Plasmid 114 (2021) 102556

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Enhancement of transgene expression by the β-catenin inhibitor iCRT14

Kyle Spivack, Christine Muzzelo, Matthew Hall, Eric Warga, Christopher Neely, Holly Slepian, Alyssa Cunningham, Matthew Tucker, Jacob Elmer *

Villanova University, Department of Chemical & Biological Engineering, United States

ARTICLE INFO

Keywords:
Non-viral gene delivery
Innate immune response
β-Catenin
TCF4
iCRT14
PEI

ABSTRACT

The innate immune response is an essential defense mechanism that allows cells to detect pathogen-associated molecular patterns (PAMPs) like endotoxin or cytosolic DNA and then induce the expression of defensive genes that restrict the replication of viruses and other pathogens. However, the therapeutic DNA used in some gene therapy treatments can also trigger the innate immune response, which activates host cell genes that may inhibit transgene expression. The goal of this study was to enhance transgene expression by inhibiting key components of the innate immune response with small molecule inhibitors (iCRT14, curcumin, Amlexanox, H-151, SC-514, & VX-702). Most of the inhibitors significantly increased transgene (luciferase) expression at least 2-fold, but the β-catenin/TCF4 inhibitor iCRT14 showed the highest enhancement (16 to 35-fold) in multiple cell lines (PC-3, MCF7, & MB49) without significantly decreasing cellular proliferation. Alternatively, cloning a β-catenin/TCF4 binding motif (TCAAAG) into the EF1α promoter also enhanced transgene expression up to 8-fold. To further investigate the role of β-catenin/TCF4 in transgene expression, mRNA-sequencing experiments were conducted to identify host cell genes that were upregulated following transfection with PEI but down-regulated after the addition of iCRT14. As expected, transfection with plasmid DNA activated the innate immune response and upregulated hundreds (687) of defensive genes, but only 7 of those genes were down-regulated in the presence of iCRT14 (e.g., PTGS2 & PLA1A). Altogether, these results show that transgene expression can be enhanced by inhibiting the innate immune response with SMIs like iCRT14, which inhibits β-catenin/TCF4 to prevent the expression of specific host cell genes.

1. Introduction

The innate immune response (IIR) is the cell's first line of defense against infection (Flajnik and Kasahara, 2010; Buchmann, 2014; Fearon and Locksley, 1996). It consists of several enzymes that actively monitor the cell for pathogen-associated molecular patterns (PAMPs) that appear during infections. For example, cytosolic plasmid DNA is a PAMP that is interpreted as a sign of bacterial infection because host cell DNA is usually restricted to the nucleus (Holm et al., 2013). Cytosolic DNA may be detected by one of more redundant DNA sensor proteins (see Fig. 1), which then recruit adaptor proteins (e.g., MyD88 and STING) to activate signaling cascades of kinases and transcription factors that ultimately induce the expression of cytokines (e.g. IFN β). These cytokines then propagate the innate immune response in the same cell or neighboring cells by triggering subsequent signaling cascades that induce the expression of additional cytokines and cytokine-stimulated genes (CSGs) that help defend the cell against the pathogen by inducing apoptosis, inhibiting protein translation, or other mechanisms (Paludan and Bowie, 2013). For example, when the DNA sensor IFI16 binds to cytosolic DNA, it coordinates with STING to phosphorylate the IKK and TBK kinases, which then activate the transcription factors IRF3 and NF-κB to induce the expression of cytokines and CSGs (de Veer et al., 2001).

Unfortunately, while these pathways provide a crucial defense against foreign pathogens, they can also hinder gene therapy, since most gene delivery techniques introduce plasmid DNA or other foreign nucleic acids into the cytosol (Perez Ruiz de Garibay, 2016; Yasuda et al., 2002; Yi and Krieg, 1998). In addition, since plasmid DNA is produced in bacteria, it lacks methylation on CpG motifs, which can be specifically recognized by the DNA sensors TLR9, DHX9, and DHX36 (Fig. 1). Indeed, transfection of plasmid DNA has been shown to increase the expression of inflammatory cytokines (e.g., TNF-α) that can significantly decrease transgene expression (Holm et al., 2013; Baker et al., 2013; Qin et al., 1997). Therefore, the innate immune response is an important factor to consider in the development of new techniques for

* Corresponding author at: Villanova University, 800 East Lancaster Avenue, White Hall Room 119, Villanova, PA, 19085, United States.
E-mail address: jacob.elmer@villanova.edu (J. Elmer).

https://doi.org/10.1016/j.plasmid.2021.102556
Received 1 August 2020; Received in revised form 8 October 2020; Accepted 14 October 2020
Available online 17 January 2021

gene therapy.

Many previous attempts to improve gene therapy via inhibition of the IIR have focused on TLR9 (Unterholzner, 2013; Hyde et al., 2008). For example, since TLR9 is activated by unmethylated CpG motifs, Hyde et al. removed all of the CpG motifs from a plasmid DNA sequence. The resulting "CpG-free" plasmid did not activate TLR9 and did not induce inflammation in mice (Hyde et al., 2008). However, reintroducing a single CpG motif into the plasmid was sufficient to activate TLR9. Alternatively, in-vitro methylation of CpGs in plasmid DNA was also shown to prevent TLR9 activation and prolong transgene expression by up to four weeks when compared to unmethylated CpGs (Reyes-Sandoval and Ertl, 2004). In addition to TLR9, another recent study showed that inhibition of TBK-1 kinase with the small molecule inhibitor BX-795 enhanced lentiviral transgene expression in natural killer (NK92) cells by an average of 3.8-fold (Sutlu et al., 2012). Finally, silencing of the receptor for IFNα/γ (IFNAR) with shRNA has also been reported to enhance transgene expression (Takahashi et al., 2010; Bauer et al., 2009).

These previous studies clearly show that transgene expression can be improved by inhibiting different parts of the IIR. The aim of this work was to further improve gene therapy by targeting additional components of the IIR with the small molecule inhibitors (SMIs) shown in Figure 1 (Sutlu et al., 2012; Lamphier et al., 2014; Jurenka, 2009; Gonsalves et al., 2011; Ding, 2006). DNA sensors were not targeted for inhibition due to their redundancy, but the downstream adaptor STING was inhibited with H-151. Downstream kinases in the IIR pathways were also inhibited with Amlexanox (TBK-1i) (Reilly et al., 2013), VX-702 (p38MAPKi) (Ding, 2006), and SC-514 (IKK-2i) (Kishore et al., 2003). Curcumin was also tested as a general anti-inflammatory drug, since it has been shown to inhibit cytokine production (TNF-α, IL-6, IL-12) by suppressing the activation of transcription factors (e.g., NF-κB and AP-1) associated with the innate immune response (Jurenka, 2009). Finally, iCRT14 was also used to inhibit the assembly of the β-catenin/TCF4 (a.k.a. TCF7L2) transcriptional complex, which would otherwise be activated by the cytosolic DNA sensor LRRFIP1 to co-activate cytokine and CSG expression with IRF3 (Yang et al., 2010a; Liu et al., 2015; Marcato et al., 2016). Each of these inhibitors was initially screened in prostate cancer (PC-3) cells to identify a lead compound that provided the highest transgene expression, then the lead (iCRT14) was further investigated to determine its effects in other cell lines (MB49, MCF7) and elucidate the mechanism of its enhancement.

2. Materials and methods

2.1. Reagents and materials

Polyplexes were prepared by mixing branched polyethyleneimine (PEI) from Sigma Aldrich (average MW = 25,000) with a luciferase expression plasmid (pGL4.50) driven by a cytomegalovirus (CMV) promoter from Promega (Madison, WI). Small molecule inhibitors were purchased from Sigma Aldrich (iCRT-14, Cat# SML0203 and curcumin, Cat# C1386), Tocris Bioscience (Amlexanox, Cat# 4857 and SC-514, Cat# 3318), Cayman Chemical (VX-702, Cat# 13108), and Invivogen (H-151, Cat# inh-h151). All small molecule inhibitors were dissolved in DMSO and used immediately thereafter when possible or stored at −72 °C until needed.

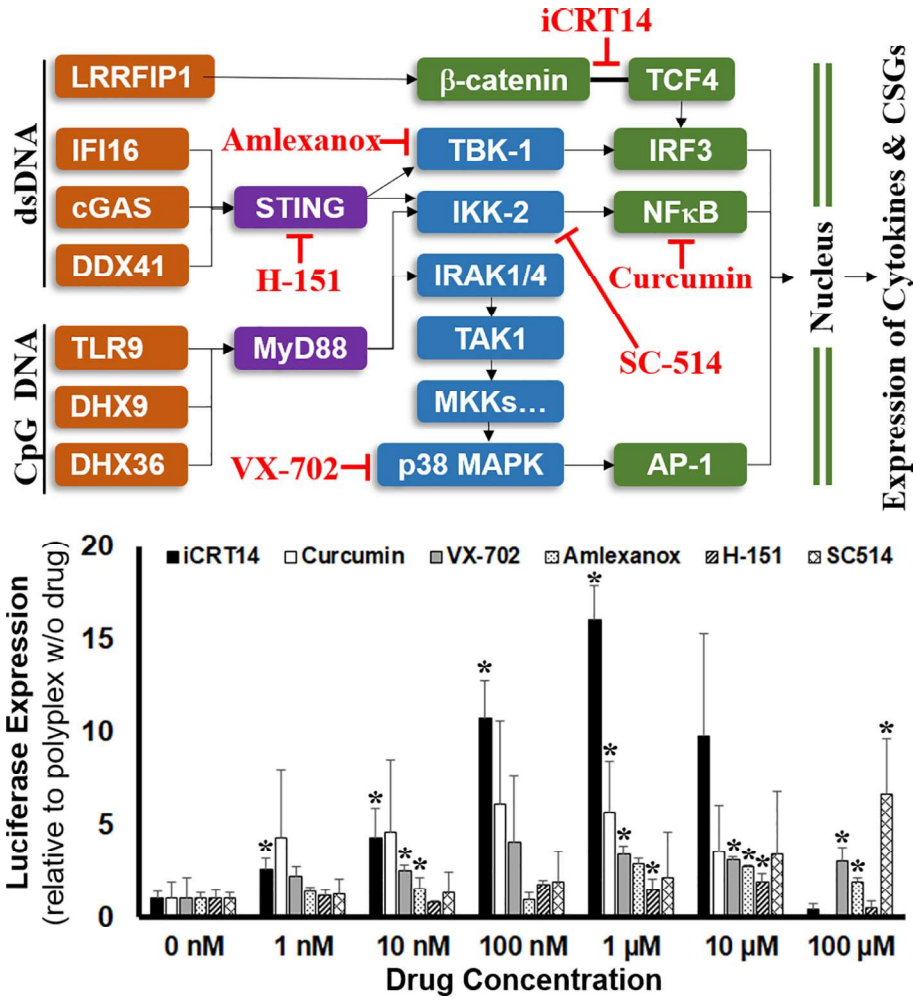

Fig. 1. Inhibition of key points in cytosolic DNA sensing pathways with small molecule inhibitors enhances transgene (luciferase) expression in PC-3 cells. **Top –** Plasmid DNA may be recognized by cytosolic DNA sensors (brown) that specifically bind CpG DNA (TLR9, DHX9, or DHX36) or nonspecifically bind dsDNA in the cytosol (e.g., IFI16, cGAS, DDX41, et al.). When these sensors bind DNA, they bind adaptor proteins (purple) which then activate a signaling cascade of kinase (blue) and transcription factors (green) that culminates in the transcription of cytokines and cytokine stimulated genes that can lead to changes in the cell that inhibit transgene expression (e.g., inflammation, apoptosis, or transcriptional/translational arrest). **Bottom –** Enhancement of transgene expression (relative to a polyplex control with no drug – 0 nM) in PC-3 cells by small molecule inhibitors for various targets in the DNA sensing pathways. Error bars represent standard deviations from $n = 3$ separate experiments, while asterisks (*) indicate significant increases in luciferase expression relative to the polyplex control (0 nM drug). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Cell lines

Human prostate cancer PC-3 (Cat# CRL-1573) and breast cancer MCF7 (Cat# HTB-22) cells were purchased from ATCC. Murine bladder cancer cells (MB49) were generously provided by Dr. Christina Voelkel-Johnson of the Medical University of South Carolina. PC3 and MB49 cells were cultured in RPMI-1640, while MCF7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM).

2.3. PEI transfections

MB49, PC-3, and MCF7 cells were separately seeded onto 24-well plates at a density of 50,000 cells/well in fetal bovine serum-containing media (SCM) 24 h prior to transfection. Polyplexes were prepared by mixing PEI and the luciferase expression plasmid pGL4.50 in a 5:1 *w*/w ratio (1000 ng PEI + 200 ng DNA/well) and then incubating the mixture for twenty minutes at room temperature. Meanwhile, the SCM in each well was aspirated and replaced with serum-free media (SFM). Polyplexes were added to each well and then divided into triplicates of wells that each received a different concentration (1 nM - 100 μM) of each inhibitor, while controls received polyplex and 0.5% DMSO, but no inhibitors. Cells were incubated for an additional six hours at 37 °C in SFM, then the media was exchanged again with fresh SCM containing the inhibitors. Cells were then incubated for 48 h before luciferase expression was measured with a luminescence assay, while total protein concentrations were measured with a BCA assay. The relative luminescence values displayed in Figs. 1 and 2 were calculated by dividing the raw luminescence values for each sample by the luminescence of the polyplex/DMSO control in each experiment.

2.4. MTT assay

In addition to transgene expression, cell proliferation was also measured with an MTT (3-(4,5-dimethylthiazol-2-yl)-2,4-dipehnyltetrazolium bromide) assay to quantify the effects of transfection and the inhibitors on cell proliferation. Specifically, 10 μL of MTT (5.0 mg/mL) was added to the cells and incubated for two hours at 37 °C to allow viable cells to reduce the yellow MTT dye into purple formazan. Cells were then lysed with 150 μL of DMSO per well and mixed to dissolve the formazan, which was quantified by measuring the sample absorbance at 590 nm. Relative proliferation was then calculated by dividing the absorbance of each sample by the absorbance of the live cell control, in which no polyplex or drug was added.

2.5. Cloning of the TCF4 motif

The luciferase expression plasmids evaluated in Fig. 3 were initially prepared by cloning the luciferase gene into the pEF-GFP plasmid from Addgene (plasmid #11154) to obtain pEF-LUC, a luciferase expression plasmid driven by the EF1α promoter. The EF1α promoter was then excised and replaced with a CMV promoter to yield pCMV-GFP. Oligo annealing cloning was then used to introduce TCF4 binding sites (ACATCAAAGG or CCTTTGATGT) between *Sph*I/*Xba*I sites upstream *Eco*RI/*Kpn*I sites downstream (respectively) of both promoters. The exact location of the TCF4 sites relative to the transcription start site (TSS) of each promoter is shown in Fig. 3. All plasmids were sequenced to confirm the insertion of the sequences and the sequence of the corresponding promoter, then transfections were performed with PEI.

2.6. mRNA sequencing

To detect changes in host cell gene expression induced by transfection and iCRT14, nine parallel cultures of PC-3 cells were grown in RPMI media in T-75 flasks. A triplicate of control flasks was untreated, while another triplicate was transfected with PEI, and the last triplicate was transfected and treated with 1 μM iCRT14. All the flasks were then incubated at 37 °C for an additional 24 h. A small fraction of the transfected cultures was lysed and tested with a luciferase assay to confirm enhancement of luciferase expression by iCRT14, then total RNA samples were extracted from the remaining cells with a Qiagen RNEasy kit. The RNA samples were then submitted to the Beijing Genomics Institute (BGI, Hong Kong, China) for library preparation, mRNA-sequencing, and data analysis to obtain the gene expression levels shown in Table 1. The complete mRNA-seq data (fastq files and a spreadsheet of FPKM values) from these experiments are available at the NCBI GEO repository (GEO Accession# GSE155560).

3. Results and discussion

3.1. Initial screening of IIR inhibitors reveals potent enhancement by iCRT14

The effects of each inhibitor on luciferase expression in PC-3 cells are shown in Fig. 1. It is worth mentioning that additional inhibitors for other targets (e.g., the IRAK 1/4 inhibitor IRAK1/4i, the MNK1 inhibitor CGP 57380, and the TBK-1 inhibitor BX-795) (Rowlett et al., 2008) were also tested, but they did not significantly enhance transgene expression (data not shown) and are consequently not included in Fig. 1. Nonetheless, luciferase expression was significantly enhanced (relative to the controls only treated with polyplex) by several inhibitors at different optimum concentrations, including 10 μM H-151 (1.9 ± 0.4-fold), 10 μM Amlexanox (2.8 ± 0.4-fold), 1 μM VX-702 (3.4 ± 0.4-fold), 1 μM curcumin (5.7 ± 2.8-fold), and 100 μM SC-514 (6.6 ± 3.0-fold).

Overall, iCRT-14 provided the highest enhancement of transgene expression in PC-3 cells (Fig. 1) and 2 other cell lines (MCF7 and MB49, see Fig. 2A). In PC-3 cells, iCRT14 significantly enhanced luciferase expression by a factor of 16.1 ± 1.8 at an optimum concentration of 1 μM. The same optimum concentration of 1 μM was also observed in MCF7 and MB49 cells, in which iCRT14 provided an even higher enhancement of 32 to 35-fold (Fig. 2A).

In addition to luciferase expression, the effects of iCRT14 on cell proliferation were also tested with an MTT assay (Fig. 2B). First of all, it is important to note the decrease in cell viability of 10–25% that was observed in the cells that were only treated with polyplex and 0.5% DMSO (bars labeled 0 nM iCRT14). This observation aligns with previous reports that branched PEI is mildly toxic to cells (Christensen et al., 2015; Elmer et al., 2016). The highest concentration of iCRT14 tested (100 μM) also significantly decreased cell viability in all three cell lines. Significant decreases in cell viability were also observed at lower concentrations of iCRT14 in PC-3 cells, but the optimum concentration of iCRT14 that provided the highest transgene expression (1 μM) showed no significant effects on cell viability relative to the polyplex/DMSO control cells in all 3 cell lines.

The data shown in Figs. 1 and 2 demonstrate that iCRT14 can be used to significantly enhance transgene expression in multiple cell lines without decreasing cell viability. Therefore, iCRT14 was further investigated in subsequent experiments to determine the exact mechanism by which it enhances transgene expression. Previous reports have already shown that iCRT14 and similar thiazolidinediones can inhibit the Wnt/β-catenin pathway by directly binding to β-catenin to prevent its interaction with the transcription factor TCF4 (a.k.a. TCF7L2) (Gonsalves et al., 2011; Wang et al., 2009a). This phenomenon is directly relevant to gene therapy, since cytosolic plasmid DNA has been shown to trigger the DNA sensor LRRFIP1, which then activates β-catenin, thereby allowing it to bind TCF4. This complex then translocates to the nucleus, where it works with IRF3 and p300 acetyltransferase to synergistically co-activate cytokines and cytokine-stimulated genes that may inhibit transgene expression. (Yang et al., 2010b; Parekh and Maniatis, 2017; Merika et al., 2017) However, the exact roles of the β-catenin/TCF4 complex and its target genes in non-viral transgene delivery and expression have not yet been elucidated.

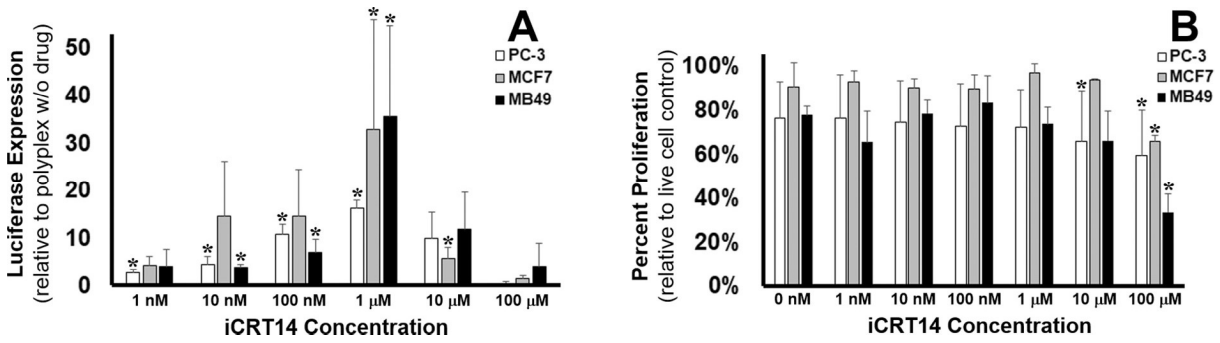

Fig. 2. Effects of iCRT14 at various concentrations on proliferation (measured with an MTT assay) and transgene expression in PC-3 (human prostate cancer), MCF7 (human breast cancer), and MB49 (murine bladder cancer) cells. **A –** Enhancement of transgene (luciferase) expression by iCRT14 relative to cells transfected without drug. **B –** Percent proliferation of transfected cells treated with 0–100 μM iCRT14 relative to untransfected cells that were not exposed to drug (100% proliferation). The error bars in each panel represent standard deviations from $n = 3$ separate experiments, while asterisks (*) indicate significant increases in luciferase expression (A) or significant decreases in percent proliferation (B) relative to the polyplex control.

3.2. Addition of a TCF4 motif enhances the EF1α promoter

If the enhancement provided by iCRT14 is due to inhibition of the β-catenin/TCF4 transcriptional complex, then it is highly likely that β-catenin and TCF4 are present and actively inducing the transcription of host cell genes following transfection in PC-3 cells. To test the hypothesis that β-catenin and TCF4 can induce transcription in PC-3 cells, we prepared a set of plasmids in which luciferase expression was driven by two commonly used promoters (CMV and EF1α) and then added a TCF4 motif (ACATCAAAGG) to the (+) or (−) strands, either upstream or downstream of each promoter (see Fig. 3 for the exact placement of the motifs). TCF4 motifs were not inserted directly into the promoter to avoid interfering with existing transcription factor binding sites (TFBS) in the promoter sequence.

Fig. 3 shows that insertion of the new TCF4 motif increased transgene expression by 1–3 fold in some positions and orientations, but the only significant enhancement was achieved by inserting the TCF4 motif downstream of the EF1α promoter on the (−) strand. In contrast, no enhancement was observed when the TCF4 motif was cloned into the (+) strand at the same location. This result shows that it may be possible to enhance transgene expression by recruiting transcription factors that are activated in the innate immune response to plasmid DNA. Indeed, it has been previously shown that human papilloma virus (HPV) contains a binding site for the transcription factor IRF1, which enhances transcription from the HPV promoter in the presence of IFN-γ (Reilly et al., 2013). However, it is important to note that the effects of a TFBS are highly dependent upon its position and the promoter being used. For example, no significant enhancement was observed in any CMV promoters with TCF4 motif inserts. This may be due to suboptimal positioning of the TCF4 motif relative to the TSS or the fact that the CMV promoter already contains 5 NF-κB sites, which may obviate the need for additional motifs for TCF4 and other transcription factors.

3.3. Effects of transfection and iCRT14 on host cell gene expression

In our next experiments, we sought to identify the mechanism by which iCRT14 enhances transgene expression. Initially, we used ELISAs to detect changes in specific cytokine levels following transfection and/or addition of iCRT14, since previous reports had suggested that β-catenin and TCF4 can increase expression of IFNβ (Yang et al., 2010b). Those experiments did detect significantly higher levels of IFNβ and IL-6 in transfected cells relative to untransfected controls, but adding iCRT14 had no effect on the levels of these cytokines in transfected cells (data not shown).

Nonetheless, we still sought to identify host cell genes that are down-regulated when iCRT14 is used to inhibit the assembly of the β-catenin/TCF4 transcriptional complex, so we used mRNA sequencing to survey the entire transcriptome for changes in host cell gene expression. Hypothetically, genes that are upregulated following transfection and down-regulated in the presence of iCRT14 would be the most likely

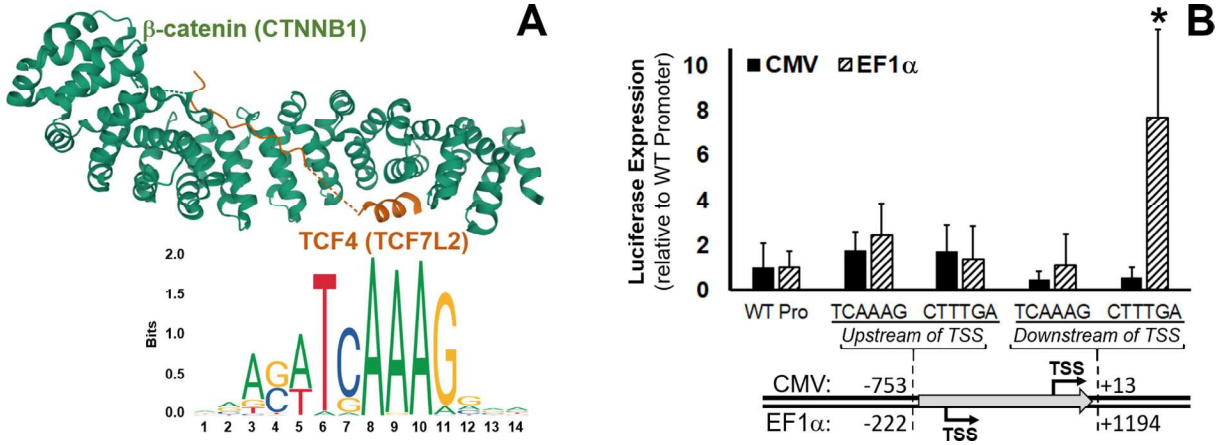

Fig. 3. Insertion of a TCF4 (TCF7L2) binding site into the CMV and EF1α promoters. **Left –** β-catenin can bind to TCF4 (TCF7L2) to form a complex (PDB ID 1JPW) that selectively binds and activates promoters with one or more SATSAAARN (JASPAR motif MA0532.1) sites. **Right –** Transgene expression from CMV and EF1α promoters containing a TCF4 binding site (ACATCAAAGG) on the coding or non-coding strand, either upstream or downstream of the transcription start site (TSS), relative to the wild type promoters. Error bars represent standard deviations from $n = 3$ separate experiments, while asterisks (*) indicate significant ($p < 0.05$) increases in luciferase expression relative to the wild type promoter. Note: The EF1α promoter used in this study contains an intron that spans from +33 to +976.

repressors of transgene expression.

Plots showing changes in gene expression patterns measured 24 h after transfection (Fig. 4A) and with or without 1 mM iCRT14 (Fig. 4B) in PC-3 cells are shown in Fig. 4. First of all, it is important to mention that the targets for all of the inhibitors evaluated in this study were detected in PC-3 cells, including LRRFIP1, β-catenin (CTNNB1), TCF4 (TCF7L2), and IRF3. However, the endosomal DNA sensor TLR9 was not detected.

As expected, transfection of plasmid DNA into the cytoplasm with PEI activated the innate immune response and induced or upregulated the expression of hundreds of host cell genes (Fig. 4A). A complete list of the upregulated genes is shown in the supplementary information (Table S1), but the observed results are mostly similar to lists of upregulated genes reported in previous studies (Shayakhmetov et al., 2010). For example, several cytokine genes were upregulated, including interferons (IFNA7/10/16, IFNB1, and IFNL1/2/3), interleukins (IL7/24/12A/1A/15), and chemokines (CCL20/5/26, CXCL11/9/10/16). Many cytokine stimulated genes were also significantly upregulated, including the DNA sensor IFI16, transcription factors (e.g., IRF7/1/9/2, STAT1/2/6), and other genes with known anti-viral functions that could potentially interfere with transgene expression (e.g., ISG15, MX1/2, RSAD2, and oligoadenylate synthetases) (Gariano et al., 2012; Perng and Lenschow, 2018; Verhelst et al., 2013; Honarmand Ebrahimi, 2018; Hovanessian and Justesen, 2007).

Addition of iCRT14 to the transfected cells affected a much smaller set of genes, including 22 genes that were down-regulated (Tables 1 and S2) and 3 genes that were upregulated (Table S3) in PC-3 cells transfected with PEI. It is possible that any of these differentially expressed genes may be responsible for the enhancement observed with iCRT14. However, it is important to note that many of the CSGs that are activated during the innate immune response have highly specific functions that potently inhibit the transduction and/or replication of some specific viruses, but have no effect on non-viral gene delivery of plasmid DNA with PEI or episomal transgene expression. For example, PLA1A (Phospholipase A1 Member A) is one of the genes shown in Table 1 that were upregulated after transfection and down-regulated in the presence of iCRT14. PLA1A is also induced during Hepatitis C infection and its overexpression has been shown to strengthen the antiviral response to Sendai virus (SeV) by increasing cytokine expression. Knockdown of PLA1A has been shown to prevent the association of TBK-1 and MAVS, an adaptor protein that is involved in the detection of RNA viruses like SeV. PLA1A knockdown also leads to a decrease in TBK-1 phosphorylation during SeV infection, which results in a concomitant decrease in IRF3 activation and IFNB1 expression (Gao et al., 2018). In contrast, knockdown of PLA1A does not influence the association of TBK-1 and STING, an adaptor protein that is involved in the detection of cytosolic dsDNA and DNA viruses. This specificity of PLA1A for MAVS may explain why our mRNA sequencing experiments did not show a decrease in IFNB1 expression in the cells transfected with plasmid DNA (which would have been recognized by a DNA sensor that binds STING instead of MAVS). Therefore, while PLA1A may play an important role in the response to RNA viruses, it is less likely that it is involved in the response to cytosolic DNA and the enhancement of episomal transgene expression provided by iCRT14.

Many of the other genes listed in Table 1 also have known anti-viral functions, but it is unclear how they might affect transgene expression. For example, the chemokine receptor CCR1 and the interleukin receptor IL7R play important roles in the adaptive immune response to viruses and other pathogens by recruiting immune cells to the site of infection in vivo (Melchjorsen et al., 2003; Sørensen and Paludan, 2004; Domachowske et al., 2000; Osugui et al., 2018), but the lack of immune cells in our in vitro experiments makes it unlikely that their down-regulation enhances transgene expression. SCN3A and HSPA6 are also induced during viral infection, but their anti-viral mechanisms have not yet been determined (Pinkham et al., 2017; Braga et al., 2017; Zhong et al., 2019). Multiple anti-viral mechanisms for IGFBP3 have been demonstrated, including the binding and sequestration of the growth factor IGF-1, which can prevent cell growth and potentially induce apoptosis (Mohseni-Zadeh and Binoux, 1997; Baege et al., 2004). However, the expression levels of IGF-1 transcription detected in this study were negligible. Alternatively, IGFBP3 may also be required for activation of the potent viral restriction factor MxA, but the majority of studies on MxA suggest that it prevents viral replication by either inhibiting the cytoplasmic trafficking or assembly of viral capsids (Verhelst et al., 2013; Dornfeld et al., 2018). Therefore, like PLA1A, it is unclear how inhibition of IGFBP3 and MxA by iCRT14 might influence the expression of a plasmid that was delivered with a non-viral vehicle like PEI.

Out of all the down-regulated genes listed in Table 1, PTGS2 (Prostaglandin Synthetase 2) is the most likely candidate to impact transgene expression. PTGS2 is also upregulated during HIV-1 infection and its overexpression inhibits HIV-1 replication in human peripheral blood mononuclear cells (PMBCs). Specifically, PTGS2 inhibits NFκB signaling, which leads to a decrease in transcription from HIV-1 genes with an NFκB motif (GGGSNNYYCC) (Whitney et al., 2011).

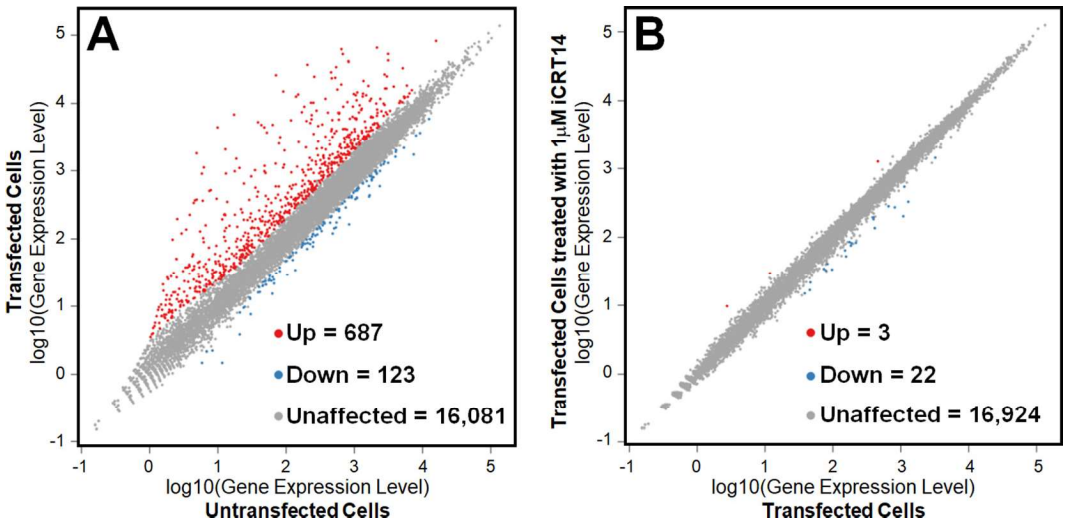

Fig. 4. Differential expression of host cell genes following transfection with polyplex (left) and addition of 1 μM iCRT14 (right). Host cell genes with expression levels that did not change significantly are shown in gray, while genes that were significantly ($P_{adj} < 0.05$) upregulated or downregulated at least 2-fold are shown in red and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Host cell genes upregulated by transfection and down-regulated by iCRT14.

	Expression Levels				Expression Levels			
Gene Symbol	Control	Polyplex	Fold-change	P adj	Polyplex	Polyplex +iCRT14	Fold-change	P adj
CCR1	3	195	77.1	1.1E-37	188	82	0.4	1.2E-04
PLA1A	18	226	12.7	1.5E-31	218	83	0.4	1.5E-06
SCN3A	24	191	7.96	2.2E-17	184	76	0.4	2.9E-04
HSPA6	145	1152	7.93	9.8E-19	1088	203	0.2	6.8E-12
IL7R	373	1334	3.58	7.6E-06	1292	329	0.3	2.6E-10
PTGS2	159	435	2.74	3.5E-02	414	136	0.3	2.7E-03
IGFBP3	1529	3291	2.15	5.2E-03	3226	1435	0.4	1.2E-06

Consequently, the upregulation of PTGS2 during transfection may lead to a decrease in expression from some of the viral promoters used in gene therapy, like the CMV promoter in pGL4.50 that was used to drive luciferase expression in our experiments with iCRT14 (Figs. 1 and 2). Indeed, the CMV promoter contains 5 NFκB binding sites and it has been previously shown that NFκB activates transcription from the CMV promoter (He and Weber, 2004).

Finally, it is also worth mentioning that there were several host cell genes that were downregulated by iCRT14, but unaffected by transfection (Table S3). Some of these genes have been reported to inhibit viral replication (e.g., FGF1 (van Asten et al., 2018) and CEACAM6 (Wang et al., 2009b)), but none of them have known functions that appear to be relevant to non-viral gene delivery or expression. It is also worth noting that 3 genes (HIST1H4K, DHRS2, and NKX2-8) were upregulated 2 to 3-fold in the presence of iCRT14 (Table S3), but they do not have any known anti-viral functions that would affect transgene expression. Therefore, out of the 25 genes that were differentially expressed in response to iCRT14 treatment in transfected PC-3 cells, it appears that down-regulation of PTGS2 may be responsible for the enhancement provided by iCRT14.

4. Conclusion

Overall, this study shows that inhibition of the innate immune response can significantly improve transgene expression in a variety of cell lines. The most potent inhibitor in this study was iCRT14, which inhibits β-catenin and TCF4 to affect significant changes in host cell gene expression. Most importantly, it appears that iCRT14 may enhance transgene expression by down-regulating PTGS2, a host cell gene that may repress the CMV promoter by inhibiting the NFκB transcription factor. Additional studies involving the knockdown or knockout of PTGS2 are needed to confirm its potentially repressive role in transgene expression, but the results clearly show that iCRT14 is a potent enhancer of transgene expression in non-viral systems using PEI to deliver plasmid DNA.

Declaration of Competing Interest

None.

Acknowledgements

The authors would like to gratefully acknowledge Dr. Christine Voekel-Johnson, Dr. Janice Knepper, and Dr. Noelle Comolli for sharing materials (e.g., cell lines) and helpful advice. This work was supported by the U.S. National Science Foundation [Grant 1651837].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plasmid.2021.102556.

References

Baege, A.C., Disbrow, G.L., Schlegel, R., 2004. IGFBP-3, a marker of cellular senescence, is overexpressed in human papillomavirus-immortalized cervical cells and enhances IGF-1-induced mitogenesis. J. Virol. 78, 5720–5727.
Baker, J.R., Orr, B.G., Holl, M.M.B., 2013. Polyplex Exposure Inhibits Cell Cycle, Increases Inflammatory Response, and Can Cause Protein Expression without Cell Division.
Bauer, M., Kinkl, N., Meixner, A., Kremmer, E., Riemenschneider, M., Förstl, H., Gasser, T., Ueffing, M., 2009. Prevention of interferon-stimulated gene expression using microRNA-designed hairpins. Gene Ther. 16, 142–147.
Braga, A.C.S., Carneiro, B.M., Batista, M.N., Akinaga, M.M., Bittar, C., Rahal, P., 2017. Heat shock proteins HSPB8 and DNAJC5B have HCV antiviral activity. PLoS One 12, e0188467.
Buchmann, K., 2014. Evolution of innate immunity: Clues from invertebrates via fish to mammals. Front. Immunol. 5, 1–8.
Christensen, M.D., Elmer, J.J., Eaton, S., Gonzalez-Malerva, L., LaBaer, J., Rege, K., 2015. Kinome-level screening identifies inhibition of polo-like kinase-1 (PLK1) as a target for enhancing non-viral transgene expression. J. Control. Release 204, 20–29.
de Veer, M.J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J.M., Silverman, R.H., Williams, B.R.G., 2001. Functional classification of interferon-stimulated genes identified using microarrays. J. Leukoc. Biol. 69, 912–920.
Ding, C., 2006. Drug evaluation: VX-702, a MAP kinase inhibitor for rheumatoid arthritis and acute coronary syndrome. Curr. Opin. Investig. Drugs 7, 1020–1025.
Domachowske, J.B., Bonville, C.A., Gao, J.-L., Murphy, P.M., Easton, A.J., Rosenberg, H. F., 2000. The chemokine macrophage-inflammatory protein-1α and Its receptor CCR1 control pulmonary inflammation and antiviral host defense in paramyxovirus infection. J. Immunol. 165, 2677–2682.
Dornfeld, D., Dudek, A.H., Vausselin, T., Günther, S.C., Hultquist, J.F., Giese, S., Khokhlova-Cubberley, D., Chew, Y.C., Krogan, N.J., Garcia-Sastre, A., Schwemmle, M., Shaw, M.L., 2018. SMARCA2-regulated host cell factors are required for MxA restriction of influenza A viruses. Sci. Rep. 8, 1–13.
Elmer, J.J., Christensen, M.D., Barua, S., Lehrman, J., Haynes, K.A., Rege, K., 2016. The histone deacetylase inhibitor Entinostat enhances polymer-mediated transgene expression in cancer cell lines. Biotechnol. Bioeng. 113, 1345–1356.
Fearon, D.T., Locksley, R.M., 1996. The instructive role of innate immunity in the acquired immune response. Science (80-.). 272, 50–54.
Flajnik, Martin, Kasahara, Masanori, 2010. Origin and evolution of the adaptive immune system: genetic events and selective pressures. Nat Rev Genet 11, 47–59.
Gao, X., Chen, D., Hu, X., Zhou, Y., Wang, Y., Wu, C., Chen, J., Wang, Y., Pei, R., Chen, X., 2018. PLA1A participates in the antiviral innate immune response by facilitating the recruitment of TANK-binding kinase 1 to mitochondria. J. Innate Immun. 10, 315–327.
Gariano, G.R., Dell'Oste, V., Bronzini, M., Gatti, D., Luganini, A., De Andrea, M., Gribaudo, G., Gariglio, M., Landolfo, S., 2012. The Intracellular DNA Sensor IFI16 Gene Acts as Restriction Factor for Human Cytomegalovirus Replication. PLoS Pathog. 8, e1002498.
Gonsalves, F.C., Klein, K., Carson, B.B., Katz, S., Ekas, L.A., Evans, S., Nagourney, R., Cardozo, T., Brown, A.M.C., DasGupta, R., 2011. An RNAi-based chemical genetic screen identifies three small-molecule inhibitors of the Wnt/wingless signaling pathway. Proc. Natl. Acad. Sci. U. S. A. 108, 5954–5963.
He, B., Weber, G.F., 2004. Synergistic activation of the CMV promoter by NF-κB P50 and PKG. Biochem. Biophys. Res. Commun. 321, 13–20.
Holm, C.K., Paludan, S.R., Fitzgerald, K.A., 2013. DNA recognition in immunity and disease. Current Opinion in Immunology 25, 13–18.
Honarmand Ebrahimi, K., 2018. A unifying view of the broad-spectrum antiviral activity of RSAD2 (viperin) based on its radical-SAM chemistry. Metallomics 10, 539–552.
Hovanessian, A.G., Justesen, J., 2007. The human 2′-5′oligoadenylate synthetase family: Unique interferon-inducible enzymes catalyzing 2′-5′ instead of 3′-5′ phosphodiester bond formation. Biochimie 89, 779–788.
Hyde, S.C., Pringle, I.A., Abdullah, S., Lawton, A.E., et al., 2008. CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. Nat. Biotechnol. 26, 549–551.
Jurenka, J.S., 2009. Anti-inflammatory properties of curcumin, a major constituent of *Curcuma longa*: A review of preclinical and clinical research. Alternative Medicine Review 14, 141–153.
Kishore, N., Sommers, C., Mathialagan, S., Guzova, J., Yao, M., Hauser, S., Huynh, K., Bonar, S., Mielke, C., Albee, L., Weier, R., Graneto, M., Hanau, C., Perry, T., Tripp, C. S., 2003. A Selective IKK-2 Inhibitor Blocks NF-κB-dependent Gene Expression in Interleukin-1β-stimulated Synovial Fibroblasts. J. Biol. Chem. 278, 32861–32871.

Lamphier, M., Zheng, W., Latz, E., Spyvee, M., et al., 2014. Novel small molecule inhibitors of TLR7 and TLR9: mechanism of action and efficacy in vivo. Mol. Pharmacol. 85, 429–440.
Liu, Y., Zou, Z., Zhu, B., Hu, Z., Zeng, P., Wu, L., 2015. LRRFIP1 inhibits hepatitis C virus replication by inducing type I interferon in hepatocytes. Hepat. Mon. 15.
Marcato, V., Luron, L., Laqueuvre, L.M., Simon, D., Mansuroglu, Z., Flamand, M., Panthier, J.-J., Souès, S., Massaad, C., 2016. Bonnefoy, E. β-Catenin Upregulates the Constitutive and Virus-Induced Transcriptional Capacity of the Interferon Beta Promoter through T-Cell Factor Binding Sites. Mol. Cell. Biol. 36, 13–29.
Melchjorsen, J., Sørensen, L.N., Paludan, S.R., 2003. Expression and function of chemokines during viral infections: from molecular mechanisms to in vivo function. J. Leukoc. Biol. 74, 331–343.
Merika, M., Williams, A.J., Chen, G., Collins, T., Thanos, D., 2017. Recruitment of CBP/p300 by the IFNβ enhanceosome is required for synergistic activation of transcription. Mol. Cell 1, 277–287.
Mohseni-Zadeh, S., Binoux, M., 1997. Insulin-like growth factor (IGF) binding protein-3 interacts with the Type 1 IGF Receptor, reducing the affinity of the receptor for its ligand: an alternative mechanism in the regulation of IGF action. Endocrinology 138, 5645–5648.
Osugui, L., de Roo, J.J., de Oliveira, V.C., Sodré, A.C.P., Staal, F.J.T., Popi, A.F., 2018. B-1 cells and B-1 cell precursors prompt different responses to Wnt signaling. PLoS One e0199332, 13.
Paludan, S.R., Bowie, A.G., 2013. Immune sensing of DNA. Immunity 38, 870–880.
Parekh, B.S., Maniatis, T., 2017. Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-β Promoter. Mol. Cell 3, 125–129.
Perez Ruiz de Garibay, A., 2016. Endocytosis in gene therapy with non-viral vectors. Wiener Medizinische Wochenschrift 166, 227–235.
Perng, Y.C., Lenschow, D.J., 2018. ISG15 in antiviral immunity and beyond. Nat. Rev. Microbiol. 16, 423–439.
Pinkham, C., Dahal, B., De La Fuente, C.L., Bracci, N., Beitzel, B., Lindquist, M., Garrison, A., Schmaljohn, C., Palacios, G., Narayanan, A., Campbell, C.E., Kehn-Hall, K., 2017. Alterations in the host transcriptome in vitro following Rift Valley fever virus infection. Sci. Rep. 7.
Qin, L., Ding, Y., Pahud, D.R., Chang, E., Imperiale, M.J., Bromberg, J.S., 1997. Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. Hum. Gene Ther. 8, 2019–2029.
Reilly, S.M., Chiang, S.H., Decker, S.J., Chang, L., Uhm, M., Larsen, M.J., Rubin, J.R., Mowers, J., White, N.M., Hochberg, I., Downes, M., Yu, R.T., Liddle, C., Evans, R.M., Oh, D., Li, P., Olefsky, J.M., Saltiel, A.R., 2013. An inhibitor of the protein kinases TBK1 and IKK-ε improves obesity-related metabolic dysfunctions in mice. Nat. Med. 19, 313–321.
Reyes-Sandoval, A., Ertl, H.C.J., 2004. CpG methylation of a plasmid vector results in extended transgene product expression by circumventing induction of immune responses. Mol. Ther. 9, 249–261.
Rowlett, R.M., Chrestensen, C.A., Nyce, M., Harp, M.G., Pelo, J.W., Cominelli, F., Ernst, P.B., Pizarro, T.T., Sturgill, T.W., Worthington, M.T., 2008. MNK kinases regulate multiple TLR pathways and innate proinflammatory cytokines in macrophages. Am. J. Physiol. - Gastrointest. Liver Physiol. 294.
Shayakhmetov, D.M., Di Paolo, N.C., Mossman, K.L., 2010. Recognition of virus infection and innate host responses to viral gene therapy vectors. Mol. Ther. 18, 1422–1429.
Sørensen, L.N., Paludan, S.R., 2004. Blocking CC chemokine receptor (CCR) 1 and CCR5 during herpes simplex virus type 2 infection in vivo impairs host defence and perturbs the cytokine response. Scand. J. Immunol. 59, 321–333.
Sutlu, T., Nyström, S., Gilljam, M., Stellan, B., Applequist, S.E., Alici, E., 2012. Inhibition of Intracellular Antiviral Defense Mechanisms Augments Lentiviral Transduction of Human Natural Killer Cells: Implications for Gene Therapy. Hum. Gene Ther. 23, 1090–1100.
Takahashi, Vikman, Nishikawa, Ando, Watanabe, Takakura, 2010. Persistent interferon transgene expression by RNA interference-mediated silencing of interferon receptors. J. Gene Med. 12, 739–746.
Unterholzner, L., 2013. The interferon response to intracellular DNA: why so many receptors? Immunobiology 218, 1312–1321.
van Asten, S.D., Raaben, M., Nota, B., Spaapen, R.M., 2018. Secretome screening reveals fibroblast growth factors as novel inhibitors of viral replication. J. Virol. 92.
Verhelst, J., Hulpiau, P., Saelens, X., 2013. Mx proteins: antiviral gatekeepers that restrain the uninvited. Microbiol. Mol. Biol. Rev. 77, 551–566.
Wang, P.S., Chou, F.S., Bloomston, M., Vonau, M.S., Saji, M., Espinosa, A., Pinzone, J.J., 2009a. Thiazolidinediones downregulate Wnt/β-catenin signaling via multiple mechanisms in breast cancer cells. J. Surg. Res. 153, 210–216.
Wang, Y., Gangeswaran, R., Zhao, X., Wang, P., et al., 2009b. CEACAM6 attenuates adenovirus infection by antagonizing viral trafficking in cancer cells. J. Clin. Invest. 119, 1604–1615.
Whitney, J.B., Asmal, M., Geiben-Lynn, R., 2011. Serpin induced antiviral activity of prostaglandin synthetase-2 against HIV-1 replication. PLoS One 6.
Yang, P., An, H., Liu, X., Wen, M., Zheng, Y., Rui, Y., Cao, X., 2010a. The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type i interferon via a B-catenin-dependent pathway. Nat. Immunol. 11, 487–494.
Yang, P., An, H., Liu, X., Wen, M., Zheng, Y., Rui, Y., Cao, X., 2010b. The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway. Nat. Immunol. 11, 487–494.
Yasuda, K., Ogawa, Y., Kishimoto, M., Takagi, T., Hashida, M., Takakura, Y., 2002. Plasmid DNA activates murine macrophages to induce inflammatory cytokines in a CpG motif-independent manner by complex formation with cationic liposomes. Biochem. Biophys. Res. Commun. 293, 344–348.
Yi, A.K., Krieg, A.M., 1998. Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. J. Immunol. 161, 4493–4497.
Zhong, C., Li, J., Mao, L., Liu, M., Zhu, X., Li, W., Sun, M., Ji, X., Xiao, F., Yang, L., Zhang, W., Liao, Z., 2019. Proteomics analysis reveals heat shock proteins involved in caprine parainfluenza virus type 3 infection. BMC Vet. Res. 15, 151.