Communication

Establishment of a Panel of Human Cell Lines to Identify Cellular Receptors Used by Enteroviruses to Infect Cells

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Establishment of a Panel of Human Cell Lines to Identify Cellular Receptors Used by *Enteroviruses* to Infect Cells

Anastasiia O. Sosnovtseva [1,2,*], Le T. Hoa [3], Dmitry S. Karpov [1,2], Pavel O. Vorobyev [1,2], Yana D. Gumennaya [2], Olga N. Alekseeva [2], Peter M. Chumakov [1,2] and Anastasia V. Lipatova [1,2]

[1] Center for Precision Genome Editing and Genetic Technologies for Biomedicine, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia
[2] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia;
[3] Department of Molecular Microbiology and Immunology, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA
* Correspondence: sollomia@yandex.ru

Abstract: Non-pathogenic natural and recombinant strains of human *Enteroviruses* are the subject of ongoing study, with some strains having been approved for use as anticancer agents. The efficacy of oncolytic virotherapy is dependent upon the identification of the receptor utilized by a specific strain for cell entry and the presence of this receptor on the surface of cancer cells. Accordingly, a rapid and straightforward approach for determining the enteroviral receptors is necessary for the development of effective patient-specific virus-based cancer therapy. To this end, we created a panel of seven lines with double knockouts on the background of the HEK293T cell line, which lacks the *IFNAR1* gene. In these lines, the main viral receptor genes, including *PVR*, *CXADR*, *CD55*, *ITGA2*, *SCARB2*, *ICAM1*, and *FCGRT*, were knocked out using the CRISPR/Cas9 system. A panel of lines was validated on a set of 12 *Enteroviruses*, providing a basis for studying the molecular mechanisms of enterovirus entry into cells and developing new therapeutic strains.

Keywords: CRISPR/Cas9; *Enteroviruses*; viral phylogeny; coxsackievirus; echovirus

1. Introduction

Enteroviruses are a genus of viruses in the family Picornoviridae, including positively polar single-stranded, single-stranded, non-enveloped RNA viruses with a genome length of about 7500 nt. Enterovirus infections are primarily enteric and cause infections ranging from asymptomatic and mild flu-like colds to serous meningitis and poliomyelitis [1]. According to modern classification, all viruses belonging to the genus *Enterovirus* are divided into nine distinct species: *Enterovirus* A, B, C, D, E, F, G, H, and J. Additionally, three rhinovirus species were identified: *Rhinovirus* A, B, and C [2].

Small genome of *Enteroviruses* encode regulatory proteins that manipulate with are heavily relied on a host factors to entry Different species, serotypes and individual strains of *Enteroviruses* can enter cells using a variety of cell surface proteins that act as receptors for viral attachment and subsequent endocytosis [3,4]. Polioviruses (PV) (*Enterovirus C*) gain entry to cells by binding to the surface protein CD155, a product of the *PVR* gene. This protein plays a role in the formation of intercellular adhesion junctions between epithelial cells [5] and in some intercellular regulatory interactions in immune cells [6]. Coxsackie B (CVB) and certain echoviruses (E) belonging to *Enterovirus B* employ the CAR immunoglobulin superfamily protein, a product of the *CXADR* gene [7,8]. Some members of Coxsackie A (CVA), including CVA7, CVA14, CVA16, and Enterovirus 71 (EV-A71) (*Enterovirus A*), utilize the type B2 scavenger receptor (SCARB2) [9], also known as lysosomal integral membrane protein II, LIMP-2v or CD36b-like protein-2, which is involved in vesicular transport [10]. E1 (*Enterovirus B)* has been demonstrated to utilize integrin $\alpha 2\beta 1$ (VLA-2;

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collagen and laminin receptor) [11,12], CVA9 (*Enterovirus B)* utilizes the αVβ and αVβ6 integrins [13,14], CVA21 [15,16] and CVA11 [17] (*Enterovirus C*) utilizes an integrin molecule ICAM-1, and many members of the echovirus group (*Enterovirus B)* utilize the neonatal Fc receptor FcRn, which consists of two subunits, the *FCGRT* gene product and β2-microglobulin [18]. In addition to these receptors, some enteroviruses may also bind the CD55 co-receptor, or DAF, which normally protects cells from the toxic effects of complement [15,19,20]. The binding of the virus to CD55 does not stimulate the uncoating and release of the viral genome; however, it does promote virion attachment to the cell surface, thereby increasing the likelihood of binding to another receptor on the cell surface [21].

The RNA genome of enteroviruses is unstable and subject to frequent mutations and recombinations, which occur predominantly among members of the same species and determine their evolution [22]. Recombinations and mutations can lead to the emergence of new strains [23] with altered drug resistance, virulence, antigenicity and transmissibility, and in some cases it is associated with changes in the receptor through which the virus enters the cell [24].

Non-pathogenic and recombinant strains of enteroviruses extensively investigated as potential agents for oncolytic virotherapy [25,26] and some strains are approved the for melanoma treatment in Latvia [27,28]. The high recombination ability of enteroviruses facilitates the creation of improved oncolytic strains with increased specificity to tumor cells by altering the tropism of the virus [29]. The efficacy of oncolytic therapy is contingent upon the identification of the receptor utilized by a specific strain and the presence of this receptor on the surface of cancer cells [30]. It is therefore imperative to develop a rapid and straightforward method for determining the receptor utilized by enteroviruses for cellular entry in order to accelerate the development of effective patient-specific virus-based cancer therapy.

In this study, we have established a panel of cells with knockouts of various receptors employed by enteroviruses for cellular invasion, thereby enabling us to ascertain the receptors essential for enterovirus infection of cells.

2. Results

2.1. Phylogenetic Analysis of Enteroviruses

A phylogenetic analysis was conducted on the structural protein sequences of 32 enterovirus strains. The sequences of 22 strains were obtained from the GeneBank database, and the sequences of 10 strains were obtained from the laboratory collection of live vaccine enteroviruses, including six new strains whose genomes were sequenced. It is noteworthy that the novel strains exhibited a high degree of similarity to their respective prototypes, as illustrated in Figure 1. Moreover, phylogenetic analysis facilitated the prediction of the receptor utilized by the novel viral strain for cellular entry. So, our data suggest that the novel strain Coxsackie A21 (LEV28) strain should interact with ICAM1, and the echovirus strains 7 (LEV21), 12 (LEV20), 6 (LEV22), and 30 (LEV23) should enter cells via interaction with FCGRT. The construction of the phylogenetic tree did not allow for the identification of viruses that utilize CD55 to infect cells in a discrete cluster. This may be attributed to the role of CD55 in cell infection, as it solely facilitates virion attachment to the cell surface [21].

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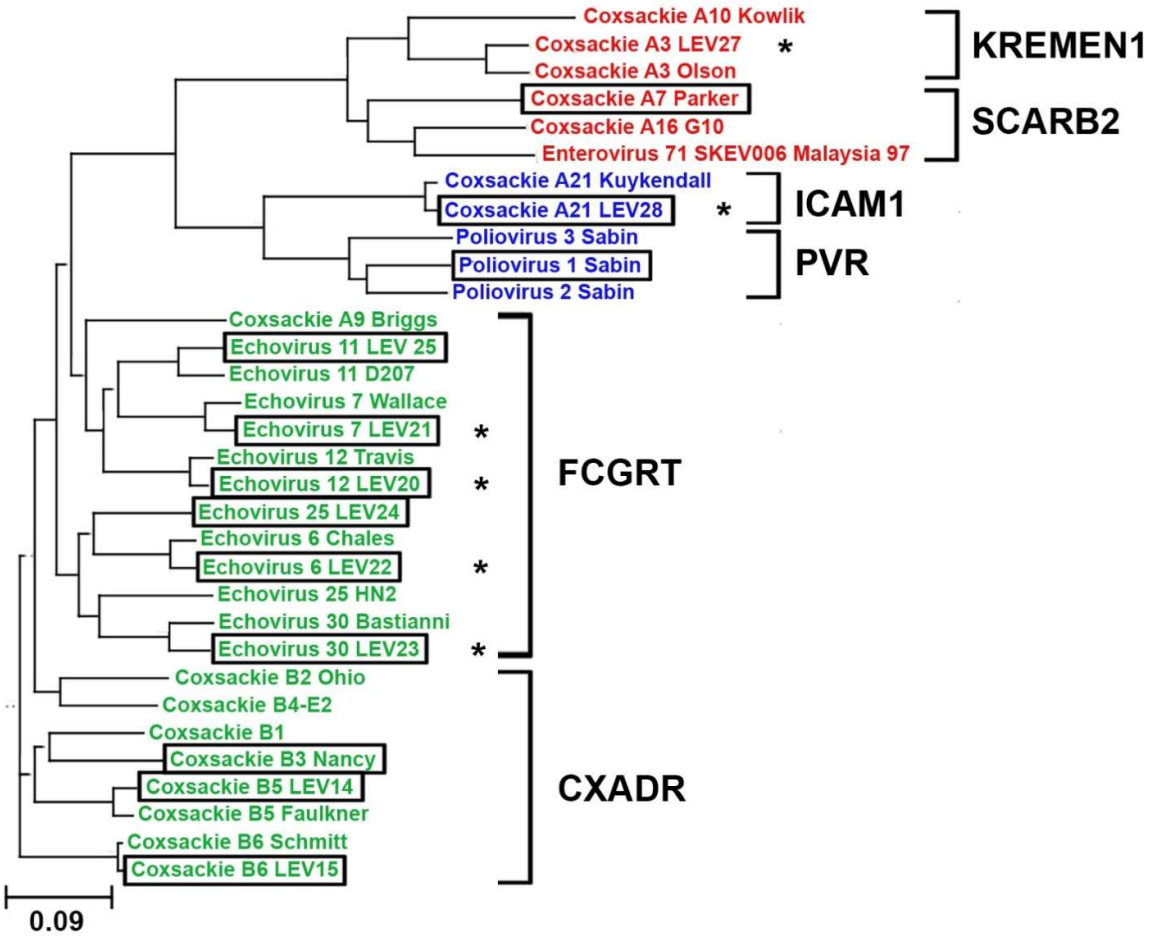

Figure 1. Phylogenetic tree of enteroviruses, including the strains used in this work. Strains belonging to groups A, B, and C are colored in red, green, and blue, respectively. New strains with sequenced genome are marked with an asterisk. Strains with names in rectangles are used in this work. The names of main viral receptors are also indicated.

2.2. Obtaining a Panel of Cell Lines with Knockouts of Different Enterovirus Receptors

The results of the phylogenetic analysis need experimental verification. Consequently, we devised an experimental approach for the validation of viral receptors. The experimental approach is based on testing the reproduction of enteroviruses in seven HEK293 lines carrying knockouts of known enteroviral receptors.

To obtain knockout lines, a CRISPR/Cas9 system was employed to target genes encoding seven surface molecules (PVR, CXADR, CD55, SCARB2, ITGA2, ICAM1, and FCGRT) for which a role as an enteroviral receptor or coreceptor has been described. Each gene knockout was introduced into the HEK293TΔIFNAR1 cell line, which had previously been generated by knockout of the IFNAR1 gene encoding the type I interferon receptor subunit [31]. This cell line, due to its impaired cellular interferon response, is capable of supporting the replication of a large number of enteroviruses, including nonpathogenic ones.

The cells were transfected with the pCas-Guide-P2A-RFP plasmid, which contains inserted sites encoding gRNA spacers targeting the corresponding gene. Following transfection, monoclonal cultures were obtained and subsequently analyzed by sequencing the Cas9-targeted gene region. The most promising clones were then selected based on the results of this sequencing. Consequently, a panel of seven HEK293T cell lines with two gene knockouts (*IFNAR1* and one of the genes encoding

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receptor proteins for enteroviruses) was obtained (Figure 2). Knockout cell lines were tested by western-blotting analysis, confirming the absence of the proteins of interest.

Figure 2. Sequences of enterovirus receptor gene regions in a panel of CRISPR/Cas9-induced knockout lines. Blue letters are PAMs. Red letters and dashes are changes in gene sequences introduced by CRISPR/Cas9 system.

2.3. Evaluation of Enterovirus Reproduction in a Panel of Cells with Viral Receptor Knockouts

The objective of this study was to assess the reproduction of 12 different enteroviruses on the HEK293TΔIFNAR1 cell line and its derivatives with an additional knockout of one of the viral receptors. The results of this assessment are presented in Figure 3.

As can be observed, the knockout of PVR resulted in a notable reduction in PV1 (Sabin) production. The replication of all other strains remained unimpaired, which is consistent with the data on the receptor properties of PVR [32].

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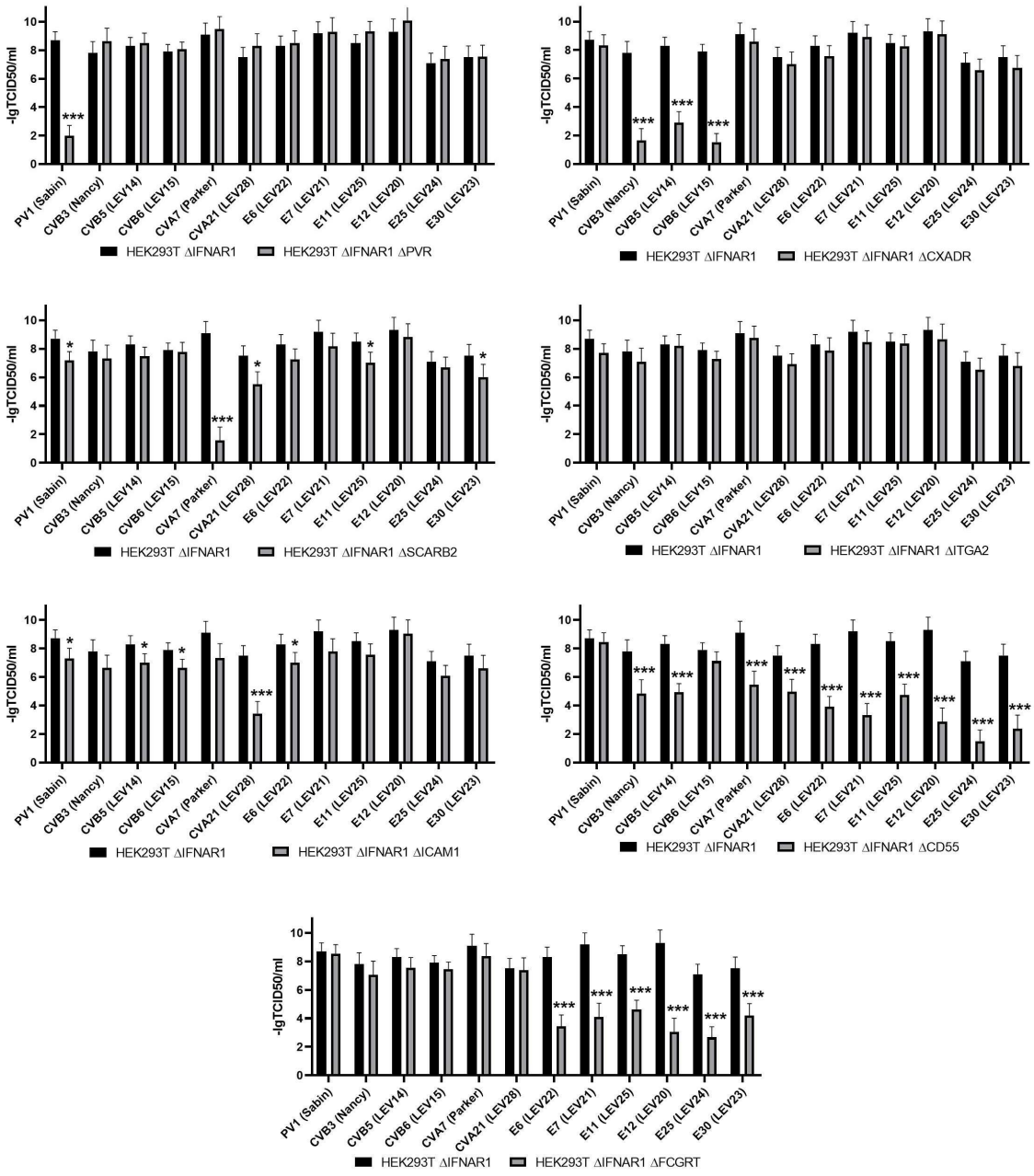

Figure 3. Reproduction of enteroviruses on cell lines with knockout of genes encoding main enterovirus receptors. Results from three independent experiments are presented. Error bars are standard deviation. Statistical significance: * p-value between 0.05 and 0.01; *** p-value < 0.001, according to Student's *t* test.

The inactivation of *CXADR* gene expression resulted in the prevention of replication only for coxsackieviruses of group B (CVB3 (Nancy), CVB5 (LEV14), and CVB6 (LEV15)), which is consistent with the available data on the role of the *CXADR* gene in the infection of cells with some representatives of coxsackieviruses of group B [33]. Previously, comparable data were obtained for CVB5 (LEV14) and CVB6 (LEV15) on the HEK293T cell line with CXADR knockout [34].

The inactivation of the *ICAM1* gene has been demonstrated to significantly reduce the replication of CVA21 (LEV28), a finding that is consistent with the existing literature [15]. We can also note that a slight effect is observed for PV1 (Sabin), CVB5 (LEV14), CVB6 (LEV15) and E6 (LEV22).

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Knockout of the *SCARB2* gene suppressed reproduction of only CVA7 (Parker) reproduction, which is consistent with previously published data [9]. Nevertheless, it is evident that a slight impact is observed in the reproduction of PV1 (Sabin), CVA21 (LEV28), E11 (LEV25), and E30 (LEV23).

The knockout of *ITGA2* did not result in a reduction in reproduction in any of the strains under study, which is also consistent with the findings of previous research.

The inactivation of the *CD55* gene had a negative effect on the reproduction of numerous enteroviruses, albeit to varying degrees. The replication of CVB3 (Nancy) and CVB5 (LEV14) viruses was reduced by three orders of magnitude, while that of CVA7 (Parker) and CVA21 (LEV28) viruses was reduced by two and a half to three orders of magnitude, and that of E6 (LEV22), E7 (LEV21), E11 (LEV25), E12 (LEV20), E25 (LEV24), and E30 (LEV23) was reduced by four orders of magnitude. The replication of E25 (LEV24) was notably diminished, exhibiting a reduction of up to seven orders of magnitude. The broad impact of CD55 on the infection of cells by a range of enterovirus strains has been previously documented, including CVA21 [15], CVB3 and CVB5 [15], as well as E6 [19], E7 [15], E11 [35], E12 [36] and E30 [37]. The observed effect of CD55 on cell infection with CVA7 or E25 strain has not been previously reported. Furthermore, our findings indicate that CD55 is not a requisite factor for the infection of cells with PV1 (Sabin), and CVB6 (LEV15).

The knockout of *FCGRT* resulted in the complete cessation of reproduction of E6 (LEV22), E7 (LEV21), E11 (LEV25), E12 (LEV20), E25 (LEV24), and E30 (LEV23), while no effect was observed on the reproduction of coxsackie and polioviruses. This finding is consistent with the existing literature on the role of the neonatal Fc receptor FCGRT in the infection of cells by certain representatives of species *Enteroviruses B* [18].

3. Discussion

The resulting panel of cells serves as a convenient tool for determining the viral receptor utilized by a specific enterovirus strain for cell entry. Upon verification of the obtained cell panel, the majority of the studied enterovirus strains exhibited results that were consistent with the phylogenetic analysis conducted and with the data previously obtained by other researchers in the field.

The availability of experimental data indicating the use or non-use by viruses of this or that receptor may prove invaluable in the development of oncolytic therapy based on enteroviruses. Due to the inherent instability of the enterovirus genome and the potential for mutation accumulation, the resulting panel of viruses can be employed to generate viruses that utilize alternative receptor molecules to infect cells through a process known as bioselection. For example, evidence suggests that FCGRT may play a role in antitumor immune recognition of tumor cells at an early stage of tumor growth [38]. Additionally, a decrease in FCGRT expression has been observed to correlate with tumor progression [39]. Consequently, the search and creation of enteroviruses with FCGRT-independent mechanisms of penetration can be achieved using this panel.

In addition, different levels of suppression of viral reproduction in knockout cell lines may indicate the degree of dependence of a given viral strain on the corresponding receptor for cell entry. We hypothesize that the stronger the level of reproduction suppression of a viral strain in a given knockout line, the stronger the dependence of that strain on a given receptor. This feature of our panel can be used for preliminary analysis of mutant viral strains with altered receptor affinity for subsequent validation by more precise methods.

Consequently, the constructed cell panel may prove advantageous for the isolation of enteroviruses exhibiting heightened oncolytic potency. This may be achieved by identifying more appropriate viruses in accordance with the receptors utilized and by further delineating alterations in the genome that result in modifications to the cell penetration pathway. This can then be employed to generate recombinant strains with defined characteristics.

4. Materials and Methods

4.1. Phylogenetic Analysis of the Enteroviruses

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The full-genome nucleotide sequences of available enterovirus strains were retrieved from the GenBank database. The genomes of novel strains were sequenced using the Illumina platform. The sequences of structural proteins were obtained and used in a multiple protein sequence alignment, followed by the construction of a phylogenetic tree, which was performed using Clustal Omega [40] on the EMBL-EBI site (https://www.ebi.ac.uk/, accessed 21 November, 2024). Visualization of the phylogenetic tree was performed in Phylogenetic tree (newick) viewer [41] (http://etetoolkit.org/treeview/, accessed 21 November, 2024).

4.2. Cell Lines and Viral Strains

The human embryonal rhabdomyosarcoma (RD) and HEK293T cell lines were procured from the ATCC bank. The IFNAR1-knockout HEK293T cell line was previously established [31]. The cells were cultured in DMEM medium containing 100 μg/mL penicillin/streptomycin and 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C.

Sabin vaccine strain of poliovirus type 1 (PV1 (Sabin),); Echovirus type 6, LEV22 (E6 (LEV22); Echovirus type 7, strain LEV21 (E6 (LEV21); Echovirus type 11, strain LEV25 (E6 (LEV25); Echovirus type 12, strain LEV20 (E6 (LEV20); Echovirus type 25, strain LEV24 (E6 (LEV24); Echovirus type 30, strain LEV23 (E6 (LEV23); Coxsackie virus A7, strain Perker (CVA7 (Parker)), Coxsackie A21 virus, LEV28 (CVA21 (LEV28); Coxsackie B3 virus, strain Nancy (CVB3 (Nancy)), Coxsackie B5 virus, strain LEV14 (CVB5 (LEV14)), and Coxsackie B6 virus, strain LEV15 (CVB6 (LEV15) were obtained from the laboratory collection. The enteroviruses were amplified on the RD cell line. The infected cells and culture supernatant were collected within 2–3 days, after which the cells were destroyed by three cycles of freezing and purified by centrifugation at 2000×g for 10 minutes. Virus titers were determined using the standard Reed-Muench method.

4.3. The HEK293T Cell Lines with Viral Receptor Gene Knockouts

The knockout system, based on CRISPR/Cas9 technology, was employed to generate lines of HEK293TΔIFNAR1 cells with an additional knockout of genes encoding receptor proteins for enteroviruses. To this end, plasmid pCas-Guide (Origene, Rockville, MD, USA) was modified by the insertion of the gene encoding red fluorescent protein RFP as the second expressed translation frame, which was placed after the Cas9 gene via element 2A, thereby facilitating polycistronic translation. The pCasGuide-2A-RFP plasmid constructs, which express sgRNA spacers specific to enterovirus receptor gene sites, were obtained by cloning the oligonucleotides from Table 1 as previously described [31]. Subsequently, the cells were transfected with calcium phosphate in accordance with the previously described methodology [42]. Cell clones were subsequently obtained as previously described [31]. Briefly, genomic DNA of cell clones was isolated using a specialized ExtractDNA Blood & Cells kit (Evrogen, Moscow, Russia) in accordance with the manufacturer's instructions. The presence of CRISPR/Cas9-mediated editing of genes encoding viral receptors was confirmed by PCR with primers indicated in Table 1, followed by Sanger sequencing.

Table 1. Sequences of sgRNA spacers, primers for PCR amplification, and antibodies used for the generation and validation of enterovirus receptor gene knockout.

Viral receptor target gene	Oligonucleotides used for cloning of sgRNA spacers	Oligonucleotides used for amplification of CRISPR/Cas9 targeted gene regions	An antibody used against a viral receptor
PVR	5′-phospho-GATCGcgggatgcccaatacgagccG 5′-phospho-AAAACggctcgtattgggcatcccgC	CCATGCCATCCTGTACCCTT GAAGCAATGCCTACAGTGCC	Abcam (ab205304)
CXADR	5′-phospho-GATCGtacgcttagtcccgaagaccG	TTCTGAATGGCTGCGGGG	Abcam (ab126250)

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	5′-phospho-AAAACggtcttcgggactaagcgtaC	ATGTGACTGGCAAGGTGATG	
CD55	5′-phospho-GATCGattggagagcactctattG 5′-phospho-AAAACaatagagtgctctccaatC	CCAGCACCACCACAAATTGA AGACCCTTCTGAGAAGCTGT	Abcam (ab253284)
SCARB2	5′-phospho-GATCGacagttgacgaattgctctggG 5′-phospho-AAAACccagagcaattcgtcaactgtC	ATCGAGGCCATGTTGAAAGC GTTAATCTGGCTTGGGGTGC	Abcam (ab176317)
ICAM1	5′-phospho-GATCGgctattcaaactgccctgatgG 5′-phospho-AAAACcatcagggcagtttgaatagcC	GCGCACATTCCCCTTGATGAA CAGTACACGGTGAGGAAGGT	Abcam (ab109361)
ITGA2	5′-phospho-GATCGattggagagcactctattG 5′-phospho-AAAACgttggtacttcggctttctcC	TGTAGCCACAAGACACTGATG ACAGCAAAAGGATTCCAGCA	Abcam (ab181548)
FCGRT	5′-phospho-GATCGctgagctacaatagcctgcgC 5′-phospho-AAAACaccggaatctcacctttcccG	TTCTCCCTCCCTGGGTATCT ACCGGAATCTCACCTTTTCCC	Abcam (ab228975)

4.4. Enterovirus RNA Purification and Genome Sequencing

Novel strains of enteroviruses were produced in the RD cell line. The harvested medium was clarified from cell debris by centrifugation at 4 °C, 2500 rpm for 15 minutes. The virus-containing supernatant was stored in 0.5 mL aliquots at –80 °C. Viral RNA was extracted using the GeneJET Viral DNA/RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. The quality and quantity of the RNA were evaluated through spectrophotometry and RNA gel electrophoresis. The cDNA synthesis was conducted using reverse transcriptase Mint (Evrogene, Moscow, Russia) with N6 random primers, in accordance with the manufacturer's instructions. Enteroviruses cDNA was used to prepare sequencing library with the Nextera DNA Flex Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Size of the genomic library was analyzed on an Agilent 2100 Bioanalyzer (Thermo Fisher Scientific, Waltham, MA, USA) was about 500 bp. The library was sequenced on an Illumina MiSeq System using the MiSeq Reagent Micro Kit v2 (300 cycles). A draft enteroviral genomes were de novo assembled using the SPAdes assembler [43] on the Galaxy web service [44] with automatic k-measure size selection; other parameters were set as default.

4.5. Determination of Enterovirus Reproduction

To determine the rate of reproduction, the double-knockout cell line and the control cell line, HEK293TdIFNAR1, were infected with enteroviruses at MOI of 0.001. After three days, the medium containing the viruses was harvested, cleared by centrifugation for five minutes at 2000 g, and used to determine the titer of the virus using the Reed-Muench method. To this end, RD cells were infected with 10-fold dilutions of the collected medium, and the titer was calculated after three days.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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