# Targeting 2A Protease by RNA Interference Attenuates Coxsackieviral Cytopathogenicity and Promotes Survival in Highly Susceptible Mice

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***Background***—Enteroviridae such as coxsackievirus B3 (CVB3) are important infectious agents involved in viral heart disease, hepatitis, and pancreatitis, but no specific antiviral therapy is available.

***Methods and Results***—The aim of the present study was to evaluate the impact of RNA interference on viral replication, cytopathogenicity, and survival. Small interfering RNA (siRNA) molecules were designed against the viral 2A region (siRNA-2A), which is considered to be highly conserved and essential for both virus maturation and host cytopathogenicity. siRNA-2A exhibited a significant protective effect on cell viability mediated by marked inhibition of CVB3 gene expression and viral replication. In highly susceptible type I interferon receptor–knockout mice, siRNA-2A led to significant reduction of viral tissue titers, attenuated tissue damage, and prolonged survival. Repeated siRNA-2A transfection was associated with a further improvement of survival. Various control siRNA molecules had no protective effect in vitro or in vivo.

***Conclusions***—RNA interference directed against the 2A protease encoding genomic region effectively confers intracellular immunity toward CVB3-mediated cell injury and improves survival, suggesting a potential role for RNA interference for future treatment options targeting enteroviral diseases. **(*Circulation*. 2005;111:1583-1592.)**

**Key Words:** coxsackievirus ■ RNA ■ enterovirus ■ myocarditis ■ protease

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Picornaviridae encompass one of the largest and clinically most important families of infectious human pathogens. In particular, rhinoviruses and enteroviruses such as poliovirus and coxsackievirus are of considerable medical and economic importance. Coxsackievirus B3 (CVB3) is considered the most important infectious agent involved in viral heart muscle disease.[1] CVB3 may induce acute and chronic forms of human myocarditis, which can be a life-threatening disease with adverse acute and long-term clinical outcome.[2] In addition, persisting CVB3 genomes may lead to dilated cardiomyopathy without obligate previous clinically apparent inflammatory involvement of the myocardium.[3,4] Besides its clinically important adverse effects on the heart, CVB3 has also been implicated as an infectious agent involved in the pathogenesis of extracardiac diseases such as hepatitis,[5] pancreatitis,[6] and aseptic meningitis.[7]

It has been shown that acute as well as persisting CVB3 infection of cardiac cells causes a direct cytopathic effect in both cultured human myocardial fibroblasts[8] and myocytes[9] and in various animal models.[4,5,10] In the intact myocardium, cleavage of dystrophin by coxsackieviral protease 2A is considered an important mechanism for CVB3-induced cardiac injury.[11] Additionally, there is evidence for CVB3-mediated apoptotic cell death.[12] Therefore, therapeutic strategies specifically targeting virus elimination can be considered the key therapeutic approach to cure or attenuate CVB3-related disease.

RNA interference is a highly conserved mechanism for silencing the transcriptional product of an activated gene. The process of posttranscriptional gene silencing is initiated by small interfering RNA (siRNA) molecules. These double-stranded siRNA molecules of 21 to 23 nucleotides in length are highly specific for the target mRNA.[13] In mammalian cells, siRNA associates with helicase and nuclease molecules to form a complex that ultimately leads to precise, highly sequence-specific degradation of the target mRNA. Because intracellular RNA interference is considered to have also evolved as an endogenous defense mechanism to cleave double-stranded viral RNA,[14] this mechanism may be used as a therapeutic tool to attenuate coxsackieviral replication and improve cell survival.

On cell entry, coxsackievirus releases its messenger-like plus stranded RNA, which is subsequently translated into a monocistronic polyprotein. The first step in subsequent

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**siRNA Sequences and Corresponding Target Genes**

| Name | 5′-3′ Sequence | Target Gene | Nucleotide Position |
|---|---|---|---|
| siRNA-2A | AAGGUGUGAGCAUGGUGUCAU | 2A protease | 3637–3657 |
| siRNA-2A$^{mut}$ | AAGGUGUGAGCGUAGUGUCAU | None | ... |
| siRNA-SCR | n/a | None | ... |

All molecules consist additionally of dTdT overhangs. Mutated nucleotides in siRNA-2A$^{mut}$ are underlined. Nucleotide position refers to the nucleotide position in the coxsackieviral genome. n/a indicates not available (commercially available scrambled siRNA obtained from Ambion).

polyprotein processing is cleavage by the viral protease 2A.[15] This protease is also involved in cytopathic processes, mediated by either cleavage of dystrophin and/or shut off from host cell translational mechanisms.[11,16] Thus, coxsackieviral protease 2A comprises an important nonstructural viral gene essential for maturation and cytopathic processes. The aim of the present study was to evaluate the impact of siRNA designed against this particular region of the virus on CVB3 replication, pathogenicity, and survival in vitro and in vivo.

## Methods

### Coxsackievirus B3

Infectious virus used in this study was derived from the cDNA copy of the Woodruff variant of coxsackievirus B3[17] as previously described.[9] Virus preparation and organ titers were determined by plaque-forming assay as previously stated.[17] For infection of animals, virus stock was diluted to a final volume of 0.1 mL and injected intraperitoneally. To monitor CVB3 infection, a green fluorescent protein (GFP) expressing full-length infectious coxsackievirus (a kind gift from R. Feuer and L. Whitton, Scripps Research Institute, La Jolla, Calif) was in vitro transcribed and propagated as described elsewhere.[7]

### Cell Culture, CVB3 Infection, and Transfection of siRNA

HeLa cells were maintained in DMEM supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin including 5% heat-inactivated FBS at 37°C in 5% $CO_2$. For siRNA transfection, cells were grown to a confluence of 30% to 40% in 6-well plates (Becton Dickinson) and transfected with the use of 4 μL Oligofectamine reagent (Invitrogen, catalog No. 12252-011), 300 pmol siRNA, and OptiMEM medium (Invitrogen, No. 31985-047) up to a final volume of 1 mL. Transfection mixtures were left on cells for 4 hours. After washing of cells and an additional 6-hour incubation period, a second transfection was performed analogously. After an additional 6 hours, cells were infected with CVB3 at multiplicity of infection (MOI) of 5.

### siRNA Design, Synthesis, and Labeling

siRNAs were designed after determination of target sequences by aligning the CVB3 2A sequence[17] to an Ambion Web-based algorithm (http://www.ambion.com/techlib/misc/siRNA_finder.html). siRNA duplex oligonucleotides were manufactured by Dharmacon (Lafayette, Colo) in the "ready-to-use" option. The 21-nucleotide duplex siRNA molecules with 3′-dTdT overhangs were resuspended in nuclease-free water according to the instructions of the manufacturer (Table). To ensure stringent controls, both a 2A-based mutated control siRNA with 2 nucleotide mismatches (siRNA-2A$^{mut}$) and a scrambled control sequence (siRNA-SCR) obtained from Ambion (Silencer Negative Control No. 1 siRNA, catalog No. 4610) were used. To study the distribution pattern of siRNA in cell culture, some duplex siRNAs were fluorescence labeled with the use of the Silencer Cy3 RNA Labeling Kit (Ambion, No. 1632) according to the protocol provided by the manufacturer. For determination of transfection efficiency in vivo, siRNA molecules were custom labeled with Cy5 fluorochrome by the manufacturer (Dharmacon).

### Assessment of Cell Proliferation and Viability (LDH Assay)

Cell proliferation was assessed by counting 5 random ×100-power fields with the use of an Axiovert 10 inverted microscope (Zeiss). Cytotoxicity was assessed by determination of LDH release from the cytosol of injured cells into the supernatant. LDH was quantified by a colorimetric assay (Roche, No. 1644793) as previously described.[18]

### Reverse Transcriptase–Polymerase Chain Reaction

To determine the expression of viral RNA in HeLa cells, reverse transcriptase–polymerase chain reaction (RT-PCR) with specific primers binding within the 5′UTR of the CVB3 genome was performed. Total RNA was isolated 5, 12, and 24 hours after infection and reverse transcribed as previously described.[9] Primers termed CP1 (5′-ACCTTTGTGCGCCTGTT-3′) at position 68 to 84 of the CVB3 genome for the detection of viral negative stranded RNA and CP2 (5′-CACGGACACCCAAAGTA-3′) (position 546 to 562) for identification of viral positive stranded RNA were used. Subsequently, cDNA was PCR amplified with the use of CP1 and CP2 primers at an annealing temperature of 52°C by Taq DNA-polymerase (Takara Bio Europe, No. R001A). Appropriate controls were used for every step to control for sensitivity and specificity of the amplification. GAPDH served as an internal control for RT-PCR (primers from Stratagene, No. 302047).

### Hydrodynamic Transfection of Type I Interferon Receptor–Deficient Mice and Determination of siRNA Transfection Efficiency In Vivo

Type I interferon receptor–deficient mice,[19] highly susceptible for CVB3 infection,[9] were obtained from B&K Universal Ltd, Aldbrough Hull, UK. To study the effect of RNA interference in this animal model, mice were inoculated with a typically lethal dose of $5\times10^3$ plaque-forming units of CVB3 in PBS. Delivery of siRNA was performed via the tail vein by high-volume injection with the use of 0.6 nmol of siRNA/g body wt. siRNA was applied in a total volume of 1.6 mL NaCl 0.9% via a permanent venous catheter (Neoflon, Becton Dickinson) inserted into the tail vein. Injection time was approximately 20 seconds. For hydrodynamic transfection, animals were anesthetized intraperitoneally as described.[20] Mice were transfected twice, 6 and 14 hours after infection, and closely monitored for determination of survival. To evaluate the effect of a third siRNA transfection exclusively on survival, 1 group of 4 mice received an additional siRNA treatment 48 hours after infection. For measurements of viral organ titers by plaque-forming assay, assessment of viral protein expression and organ damage by histology, and measurement of alanine aminotransferase (ALT) in serum by a specialized laboratory for veterinarian clinical chemistry (Synlab, Augsburg, Germany), mice were euthanized 42 to 48 hours after infection. Elevated levels of ALT, previously known as serum glutamic-pyruvic transaminase (SGPT), specifically indicate liver injury. For assessment of viral-mediated tissue damage, histological liver specimens derived from mice 48 hours after infection were stained with hematoxylin-eosin and graded by a blinded pathologist using a semiquantitative score for inflammation and cell necrosis in the liver, as follows: 0, absent; 1, mild; 2, moderate; and 3, severe. For assessment of in vivo siRNA transfection efficiency, Cy-5–labeled siRNA molecules were hydrodynamically transfected in CVB3-infected mice as described, and liver and heart were removed 10 hours after the second transfection. Control mice received NaCl 0.9% solution without siRNA (n=3 in each group). Liver- and heart-derived cells were subsequently analyzed for Cy-5 fluorescence by flow cytometry (FACSCalibur, Becton Dickinson).

### Assessment of Viral Protein Expression by Western Blotting

SDS-PAGE was performed on 4% to 20% Tris-glycine gradient gels (Invitrogen, No. EC6028) as previously described.[21] A polyclonal

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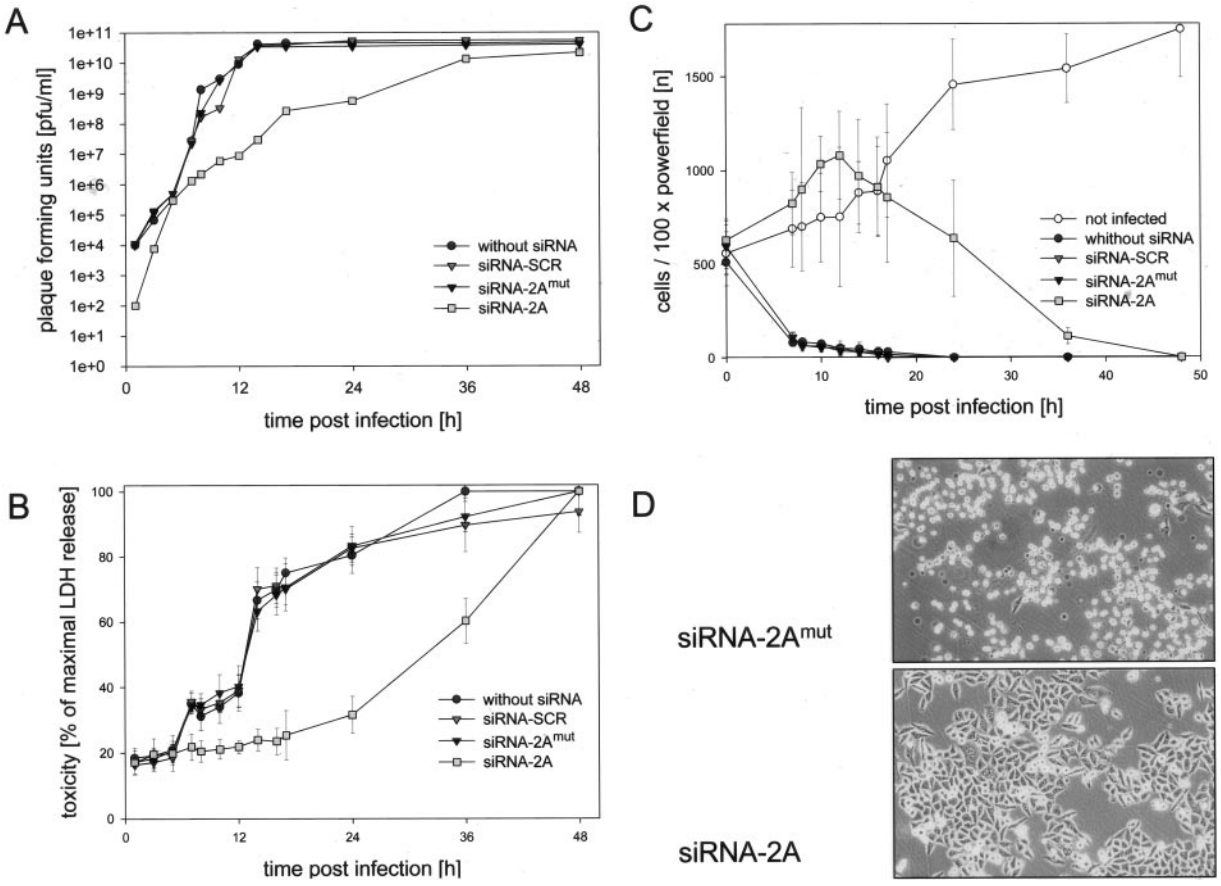


**Figure 1.** Efficacy of RNA interference directed against the 2A sequence of the coxsackieviral genome. siRNA directed against 2A protease significantly delays coxsackieviral replication in permissive HeLa cells ($P<0.001$ vs siRNA-2A$^{mut}$ control, logarithmic scale) (A) and attenuates cytotoxicity as quantified by LDH release (B) ($P<0.001$ vs siRNA-2A$^{mut}$ control). C, Consequently, siRNA-2A confers an antiviral state during early infection and permits similar cell proliferation compared with noninfected cells up to 18 hours after infection ($P$=NS between cells treated with siRNA-2A and noninfected cells up to 18 hours after infection; $P<0.001$ between cells treated with siRNA-2A and siRNA-2A$^{mut}$). Scrambled control and the mutated siRNA-2A$^{mut}$ have no appreciable effect on viral replication, viral cytotoxicity, and cellular proliferation. Data are mean±SD. D, Representative phase-contrast image of infected HeLa cells treated with siRNA-2A$^{mut}$ or siRNA-2A, respectively (magnification ×100). Whereas cells receiving siRNA-2A$^{mut}$ display a severe cytopathic effect 12 hours after infection, a viable phenotype is maintained in siRNA-2A transfected cells.

rabbit-derived antibody recognizing the VP1 epitope of CVB3 (kind gift of Andreas Henke, Jena, Germany)[22] was used as the primary antibody for immunoblotting.

### Statistical Analysis

Results are expressed as mean±SD. The significance of variability among the means of the experimental groups was determined by 1- or 2-way ANOVA, with the use of SPSS for Windows version 10.0 software. Differences among experimental groups were considered statistically significant at $P<0.05$.

## Results

### RNA Interference Directed Against Protease 2A Leads to Inhibition of Viral Replication, Attenuated Cytopathogenicity, and Increased Cellular Survival

To evaluate the efficacy of RNA interference on coxsackieviral replication and virus-mediated cell injury, siRNAs were transfected in HeLa cells, a cell line highly permissive for CVB3 infection. Transfection of siRNA in HeLa cells revealed no significant cytopathic effect, as assessed by measurement of LDH release as a measure of cell injury (data not shown). Transfection of both control siRNAs, siRNA-2A$^{mut}$ (containing 2 mutations of the original siRNA-2A sequence), and siRNA-SCR (a commercially available scrambled siRNA) did not have an appreciable effect on viral replication (Figure 1A), cell injury (Figure 1B), and cell proliferation (Figure 1C), respectively. In contrast, RNA interference directed against the 2A region proved highly effective for the control of early viral replication and virus-mediated cell injury during initial stages of infection (Figure 1A to 1C). To increase transfection efficiency, 2 subsequent transfections were performed, yielding a consistent transfection efficacy of >65% of cells, as determined by immunofluorescent assessment of Cy3-labeled siRNA molecules (Figure 3B). Despite attenuation of viral replication by >1000-fold and extension of cell survival >3-fold by siRNA-2A, at later phases of infection CVB3 was able to augment its replication as well in initially siRNA-2A–transfected cells, leading to an increase in cytotoxicity and inevitable cell death. Whether this effect was due to a lack of effectiveness of siRNA-2A itself or because of siRNA dilution

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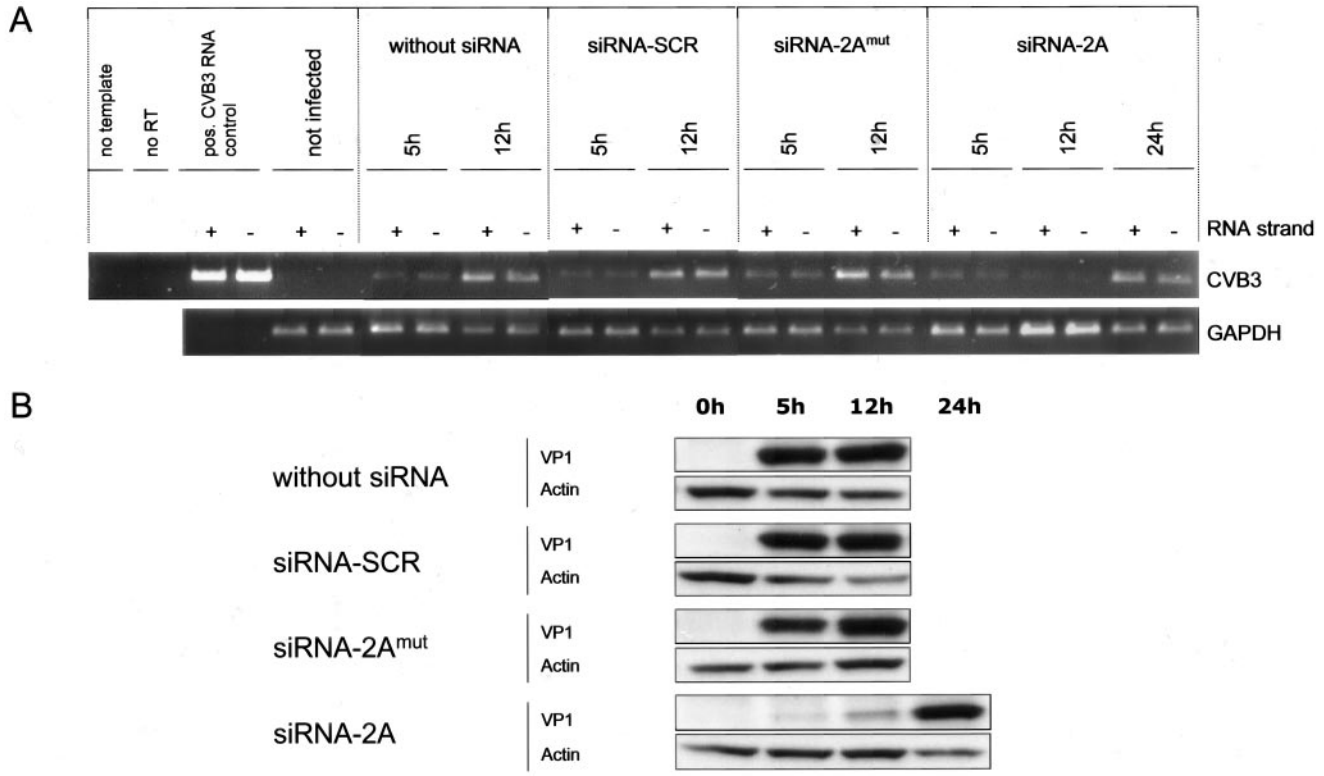


**Figure 2.** Effect of siRNA directed against protease 2A on the expression of coxsackieviral positive and negative stranded RNA and viral capsid protein VP1. A, RT-PCR analysis of viral RNA (top lane) obtained from infected, without and with siRNA-2A, siRNA-SCR– or siRNA-2A$^{mut}$–transfected HeLa cells, respectively, including appropriate controls. + indicates amplified viral positive strand; −, negative strand. For the positive control, 2 $\mu$g of coxsackieviral RNA was used as template for reverse transcription. The fraction of eukaryotic RNA within total loaded RNA was assessed by RT-PCR of GAPDH mRNA (bottom lane). RT-PCR at various time points after infection analysis shows considerable downregulation of CVB3 plus and negative strand gene expression in cells treated with siRNA-2A compared with all other controls, irrespective of siRNA-2A$^{mut}$ or siRNA-SCR treatment or no siRNA delivery. Notably, expression of both positive and negative viral strand is suppressed. Concordantly, the portion of viral RNA on total loaded RNA is increasing more rapidly in all siRNA control transfected cells compared with siRNA-2A–treated cells, visually highlighted by the steadily decreasing portion of eukaryotic GAPDH mRNA on total isolated RNA. B, Western blot analysis against the viral capsid protein VP1 for time-dependent assessment of viral protein expression levels. Whereas viral protein is readily detectable as early as 5 hours after infection, in cells treated with siRNA control constructs and cells not treated with siRNA, siRNA-2A delayed protein expression of CVB3 almost 5-fold. No assessment was feasible at 24 hours for cells not treated with siRNA-2A because no adherent viable cells were existent at this time.

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and/or degradation was addressed in an additional experiment described below.

### RNA Interference Directed Against 2A Protease Region Inhibits Viral RNA and Protein Expression

To assess whether attenuation of viral replication and cytopathogenicity were associated with decreased viral RNA and protein levels, virally infected cells receiving no siRNA or treated with either siRNA-2A, siRNA-2A$^{mut}$, or siRNA-SCR were harvested at different stages of infection, and total RNA and protein were extracted. RT-PCR revealed considerably higher levels of viral RNA at earlier time points in cells treated with siRNA-2A$^{mut}$ compared with cells transfected with siRNA-2A (Figure 2A). Importantly, both viral positive and negative strand expressions were significantly inhibited by siRNA-2A. Additionally, siRNA-2A–treated cells were protected for a considerably longer time from viral protein expression than cells treated with control siRNA or no siRNA (Figure 2B).

### CVB3 Gene Expression and Replication Are Inhibited as Long as Intracellular siRNA-2A Molecules Are Detectable

Protection against virus-mediated cell injury and death as well as inhibition of viral replication is achieved by siRNA-2A; however, the therapeutic effect is transient. To address whether this is due to a temporal distribution pattern of siRNA molecules, siRNA-2A and siRNA-2A$^{mut}$ constructs were fluorescence labeled. To allow imaging of both siRNA and viral gene expression simultaneously, cells were infected with a recombinant infectious CVB3 expressing GFP. As illustrated in the bottom 2 lanes of Figure 3A, viral protein expression is suppressed as long as siRNA-2A remains efficiently expressed in cells. Apparently, this is not the case in siRNA-2A$^{mut}$–transfected CVB3-infected cells, in which coxsackieviral gene expression is colocalizable with siRNA-2A$^{mut}$ and detectable as early as 12 hours after infection (Figure 3A, top 2 lanes). It is well known that the GFP expressing recombinant CVB3 covers a longer replication

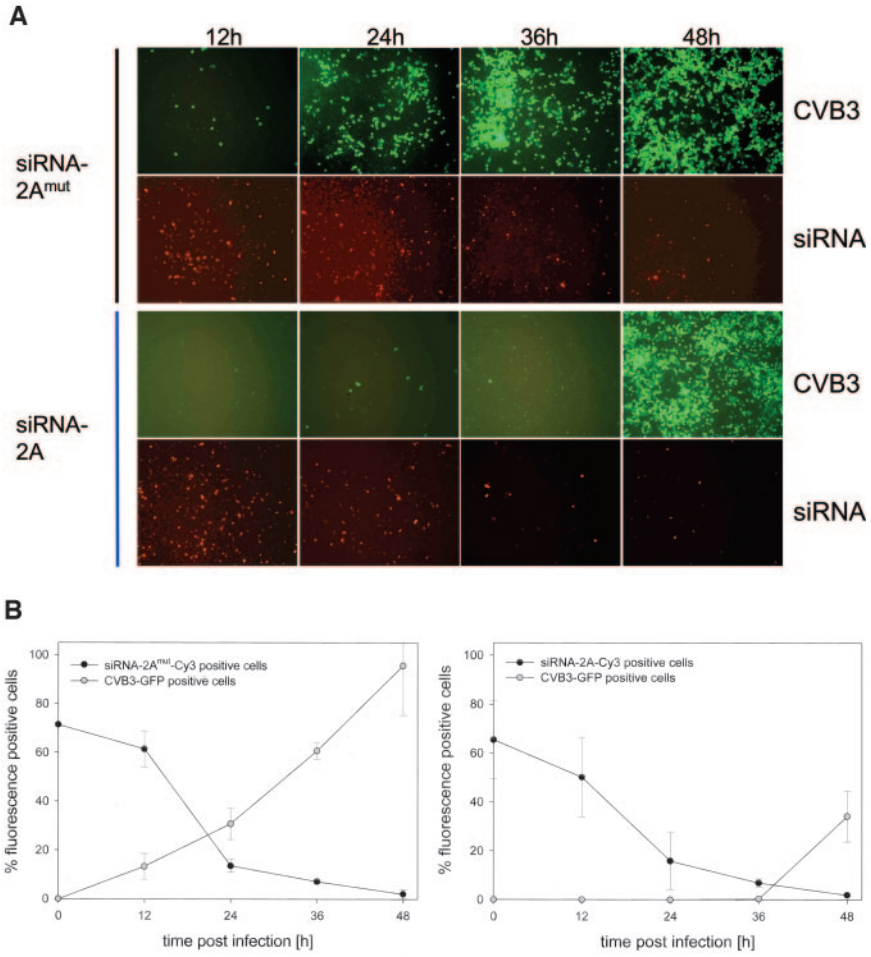


**Figure 3.** Coxsackieviral gene expression is inhibited in cells expressing siRNA-2A. To study the relationship between intracellular siRNA levels and CVB3 gene expression, siRNA molecules were labeled with Cy3, emitting red light. To monitor CVB3 gene expression, a fully infectious CVB3, recombinantly expressing GFP emitting green light, was used. Images were taken at a magnification of ×100 (A). Cells in the top 2 panels were treated with siRNA-2A$^{mut}$ as a control. Coxsackieviral gene expression (green) is steadily increasing within the observation period despite substantial levels of siRNA-2A$^{mut}$ expression (red). Both bottom lanes depict cells treated with siRNA-2A. As long as there is traceable siRNA-2A expression, CVB3 protein expression is sufficiently repressed. Just as siRNA-2A expression decreases significantly, CVB3 gene expression recovers, demonstrating the specific effect of siRNA-2A on the inhibition of CVB3 replication. This is supported by quantitative analysis of cells staining positive for siRNA and GFP expression (B) in cells treated with siRNA-2A$^{mut}$ (left) and siRNA-2A (right), respectively.

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cycle.[7] Therefore, deferred viral gene expression and cytopathic effects compared with wild-type CVB3 used in this study are reflected in this experiment.

## Transfection of siRNA-2A In Vivo Increases Survival Time and Attenuates Viral Replication and Organ Damage in Highly Susceptible Type I Interferon Receptor–Knockout Mice

Homozygous type I interferon receptor (IFNR)–deficient mice are highly susceptible to coxsackieviral infection and die early even when infected with low viral titers, most probably as a result of fulminant hepatitis.[5] To reveal the effect of RNA interference on survival, virus replication, and tissue damage, we applied hydrodynamic transfection of siRNA-2A and as a control siRNA-2A$^{mut}$ after infection with a usually lethal dose of $5\times10^3$ plaque-forming units of wild-type CVB3 virus. Transfection efficiency as assessed by fluorescence-labeled siRNA molecules was determined to exceed 80% of liver and 50% of heart cells in type I IFNR–knockout mice (n=3; Figure 4). siRNA delivery in noninfected mice did not cause increased mortality in type I IFNR-deficient mice (data not shown). Transfection of siRNA-2A$^{mut}$ had no effect on survival (Figure 5A) compared with infected but non–siRNA-treated animals. All mice died at 52±5 hours after infection, concordant with previously published results in infected animals with identical CVB3 titers.[5] In contrast, in mice receiving siRNA-2A after viral infection, life span improved significantly (Figure 5B). One mouse of 8 receiving siRNA-2A survived the infection completely. Additional transfection of siRNA-2A further improved life span (Figure 5A and 5B). Viral protein expression at 42 hours after infection was readily detectable in mice receiving siRNA-2A$^{mut}$, whereas there was no appreciable expression detectable at this stage in siRNA-2A–delivered animals (Figure 6A, top). Notably, no difference could be detected between mice receiving no siRNA or siRNA-2A$^{mut}$. At later stages, however, viral protein expression in the liver was detectable in siRNA-2A–treated mice as well, indicating incremental viral replication (Figure 6A, bottom). siRNA-2A had a profound impact on viral replication in several tissues harvested from infected mice (Figure 6B). Shortly before death of non–siRNA-2A–treated mice at 42 hours after infection, mice were euthanized to directly compare tissue virus titers by plaque-forming assay between animals receiving no siRNA, siRNA-2A, or siRNA-2A$^{mut}$, respectively. Virus titer was $10^5$-fold lower in the liver of animals that received siRNA-2A (n=3) compared with controls. Notably, no virus could be detected in the heart and lung of siRNA-2A–transfected animals, whereas considerable virus titers were detectable in animals treated with siRNA-2A$^{mut}$ (n=3)

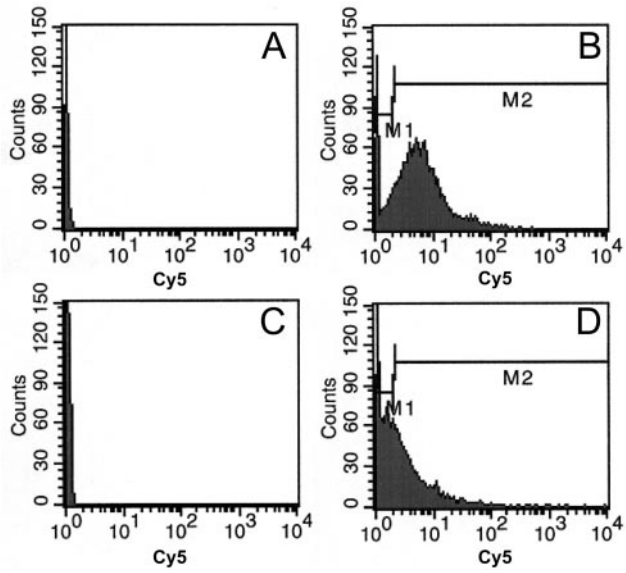


**Figure 4.** In vivo effectiveness of dual hydrodynamic siRNA transfection in liver and heart. FACS analysis of Cy5 fluorescent labeled siRNA-2A molecules in isolated liver (A and B) and heart cells (C and D) derived from previously transfected type I IFNR–knockout mice. Non–siRNA-transfected mice show no apparent fluorescence in the liver (A) and the heart (C). The number of Cy5-positive liver (B) and heart cells (D) was determined at 83.3±5.7% for the liver and 50.2±4.2% for the heart in a total of 3 mice. Cells gated under M1 were considered negative and cells gated in M2 were considered positive for Cy5 fluorescence.

(Figure 6B). Virus-mediated tissue damage was assayed by semiquantitative histological liver examination performed by an experienced, blinded pathologist. At 42 to 48 hours after infection, there was a significantly higher level of liver cell injury evident in mice receiving control siRNA-2A$^{mut}$ compared with siRNA-2A (Figure 6C and 6D, left). This observation was reflected in ALT serum levels drawn 42 to 48 hours after infection, which were significantly higher in siRNA-2A$^{mut}$–treated animals than in mice receiving the protective siRNA-2A (Figure 6D, right). Consistent with previous findings,[5] cardiac histological assessment 42 to 48 hours after infection revealed no evidence of myocyte necrosis or inflammatory cellular infiltrations of the heart, respectively. This can be explained by the observation that inflammatory cells are generally apparent at the earliest at day 4 to 5 after virus inoculation and not at this early stage of coxsackieviral infection. Additionally, previously published data indicated that CVB3-infected type I IFNR-deficient mice do not display significantly elevated CK levels at the time of death,[5] which occurs early after CVB3 infection.

## Discussion

Enteroviral diseases have considerable medical and economic impact; however, there is no specific antiviral therapy available. Coxsackieviruses can infect and damage various tissues, leading to hepatitis, pancreatitis, or viral heart disease. On CVB3 infection, cellular injury and cell death may occur, either by a direct viral cytopathic effect[9,11] and/or mediated by immununomodulatory mechanisms.[23,24] Gene silencing by double-stranded RNA is a sequence-specific, highly conserved mechanism in eukaryotes. RNA interference has been recently observed in mammalian cells, in which the system uses siRNAs (21-nucleotide double-stranded RNA intermediates) to specifically degrade cellular as well as viral RNA, thus interfering with eukaryotic and viral gene expression.[13,25,26] Hence, RNA interference may be viewed as an evolutionary highly conserved mechanism promoting viral clearance, cell survival, and intracellular immunity. Consequently, a novel therapeutic concept to limit coxsackieviral replication and cellular injury might be the use of parenterally delivered, highly specific siRNAs directed against a distinct region of the viral genome. Poliovirus is an enterovirus related to coxsackievirus. A previous study indicated that RNA interference effectively inhibits poliovirus replication.[27] This study was restricted to cell culture experiments. The authors showed significant inhibition of poliovirus replication and cytopathogenicity with siRNAs directed against target sequences within the capsid as well as the 3D polymerase. Because most published clinically relevant mutations of enteroviral genomes are located within the P1 region encoding for structural proteins,[17,28] and siRNA function may be severely attenuated by a single nucleotide mutation,[29] RNA interference was targeted toward the protease 2A region. Because of its catalytic activity, the nucleotide sequence of this protease is considered to be highly conserved and therefore an attractive therapeutic target for RNA interference. siRNA directed against the 2A protease region proved highly efficient for the inhibition of viral replication and virus-mediated cell injury and death. To ensure specificity of the results, various control siRNAs were used: 1 construct with 2 point mutations within the original siRNA-2A sequence (siRNA-2A$^{mut}$) and a commercially available scrambled control,[30,31] termed siRNA-SCR. Both control siRNA molecules had no effect on coxsackieviral replication, cytotoxicity, and viral RNA and protein expression in cell culture, demonstrating the specificity of siRNA-2A–mediated protective effects. Importantly, siRNA-2A was able to inhibit the expression of both viral positive and negative stranded RNA. Despite pronounced inhibition of viral replication and cell injury during the initial phase of viral infection, the effect of siRNA-2A diminished over time and led to viral recovery and subsequent cell death. Fluorescence labeling of siRNA and CVB3 revealed that this late recovery was due to diminished presence of siRNA-2A. This observation indicates a specific siRNA-2A–mediated inhibitory effect on CVB3 replication and cytotoxicity. Decreasing levels of siRNA-2A can be explained by dilution of siRNA concentration below effective levels as a result of cellular replication as well as nuclease-mediated degradation of siRNA.[32] Because HeLa cells are rapidly dividing cells and cellular proliferation was protected during early viral replication by siRNA-2A, siRNA dilution might be primarily responsible for the diminishing protective siRNA effect. Therefore, it is tempting to speculate that alternative treatment protocols with repeated siRNA delivery may improve outcome.

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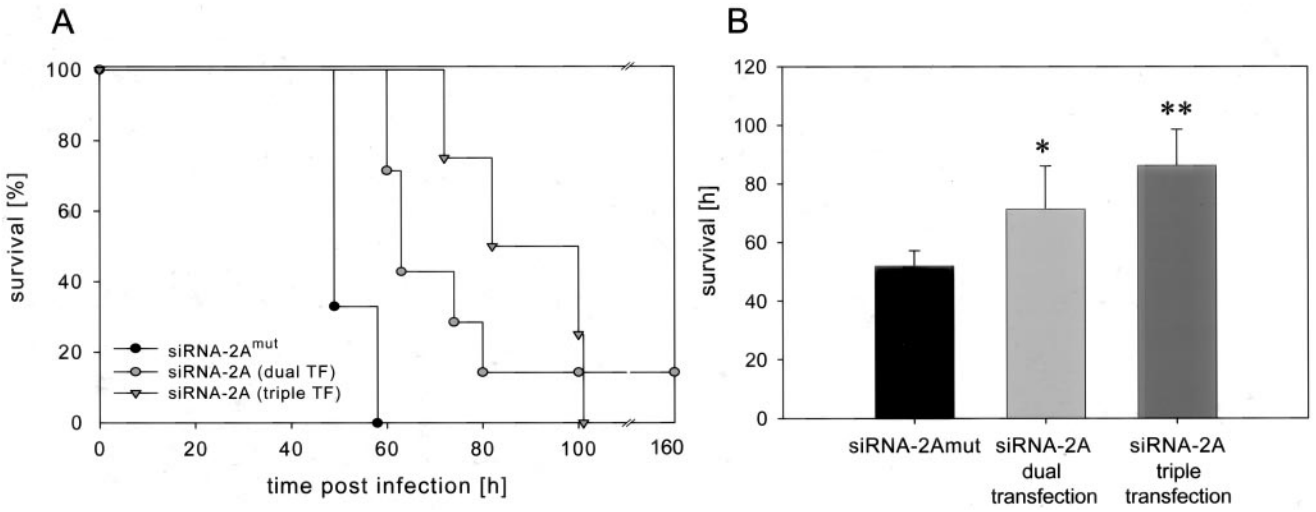


**Figure 5.** In vivo application of siRNA-2A significantly improves survival in CVB3-infected, highly susceptible type I IFNR-deficient mice. Type I IFNR-deficient mice were infected with a lethal dose of CVB3 and transfected twice hydrodynamically with siRNA-2A (n=8) and siRNA-2A$^{mut}$ (n=3) at 6 and 14 hours after infection, respectively, and monitored for survival. Additionally, another group (n=4) received a third siRNA-2A injection at 48 hours after infection. As shown in a Kaplan-Meier curve (A), siRNA-2A prolonged survival compared with siRNA-2A$^{mut}$–treated animals, which did not live longer than non–siRNA-transfected type I IFNR–knockout mice[5] ($P<0.01$ for both siRNA-2A groups compared with siRNA-2A$^{mut}$ group). TF indicates transfection. B, Median time to death after CVB3 infection of type I IFNR-deficient mice, illustrating the significant increase in life span in siRNA-2A–treated mice. One mouse of 8 treated with siRNA-2A survived and is not included in the figure. Survival could be increased by a third siRNA-2A transfection. *$P<0.05$, **$P<0.01$ vs siRNA-2A$^{mut}$ control.

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In mammalian cells, RNA interference using plasmid delivery may trigger the interferon pathway,[33] and interferons are known to inhibit CVB3 replication.[34] Nevertheless, it is unlikely that the induction of an interferon response had an effect in our experiments because the interferon pathway is usually triggered by long siRNAs of >30 nucleotides in length,[13] and all other siRNA molecules except siRNA-2A used in this study did not reveal a detectable effect on CVB3 infection.

The mouse model is recognized as an established model to study various aspects of coxsackieviral disease. Gene-modified mouse models have contributed substantially to our current understanding of this disease.[15] Type I IFNR–knockout mice[19] are highly susceptible to CVB3 infection and die early even when infected with low virus titers, most likely as a result of acute liver failure caused by unrestricted viral replication.[5] Therefore, it is an appropriate animal model to study the antiviral effect of RNA interference because it allows the assessment of the "hard" end points of mortality/survival as well as the assessment of viral replication and tissue damage. Various studies have shown that siRNA molecules can be efficiently delivered into the intact mouse[35] via hydrodynamic transfection,[36] with the highest siRNA concentrations generally deposited in the liver.[35] Therefore, RNA interference is regarded as a potential therapeutic approach, eg, for hepatic diseases.[37] Delivery of siRNA-2A after CVB3 infection of type I IFNR-deficient mice led to a significant increase of the survival period of these mice, whereas the control siRNA-2A$^{mut}$ did not appear to have any effect on survival compared with non–siRNA-transfected mice, as previously reported.[5] Notably, 1 mouse of 8 survived CVB3 infection and efficiently cleared the virus, a phenomenon not observed in any non–siRNA-2A–treated type I IFNR–knockout mouse in the past. Differences in survival time between mice treated with similar doses of siRNA-2A may be most likely attributed to the variable efficiency of hydrodynamic transfection. In CVB3-infected knockout mice, siRNA-2A led to a marked attenuation of viral tissue titers at the time when control siRNA-treated mice died on viral infection. Inhibition of viral replication translated into attenuated tissue injury, as assessed by ALT serum levels reflecting hepatic injury as well as histological grading of cell damage in liver specimens. Interestingly, 42 hours after infection, there was no detectable virus by plaque-forming assay in the heart of infected mice treated with siRNA-2A in contrast to mice who received siRNA-2A$^{mut}$, in which virus was readily detectable in every heart. Together with the observation that >50% of heart cells could be successfully targeted with siRNA molecules, it is highly suggestive that specific siRNA treatment of CVB3 may also improve outcome in viral heart disease.

siRNA-2A was highly efficient in limiting CVB3 gene expression, replication, and tissue damage at the time when control siRNA-treated animals died, thus improving overall survival. Still, during later stages of infection, the coxsackieviral cytopathic effect was aggravated and led to death in the large majority of animals. This phenomenon might be due to degradation of siRNAs by intracellular nucleases[13] or by the fact that not all hepatocytes could be transfected efficiently, creating a reservoir for CVB3 replication. In previous studies, the level of hepatocytes successfully transfected with siRNA was reported to be between 70% and 89%.[38,39] This is

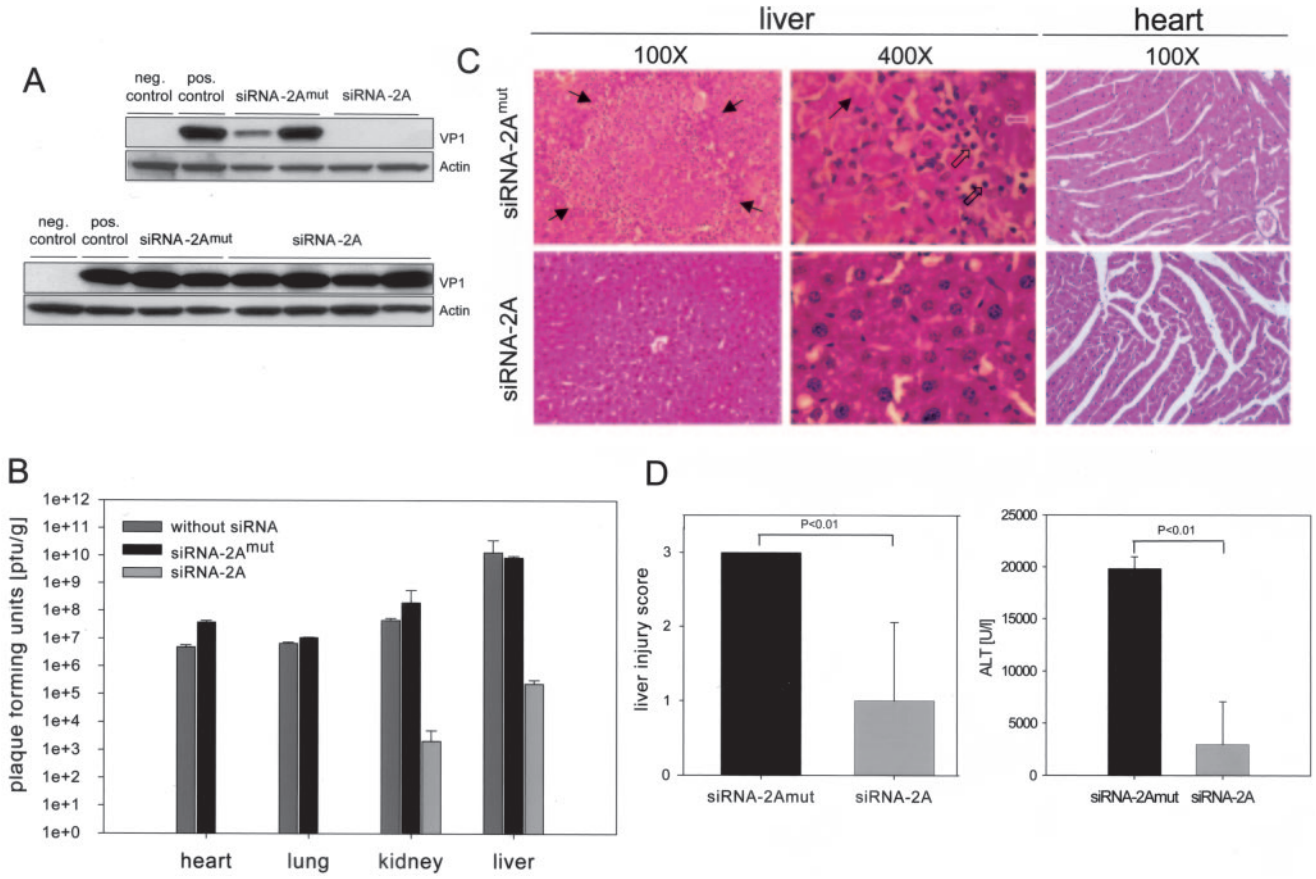


**Figure 6.** 2A RNA interference inhibits viral protein expression, replication, and tissue injury in vivo. A, Expression of the viral capsid protein VP1 in the liver is suppressed in mice treated with siRNA-2A 42 hours after infection (top). Hepatic protein derived from noninfected and infected, non–siRNA-treated type I IFNR-deficient mice 42 hours after infection served as negative and positive control, respectively. Displayed are 2 representative samples from each treatment group. The bottom panel displays hepatic viral protein expression in different animals at the time of death in the 2 treatment groups (n=2 for siRNA-2A$^{mut}$; n=4 for siRNA-2A), indicating an aggravated level of viral protein expression at the end of life, consistent with a possible hepatic cause of death due to viral infection. Notably, viral expression levels increase significantly more slowly over time in animals treated with siRNA-2A but in some cases reach levels of expression at the end of life comparable to those of control treated mice. Actin protein stain served as loading control. B, Viral replication is significantly inhibited by siRNA-2A in various tissues 42 hours after viral infection and leads to a decrease of viral titer of a factor $>10^6$ in the liver and no detectable virus in the heart and lung as determined by plaque-forming assay. Viral titers are presented on a logarithmic scale (eg, $1e+0=1\times10^0$). C, Representative hematoxylin-eosin–stained histological liver and heart specimens of mice treated with siRNA 48 hours after infection. Tissue specimens from animals receiving siRNA-2A$^{mut}$ are shown in the top panel, and histological sections from siRNA-2A–receiving mice are presented in the bottom panel. Left top image shows sharply demarcated acinar necrosis of hepatocytes (between black arrows) surrounded by viable hepatocytes. Higher magnification in the middle of the top panel reveals hepatocyte necrosis (black arrow), inflammatory cells (open black arrow), and hepatocytes without evidence of a cytopathic effect (white arrow). At this stage of infection, most cytopathic features in the liver detected in siRNA-2A$^{mut}$–treated mice were absent when siRNA-2A was delivered (left and central images, bottom panel). Histological analysis of cardiac muscle (top and bottom images on right) shows no evidence for myocyte necrosis or inflammatory infiltrates at this early stage of infection, consistent with previous observations.[5] D, Hepatic injury score assessed by a blinded pathologist is significantly decreased 48 hours after infection in mice treated with siRNA-2A (left). Liver injury score was categorized semiquantitatively as described; n=10 for animals receiving siRNA-2A and n=3 for animals receiving siRNA-2A$^{mut}$. Right panel shows ALT serum levels 48 hours after infection. ALT levels in siRNA-2A–treated mice were markedly lower than those in mice treated with control siRNA-2A$^{mut}$ (n=6 for siRNA-2A and n=3 for siRNA-2A$^{mut}$).

in good concordance with our results. The chosen animal model resembles a highly susceptible gene-targeted mouse strain, and siRNA-2A–mediated promotion of survival time can be considered an encouraging step toward a specific therapeutic approach for the treatment of enteroviral diseases. The therapeutic outcome might be improved by treatment protocols including repeated administration of siRNA. This assumption is supported by the finding that animals receiving triple siRNA transfections survived longer than those receiving 2 transfections. Additionally, even retardation of viral gene expression and concomitant cytopathic effect may give the immune system more time to mount an efficient immune response, leading to improved outcomes in conjunction with intracellular immunity conferred by specific siRNA.

To our knowledge, this is the first study reporting successful application of RNA interference directed against a picor-

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navirus in vivo as well as demonstrating prolonged survival by siRNA treatment after viral infection in highly susceptible animals. The pronounced antiviral effect of siRNA-2A indicates that designation of siRNA targets determines clinical outcome. It is well known that 2A protease is an important coxsackieviral gene both for viral protein processing and for the induction of a direct cytopathic effect in the host. The cytopathic effect may be mediated either by interference with eukaryotic translational processes[40] and/or by directly cleaving dystrophin.[11] Therefore, it is likely that 2A covers a highly conserved genomic sequence and therefore resembles an attractive target for RNA interference.

In the future, therapeutic antiviral efficiency of RNA interference in vivo may be improved by innovative expression and delivery systems.[41] Additionally, modifications within the siRNA to improve nuclease resistance may ultimately lead to prolonged as well as increased levels of siRNA present in cells[42] and eventually improved therapeutic outcome. Because of their unique specificity and therapeutic efficiency, once introduced into the infected target cell, antiviral duplex siRNA molecules have the potential to improve the outcome of coxsackieviral disease.

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# Targeting 2A Protease by RNA Interference Attenuates Coxsackieviral Cytopathogenicity and Promotes Survival in Highly Susceptible Mice

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