mut} H2030 cells to determine the anti-tumor effects of CVB3 *in vivo*. We first used non-obese diabetic (NOD) severe combined immunodeficiency (SCID) gamma (NSG)-immunodeficient mice, where immunity is completely abolished because of the lack of mature T cells, B cells, and functional natural killer (NK) cells.[35] A pilot study was performed with four different dosages of CVB3 (i.e., 5×10^4, 5×10^5, 5×10^6, and 5×10^7 plaque-forming units [PFUs]), demonstrating similar results in terms of tumor regression and mortality rate (data not shown). Figures 6A and 6B showed that intratumoral injection of WT-CVB3 at 5×10^4 PFUs resulted in a dramatic reduction in *KRAS*^{mut} xenograft tumor volumes, whereas tumor sizes continued to increase with the treatment of UV-CVB3. The tumor volume of mice exposed to WT-CVB3 on day 15 was ~12-fold smaller than that of mice exposed to UV-CVB3, suggesting that CVB3 potently kills *KRAS*^{mut} lung adenocarcinoma *in vivo*, irrespective of immune response. Despite significant regression of *KRAS*^{mut} xenograft tumors, as shown in Figure 6C, mice obtained no survival benefit after WT-CVB3 treatment, and all mice were euthanized on days 12–15 because of sickness (according to the endpoints approved by the Animal Care Committee at the University of British Columbia). To examine possible comorbidities associated with WT-CVB3, we compared virus loads in the xenograft tumors and different organs. Figure 6D showed that, in addition to tumor, viral replication was also detected in various mouse organs, in particular the heart, suggesting an active systemic viral infection following intratumoral injection of WT-CVB3.

Intratumoral Injection of CVB3 Results in a Significant Reduction in *KRAS*^{mut} Tumor Size in NOD-SCID Immunocompromised Mice

It is well documented that the host innate immune response plays a crucial role in limiting viral spread.[36] To determine whether partial recovery of innate immunity could attenuate the capability of CVB3 in killing tumors, we carried out the xenograft experiments using NOD-SCID mice, which have residual innate immunity including defective NK cells, macrophages, granulocytes, and complement.[35] To investigate whether CVB3 has local and/or systemic oncolytic effects on tumors, lung adenocarcinoma cells were injected subcutaneously into the bilateral flanks of the mice, and a subsequent one-dose injection of WT-CVB3 was administered only in the left flank tumor. We found that *KRAS*^{mut} tumor volume significantly decreased on both flanks of mice, suggesting a possible systemic effect of local

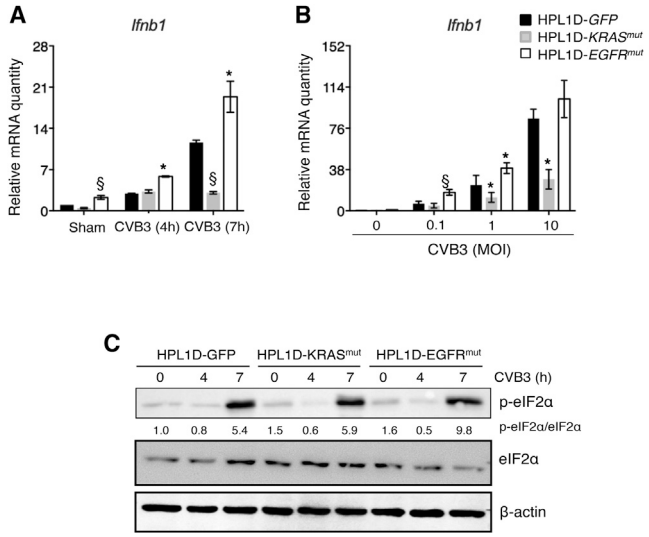

Figure 4. *KRAS* Mutation Leads to Impaired Type I Interferon Response in CVB3-Infected Human Lung Epithelial Cells

(A) HPL1D cell lines stably expressing *GFP* (control), $KRAS^{G12V}$, or $EGFR^{L858R}$ were sham or CVB3 infected (MOI = 1.0) for the indicated time points. (B) Various HPL1D stable cells were either sham treated or infected with indicated MOIs of CVB3 for 7 h. *Ifnb1* gene levels were quantified by qRT-PCR, and the results were presented as relative mRNA quantity normalized to GAPDH (means ± SD, n = 3). $^{§}p < 0.01$, $^{*}p < 0.05$ compared with sham infection. (C) HPL1D cell lines stably expressing *GFP* (control), $KRAS^{G12V}$, or $EGFR^{L858R}$ were sham or CVB3 infected (MOI = 1.0) for the indicated time points. Western blot analysis was conducted to determine protein expression of p-eIF2α, eIF2α, and β-actin. Level of p-eIF2α was quantitated by densitometric analysis, normalized to eIF2α, and presented underneath as fold changes compared with the first lane that is arbitrarily set at a value of 1.

intratumoral injection of CVB3 on distant tumors (Figures 7A and 7B). However, the survival curve showed no improvement of mouse survival after WT-CVB3 treatment (Figure 7C). Viral quantitation demonstrated active viral replication in tumors at both sides and in multiple organs, particularly the heart (Figure 7D). Future research is required to further reduce the toxicity.

Partial Recovery of the Host Innate Immunity Attenuates CVB3-Induced Pancreatic Damage

CVB3 is known to be a common causative agent for viral myocarditis and pancreatitis, especially in children and those who have defective anti-viral immunity.[18,19] As expected, in immunodeficient NSG mice, WT-CVB3 caused a significant cytotoxicity in heart and pancreas, as characterized by myocardial injury and inflammatory infiltration, as well as destruction of acinar cells of the pancreas, when compared with UV-CVB3 treatment in both groups (Figures 8A and 8B). In mice infected with WT-CVB3, pulmonary edema was observed and likely a consequence of heart failure. However, damage to the lung epithelial cells appeared to be minor. Minimal pathological changes were seen in the liver and spleen. We further showed that partial recovery of the innate immunity in NOD-SCID mice markedly attenuated CVB3-induced injury to the pancreas (Figures 8C and 8D). However, myocardial damage remained, and survival rate was not significantly improved in NOD-SCID mice, pointing to a future direction in developing CVB3 as on oncolytic virus for $KRAS^{mut}$ lung cancer therapy by genetically engineering the CVB3 genome to decrease its cardiotoxicity.

DISCUSSION

Emerging evidence has indicated that *KRAS* mutation is a negative predictor of benefit from either adjuvant chemotherapy or tyrosine kinase inhibitor treatment, and there is no effective targeted therapy currently available for $KRAS^{mut}$ adenocarcinomas.[37] Thus, alternative strategies for targeting *KRAS* mutation tumors have gained considerable attention in recent years. However, little progress has been made to develop specific RAS inhibitors effective across the mutations present in lung adenocarcinomas. Here we report that CVB3 is a natural agent that can specifically target $KRAS^{mut}$ lung adenocarcinomas, leading to significant tumor regression *in vivo*. Our findings highlight that CVB3 could be an excellent candidate to be further developed into a novel oncolytic virus for $KRAS^{mut}$ lung adenocarcinoma therapy.

CVB3 is known to subvert host signaling pathways to facilitate its own replication. Among these pathways, the mitogen-activated protein kinase (MAPK) module, which consists of RAF, MEK1/2, and ERK1/2, plays a central role.[28,29] As the upstream activator of the RAF/MEK/ERK cascade, small GTP-binding protein RAS activates the ERK1/2 pathway by binding RAF and anchoring it at the cell membrane, where it is activated by other kinases.[38] In lung adenocarcinomas, KRAS protein acquires impaired GTPase activity as a result of a point mutation in the gene, leading to a constitutive activation of ERK1/2 signaling.[39] In the present study, we found that specific inhibition of the ERK1/2 activation by MEK1/2 inhibitor U0126 results in a significant attenuation of virion production in $KRAS^{mut}$ lung adenocarcinoma cells, suggesting that viral replication within $KRAS^{mut}$ lung adenocarcinoma cells is predominantly dependent on the host ERK1/2 signaling. Despite that EGFR is also an upstream activator of the ERK1/2 signaling pathway, we showed that the extent of ERK1/2 phosphorylation is much lower in $EGFR^{mut}$ than in $KRAS^{mut}$ lung adenocarcinomas, similar to previous reports showing that constitutive EGFR activation in $EGFR^{mut}$ adenocarcinomas selectively activates the AKT and STAT signaling pathways to promote cell survival and invasion, but has less effects on the ERK1/2 pathway that is generally associated with cell proliferation and survival.[40,41] Thus, ERK1/2 signaling appears to be preferentially activated by $KRAS^{mut}$ rather than $EGFR^{mut}$, and the relative resistance of $EGFR^{mut}$ lung adenocarcinomas to CVB3 is likely due to attenuated activation of the ERK1/2 signaling pathway.

Type I interferon is induced upon viral infection as the first line of antiviral response. In this study, we showed that CVB3-induced IFN-β gene (*Ifnb1*) production is significantly suppressed in cells expressing $KRAS^{mut}$ as compared with normal lung epithelial cells and cells expressing $EGFR^{mut}$. These results suggest an additional mechanism by which CVB3 selectively infects and kills $KRAS^{mut}$ lung adenocarcinoma cells while sparing the other two cell types. The exact

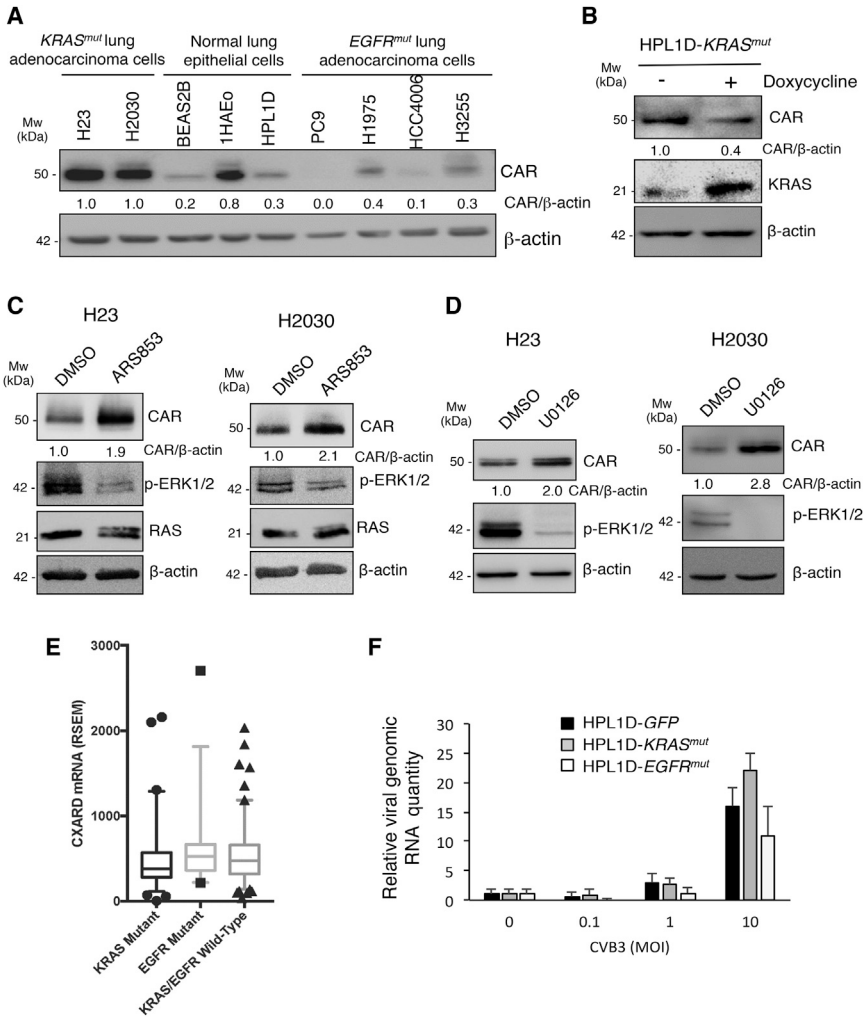

Figure 5. CAR Expression in *KRAS*^{mut} Cells Is Not the Major Determinant of Increased Susceptibility of Adenocarcinomas to CVB3

(A and B) Protein levels of CAR in various adenocarcinoma and normal lung epithelial cells (A) or HPL1D cells stably expressing *KRAS*^{G12V} in the presence or absence of doxycycline (100 ng/mL, 48 h) (B) by western blot analysis. CAR expression was quantitated by densitometric analysis using NIH ImageJ, normalized to β-actin, and presented underneath as fold changes compared with the first lane that is arbitrarily set at a value of 1. (C and D) Inhibition of KRAS (C) or ERK1/2 (D) activation results in increased CAR expression in *KRAS*^{mut} lung adenocarcinoma cells. H23 and H2030 cells were treated with or without the KRAS^{G12C} inhibitor ARS853 (50 nM) or MEK1/2 inhibitor U0126 (20 μM) as indicated for 24 h, followed by western blot and densitometric analysis of CAR as above. (E) *CXARD* mRNA levels (RSEM scaled estimate values) in various lung adenocarcinomas. A total of 230 human lung adenocarcinoma tumors were grouped by genotype (*KRAS*^{mut}, *EGFR*^{mut}, or *EGFR*/*KRAS*$^{wild\text{-}type}$), and expression values were compared between groups using the Mann-Whiney U test in Prism 7 (GraphPad). No statistical significance between groups was observed. (F) HPL1D cell lines stably expressing *GFP*, *KRAS*^{G12V}, or *EGFR*^{L858R} were infected with CVB3 at different MOIs as indicated for 30 min. After PBS washing, cells were collected for RNA extraction. Virus uptake was determined by qPCR analysis of viral genomic RNA (means ± SD, n = 3). There are no statistical differences in virus uptake between control and *KRAS*^{mut} or *EGFR*^{mut} cells.

mechanism by which KRAS^{mut} disrupts CVB3-induced Ifnb1 expression is currently unclear and warranted future investigations.

CAR is the primary receptor responsible for CVB3 internalization and is hence a determinant of virus tropism.[32] Although protein levels of CAR are usually low in cancerous cells, for example, in prostate cancer, gastrointestinal cancer, and glioma,[42–44] it is highly expressed in various lung cancer cells.[21,45] Multiple mechanisms, including transforming growth factor-β signaling cascade, epithelial-mesenchymal differentiation, histone deacetylation of the CAR gene promoter, hypoxia-inducible factor-1α-dependent hypoxia, and the MAPK signaling pathways, have been proposed to be involved in the regulation of CAR.[33,46] In the present study, increased protein expression of CAR was detected in two *KRAS*^{mut} cell lines (i.e., H23 and H2030) as compared with *EGFR*^{mut} and normal cell lines, suggesting that CAR level is a potential determinant for enhanced sensitivity of *KRAS*^{mut} lung adenocarcinomas toward CVB3 infection. However, further investigation revealed that KRAS negatively regulates CAR expression, indicating that additional factors and/or gene mutations, other than KRAS, mediate the observed upregulation of CAR in H23 and H2030 cells. Nonetheless, our data suggest that *KRAS*^{mut} lung adenocarcinomas have two attributes that contribute to CVB3 susceptibility, CAR expression and enhanced ERK signaling, with the latter being the main determinant in mediating the oncolytic effects.

It is well documented that the host immune system plays a dual role in oncolytic virotherapy. On the one hand, early innate immune responses to viruses result in rapid viral clearance; on the other hand, viral infection elicits a significant anti-tumor immune response that breaks immune tolerance and allows for long-term cancer destruction. Both direct oncolysis and anti-tumor immunity triggered by virus infection are believed to contribute to the efficacy of cancer virotherapy.[47,48] Thus, maintaining a delicate balance between the anti-viral response and anti-tumor immunity will be crucial in mediating successful anti-cancer virotherapy. In this study, we showed that the cytotoxicity caused by CVB3, especially to the pancreas, is greatly attenuated in *KRAS*^{mut} NOD-SCID mice when compared

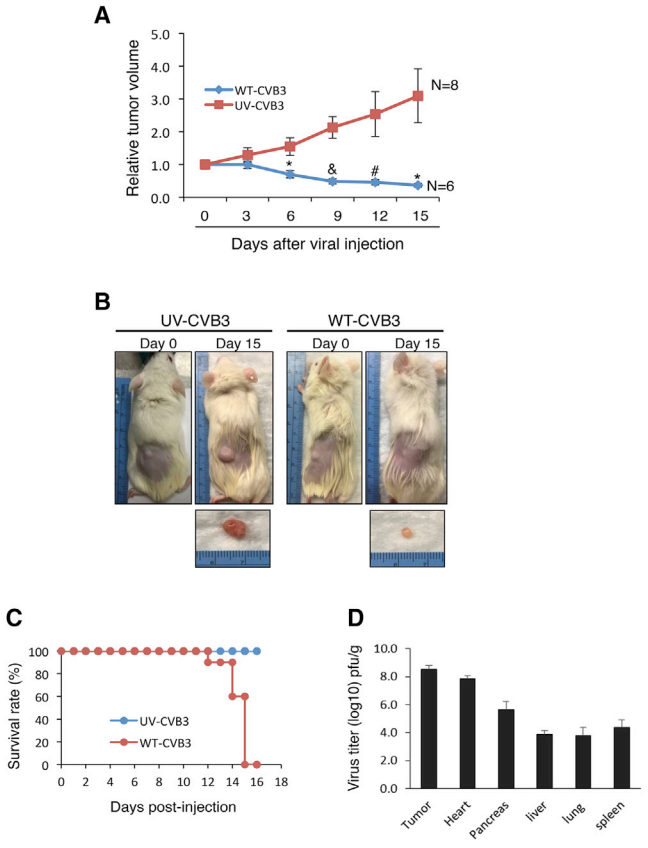

Figure 6. Intratumoral Injection of CVB3 Leads to a Significant Regression of *KRAS^{mut}* Xenograft Lung Tumors in NSG Immunodeficient Mice

(A) Patient-derived *KRAS^{mut}* H2030 cells (5×10^6 cells) were injected subcutaneously into the left flank of NSG immunodeficient mice. When tumors reached a palpable size, mice were intratumorally injected with a single dose of WT-CVB3 (5×10^4 PFU) or UV-CVB3 in the left flank. Tumor volumes of *KRAS^{mut}* xenografts were measured every 3 days and expressed as means ± SEM; $^{*}p < 0.05$; $^{\&}p < 0.005$; $^{\#}p < 0.001$ as compared with UV-CVB3 controls. (B) Representative images of mice with *KRAS^{mut}* xenograft tumors treated with UV- or WT-CVB3 for different days as indicated. (C) Kaplan-Meier plot was used to show survival rate. (D) Virus titers in the different organs and tumors collected from *KRAS^{mut}* xenograft mice treated with WT-CVB3 at the end of the experiment. Results are presented as means ± SD (n = 3).

with *KRAS^{mut}* NSG mice, suggesting a protective function of the host innate immunity in limiting viral spread and replication. Meanwhile, we found that CVB3 injection causes a similar rate of tumor regression in both NOD-SCID and NSG mice, indicating that CVB3-mediated direct oncolytic lysis plays a predominant role in tumor reduction. It is noteworthy that CVB3 inoculation into one side of the bilateral *KRAS^{mut}* xenografts results in a significant tumor regression of both sides, suggesting a potential application of CVB3 in the patients with metastatic tumors.

CVB3 is known to have a high tropism toward cardiac and pancreatic tissues.[18,19] Despite the potent anti-tumor effects of WT-CVB3, virus-induced myocarditis and pancreatitis are a concern for its application as an oncolytic agent, especially for those who are immunocompromised. Our *in vivo* study revealed that WT-CVB3 causes damage to multiple organs, particularly heart and pancreas in NSG immunodeficient mice, which is likely a result of uncontrolled viral growth. Partial recovery of innate immunity in NOD-SCID mice attenuates CVB3-induced injury to the pancreas; however, cardiac damage remains. The exact mechanism by which the pancreas, but not the heart, was protected from CVB3-induced damage in NOD-SCID mice is currently unclear. Host innate immunity is known to play a dual role during viral infection.[18] On the one hand, the innate immune response constitutes the first line of host defense against invading viruses; on the other hand, aberrant immune response can be detrimental, contributing to further tissue damages. A balance between the protective and harmful effects will ultimately determine disease progression and tissue toxicity. Thus, we speculate that decreased pancreatic toxicity in NOD-SCID mice is a result of more effective viral clearance (Figure 7D) and/or lower sensitivity to immune-mediated tissue damage in the pancreas.

Future studies are urgently needed to genetically engineer the CVB3 genome to further enhance its tumor specificity toward lung adenocarcinomas and decrease its toxicity to normal tissues. Recent evidence has suggested that microRNAs (miRNAs) play a key role in the development of cancers.[49–51] miRNAs are frequently detected to be downregulated in diverse types of cancer tissues compared with normal tissues.[52] This unique feature of cancer cells can be employed to develop tumor-specific oncolytic viruses. For example, it is expected that insertion of tumor-suppressive miRNA target sequences into the UTR of the CVB3 genome will lessen its toxicity to normal tissues. While preparing for re-submission of this manuscript, there was a new publication that corrected their previous report regarding the safety of WT CVB3[21] and showed that CVB3 modified by insertion of miR-34 target sequences results in reduced toxicity of WT CVB3 in normal cells in mice.[53] Another potential strategy to reduce CVB3 replication and toxicity in normal cells is to engineer CVB3 to re-direct its tissue tropism through receptor binding. For example, Hazini et al.[54] recently reported that a specific CVB3 variant (PD strain) that uses heparan sulfate, rather than CAR, for viral entry, specifically replicates in colorectal cancer cells, but not in normal tissues.

Why does CVB3 still infect IFN-intact cardiomyocytes? It should be noted that a successful viral infection needs multiple factors, including the expression of cell surface receptors (e.g., CAR), activation of oncogenic signaling pathways (e.g., ERK1/2 pathway), defects in host immunity (e.g., defective type I interferon immune response), and low abundance of intracellular anti-viral proteins (e.g., intracellular proteins involved in cap-dependent protein translation). Although cardiomyocytes are generally non-proliferating, they express a high level of CAR, especially in children, and a low level of host anti-viral molecules. In addition, like many other RNA viruses, CVB3 has evolved mechanisms to suppress this antiviral host response. For example, virus-encoded proteases (2A and/or 3C) have been shown to cleave MDA5, RIG-I, and MAVS, critical innate immune signaling proteins, leading to impaired type I IFN

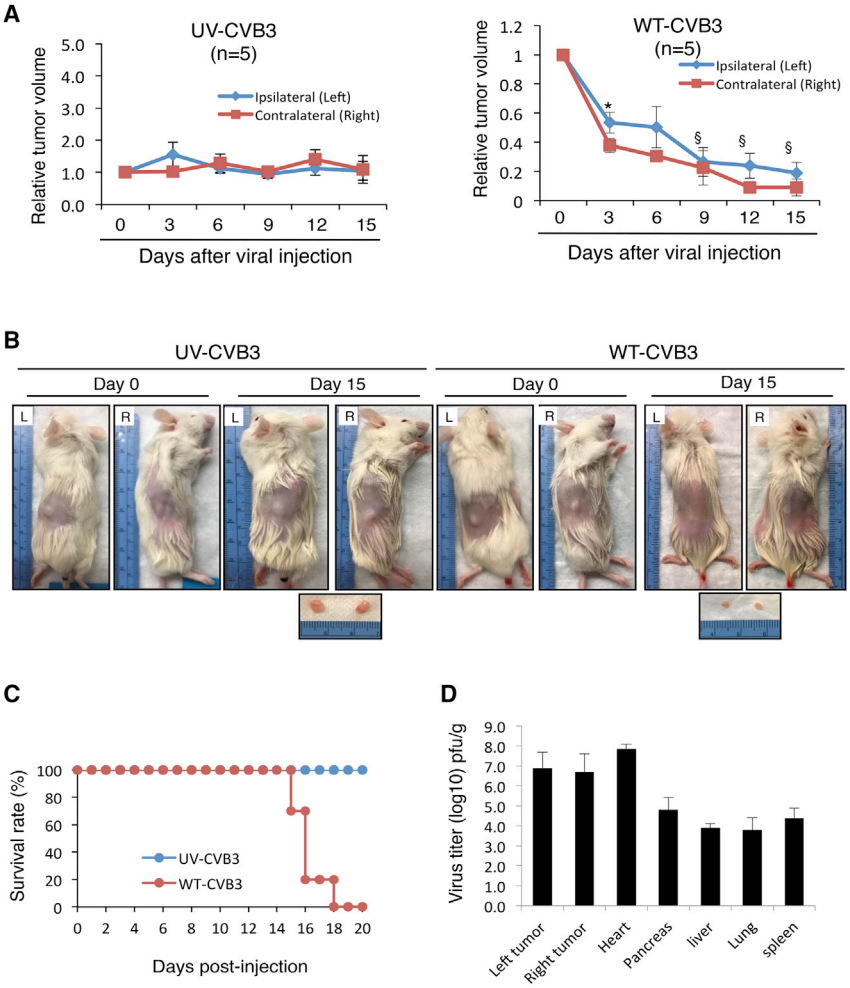

Figure 7. Intratumoral Injection of CVB3 Results in a Significant Reduction in *KRAS*^{mut} Tumor Size in NOD-SCID Immunocompromised Mice

(A) Patient-derived *KRAS*^{mut} H2030 (5×10^6 cells) were injected subcutaneously into both flanks of NOD-SCID mice. When tumors reached a palpable size, mice were intratumorally injected with a single dose of WT-CVB3 (5×10^4 PFU) or UV-CVB3 in the left flank. Tumor volumes of *KRAS*^{mut} xenografts were measured every 3 days and expressed as means ± SEM. $^{*}p < 0.05$; $^{\S}p < 0.01$ as compared with UV-CVB3 controls. (B) Representative images of animals with *KRAS*^{mut} xenograft tumors treated with UV- or WT-CVB3 on days 0 and 15 after CVB3 injection. (C) Kaplan-Meier plot of mouse survival rate. (D) Virus titers (means ± SD, n = 3) in the xenograft tumors and different organs from mice inoculated with WT-CVB3.

response.[55,56] Collectively, all of these factors enable a productive viral infection within this type of cell.

In conclusion, our study suggests that CVB3 selectively kills *KRAS*^{mut} lung adenocarcinomas mainly via the virus self-replication process. The potential application of CVB3 as an oncolytic therapy may provide a new direction for refractory *KRAS*^{mut} lung adenocarcinomas.

MATERIALS AND METHODS

Mice

NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (also known as NSG) and NOD.CB17-*Prkdc*^{scid}/J (also known as NOD-SCID) immunocompromised mice were purchased from the Jackson Laboratory and bred at the Animal Resource Centre of BC Cancer Research Centre. All animal experiments were performed in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of British Columbia (A15-0015).

Cell Lines

Three *KRAS*^{mut} (A549, H2030, and H23), four *EGFR*^{mut} (H1975, PC-9, HCC4006, and H3255) lung adenocarcinoma cell lines, and three normal lung epithelial cells (HAE, BEAS-2B, and HPL1D) were used in this study: A549 cell line derived from adenocarcinomic human alveolar basal epithelial cells (American Type Culture Collection [ATCC] catalog number CCL-185); H2030 cell line derived from metastatic lymph node of stage III lung adenocarcinoma (CRL-5914; ATCC); H23 cell line derived from lung adenocarcinoma of epithelial origin (CRL-5800; ATCC); H1975 cell line derived from lung adenocarcinoma of epithelial origin (CRL-5908; ATCC); PC-9 cell line derived from undifferentiated type of lung adenocarcinoma (90071810; Sigma-Aldrich); HCC4006 cell line derived from metastatic pleural effusion of lung adenocarcinoma (CRL-2871; ATCC); H3255 cell line derived from metastatic pleural effusion of lung adenocarcinoma (ATCC, CRL-2882); 1HAEo, a post-crisis SV-40 T antigen transformed epithelial cell line (obtained from Dr. Dieter Gruenert, California Pacific Medical Center, University of California, San Francisco, San Francisco, CA, USA)[57]; BEAS-2B cell line expressing keratins and SV40 T antigen derived from normal human bronchial epithelium (CRL-9609; ATCC); and HPL1D cell line expressing SV40 T antigen derived from normal human small airway epithelium (originally generated by Takashi Takahashi from Nagoya University, Japan). All cells were grown in RPMI 1640 medium (Cat. #11875093; Thermo) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. Stable HPL1D cell lines expressing tetracycline-inducible genes (HPL1D-GFP, HPL1D-*KRAS*^{G12V}, and HPL1D-*EGFR*^{L858R}) were generated and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum as with constructs previously described.[58] To induce transgene expression, we

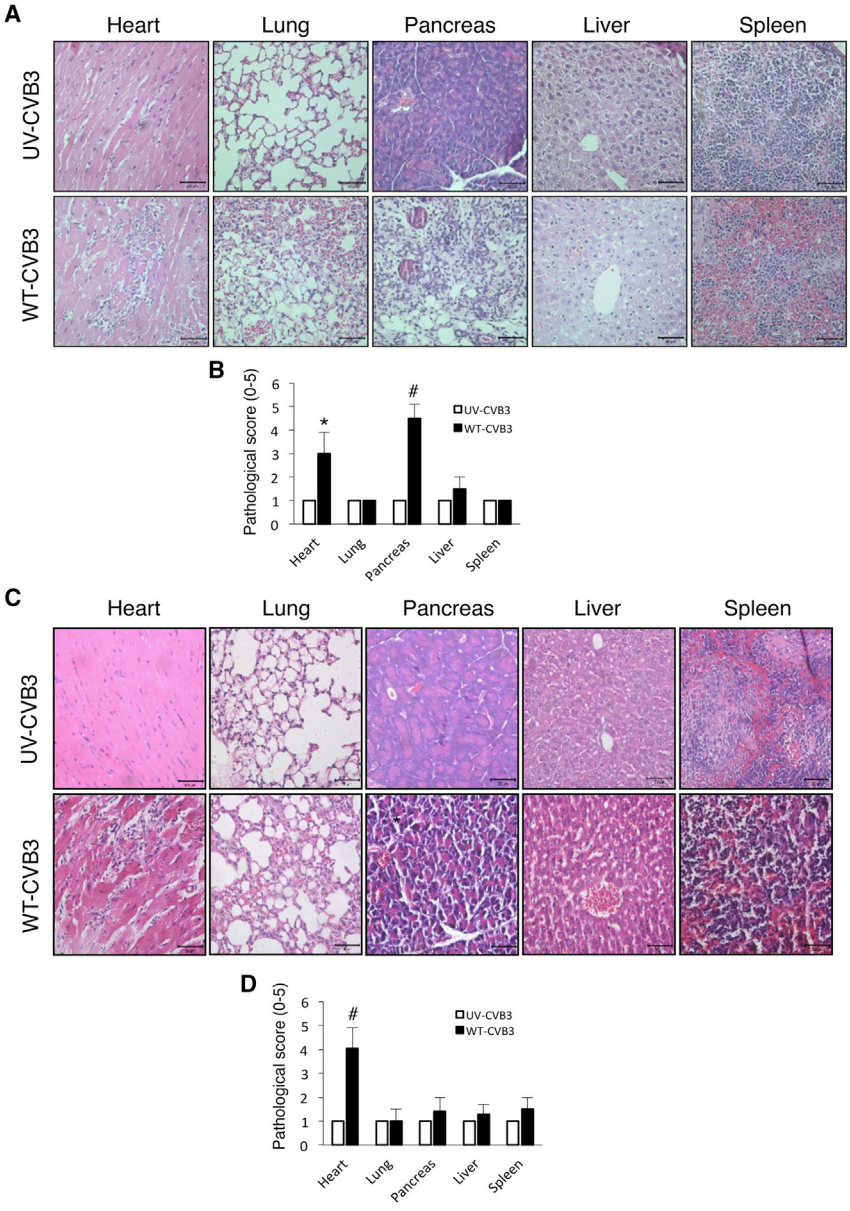

Figure 8. Partial Recovery of the Host Innate Immunity Attenuates CVB3-Induced Pancreatic Damage

(A and C) H&E staining of different organs harvested from *KRAS*^{mut} xenograft NSG (A) or NOD-SCID (C) mice at the end of the experiment. Images were taken using the SPOT Insight camera and Nikon ECLIPSE E600 microscope at original magnification ×40. Scale bars, 50 μm. (B and D) The extent of tissue damage in NSG (B) or NOD-SCID (D) mice was histologically graded based on the intensity and character of injury and/or inflammatory infiltration, and expressed as pathological scores (0–5, means ± SEM). $^{\#}p < 0.001$ as compared with UV-CVB3 control.

added doxycycline hyclate (Cat. #324385; Sigma-Aldrich) at 100 ng/mL at the time of cell seeding for 48 h. Human primary airway epithelial cells, a gift from Dr. Tillie Hackett at the University of British Columbia, were isolated from normal donors and cultured as previously described.[59]

Viruses and Viral Infection *In Vitro*

CVB3 (Nancy strain) was propagated in HeLa cells and stored at −80°C. UV irradiation was performed using UV Stratalinker 1800 (Stratagene) for 4 h with the virus container kept 5 cm from the UV bulb. For viral infection, cells were incubated with serum-free medium containing either CVB3 at different MOIs or PBS (sham infection) for different periods of time as indicated.

Crystal Violet Staining

CVB3-induced cytotoxicity was evaluated by crystal violet staining as previously described.[60] In brief, after wash with PBS, viable cells attached to the bottom of the plates were fixed and stained with 0.4% crystal violet solution in methanol for 30 min.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium Salt (MTS) Assay

Cell viability was determined using a CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Cat. #G5421; Promega) according to the manufacturer's protocols. In brief, 20 μL of the combined MTS/phenazine methosulfate (PMS) solution was added into each well of the 96-well assay plate containing ~1 × 10^5 cells in a final volume of 100 μL culture medium, and the plate was incubated at 37°C for 4 h. Subsequently the absorbance at 490 nm was recorded on a microplate reader. The absorbance of sham-infected cells was defined as a value of 1. Cell viability in CVB3-treated cells is presented as the ratio to that of sham-infected cells.

Western Blot Analysis

Cells were harvested using modified oncogene science lysis buffer (250 mM NaCl [pH 7.2], 50 mM Tris-HCl, 0.1% Nonidet P-40 [NP-40], 2 mM EDTA, and 10% glycerol) supplemented with protease inhibitors. Western blot analysis was performed as previously described.[61] In brief, equal amounts of proteins were resolved by SDS-PAGE and then transferred to nitrocellulose membranes. The resulting membranes were incubated with primary antibodies at 4°C for overnight, followed by incubation with HRP-conjugated secondary antibodies at room temperature for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence. Primary antibodies used in this study were: anti-rat sarcoma viral oncogene (RAS) (#3965; Cell Signaling), anti-EGFR (#4267; Cell Signaling), anti-phospho-eIF2α (#9721; Cell Signaling), anti-eIF2α (#9722; Cell Signaling), anti-CAR (#16984; Cell Signaling), anti-viral capsid protein VP1 (NCL-ENTERO; Leica

Biosystems), anti-phospho-ERK1/2 (#4370; Cell Signaling), and β-actin (#2228; Sigma-Aldrich).

Viral Plaque Assay

The viral titers in CVB3-infected cells or mouse organs were evaluated by plaque assay as previously described.[61] In brief, culture media collected from CVB3-infected cells or homogenized tissue supernatants were serially diluted and overlaid on a monolayer of HeLa cells. After 1-h incubation, the medium was replaced by complete DMEM containing 0.75% agar. After 3-day incubation, cells were fixed with Carnoy's fixative (75% ethanol and 25% acetic acid) for 30 min, followed by crystal violet staining. The plaques were counted, and the viral titers were calculated and represented as plaque-forming units per milliliter or per gram.

Quantitative Real-Time PCR

qPCR was conducted to determine mRNA levels of *Ifnb1* gene as previously described.[62] In brief, total RNA was extracted using the Monarch Total RNA Miniprep kit (#T2010S; New England Biolabs). qPCR targeting *Ifnb1* gene (forward primer: 5′-GTC TCC TCC AAA TTG CTC TC; reverse primer: 5′-ACA GGA GCT TCT GAC ACT GA-3′) was performed using the Luna Universal One-Step RT-qPCR kit (#E3005S; New England Biolabs) and normalized to GAPDH mRNA (forward primer: 5′-AAT CCC ATC ACC ATC TTC CA-3′; reverse primer: 5′-TGG ACT CCA CGA CGT ACT CA-3′) following the manufacturer's recommendations.

Virus Uptake Assay

Viral particle uptake experiment was performed as described previously.[63] In brief, following incubation with doxycycline for 36 h, HPL1D stable cells were infected with CVB3 at an MOI of 0.1, 1, and 10 for 30 min. After three washes with ice-cold PBS, cells were harvested and subjected to RNA extraction. qPCR was performed as above to determine levels of viral genomic RNA using primer pairs of CVB3 2A (forward: 5′-GCT TTG CAG ACA TCC GTG ATC-3′; reverse: 5′-CAA GCT GTG TTC CAC ATA GTC CTT CA-3′). The values were normalized to β-actin mRNA (ACTB, forward primer: 5′-ACT GGA ACG GTG AAG GTG AC-3′; reverse primer: 5′-GTG GAC TTG TTG GGA GAG GAC TG-3′) levels.

Mouse Xenograft Models

H2030 ($KRAS^{mut}$) cells were used to establish lung adenocarcinoma xenograft mouse models. In brief, H2030 cells (5×10^6 cells) were injected subcutaneously into the left flank of male NSG mice or both flanks of male NOD-SCID mice. When tumors reached a palpable size (~30–60 mm^3), mice were intratumorally injected with either WT- or UV-inactivated CVB3 for a single dose (5×10^4 PFU). Mice were monitored daily for general appearance, behavior, weight, and any signs of infection at the tumor cell injection site. Tumor size was measured every 3 days, and tumor volume was calculated as length × width × width/2. Mice were euthanized when they manifested severe symptoms related to CVB3 injection or the tumor diameter exceeded 2.0 cm.

H&E Staining

The tissues were harvested and fixed in 10% formalin, followed by embedding in paraffin and sectioning for standard H&E staining.

Gene Expression Profiling

RNA sequencing (RNA-seq) data (relative SEM [RSEM] scaled estimate expression values) for *CXADR* that encodes human coxsackievirus-adenovirus receptor (CAR) for 230 lung adenocarcinomas from The Cancer Genome Atlas were downloaded along with *KRAS* and *EGFR* mutation status from the cBio Portal (http://www.cbioportal.org/).[64,65] Tumors were grouped by genotype ($KRAS^{mut}$, $EGFR^{mut}$, or $EGFR/KRAS^{wild\text{-}type}$), and expression values were compared between groups using the Mann-Whiney U test in Prism 7 (GraphPad). One tumor with concurrent *KRAS* and *EGFR* mutation was excluded from analysis.

Inhibitor Treatments

For inhibition experiments, cells were treated with ARS853 (#550377; Selleckchem, Houston, TX, USA), a selective, covalent $KRAS^{G12C}$ inhibitor, or U0126 (#9903; Cell Signaling, Beverly, MA, USA), an MEK1/2 inhibitor for various concentrations as indicated.

Statistical Analysis

All results presented are representative of at least three independent experiments. Results generated from *in vitro* experiments are expressed as mean ± SDs, and results from *in vivo* mouse studies are presented as mean ± SEMs. Statistical analysis was conducted using unpaired Student's t test. The survival curve was plotted by the Kaplan-Meier approach. Values of $p < 0.05$ were considered to be statistically significant.

AUTHOR CONTRIBUTIONS

H.D., W.W.L., W.W.G.J., and H. Luo designed the studies. H.D., H. Liu, T.d.S., Y.C.X., Y.M., C.S.N., J.Q., and J.Z. performed the experiments. H.D., W.W.L., 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. No conflicts of interest were disclosed by others.

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