

**Modeling infection and antiviral therapy of
enteric viruses using primary intestinal
organoids**

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Modeling infection and antiviral therapy of enteric viruses using primary intestinal organoids

Modeleren van infectie en antivirale therapie van enterische virussen met primaire intestinale organoiden

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CHAPTER 1

General introduction and outline of the thesis

GENERAL INTRODUCTION

Infectious diarrhea

Diarrhea, also written as diarrhoea, is the situation of exhibiting at least three liquid or loose bowel movements *per diem*. It often lasts for a couple of days and can provoke dehydration as a consequence of fluid loss. In its essence diarrhea is an altered movement of ions and water that follows an osmotic gradient (2). Consequently, diarrhea is caused either by increased secretion or by decreased absorption of fluids and electrolytes. The molecular elements related to the dysregulation of ion transport processes during diarrhea prominently include the cystic fibrosis transmembrane conductance regulator (CFTR, which serves as a chloride channel) and the calcium activated chloride channel (CLCA, which is a Na⁺/H⁺ exchanger isoform), the NHE3 (involved in Na⁺ absorption), alterations in tight junctions (which create a shunting pathway for the movement of both ions and water into the lumen), downregulated in adenoma (DRA, which is responsible for chloride absorption and is associated with congenital chloride diarrhea), aquaporins (contributes to diarrhea) and the sodium-dependent glucose transporter (SGLT-1, which is responsible for transporting sodium and glucose, and it is tightly coupled with water movement and it is the basis for oral rehydration using glucose to enhance sodium absorption) (Figure 1) (2).

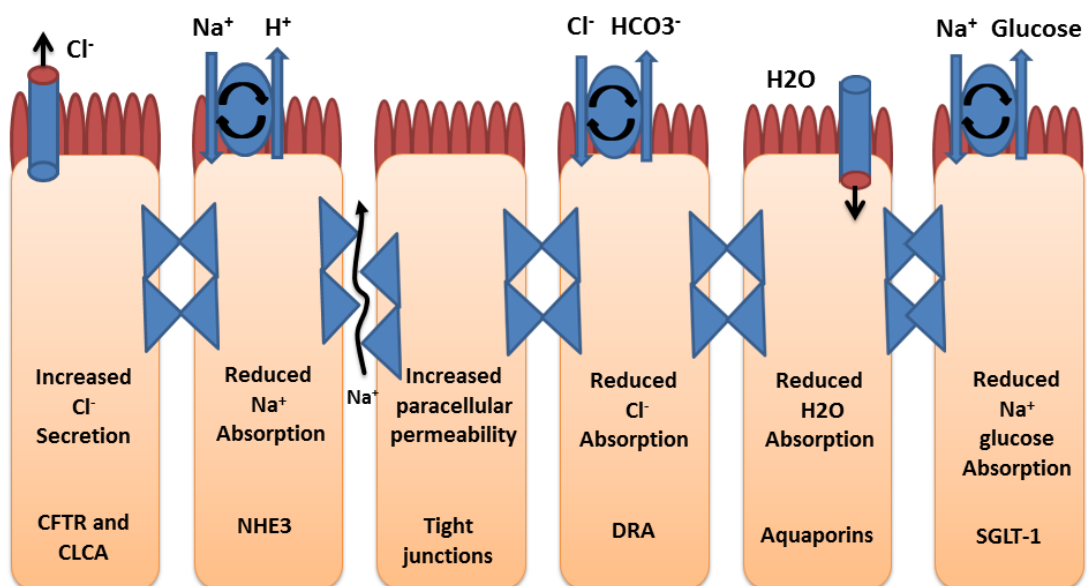


Figure 1. The schematic diagram of general mechanisms causing diarrhea (2).

Infectious diarrhea is defined as the diarrhea caused by infectious pathogens (2). Infectious diarrhea still causes a heavy burden to public health causing high morbidity and mortality throughout the world (3). Pathogens causing infectious diarrhea mainly include bacterial, viruses, and parasites (2). With respect to the latter, rotavirus and norovirus are two of major causative agents of severe infectious diarrhea. Rotavirus is one of the most important causative factors of diarrhea hospitalization for those infants younger than five years old (4). This virus mainly infects villus of intestine to result in serious diarrhea (2). Similar to the rotavirus, the norovirus often causes the epidemic of gastroenteritis (5). The the norovirus often causes sporadic gastroenteritis irrespective to ages. Clinical symptoms of norovirus infection represent themselves as vomiting at first, followed by abdominal cramps, fever, diarrhea, and some constitutional symptoms (5). Until now, there are still no specific treatments in response to rotavirus and norovirus gastroenteritis (5). Hereto, the effective and the high cost-performance medicines are desiderate for the reduction of the burden of diarrhea brought by these two viruses.

The discovery, pathogenesis, and virology of rotavirus

Rotavirus was discovered by Dr Ruth Bishop and colleagues from the royal children's hospital (RCH), Australia in 1970. But at that time, the name of this virus was not determined yet. These investigators found, however, that there were numerous viral particles in the epithelial cells of the intestine. By using electron microscopy, these investigators observed the virus to be reovirus-like or orbivirus-like but it was different from known viruses, and the particle size of the virus is 70 nm. Bishop *et al* suggested that those viruses might be orbiviruses (6). In 1974, Thomas Henry Flewett from east Birmingham hospital suggested that the rotavirus should be named as "rotavirus" based on a Latin word "rota" that means wheel, since the viral particle seems like a wheel (Figure 2). Rotavirus was officially defined in 1978 (7).

Rotavirus belongs to the family *Reoviridae*, and it is considered as a major factor of hospitalizations for acute gastroenteritis in infants younger than five years old. The mature virus particles of the rotavirus are non-enveloped, and the genome of the virus is surrounded by multilayered icosahedral protein capsids, the diameter of the viral particle is around 75 nm. The genome of rotavirus is a double-stranded RNA, and it consists of 11 segments encoding 12 proteins including six viral and six non-

structural proteins (8). For VP1, it is one of components of the core of viral particle, and it is the RNA-dependent RNA polymerase (RdRP) of rotavirus to catalyze viral RNA synthesis (9). VP2 is formed to the single layered particle (SLP= core shell, also called innermost capsid) that consists of 120 molecules (10). VP3 is the guanylyl transferase (the capping enzyme) (11). VP4 is the 84 KDa spike protein containing 775 or 776 amino acids, and it is involved in the outer capsid (12). VP4 is the protease cleaved protein, and can be cleaved into VP5* and VP8*, respectively, which can promote activation of the virus to increase virus infectivity and allow the virus to penetrate into the cell (12). As a main capsid protein, VP6 comprises around 51% of total viral protein, and it oligomerizes into trimers that constitute the intermediate shell of the virions. VP7 is the rotavirus outer capsid that forms an icosahedral lattice and a trimer (13). As one target of protective antibodies, VP7 also exerts an important role in uncoating of the virus when it enters host (13).

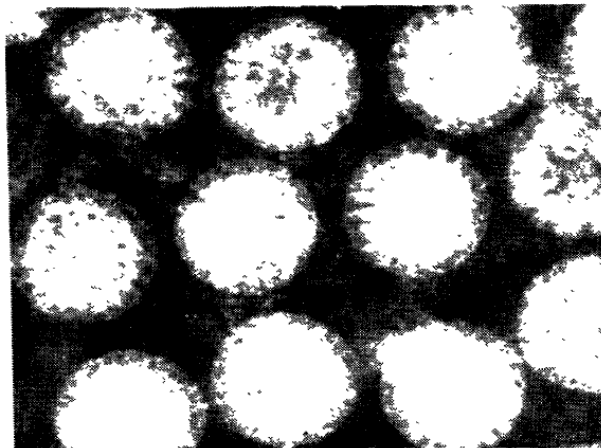


Figure 2. The morphology of the rotavirus looks like a wheel (x 296, 000) (7).

Compared with these VPs, the functions of NSPs are less well defined. Among them, NSP1 is localized throughout the cytoplasm and concentrates in viroplasms, and it is associated with the cytoskeleton but not required for rotavirus replication. Furthermore, NSP1 was reported to be vital for modulating the efficiency of viral gene expression or in regulating host cell responses (14). As a homo-octomer, NSP2 exerts helix-destabilizing activities (15), and it is very abundant in cytoplasmic inclusions (viroplasms) (16). NSP2 plays a vital role in viral genome replication (16). NSP3 has two main domains, one domain is a considerable conserved basic region, and this domain has one α -helical structure. Functionally it can specifically bind to

dsRNA. For the second domain, it has heptapeptide repeats, and this domain is situated in the carboxy-half of the NSP3 protein. NSP3 plays an important role in replicating genome and the early process of assembling virus (17). NSP4 is the viral enterotoxin being related to the viral pathogenesis and morphogenesis. NSP4 causes diarrhea, because extracellular NSP4 could stimulate phospholipase C activation, which causes the increase of the Ca^{2+} amount and the release of Cl^- in the cells (18). Similar to NSP2, NSP5 is also located in viroplasm, and it is changed into various isoforms by a complicated phosphorylation procedure (19). NSP2 might also play an important role in posttranslational modification of the NSP5 protein (16). NSP2 is able to cooperate with NSP5 to form viroplasm-like structures, and the structure can regulate RNA packaging and replication (16). Compared to other VPs and NSPs of the rotavirus, there is paucity limited of information about NSP6. NSP6 is a 12 kDa protein that can be capable of interacting with the C-terminal region of NSP5 protein (20). NSP6 might be involved in a non-essential monitoring effect upon viral infections (20).

There are multiple mechanisms to cause diarrhea by rotaviruses (21). The viruses are able to destroy the intestinal enterocytes, resulting in the shortening of intestinal villus, the augmentation of depth of crypts, and the reduction of some enzymes including sucrose, lactase, and Na^+ , K^+ -ATPase in mucus of intestinal villus (22). NSP4 of rotavirus is an enterotoxin; it is very different from bacterial enterotoxins (23). NSP4 is synthesized in villus enterocytes upon virus infections, subsequently it is split into cleavage products NSP4-(112-175), and then these cleavage products are secreted into the outside of cells, further to lumen of intestine. In addition, NSP4 protein and some of its peptides can cause diarrhea without damaging any intestinal mucosa (23). It is plausible that activation of the ENS by rotavirus infection results in the secretion of the fluid/electrolytes to cause diarrhea upon rotavirus infections (21).

Although, numerous investigations have been executed to study the virology of rotavirus and the associated pathogenesis, there are still considerable issues of uncertainty remaining, especially about subtle aspects of VPs and NSPs biochemistry (e.g. about NSP6 much remains obscure at best), the nature of virus-host interactions, and how the virus causes diarrhea. The lack of effective antivirals represents an urgent need for further investigation.

Clarification of rotavirus

Rotaviruses are the members of *Reoviridae*. Currently, there are several classification systems for rotaviruses. Based on serology, rotavirus was classified into six different groups (A, B, C, D, E, and F) (8). Among, groups including A, B and C were reported to infect humans and animal, while group D to F rotaviruses were found to only infect animals (8). Among them, group A is most often reported to cause serious diarrhea with quite considerable morbidity/mortality in children less than five years old globally (24). A binary classification system was developed based on plaque reduction or fluorescent-focus reduction neutralization assays (8). This method is based on purification of virus particles by utilizing antisera (8), which categorizes rotaviruses to be G-serotypes and P-serotypes (25). With this method, G-serotypes are corresponding to VP7, while P-serotypes are corresponding to VP4 based on their antigenic properties (25). Subsequently, it was reported one full genome-based classification of rotavirus by determining gene sequences for group A rotavirus strains to refine the genome of individual rotavirus strain to be the complete descriptor of Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (26). Based on this conception, the Rotavirus Classification Working Group (RCWG) proposed a uniformity of rotavirus strain nomenclature as rotavirus group/species of origin/country of identification/common name/year of identification/G- and P-type. With this approach, until April 2011, there were 51 completely new genotypes including VP7 (G20-G27), VP4 (P[28]-P[35]), VP6 (I12-I16), VP1 (R5-R9), VP2 (C6-C9), VP3 (M7-M8), NSP1 (A15-A16), NSP2 (N6-N9), NSP3 (T8-T12), NSP4 (E12-E14), and NSP5/6 (H7-H11) for rotavirus strains recovered from humans, cows, pigs, horses, mice, South American Camelids (guanaco), chickens, turkeys, pheasants, bats, and a sugar glider being ratified (25).

The burden of rotavirus infection

To date, the burden of rotavirus infection remains considerable. Based on findings from the WHO, in 2008, diarrhea caused by the rotavirus infections leads to around half million deaths in children < 5 years old globally, and mainly in non-developed countries (above half of total deaths caused by the rotavirus are from five countries including the Democratic Republic of the Congo, Ethiopia, India, Nigeria, and Pakistan) (27), among these countries, 25% of the deaths come from India (Figure 3) (1). Rotavirus infections are frequently transmitted from person to person, and

improvements in hygiene and sanitation are not effective to prevent this pathogen, which is very important reason for causing the augment of the etiology of serious diarrhea in children caused by rotavirus infections (1). Rotavirus infections have the characteristics of seasonality, implying that winter and spring are prevalent seasons for rotavirus caused diarrhea, whilst the risks for the rotavirus causing enterogastritis in the summer are minimum, and higher temperature and humidity were reported to benefit the decrease of rotavirus infection (28). Besides in developing countries, rotavirus infections bring a large burden for many developed countries. Since the environmental interventions including sanitation and purifying water preventing infections of many bacterial and parasites whilst they were reported to be ineffective for preventing rotavirus infection, which might be the reason why the proportion of rotavirus causing serious diarrhea admissions is higher in industrialized countries compared to developing countries (29). In America, it was reported that there were ~55,000 – 70,000 hospitalizations being caused by rotavirus and ~600,000 rotavirus related physician visits in infants younger than five years old annually (29). In the European Union, there were 231 deaths due to rotavirus infections, more than 87,000 hospitalizations and around 700,000 outpatient visits being caused by rotavirus infections annually (30). In Canada, rotavirus infections were estimated to result in between 4500 and 10000 hospitalizations (31). In fact, a limited number of patients are routinely tested for rotavirus in the clinic, which sometimes results in underestimating rotavirus caused hospitalizations in administrative databases (31). Hereto, more attentions should be paid to rotavirus infections to accumulate comprehensive databases to increase understanding of rotavirus infections, which will attribute to minimizing the burden of this pathogen.

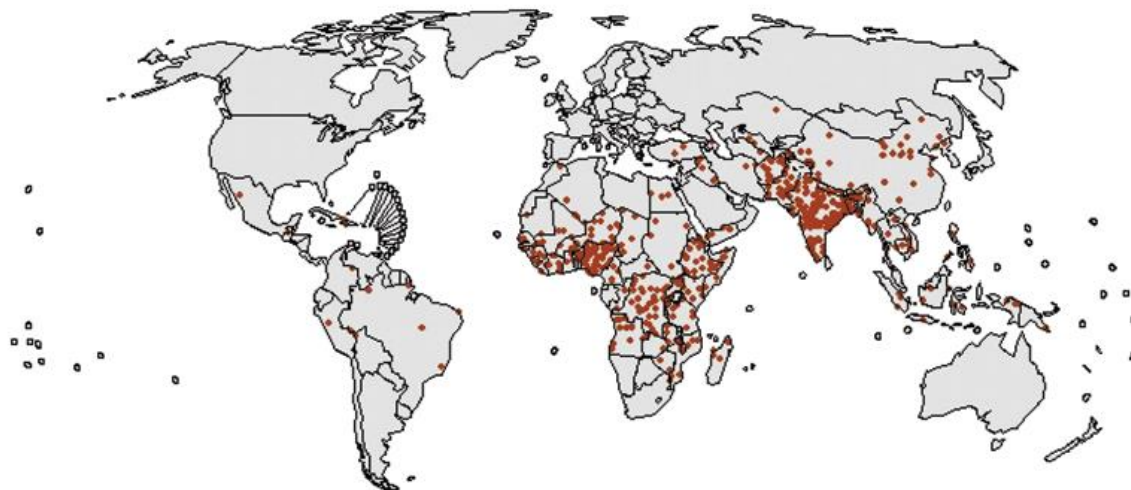


Figure 3. The distribution of deaths caused by rotavirus infections in infants younger than five years old in the world. 1000 rotavirus corresponding deaths per dot (1).

Rotavirus infection in organ transplanted and IBD patients

Normally, the rotavirus does not infect adults or is asymptomatic, while organ transplantation patients were reported to be sensitive to a rotavirus infection irrespective of their ages (32). It was confirmed that the average incidence of rotavirus infections in organ recipients was 3.03%, and intestinal transplant patients, live transplant patients, as well as hematopoietic stem cell transplantation (HSCT) recipients are most susceptible to rotavirus infection (32). Rotavirus caused diarrhea is often acute and self-limiting, while it is capable of resulting in chronic diarrhea even organ transplantation fail in organ transplantation patients (32). Eight deaths caused by rotavirus infection were reported out of 6176 organ recipients (32). The clinical consequences caused by a rotavirus infection in particular after organ transplantation still remain unclear, since very less cohorts are reported in developing countries and most rotavirus infected organ transplantation cares are from industrialized countries (32), which hampers the understanding of pathogenesis of a rotavirus infection in organ recipients and development of effective treatments to heal this disease. Additionally, it is plausible that the rotavirus is one of causal agents resulting inflammatory bowel disease (33).

The models of studying rotavirus

Valid and proper models are vital if we hope to increase our understanding of the pathogenesis and virology of any virus for developing novel effective antiviral therapy against viruses' infections. The models for investigating viruses include immortalized cells, primary cells, and animals. The advantages of immortalized cells include (i) they can be passaged for quite long time; (ii) they are more sensitive for virus infection compared to other models to make it easier to investigate virus-host interactions. However, these cell lines have many unsatisfied features such as (i) they are cancer cells that cannot exactly represent *in vivo* situation; (ii) they only consist of a single cell population, which makes them difficult to mimic complicated context in the body. For rotavirus investigations, the most commonly used cell model is Caucasian colon adenocarcinoma cell line (Caco2) that has been utilized as a classical model for studying the physiological functions of the intestine since 1989, since Caco2 cells retain the features of permeability and mucosal transport of intestine *in vivo* (34). However, it was pointed out that there were numerous sub-cloned derivatives of Caco2 cells, they displayed a large difference of protein expression and localization, and these derivatives caused uniform results in different laboratories (34). Animal models are often considered to be an optimal model for investigating pathogens, since they are thought to be closest to mimic complex physiological context in the human body. Nevertheless, animal models themselves have many unsatisfied aspects, because they often cannot reflect human physiology, for instance, it has a great difference in human population based on the results of preclinical testing (35). Animal right activists are appealing to reduce the number of animals used in the research. Furthermore, as a dsRNA virus, the rotavirus contains 11 considerably variable segments representing more than 110,000 strains, even the distinct strains are continuously emerging (36). Until now, only a few well-adapted laboratory strains are utilized as experimental platforms, this means that current using models for studying rotavirus are unsatisfied (36). Thus, a model with features that include the potential for long-term culture and encapsulating the presence of multiple cell types as are present *in vivo* while being non-transformed and having the possibility to easily capture interindividual differences would be of great value.

In 2009, the group of Prof. dr. Hans Clevers from Hubrecht institute (Utrecht, Netherlands) generated a primary intestinal organoids from the intestinal stem cells (ISCs) (37). They utilized features that epithelial cells of small intestine have higher self-renewal rates to culture intestinal organoids those are called MIMI-gut. Four well-

characterized signaling pathways are involved in intestinal homeostasis (Figure 4) including: (i) Wnt factors engage frizzled-lrp5/6 co-receptors to stabilize its downstream target β -catenin, subsequently trigger stimulation of the transcription factor Tcf4 to support stemness; (ii) Notch plays a vital role in maintaining the undifferentiated state. For intestinal stem cells, their Notch1/2 is able to augment neighboring Dll1⁺Dll4⁺ Paneth cells to initiate Notch signaling to prevent these cells from secretory differentiation. The ligand of Notch ligand Dll1/4 is capable of engaging Notch, which results in releasing Notch intracellular domain, then trigger the inhibition of the gene expression to monitor secretory differentiation under involvement of the nuclear effector RBP-J; (iii) EGF is engaged by its receptor (EGFR) to activate cellular Ras/Raf/Mek/Erk pathways to promote stemness; (iv) BMP is engaged by its receptor (BMPR) to drive the generation of Smad1/5/8 and Smad4 to suppress stemness. Inhibition of BMP by Noggin to benefit for stemness (38). This organoid model has many advantages compared with immortalized cancer cells and animal models, (i) it is considered to recapitulate the various cell types of the *in vivo* epithelium of the intestine, which can circumvent many shortcomings of tumor cells; (ii) it is more time saving and more cost-effective for developing novel medicines; (iii) it reduces the use of animals, which can circumvent issues of animal welfare (34). The organoids model has promising implementations as the platform for the experiments, diagnosing diseases and novel therapies for serious diseases (38). It has not, however, been assessed as a potential tool in rotavirus research.

The current treatments and antivirals of rotavirus infection

Until now, there has no specific medicine treating a rotavirus infection except supportive care including rehydration, maintenance of fluid and electrolyte balance (36, 39). Currently, there are two effective vaccines approved by the FDA named RotaTeq (RV5) and Rotarix (RV1), respectively. These vaccines indeed reduced the burden of a rotavirus infection, since the deaths caused by rotavirus infections globally for infants less than five years old was reduced to approximately 200,000 from half a million deaths in 2008 (40). Nevertheless, vaccines are not fully effective and new episodes may occur even in vaccinated children (41). For most non-developed countries, the protective efficiency from the vaccines is still less than

optimal (42). Hence, antiviral therapy represents an important complement to combat rotavirus infection that cannot be substituted by vaccines. For those resource-limited regions, it is more accessible and cost-effective to identify effective treatment with the existing approved medications.

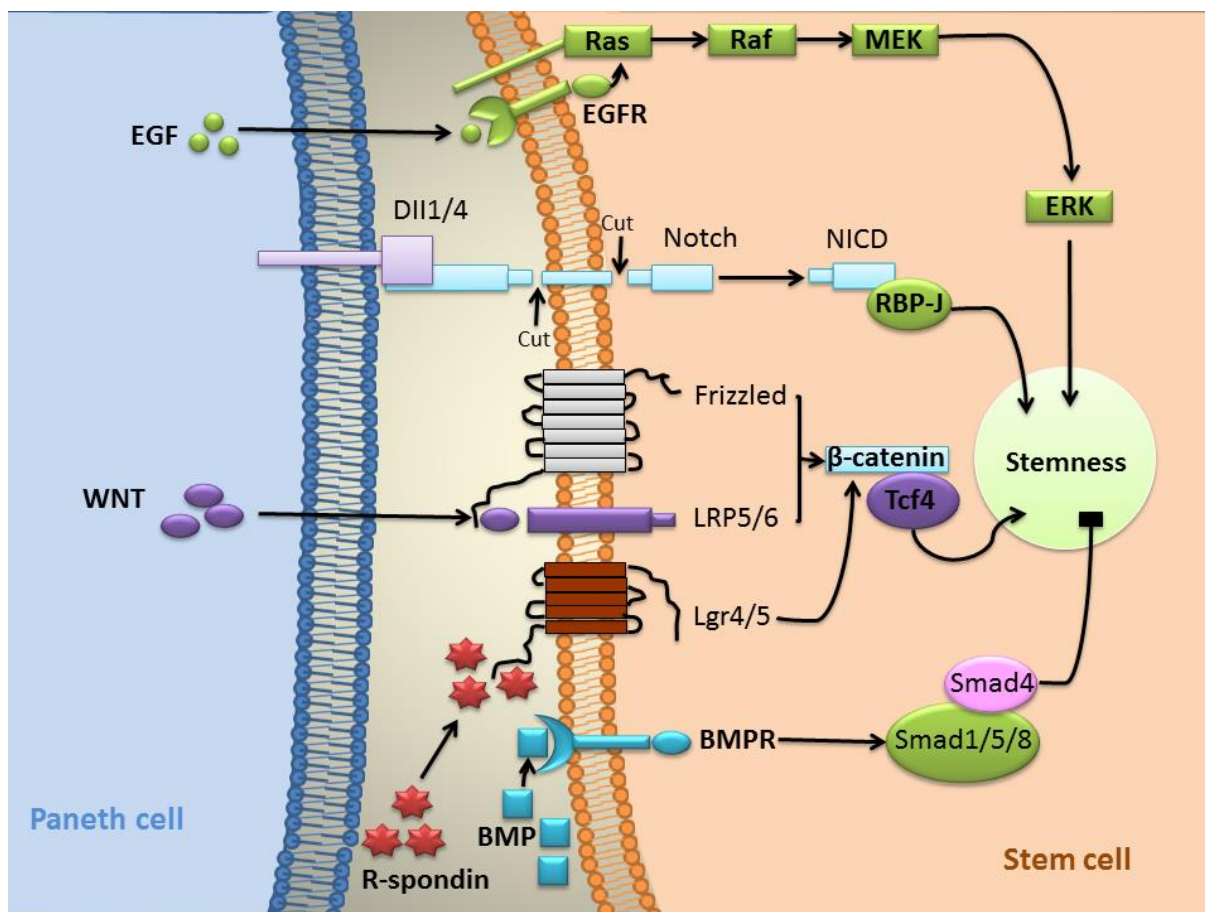


Figure 4. EGF, Notch, and Wnt signaling pathways are mainly involving in regulating the stemness of intestinal epithelium. For BMP, it is a negative regulator of stemness. R-spondin-Lgr4/5 signal plays a vital role in stimulating Wnt in the epithelial cells of intestine. R-spondin protein is the activator in the signaling (38).

AIM AND OUTLINE OF THIS THESIS

Rotavirus infection still brings a serious burden for infants below five years old especially in developing countries, and has been recognised as an important complication in organ transplantation patients irrespective of their ages. Current models including immortalized cancer cells and animal models are far from

satisfaction and the lack of proper models hampers the understanding of virus-host interactions and development of effective vaccines and antivirals. Many cellular signaling pathways are essential for regulating the course of viral infections, and many genes of particular signaling might be a target for development of novel antivirals.

Aims

The aims of the work presented in this thesis were to highlight the importance of rotavirus infection in organ transplantation, to increase our understanding of rotavirus-host interactions, to investigate the effects of various common immunosuppressants with respect to a rotavirus infection and characterize the potency of widely used antivirals, including interferon and ribavirin on most common gastrointestinal viruses including rotavirus and norovirus. Furthermore, I aim to get further insight in the effects of some important cellular signalings including PI3K-Akt-mTOR pathway and Rac1 signaling in the biology of rotavirus, as also with a view to their suitability for therapeutic targeting.

Focus

The work is focused on two aspects that have received relatively little attention hitherto in the field: the importance of rotavirus and norovirus on organ transplantation patients, to this end intestinal primary intestinal organoids are used as novel platforms for the study rotavirus infections and antiviral therapies, and how PI3K-Akt-mTOR pathway and Rac1 signaling regulates a rotavirus infection.

Outline

In this thesis, data are presented on the importance of rotavirus in organ transplantation patients and the investigation of intestinal organoids model as a potent novel platform for rotavirus infection and for discovering antiviral therapy (Figure 5). The thesis also describes how commonly used immunosuppressive medicines and several cellular signaling pathways regulates the course of infection of enteric viruses including rotavirus and norovirus (Figure 5). **In chapter 2**, it describes that rotavirus infection could cause serious gastroenteritis complications and occasionally result in the loss of grafts in organ transplantation settings, and it was

found an average incidence of 3% (there are in total 187 rotavirus infections being found from 6176 transplantation patients). The aim of this chapter is to comprehensively evaluate the impact from a rotavirus infection in a particular group of organ recipients and to gain understanding about rotavirus-host-immunosuppressants. **In chapter 3**, both primary organoids models from two species including mouse and human were successfully cultured, and they were confirmed to be easily infected by rotavirus. It was also demonstrated that human organoid models are considerably sensitive for the infection of patient-derived rotavirus strains. Human primary intestinal organoids might bring insight in individualized assessment of the efficacy of antiviral drugs and be potentially useful for developing novel antivirals. **In chapter 4**, we profiled the effect of different immunosuppressive medicines on laboratory and patient-derived rotavirus strains, and found that mycophenolic acid (MPA) resulted in a potent inhibitory effect on both rotavirus strains with a high barrier to drug resistance development. We proposed that the choice of MPA as an immunosuppressive agent appears rational. **In chapter 5**, a commonly used drug as an immunosuppressive medicine in organ transplantation patients and for treating inflammatory bowel disease (IBD) patients, 6-TG was demonstrated to potently inhibit the rotavirus infection with a high barrier for drug resistance emergence. Mechanistically, as the cellular target of 6-TG, Rac1 is essential in supporting rotavirus infection. 6-TG could effectively inhibit the active form of Rac1 (GTP-Rac1), which was involved in antiviral machinery. **Chapter 6** describes an important cellular signaling pathway, PI3K-Akt-mTOR-4EBP1 signaling is essential for regulating the infection course of the rotavirus. This effect involves 4E-BP1 mediated induction of autophagy, which in turn exerts anti-rotavirus effects. This study reveals new insight on rotavirus-host interaction and provides new avenues for antiviral drug development. **In Chapter 7**, three components of the eIF4F including eukaryotic translation initiation factor 4A (eIF4A), eukaryotic translation initiation factor 4E (eIF4E) and eukaryotic translation initiation factor 4G (eIF4G) exert anti-rotavirus activities, which implies that targeting substantial components of eIF4F might bring bright sights in developing creative antivirals against rotavirus. **Chapter 8** describes the direct effects of immunosuppressants on norovirus and underlying mechanism-of-action. Calcineurin inhibitors including cyclosporine A (CsA) and tacrolimus (FK506) moderately inhibit human norovirus replication through calcineurin. Mycophenolic

acid (MPA) could potentially inhibit norovirus infection via suppressing IMPDH1 and IMPDH2 with high drug resistance development.

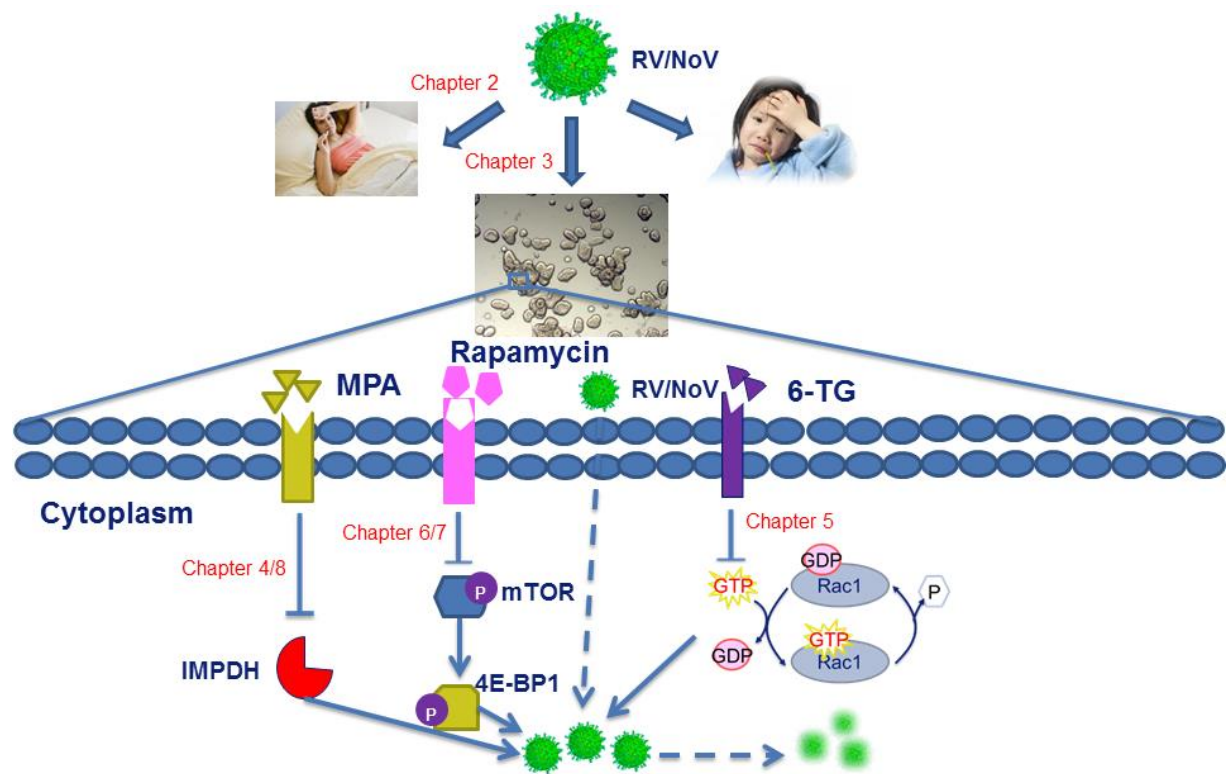


Figure 5. The outline of this thesis.

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CHAPTER 2

Rotavirus in organ transplantation: drug-virus-host interactions

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Abstract:

Although rotavirus is usually recognized as the most common etiology of diarrhea in young children, it can in fact cause severe diseases in organ transplantation recipients irrespective of pediatric or adult patients. This comprehensive literature analysis revealed 199 cases of rotavirus infection with 8 related deaths in the setting of organ transplantation been recorded. Based on published cohorts studies, an average incidence of 3% (187 infections out of 6176 organ recipients) was estimated. Rotavirus infection often causes severe gastroenteritis complications and occasionally contributes to acute cellular rejection in these patients. Immunosuppressive agents, universally used after organ transplantation to prevent organ rejection, conceivably play an important role in such a severe pathogenesis. Interestingly, rotavirus can in turn affect the absorption and metabolism of particular immunosuppressive medications via several distinct mechanisms. Even though rotaviral enteritis is self-limiting in general, infected transplantation patients are usually treated with intensive care, rehydration and replacement of nutrition, as well as applying preventive strategies. This article aims to properly assess the clinical impact of rotavirus infection in the setting of organ transplantation and to disseminate the interactions among the virus, host and immunosuppressive medications.

Key words: rotavirus; organ transplantation; incidence; immunosuppressive agents; pathogenesis

Introduction:

Rotavirus is a member of the *Reoviridae* family. It is a non-enveloped double stranded RNA (dsRNA) virus containing 11 genomic segments, which are surrounded by three concentric protein layers (1). The structure of the virus particle is constituted of six structural proteins (VP1 to VP4, VP6 and VP7), and its lifecycle in the host is supported by six non-structural proteins (NSP1 to NSP6) (2).

Rotavirus infection is the main cause of severe dehydrating enteritis diarrhea in infants and young children under age five and is characterized by acute vomiting, diarrhea and fever (3). Based on an estimation by the WHO, rotavirus-related severe diarrhea leads annually to approximately 2 million hospitalizations and 600,000 deaths, mainly in developing countries (4). In contrast, infections in adults are often asymptomatic or mild (5). However, emerging evidence indicates that organ transplantation patients, both with respect to pediatric and adult organ recipients, are particularly sensitive to rotavirus infection. In adult recipients, even, severe gastroenteritis and related morbidity and mortality have been reported (6, 7).

Although the etiologic agents of infectious diarrhea in transplantation patients have already been aware of, which include bacteria, parasites and viruses as such cytomegalovirus, norovirus, adenovirus and rotavirus, these causative agents are largely underestimated and poorly investigated in this particular setting (7). Given the limited information available about the epidemiology, etiology, clinical consequence as well as management of gastroenteritis caused by rotavirus in organ recipients, we have endeavored to extract from the literature all available information. To this end, we performed a comprehensive analysis by collecting information from series of case and cohort studies, allowing us to properly assess its clinical impact of rotavirus infection in organ transplantations and understand its pathogenesis. We feel more attention for this important health issue is warranted.

Incidence of rotavirus infection in organ transplantation patients

Severe gastrointestinal symptoms, comprising mainly of diarrhea, were reported to be frequent complications (up to 50%) in organ transplantation patients (7-9). Viruses are the most common underlying pathogens and are associated with 70% of cases (7, 9). Given that rotavirus infection is the leading cause of diarrhea in children, we performed systematic analysis to first assess its incidence in organ recipients.

Data were collected and extracted from published cohort and case studies dated back from 1982 until recently and originated from Australia (10), Austria (6, 8, 11-15), Canada (16), France (17, 18), Germany (19-21), India (22), Iran (23), Italy (24), Japan (25, 26), Netherlands (27), Turkey (28, 29), UK (9), (30), and USA (31-40) (Table 1). These patients were ranged in different age categories from very young to very old. Rotavirus infection was diagnosed in both solid organ transplant (SOT) recipients (6) and bone marrow (9, 38) or hematopoietic stem cell transplantation (HSCT) recipients (6, 19, 31, 34). In total, 199 cases with 8 related deaths were reported. Based on cohort studies only, an average incidence of 3.03% (187 infections out of 6176 organ recipients) was estimated (Table 1). Such infection can occur at any stage as early as 1 month following transplantation to as late as 8 years after the operation (41). Intestine transplant patients (12.95%), liver transplant patients (4.44%) and HSC transplant patients (4.40%) were more susceptible to rotavirus infection compared with other types of transplantation (Figure 1), although the reason behind this discrepancy remains unclear.

In analog to the burden of rotavirus infection in children, organ transplantations are mainly implemented in industrialized countries and thus data reporting rotavirus infection in organ recipients are predominately from the Western world. Intriguingly, epidemics of rotavirus infection in young children have seasonality and often occur in winter and spring (42, 43). It is inconclusive whether this is similar in organ transplant patients, although five studies observed a preferential incidence in winter and spring in their cohorts (9, 17, 18, 26, 40). It is still a mystery how these patients get infected. Fecal-oral infection is the most common transmission route and poor hygiene accounts for spreading of the virus among children in developing world (44). In the setting of transplantation, it is possible that the virus is transmitted from infected children or healthcare workers to organ transplant patients during an outbreak, since rotavirus easily presents with nosocomial infection (20, 45). Indeed, hospitalization and frequent hospital visiting may be important risk factors (15, 17, 45). Overall, the incidence of rotavirus infection in transplantation setting is considerably high according to these reported cohort studies (Table 1). This may be due to the relatively low number of reported infections or unawareness and under-diagnosis of rotavirus infection in transplantation patients. Furthermore, the use of immunosuppressive agents is conceivably an important risk factor, which will be extensively emphasized in the following sections.

Reference	Year	Total Patients	Patients	Incidence (%)	Type of Tx infected by RV	Immunosuppressants	Symptom	Death	Diagnosis	Region
Blakley <i>et al</i> (10)	1989	12	1	8.33%	BMTx	NP	Diarrhea		ELISA	Australia
Berger <i>et al</i> (11)	2006	1	1		PTx	ATG/TAC/MMF	NP		NP	Austria
Frühwirth <i>et al</i> (12)	2001	15	3	20%	LTx/KTx/Lung Tx	TAC/AZA/Pred	Vomiting /Diarrhea		ELISA/qPCR	Austria
Hochleitner <i>et al</i> (13)	2001	1	1		LTx	TAC/Steroids/AZA	Gastroenteritis		NP	Austria
Steizmueller <i>et al</i> (8)	2005	4	4	4.70%	LTx /KTx	TAC/Steroids/AZA	Diarrhea /Blood & Weight loss		ELISA	Austria
Steizmueller <i>et al</i> (14)	2006	344	16	4.70%	LTx	TAC	Diarrhea		IA/ELISA	Austria
Steizmueller <i>et al</i> (6)	2007	1303	19	1.50%	LTx/KTx/PTx-KTx	MMF/TAC	Diarrhea /Gastrointestinal bleeding	2	IA/ELISA	Austria
Steizmueller <i>et al</i> (15)	2007	2799	21	0.75%	LTx/KTx/PTx	CNI based triple-therapy/TAC	Diarrhea/Abdominal pain	1	ELISA	Austria
Adeyi <i>et al</i> (41)	2010	71	23	32.40%	MTx/SBTx	TAC	Diarrhea /Chronic rejection/ACR		ELISA	Canada
Peigue-Lafeuille <i>et al</i> (17)	1991	20	4	20%	KTx	NP	Diarrhoea/Fever/Abdominal pains		ELISA/EM	France
Troussard <i>et al</i> (18)	1993	94	8	8.51%	BMTx	NP	Diarrhea/Vomiting/Abdominal pains		LAT	France
Ernst <i>et al</i> (19)	2012	1	1		HSCTx	NP	Gastroenteritis		ELISA	Germany
Kruger <i>et al</i> (20)	1991	1	1		BMTx	NP	Diarrhea/Fever		ELISA	Germany
Pascher <i>et al</i> (21)	2004	11	1	9.09%	ITx	TAC/Sirolimus/Pred	ACR/Enteritis		ELISA	Germany
Kang <i>et al</i> (22)	2002	65	8	12.30%	BMTx	NP	Gastroenteritis	1	EM/LAT	India
Yaghoobi <i>et al</i> (23)	2011	22	1	4.55%	LTx	NP	NP		qPCR	Iran
Giovanelli <i>et al</i> (24)	2008	1	1		LTx-SBTx	TAC/Sirolimus/Steroids	Gastroenteritis /ACR		NP	Italy
Sakamoto <i>et al</i> (25)	2008	15	2	13.30%	LTx	AZA/MMF/Pred/TAC	Enteritis	2	NP	Japan
Sugata <i>et al</i> (26)	2012	62	8	12.90%	BMTx /CBTx/PBSCTx	CsA/TAC	Diarrhea /Toxic Megacolon		ELISA	Japan
van Kraaij <i>et al</i> (27)	2000	60	4	6.70%	BMTx	CsA	Diarrhoea/Fever/Nausea/Vomiting		ELISA	Netherlands
Arslan <i>et al</i> (28)	2007	43	1	2.32%	LTx	Pred/TAC/MMF/CsA/AZA	Abdominal pain/Fatigue/Nausea/Loos appetite/Fever/Vomiting		IA	Turkey
Tumgor <i>et al</i> (29)	2008	1	1		LTx	TAC/ Steroids	Gastroenteritis		ELISA	Turkey
Liakopoulou <i>et al</i> (9)	2005	500	21	4.20%	BMTx	CsA/TAC/Steroids	Diarrhoea/Vomiting /Abdominal pain/ Weight & appetite loss	1	EM/ELISA	UK
Papadopoulou <i>et al</i> (30)	1997	21	2	9.52%	BMTx	NP	Diarrhoea		NP	UK
Cox <i>et al</i> (31)	1994	296	2	0.68%	BMTx	NP	Diarrhoea		NP	USA
Fitts <i>et al</i> (32)	1995	12	12		LTx	NP	Vomiting/blood stool		NP	USA
Fleenor <i>et al</i> (33)	2002	116	3	2.60%	HTx/LTx	Steroid/TAC/CsA/AZA/MMF	Bloody diarrhear/Abdominal pain		ELISA	USA

Continued

Kamboj <i>et al</i> (34)	2007	169	2	1,18%	HSCTx	NP	Diarrhea	ELISA	USA
Kaufman <i>et al</i> (35)	2003	1	1		LTx-ITx	Steroids/TAC/Sirolimus	Diarrhea	ELISA	USA
Patel <i>et al</i> (41)	2012	1	1		HSCT	NP	Diarrhea	EM/qPCR	USA
Toomey <i>et al</i> (36)	1988	7	4	57,14%	HTx	CsA/Pred/AZA	Jejunal folds/Transient intussusception	NP	USA
Williams <i>et al</i> (37)	2014	8	4	50,00%	BMTx	NP	Diarrhea	ELISA	USA
Willoughby <i>et al</i> (38)	1988	1	1		BMTx	NP	Vomiting/Abdominal pain/Bloody stools	ELISA	USA
Yolken <i>et al</i> (39)	1982	78	9	11,54%	BMTx	NP	NP	NP	USA
Ziring <i>et al</i> (40)	2005	33	8	24,20%	ITx	Steroids/TAC/MMF	Enteritis/ACR	ELISA	USA
Total		6189	200						8

Table 1. Incidence of rotavirus infection in organ transplantation patients. RV: Rotavirus; LTx: liver transplantation; HTx: heart transplantation; KTx: kidney transplantation; BMTx: bone marrow transplantation; Lung Tx: lung transplantation; ITx: intestinal transplantation; PTx: pancreas transplantation; HSCTx: hematopoietic stem cell transplantation; CBTx: cord blood transplantation; PBSTx: peripheral blood stem cell transplantation; MTx: multiple organ transplantation; SBTx: small bowel transplantation; AZA: azathioprine; TAC: tacrolimus; Pred: Prednisolone; MMF: mycophenolate mofetil; CNI: calcineurin inhibitor; CsA: cyclosporin A; qPCR: quantitative real-time polymerase chain reaction; EM: electron microscopic; IA: Immunochromotographic assay; LAT: Latex agglutination test; ACR: acute cellular rejection; NP: information not present.

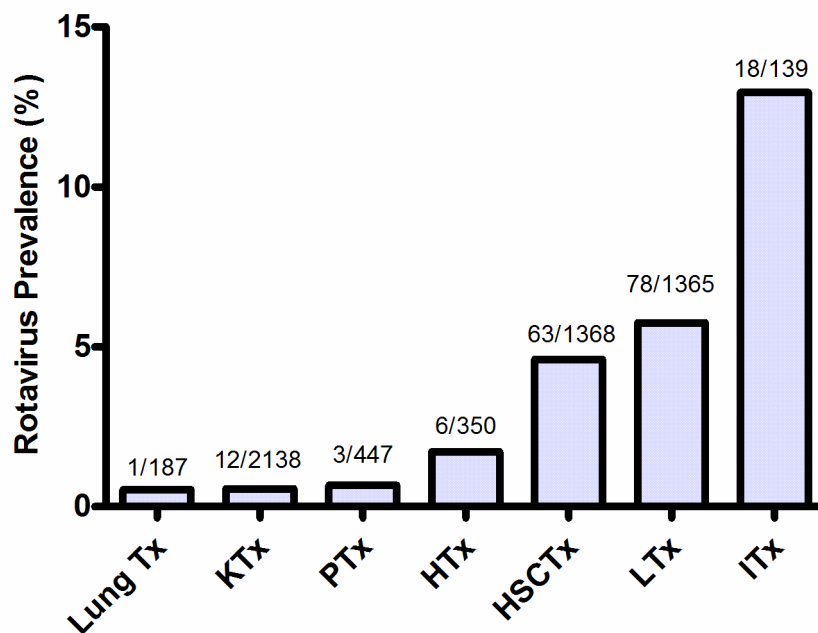


Figure 1. Rotavirus prevalence in various types of transplantation patients. HTx: heart transplantation; KTx: kidney transplantation; Lung Tx: lung transplantation; HTx: heart transplantation; LTx: live transplantation; ITx: intestinal transplantation; HSCTx: hematopoietic stem cell transplantation; including bone marrow transplantation, cord blood transplantation and peripheral blood stem cell transplantation.

Diagnosis of rotavirus infection in organ recipients

The cause of diarrhea in transplantation patients may not be so straightforward to be diagnosed. In HSCT patients, rotavirus caused diarrhea is difficult to differentiate from graft-versus-host disease (GVHD) (9). Thus, multiple approaches are used for rotavirus diagnosis, including an enzyme-linked immunosorbent assay (ELISA) (6, 12, 26, 41), (transmission) electron microscopy (EM) (9), a latex agglutination test (22), immunochromatography (6) and quantitative polymerase chain reaction (qPCR) (12, 40). ELISA is the most widely used assay for rapidly detecting rotavirus antigens in stool samples (Table 1). However, PCR has much higher specificity and sensitivity (7), which can even be used for strain identification to provide more detailed virological characterization (19, 46). Therefore, it is probably a viable option for routine diagnosis in transplantation patients (7). An urgent unresolved issue though is who should be evaluated: pediatric, adult, or all organ recipients with diarrhea? As a starting point, all stool specimens from organ recipients with diarrhea, irrespective of whether they are children or adults, probably should be routinely tested for rotavirus

to avoid underestimation and to take appropriate measures to prevent spreading of this highly contagious disease, especially in the context of hospitalization (15).

Pathogenesis

Rotaviruses are further classified into seven serogroups (A - G) based on their antigenic profiles (47). Group A is the most common cause of gastroenteritis in young children (48) and in transplantation patients (12). With respect to the mechanism, a non-structural protein, NSP4, is an essential enterotoxin causing diarrhea (49). Rotavirus can alter organization of the enterocyte cytoskeleton, trigger structural and functional alterations of the tight junctions, and induce cell injury and cell death by apoptosis (50). Especially, rotavirus infects superficial mature enterocytes close to the villi's tips of the small intestine, and replicates inside these cells, which results in functional alteration of the epithelial lining, thereby causing diarrhea (1, 4, 44). The severity in organ recipients was associated with lymphopenia, immunosuppression, GVHD and co-infection of other viruses, in particular in HSCT patients (9, 51).

In addition to severe diarrhea, rotavirus may also promote acute cellular rejection (ACR), especially in intestinal transplant patients (21, 40, 41, 52). In a cohort of 23 rotavirus-infected patients with small bowel transplantation (including 2 patients with multiple organ transplant), 16 patients (70%) developed ACR (41). It occurred late after transplantation at an average of 562 days after surgery (41). The possible explanation is that rotavirus infection may stimulate the host immune system. This is probably related to cellular infiltration of gut-associated lymphoid tissue (GALT). And the infiltrated GALT areas are sites of intense immune activation (52). Many viruses including hepatitis C virus, measles virus and HIV are known to cause diseases by nonspecific immune dysregulation, although it is still unclear whether rotavirus causes ACR via similar mechanisms (41). On the other hand, episodes including vomiting and diarrhea can result in poor immunosuppressant absorption and an associated drop in the efficacy of the immunosuppressive medication (41). Rotavirus caused ACR may lead to graft loss and requirement of re-transplantation (40, 41, 52).

The unique role of immunosuppressive agents

Immunosuppressive agents with distinct mechanism-of-action include corticosteroids (prednisolone and dexamethasone), calcineurin inhibitors (cyclosporine A (CsA) and

tacrolimus), mammalian target of rapamycin (mTOR) inhibitors (rapamycin and everolimus), selective antiproliferative agents (mycophenolate mofetil (MMF), mycophenolate acid (MPA) and azathioprine) and biological agents (monoclonal or polyclonal antibodies). It is not surprising that suppression of the immune system to prevent organ rejection will facilitate virus infection in general, since immune surveillance plays an essential role in defense against infection. In terms of rotavirus, T cells (CD8 and/or CD4) play crucial role in clearance of the virus (26, 53). In an infant with Severe Combined Immunodeficiency (SCID), successful T-cell engraftment after HSCT was reported to be associated with the clearance of rotavirus despite absent donor B cells (54). Since T cells are the primary targets of immunosuppressants, it is conceivable that these agents are associated with the severe pathogenesis of rotavirus infection in transplant patients. Although MMF (55) and tacrolimus (12, 29) were reported to be associated with a high risk of rotavirus-related enteritis among organ transplant recipients, caution still should be taken to interpret these clinical data because of limited patient number and other potential biases.

Apart from the general immunosuppressive consequences favoring infection, immunosuppressive agents could also directly affect the viral life cycle. The establishment of cell culture and small animal-based models has enabled investigation of these immunosuppressive agents on rotavirus infection (56, 57). CsA binds to cyclophilin to form a complex that can inhibit calcineurin, resulting in immunosuppression. Many viruses take the advantage of cyclophilins as host factors to support their infections (58). In contrast, cyclophilin was reported to suppress the infections of some other viruses, including rotavirus (59). Still, inhibition of cyclophilin by CsA has been shown to be antiviral against rotavirus *in vitro* and in mouse model, which was thought to be associated with restoration of the antiviral interferon signaling (60, 61). Other immunosuppressants including MPA, steroids and mTOR inhibitors have been implicated in modulating the infection of many viruses (58, 62, 63), although these have not been studied in rotavirus infection so far. Understanding such direct effects on a virus life cycle by different immunosuppressive agents is certainly relevant to the management of infected transplantation patients, as well as to the development of potential new antivirals. Thus, such type of translational research on rotavirus is urgently needed, because of the limited clinical data available to draw irrefutable conclusion.

The interaction between rotavirus and particular immunosuppressive agents

Symptoms of rotavirus infection like vomiting and diarrhea can cause poor immunosuppressant absorption (41). However, rotavirus infection is known to enhance the serum level of particular immunosuppressive agents in organ transplant recipients, which subsequently induces severe side effects including nephrotoxicity and neurotoxicity, and over immunosuppression with risk of infections (8, 64). The trough serum level of tacrolimus has been reported to be boosted by rotavirus infection in multiple settings of transplantation including liver transplant (12, 29, 64), kidney transplant (12) and lung transplantation (12). Substantial evidence suggests that the trough level in liver transplant recipients is more susceptible to be influenced by rotavirus infection compared with other organ transplantation (6). Although enhanced trough serum level of CsA was also observed in pediatric liver transplantation, it was found out that bacterial but not rotavirus related enteritis caused such an elevation of CsA level (64).

Four possible mechanisms have been proposed regarding the effects on tacrolimus trough level by rotavirus: 1) increased intestinal permeability (8, 12, 29, 65); 2) reduced hepatic metabolism (8, 13, 29, 65, 66); 3) decreased gastrointestinal transit time (12); 4) decreased activity of cytochrome p450 3A (CYP3A, a major phase I xenobiotic enzyme) and P-glycoprotein (P-gp) (12, 29, 66) (Figure 2).

Increased intestinal permeability by rotavirus is thought to be caused by diminished mitochondrial energy production (12). Decreased hepatic metabolism includes hepatic dysfunction and reduced hepatic blood flow due to sub-acute dehydration (8, 29, 65, 66), which may lead to decreased hepatic clearance of tacrolimus (66).

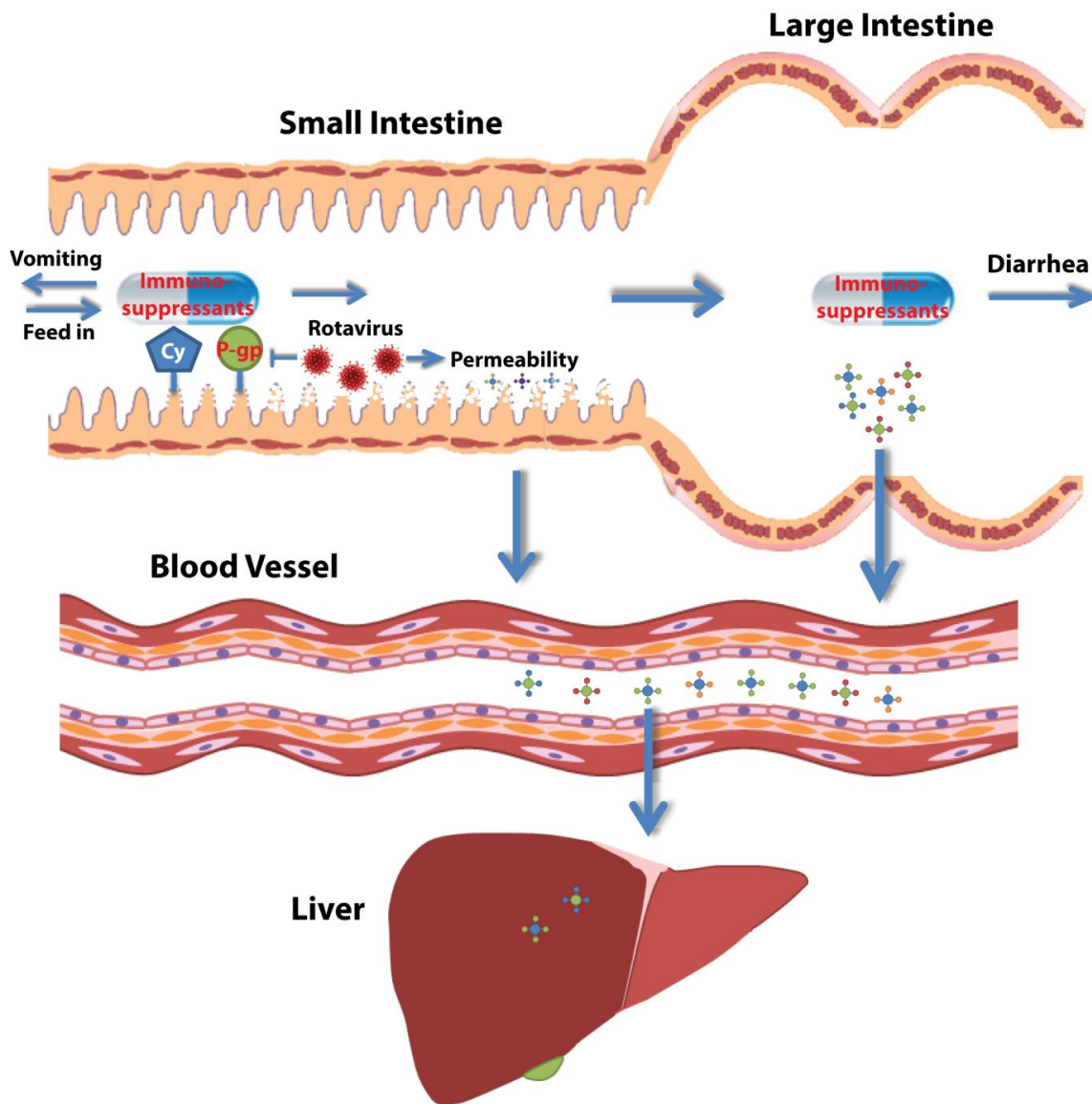


Figure 2. Rotavirus infection affects the absorption and metabolism of immunosuppressive agents in organ transplant recipients. Upon oral administration of immunosuppressive agents, rotavirus induced vomiting and diarrhea can cause poor absorption, resulting in potentially insufficient immunosuppression. On the other hand, rotavirus can increase epithelial permeability, impair pharmacokinetic enzymes/transporters such as CYP3A4 and P-gp, decrease transit time in small intestine, and attenuate hepatic metabolism, which eventually enhance the trough level of particular immunosuppressive agents.

Metabolite formation within the gastrointestinal track descends in the following order: duodenum > jejunum > ileum > colon > stomach (66). This means that a decreased gastrointestinal transit time caused by rotavirus infection forces the metabolism of tacrolimus in lower intestinal tissue (12, 66). Oxidation of 50-60% of clinical drugs is through CYP3A4 enzyme (67). CYP3A4 is highly expressed at the apex of the villus enterocytes adjacent to the microvillus border, whereas the crypt cells have null expression. Rotavirus severely destroys the tip of the villi in small

intestine, thereby diminishing activity of CYP3A4 (65). As a multidrug transporter, P-gp activity is also lessened following destruction of small intestinal epithelial cells by rotavirus (66). Both CYP3A4 and P-gp are barriers to drug absorption in small intestine and both tacrolimus and CsA are dual substrates for CYP3A and P-gp (64). Experimental evidence has confirmed the notion that the enhancement of tacrolimus trough level is mediated by a rotavirus-induced reduction of CYP3A4 enzymatic activity in conjunction with reduced P-gp function (64). Taken together, rotavirus infection increases small intestinal permeability, destroys pharmacokinetic elements, decreases gastrointestinal transit time and disturbs hepatic mechanisms to increase the amount of immunosuppressive agents entering the circulatory system in organ transplant recipients (Figure 2). Of note, the definitive proof of this notion still requires prospective studies in patients in which the factors involved are measured and are related to incidental rotavirus infection. Until such time other possibilities should be kept in mind. Since acute rejection is as mentioned earlier, a serious complication of rotavirus infection in some transplant patients, the balance between poor absorption and metabolic changes leading to increased serum levels requires further confirmation from additional experimental and clinical investigations.

Management of rotavirus infection in organ recipients

Preventive strategies such as rigorous hand washing and disinfectants with proven virucidal activity according to the manufacturer's specification have been implied to avoid nosocomial transmission (6, 7, 9, 15, 27). Effective vaccines, such as RotaTeq (RV5) and Rotarix (RV1), were recommended to be used prior to transplantation for pediatric transplantation patients in particular cases (6, 7, 68, 69), since vaccine is often poorly immunogenic after transplantation. But for adult recipients, little data is available (68, 69). The Centers for Disease Control and Prevention (CDC) recommends caution of using rotavirus vaccine in immunodeficient patients; whereas the WHO contraindicates it in severely immunosuppressed patients (68). Thus, more studies are needed to determine safety and immunogenicity of these live rotavirus vaccines in this immunocompromised population.

In general, diarrhea caused by rotavirus is often self-limiting and lasts only for several days. Therefore specific treatment is not necessary (41). Nevertheless, when confronted with rotavirus-infected transplantation patients, treatment with intensive

care, rehydration, and replacement of nutrition and electrolytes is indicated (6, 8, 14), precisely because of the absence of specific therapy (7-9). Loperamide (one to two tablets and another tablet after every bowel movement) has been suggested for diarrhea control in adult organ transplant patients (6, 15). Enteral immunoglobulins (IVIG) have been reported to improve rotaviral diarrhea in HSCT patients (9, 37), although others reported failure of using IVIG prior to transplantation to eliminate rotavirus (54). The persistence and severity of rotaviral diarrhea was also thought to be associated with GVHD, although no clear correlation between rotavirus infection and the conditioning regimen was observed (9, 26). Co-infection with other viruses could result in prolongation of symptomatic period and secretion of the virus. These patients often require prolonged hospitalization and nutritional supplementation (9).

ACR caused by rotavirus should be promptly diagnosed and treated (40), since ACR is refractory and can cause allograft loss, and even death (25). Prompt and accurate therapies are necessary for successful treatment of ACR, and ribavirin and intravenous immunoglobulin were recommended to be administered following discontinuation of ACR therapy (40). It was worthwhile to analyze the specimens and discuss each case with transplant pathologists for patients with ACR (25). The immunosuppressant OKT3, a monoclonal antibody targeting the CD3 receptor, was suggested to be used when rejection is steroid-resistant (25, 40, 41). In addition, these rejections are characterized by a rich plasmacytic component in lamina propria inflammation, therefore antilymphocytes regimen was implemented in one-third of rejecting patients (41).

In case one encounters an increase in the trough level of immunosuppressants, and particularly in the case of tacrolimus, during rotavirus-induced diarrhea (15), this may result in nephrotoxicity, neurotoxicity and over immunosuppression (8, 12). Thus, lowering dosage of immunosuppressants is an option (6, 15, 66), although low dose should only be maintained until the infection is resolved (66). Alternatively, switching immunosuppressants might be a better choice to rehabilitate trough level (8, 12, 13).

Conclusions

Although rotavirus has been well-recognized as an important cause of severe enteritis in young children, its clinical consequences in the setting of organ transplantation remain largely under recognized, despite an estimated prevalence or

incidence of 3% in organ recipients with several related deaths as its consequence having been identified. The infection and subsequently caused pathology include severe diarrhea and ACR that can occur in patients irrespective of whether they are pediatric or adult recipients. Disease course, however, may vary substantially among different patients.

A unique feature of transplantation patients is an immunocompromised condition as a consequence of the use of immunosuppressive medication. This is presumably the most important reason why rotavirus causes such severe disease in these patients. In turn, rotavirus could affect the absorption and metabolism of certain immunosuppressive agents, which causes other complications. However, the interactions between rotavirus with immunosuppressive agents are likely to depend on the particular types of drug and exact elucidation of the interactions involved remains a compelling subject for experimental and clinical research.

Finally, we feel that enhanced moment to call awareness of the clinical issues at hand is called for. We hope that the transplantation community will soon reach consensus on the diagnosis and management of rotavirus infected organ recipients.

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CHAPTER 3

Modeling rotavirus infection and antiviral therapy using primary intestinal organoids

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Abstract

Despite the introduction of oral vaccines, rotavirus still kills over 450,000 children under five years of age annually. The absence of specific treatment prompts research aiming at further understanding of pathogenesis and the development of effective antiviral therapy, which in turn requires advanced experimental models. Given the intrinsic limitations of the classical rotavirus models using immortalized cell lines infected with laboratory-adapted strains in two dimensional cultures, our study aimed to model infection and antiviral therapy of both experimental and patient-derived rotavirus strains using three dimensional cultures of primary intestinal organoids. Intestinal epithelial organoids were successfully cultured from mouse or human gut tissues. These organoids recapitulate essential features of the *in vivo* tissue architecture, and are susceptible to rotavirus. Human organoids are more permissive to rotavirus infection, displaying an over 10,000-fold increase in genomic RNA following 24 hours of viral replication. Furthermore, infected organoids are capable of producing infectious rotavirus particles. Treatment of interferon-alpha or ribavirin inhibited viral replication in organoids of both species. Importantly, human organoids efficiently support the infection of patient-derived rotavirus strains and can be potentially harnessed for personalized evaluation of the efficacy of antiviral medications. Therefore, organoids provide a robust model system for studying rotavirus-host interactions and assessing antiviral medications.

Key words: Rotavirus, Intestinal organoids, Interferon, Ribavirin

1. Introduction

Rotavirus, a member of the *Reoviridae* family, is a leading cause of severe gastroenteritis in young children worldwide. It yearly causes 1.4 billion diarrhea episodes and over 450,000 deaths of children under five years of age (1). Although infections in adults are often asymptomatic or mild, emerging evidence indicates that organ transplantation patients, both pediatric and adult organ recipients, are particularly sensitive to rotavirus infection with severe gastroenteritis as well as other complications (2). Unfortunately, no specific antiviral drug against rotavirus is available except supportive care (3). The potential off-label use of general antiviral drugs, such as interferon-alpha (IFN- α) and ribavirin, have been poorly investigated (4).

The absence of a robust experimental *in vitro* system allowing investigation of rotavirus infection is a bottleneck hampering development of novel rational strategies aiming at combating rotavirus-associated diseases. It is well-known that rotavirus primarily infects complexly organized epithelium of the small intestine (5), while currently used cell culture models based on simple two dimensional (2D) cultures of immortalized cell lines (6-8) that do not capture the dynamics nor individual variation existing in the patient mucosa (6, 8). Complicating further is the genomic diversity in patient-derived rotavirus strains. Because rotavirus is a double stranded RNA (dsRNA) virus containing eleven highly variable segments (9). In total, more than 110,000 strains have been genotyped originating from 100 different countries and relative prevalence of different strains is continuously evolving (10). In contrast, the difficulty in setting up *in vitro* models has led to the situation that only a limited number of well-adapted laboratory strains are almost exclusively used for experimental research. Therefore, current approaches for modeling rotavirus infection are far from satisfactory (6), which prompts development of novel experimental avenues.

Three dimensional (3D) cultured primary intestinal organoids are currently innovating research of intestinal physiology and pathology. They contain various types of cells and recapitulate most if not all aspects of *in vivo* tissue architecture (11-13). Accordingly, organoids have been successfully used to model a plethora of diseases including cystic fibrosis (CF, an intestinal disease that has been usually difficult with respect to *in vitro* investigation) (14) and cancer (15). In the present

study, we explored the feasibility of using primary intestinal organoids to model rotavirus infection and antiviral therapy. We demonstrated that organoids, in particular of human origin, effectively support rotavirus infection of both laboratory-adapted and patient-derived strains, as well as production of infectious virus particles. Most interestingly, human organoids were able to assess the responsiveness of patient-derived rotavirus to different antivirals on an individual basis.

2. Materials and methods

2.1. Viruses

Simian rotavirus SA11, a well-characterized and broadly used laboratory strain was used (16). SA11 rotavirus used in the study was prepared as described previously (6). Stool samples (stored at -80°C freezer) from nine rotavirus patients were obtained from the Erasmus MC biobank, Department of Viroscience, Erasmus Medical Center Rotterdam. These samples were taken during diarrhea period and tested for enterovirus, parechovirus, norovirus genogroups I and II, rotavirus, adenovirus, astrovirus and sapovirus by qRT-PCR described by previous studies (10, 17, 18).

2.2. Cell culture

Caco2 cell line (Human Caucasian colon adenocarcinoma; ECACC) was cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 20% (vol/vol) heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis USA) and 100 U/mL of Penicillin-Streptomycin (Gibco, Grand Island, USA) solution. Cells were maintained in 5% CO₂ at 37°C in a humidified incubator. Cell genotyping analysis was performed at the department of Pathology, Erasmus Medical Center Rotterdam. Mycoplasma was routinely examined in our laboratory by the MycoAlert™ Mycoplasma Detection Kit (Lonza, Rockland, ME USA) according to the manufacturer's instruction. All cells were confirmed to be mycoplasma negative.

2.3. Culture of primary mouse intestinal organoids

Mice were sacrificed by cervical dislocation following euthanasia with sodium pentobarbital (100 mg per kilogram body weight; IP injection). Subsequently, mouse

small intestine (jejunum and ileum) was harvested. Stool was flushed out with ice-cold PBS. The small intestine was dissected and cut longitudinally and washed with ice-cold PBS. Villus was scraped off with a coverslip and the remaining parts of fat were removed. Small intestine was then cut into small pieces (0.2 cm~0.5 cm) and transferred to a 50 ml tube, and washed 3 times with ice-cold PBS. Then, the tissues were rocked with 2 mM EDTA for 30 min at 4°C. Afterwards, the tissues were thoroughly suspended by pipetting up and down for 10 times with a 10 ml tip in order to loosen crypts, and then were filtered through a 70 µm cell strainer. Crypt suspension was added with 10% (vol/vol) FCS and spin down at 300 g for 5 min. Supernatant was discarded and crypts were re-suspended in 10 ml complete medium growth factor (GF)- (CMGF-, advanced DMEM/F12 was supplemented with 1% (vol/vol) of GlutaMAX™ Supplement (Gibco, Grand island, USA), 10 mM of Hepes and 100 U/mL of Penicillin-Streptomycin), and then crypts were collected by centrifugation at 150 g for 2 min at 4°C. Approximately 500 crypts were suspended in 40 µl growth factor reduced phenol-red free Matrigel (Corning, Bedford, USA). Afterwards, a 40 µl droplet of Matrigel/crypts mix was placed in the center of each well of a 24-well plate, and was subsequently incubated at 37°C with 5% CO₂ for 15 min. 500 µl of culture medium was added per well after Matrigel got solidification. The culture medium was supplemented with CMGF-, 2% (vol/vol) of B-27® Supplements (Gibco, Grand Island, USA), 1% (vol/vol) of N2® Supplements (Gibco, Grand Island, USA), 500 pg/L of epidermal growth factor (EGF), 20% (vol/vol) of R-Spondin 1 (conditioned medium), and 10% (vol/vol) of Noggin (conditioned medium). The medium was maintained until passaging organoids (it depends on the density of organoids whether medium should be refreshed during culture). To this aim, organoids were removed from Matrigel and broken up mechanically by passing through a 5 ml tip inserting a 200 µl tip, and then were transferred to fresh Matrigel. The passaging was performed every 5~6 days with a 1:3 split ratio. Each well contains 10 or more organoids.

2.4. Culture of primary human intestinal organoids

Intestinal biopsies or surgically resected intestinal tissues were transferred into a 15 ml tube containing 10 ml complete chelating solution (CCS, MilliQ H₂O was supplemented with 1.0 g/L of Na₂HPO₄·2H₂O, 1.08 g/L of KH₂PO₄, 5.6 g/L of NaCl,

0.12 g/L of KCl, 15 g/L of Sucrose, 10 g/L of D-Sorbitol and 80 µg/L of DL-dithiothreitol). Biopsies/tissues were washed three times by pipetting up and down 8~10 times and then were rocked with 8 mM EDTA for 15 min at 4°C. Supernatant with EDTA was discarded and 5 ml fresh CCS solution was added. It was thoroughly suspended by pipetting up and down with 10 ml tip for 8~10 times to loosen crypts, and 2 ml FCS was added. Supernatant with crypts was transferred into a 50 ml tube and biopsies were re-used for repeatedly collecting more crypts (2~3 times). Then, crypt suspension was centrifuged at 300 g for 5 min. Supernatant was discarded and crypts were re-suspended in 2 ml CMGF-, and then crypts were collected by centrifugation at 130 g for 5 min at 4°C. Crypts were finally suspended in 40 µl growth factor reduced phenol-red free Matrigel (Corning, Bedford, USA). Then, a 40 µl droplet of Matrigel/crypt mix was placed in the center of each well of a 24-well plate, and was subsequently incubated at 37°C with 5% CO₂ for 15 min. 500 µl of culture medium was added per well after Matrigel got solidification. The culture medium was supplemented with CMGF-, 2% (vol/vol) of B-27® Supplements (Gibco, Grand Island, USA), 1% (vol/vol) of N2® Supplements (Gibco, Grand Island, USA), 500 pg/L of EGF, 1 mM n-Acetyl Cysteine, 10 mM Nicotinamide, 0.5 µM A83-01 (TGF-β inhibitor), 3 µM SB202190 (p38 inhibitor), 20% (vol/vol) of R-Spondin 1 (conditioned medium), 10% (vol/vol) of Noggin (conditioned medium) and 50% (vol/vol) of Wnt3a (conditioned medium). Culture medium was refreshed every 2~3 days, and organoids were passaged every 6~7 days. Passaging human organoids was done as described for the mouse intestinal organoids. Each well contains 10 or more organoids.

2.5. Inoculation of SA11 rotavirus

Caco2 cells in T75 flask were washed and suspended, and subsequently seeded into a 48-well plate (5×10^4 cells/well). When cell confluence was approximately 80%, culture medium was discarded and cell monolayers were washed twice with PBS. 100 µl of serum-free DMEM medium supplemented with 5 µg/mL of Trypsin (Gibco, Paisley, UK) and SA11 rotavirus were added and incubated at 37°C with 5% CO₂ for 60 min for infection, followed by washing with PBS to remove free viruses (washed 4 times). Then, cells were added with culture medium containing 5 µg/mL of trypsin and incubated at 37°C with 5% CO₂.

For organoids, SA11 rotavirus (contain 5,000 genome copies) was activated with 5 µg/mL of trypsin (Gibco, Paisley, UK) at 37°C with 5% CO₂ for 60 min, and subsequently inoculate for 60 min at 37°C with 5% CO₂, followed by washing with PBS to remove free virus (four times). Then, organoids were re-embedded in Matrigel, maintained in culture medium without trypsin at 37°C with 5% CO₂.

2.6. Inoculation of patient rotavirus

Suspensions (10% [vol/vol]) of stool were prepared in CMGF- medium and subjected to low-speed centrifugation to pellet debris. Supernatants were then filtered through 0.45-µm-pore-size filters. Inoculation protocol is similar to inoculation of SA11 rotavirus.

2.7. Viral production assay

To investigate virus replication and production in 2D culture of Caco2 cell line, cells were inoculated with SA11 rotavirus (contain 5,000 genome copies). Culture medium was harvested at indicated time points to detect and quantify secreted rotavirus.

In parallel, human primary intestinal organoids were infected with SA11 rotavirus (contain 5,000 genome copies), followed by washing with PBS to remove free virus (four times). Afterwards, organoids were adhered on the bottom of 24-well plate coated with Collagen R solution (SERVA, Heidelberg, Germany) by spin down at 500 g for 10 minutes. Organoids culture medium was gently added and organoids were subsequently incubated at 37°C with 5% CO₂. Culture medium was harvested at indicated time points to detect and enumerate secreted rotavirus.

To confirm the infectivity of secreted rotavirus, medium harvested from infected human organoids at 1 hr, 24 hr, and 48 hr were used to inoculate Caco2 cells. The infection was determined by qRT-PCR.

2.8. Neutralization assay

Neutralizing monoclonal antibody (MAb) named HS1 was gifted by Professor Harry Greenberg from Stanford University School of Medicine, USA. Neutralization assay was performed according to previous study (19). Briefly, rotavirus were activated by 5 µg/mL of trypsin, followed by adding a series of diluted MAb (1:1000, 1:250, 1:100, 1:50) from stock in the virus and keeping neutralization for 2 h at 37°C and overnight

at 4°C. After 24 h inoculation of MAb treated and non-treated SA11 rotavirus, rotavirus genomic RNA in Caco2 cells or human organoids was detected by qRT-PCR.

2.9. qRT-PCR

Total RNA was isolated using a Machery-NucleoSpin RNA II kit (Bioke, Leiden, Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis kit (TAKARA BIO INC) with random hexamer primers. Real-time PCR reactions (50°C for 2 min, 95°C for 10 min, followed by 50 or 60 cycles of 95°C for 15 s and 58°C for 30 s and 72°C for 10 min) were performed with SYBRGreen-based real-time PCR (Applied Biosystems®, Austin, USA) according to the manufacturer's instruction. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as reference. Relative gene expressions were normalized to GAPDH using the formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{control}}$). The SA11 rotavirus sense primer and anti-sense primers are targeting 564-585 and 719-699 of the SA11 genome, respectively. Human rotavirus sense and anti-sense primers are targeting 963-982 and 1046-1027 of group A rotavirus genome, respectively. All primers are listed in supplementary Table S1.

2.10. Quantification of rotavirus genome copy numbers

In order to generate a template for quantifying rotavirus genome copy number, an amplicon of the SA11 was cloned into the pCR2.1-TOPO vector (Invitrogen, San Diego, CA). The plasmid was extracted by Qiuck Plasmid Miniprep Kit (Invitrogen, Lohne, Germany) following manufacturer's instructions. A series of dilutions, from 10^2 to 10^{-10} , were prepared and then were amplified and quantified by qRT-PCR to generate a standard curve. Standard curve was generated by plotting the log copy number versus the cycle threshold (C_T) value (Supplementary Figure 1). Copy numbers were calculated by using the following equation:

$$\text{Copy number (molecules}/\mu\text{L)} = [\text{concentration (ng}/\mu\text{L)} \times 6.022 \times 10^{23} \text{ (molecules/mol)}] / [\text{length of amplicon} \times 640 \text{ (g/mol)} \times 10^9 \text{ (ng/g)}].$$

2.11. Immunohistochemistry

Organoids were transferred in a 15 ml tube and suspended in ice-cold PBS, followed by spinning down at 150 g for 5 min at 4°C. Supernatant was discarded and pellet was added 500 µl of 4% formalin to fix for 0.5 to 1 hr at 4°C. Organoids were then treated with 70% of ethanol containing some eosin for 30 min, followed by dehydration with 96% ethanol - 96% ethanol - 100% ethanol - 100% ethanol – xylene - xylene, with each step for 5 min. Xylene was carefully removed and paraffin was added on the organoids, maintaining for 2 hrs at 62°C, and cooled down at room temperature. Sections were cut and incubated at 37°C overnight.

For immunohistochemistry analysis, organoid sections (4 µm) were deparaffinized (xylene – xylene - 100% ethanol - 100% ethanol - 70% ethanol with each step for 5 min), followed by washing with 0.05% Tween20 (MERCK, Hohenbrunn, Germany). Antigen was retrieved by boiling sections in Tris/EDTA buffer (ph= 9) for 14 min, and then the sections were cooled down by tap water. The sections were rinsed with 0.05% Tween20, and blocked by incubation with 5% skimmed milk solution for 1 hr at room temperature. Sections were incubated with primary antibody (mouse monoclonal antibody named HS-2 against SA11 rotavirus VP4; gifted by Professor Harry Greenberg from Stanford University School of Medicine, USA) diluted in 5% milk solution (1:100) at 4°C overnight. Subsequently the sections were incubated with secondary antibody, using the Dako EnVision + System-HRP Labelled Polymer Anti-mouse antibody (Dako, Carpinteria, USA) and the sections were counterstained with hematoxylin for 15 seconds, followed by rinsing slides with tap water. Staining was visualized by the Nikon light microscope (Nikon, Amsterdam, The Netherlands).

2.12. Statistics

Data are presented as Mean ± SEM. Comparisons between groups were performed with Mann-Whitney test, except for the analysis of ISG expression, which was analyzed with t test (with data of normal distribution) using GraphPad Prism (GraphPad Software Inc). Differences were considered significant at a *p* value less than 0.05.

2.13. Study approval

Animal study was approved by the institutional animal ethics committee (Dier Experimenten Commissie).

Human biopsies from the colon were completed with forceps and collected from healthy adult volunteers. Other human intestinal tissues were obtained during surgical resection. The volunteers or patients agreed to participate by written informed consent, and the study was approved by the Medical Ethical Committee of the Erasmus Medical Center (Medisch Ethische Toetsings Commissie Erasmus MC).

3. Results

3.1. Primary mouse intestinal organoids support rotavirus infection

Before embarking on human experimentation, we first attempted to set up a rotavirus infection model using 3D cultured mouse intestinal organoids employing the laboratory rotavirus strain SA11. To this end, murine intestinal organoids were cultured from isolated mouse crypts. Upper openings of crypts rapidly sealed, and swelling and budding events occurred within few days of culture. As expected, organoids were composed of a single layer of polarized epithelial cells. Murine organoids require passaging after five days of culture. We therefore opted to use organoids for rotavirus infection within four days of the primary culture or use the passaged organoids (Figure 1A).

Upon inoculation of SA11 rotavirus for 1 hour (hr), organoids were thoroughly washed to remove the residual virus particles and subsequently re-embedded in Matrigel for an additional 24 hrs. As shown by positive immunohistochemical (IHC) staining against the viral protein VP4, viral infection was observed in organoids as early as 24 hrs post-inoculation (Figure 1B). To further confirm active infection, qRT-PCR was performed to detect viral genomic RNA. As expected, no signal was detected in un-infected samples, but viral RNA expression was readily detected in virus-inoculated samples. By normalizing to the housekeeping gene GAPDH, viral RNA was significantly increased by more than 70-fold ($n = 5$, $P < 0.05$) after 24 hrs of infection as compared to 1 hr inoculation (Figure 1C). Thus murine intestinal organoids are susceptible to the SA11 rotavirus infection, which prompts further investigation into the susceptibility of human intestinal organoids to rotavirus.

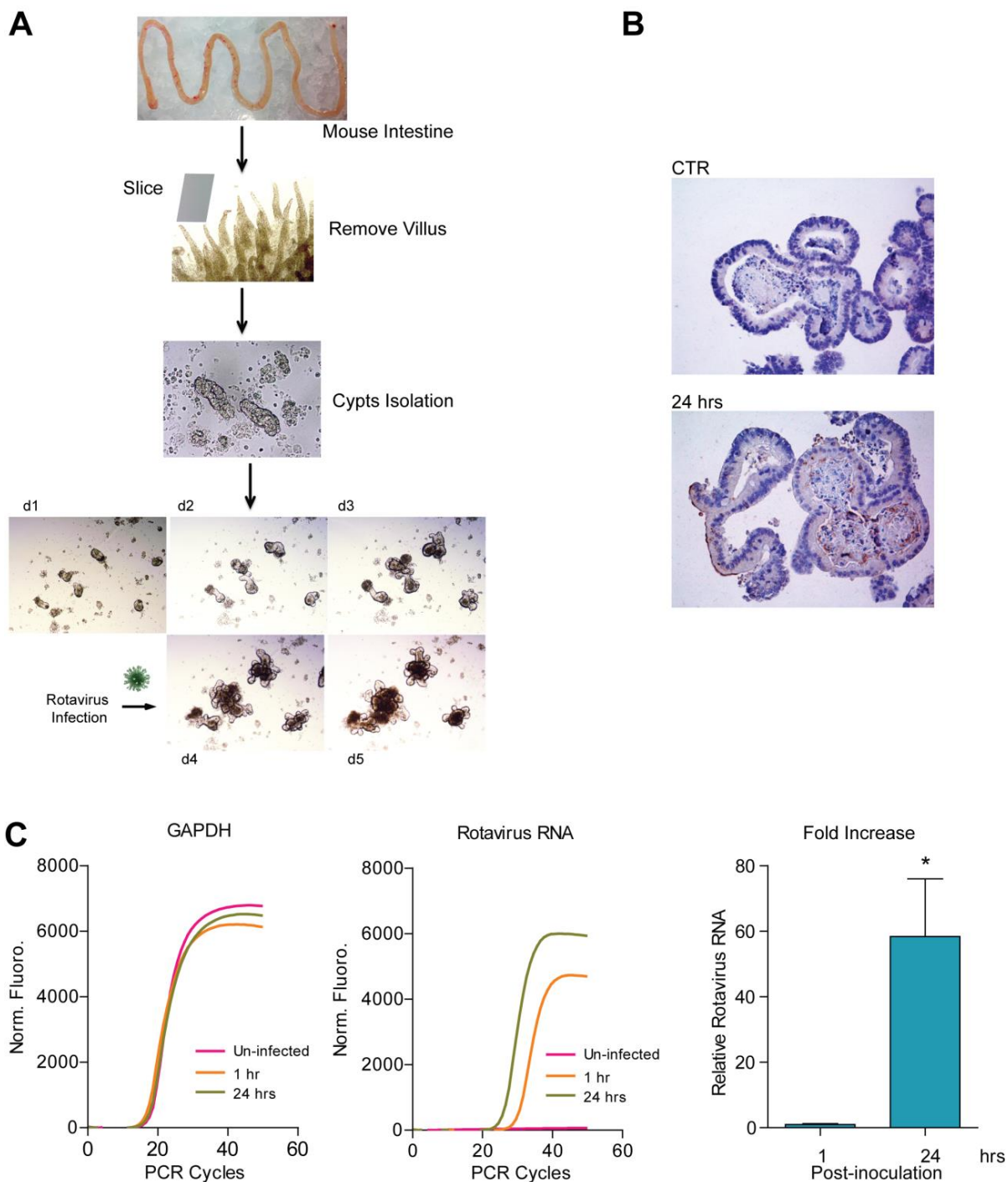


Figure 1. Mouse intestinal organoids support infection of the SA11 rotavirus strain. (A) The procedure of culturing primary mouse intestinal organoids and their infection with rotavirus. (B) Immunohistochemical staining of SA11 rotavirus VP4 protein in un-infected (CTR) and infected (24 hrs post-infection) organoids (magnification: 400 \times). (C) Detection and quantification of rotavirus RNA by qRT-PCR at indicated time points after rotavirus inoculation. Mouse GAPDH was used as a reference gene. * $P < 0.05$, Mann-Whitney test; $n = 5$.

3.2. Primary human intestinal organoids are more permissive for rotavirus infection

For testing rotavirus infection in primary human intestinal organoids, intestinal biopsies or surgically resected tissues were used as a source of human intestine (Figure 2A). Organoids were successfully cultured and expanded from isolated crypts. These organoids usually need passaging after seven days of culture (Figure 2A). Thus, human organoids within five days of primary culture or passaged organoids were used for rotavirus infection (Figure 2A).

Similar to infecting mouse organoids with rotavirus, human organoids were inoculated with SA11 rotavirus for 1 hr and subsequently thoroughly washed to remove the residual virus particles, followed by being re-embedded in Matrigel and incubated for 24 hrs. An IHC assay showed strong immunoreactivity towards viral protein VP4 in these organoids (Figure 2B). Remarkably, qRT-PCR results revealed that 24 hrs infection resulted in a more than 15,000-fold ($n = 5$, $P < 0.01$) increase in viral RNA compared to 1 hr infection (Figure 2C). These data convincingly demonstrate that human intestinal organoids, compared to mouse organoids, are much more permissive for rotavirus infection.

3.3. Kinetics of viral replication and production in organoids

To further characterize the kinetics of rotavirus infection in human organoids, we quantified intracellular viral RNA and secreted virus at consecutive time points to profile the dynamics of viral replication and production, in comparison to the infection in Caco2 cell line. Genome copy number of secreted rotavirus was quantified by qRT-PCR referring to a plasmid template using a standard curve calculation method (Supplementary Figure S1). Similar to the infection of Caco2 cells (Figure 3A), intracellular viral RNA in organoids and the production of rotavirus were increased drastically overtime. Of note, the kinetics of viral replication appears different that it is slower and less efficient in human organoids compared to Caco2 cells (Figure 3B). Furthermore, these secreted viruses are capable of infecting naïve Caco2 cells (Figure 3C). As shown in Figure 3B, the medium collected from 24 or 48 hr post-inoculation of human organoids contained much abundant amount of infectious viruses, compared to the sample collected at 1 hr post-infection. Taken together, human organoids are highly permissive for rotavirus infection comparable to conventional 2D culture of Caco2 cells, and support robust viral replication and infectious virus particle production.

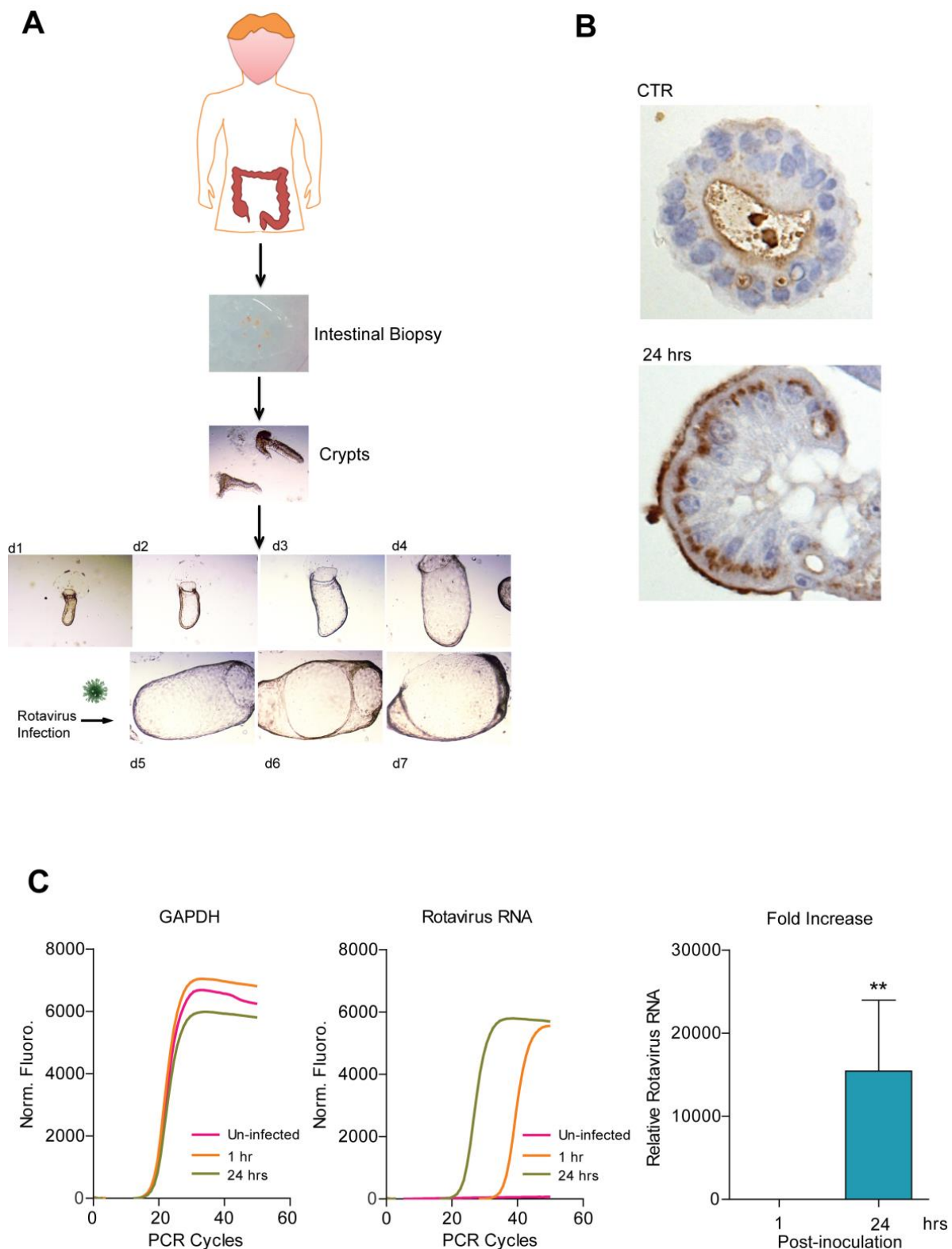


Figure 2. Human intestinal organoids are highly permissive for the infection of the SA11 rotavirus strain. (A) The procedure of culturing primary human intestinal organoids and their infection with rotavirus. (B) Immunohistochemical staining of SA11 rotavirus VP4 protein in un-infected (CTR) and infected (24 hrs post-infection) organoids (magnification: 400 \times). (C) Detection and quantification of rotavirus RNA by qRT-PCR at indicated time points after rotavirus inoculation. Human GAPDH was used as a reference gene. ** $P < 0.01$, Mann-Whitney test; $n = 5$.

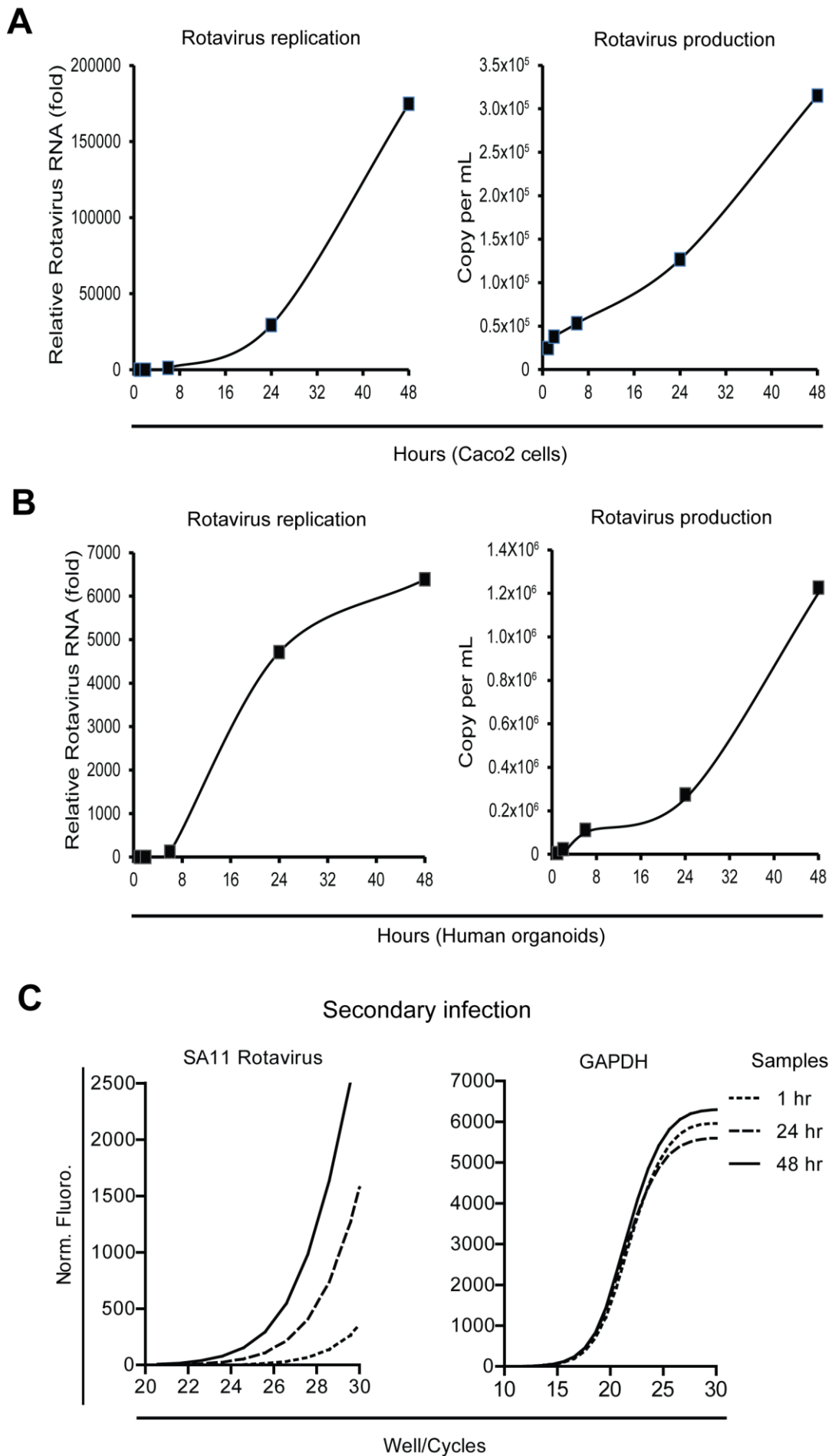


Figure 3. Production of infectious rotavirus by human organoids. (A) Genome copy number per mL of medium harvested from SA11 rotavirus infected organoids at indicated time points. (B) Secondary infection of Caco2 cell line with culture medium corrected at 1 hr, 24 hr, and 48 hr post-infection of SA11 rotavirus-inoculated human organoids. Rotavirus genome RNA and GAPDH were quantified by qRT-PCR.

3.4. Neutralization of SA11 by MAb

To investigate whether rotavirus infection in organoids can be neutralized by antibody, we tested a MAb targeting VP7 (19). It was depicted that 100 and 50-time diluted this antibody can effectively neutralize SA11 rotavirus in Caco2 cells, resulting in $44 \pm 10\%$ (Mean \pm SEM, $n = 5$, $P < 0.01$) and $94 \pm 1\%$ (Mean \pm SEM, $n = 6$, $P < 0.001$) inhibition of viral infection (Figure 4A), respectively. Similarly, different concentrations of this MAb (1000, 250, 100 and 50-time diluted) significantly inhibited SA11 rotavirus infection by $56 \pm 8\%$ (Mean \pm SEM, $n = 4$, $P < 0.01$), $38 \pm 28\%$ (Mean \pm SEM, $n = 4$, $P < 0.05$), $90 \pm 3\%$ (Mean \pm SEM, $n = 4$, $P < 0.01$) and $94 \pm 3\%$ (Mean \pm SEM, $n = 5$, $P < 0.01$), respectively, in 3D culture of human primary intestinal organoids (Figure 4B).

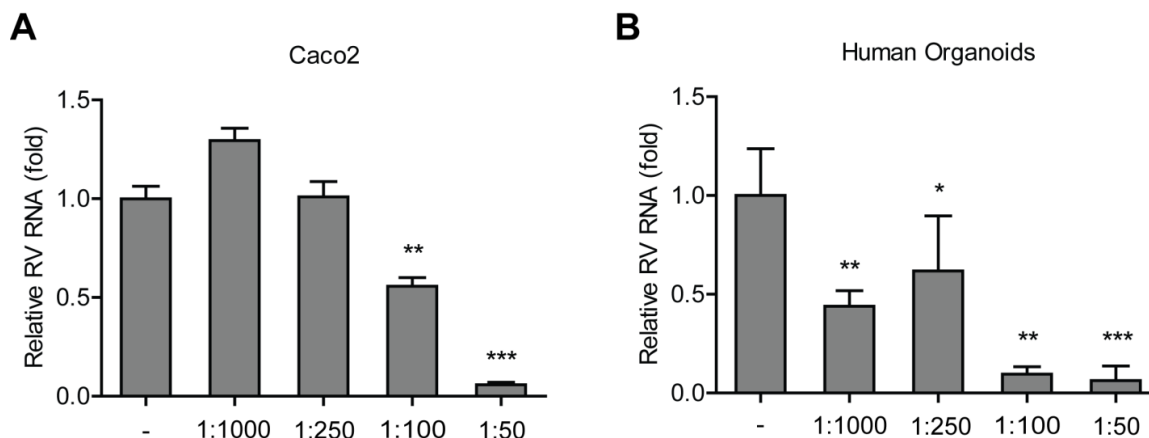


Figure 4. Neutralization of SA11 rotavirus by neutralizing MAb in Caco2 cells and Human intestinal organoids. Trypsin-treated SA11 rotavirus was mixed with four different concentrations (1000, 250, 100 and 50-time diluted) MAb from stock. Neutralization was performed at 37°C for 2 h and at 4°C overnight. Virus-antibody cocktail was inoculated onto Caco2 cells and human primary intestinal organoids in 48-well plate. After 24 h post infection, viral genomic RNA in Caco2 cells (A) and organoids (B) were examined by qRT-PCR and normalized to the reference gene GAPDH. ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney test; $n = 5\sim 6$.

3.5. Induction of ISG expression in organoids in response to rotavirus

IFN signaling is a first-line of antiviral defense mechanism and exerts the action through the induction of a variety of interferon-stimulated genes (ISGs) that mediate the subsequent antiviral response (4, 20). Many viruses can trigger the activation of this pathway, but conversely viruses have also developed strategies to corrupt this intracellular defense mechanism (4). As knowledge about interaction of rotavirus infection with the interferon system is urgent, we investigated the effects of rotavirus on ISG induction in our experimental system. We selected a panel of ISGs that are well-known for playing important roles in antiviral defense (21). These ISGs include interferon regulatory factor (IRF) 1, IRF7, IRF9, interferon-induced transmembrane protein (IFITM) 1, IFITM2, IFITM3, signal transducer and activator of transcription (STAT) 1, protein kinase R (PKR) and ISG15. We first investigated in the Caco2 cell line that is widely used for rotavirus infection. Inoculation of the SA11 strain indeed triggered the up-regulation of several ISGs, in particular IRF9 at 24 hrs post-infection (Figure 5A). Induction of ISGs in both mouse and human organoids was also observed at 24 hrs after infection, although the induction patterns were different among these models (Figure 5B and 5C). These results indicate that active virus-host interactions occur in organoids upon rotavirus infection.

3.6. Modeling the anti-rotavirus activity of IFN- α and ribavirin

IFN- α , a type I IFN, is a potent activator of the JAK-STAT cascade to induce antiviral ISG expression. Clinically, this cytokine is used successfully for treating chronic hepatitis B (HBV) and C (HCV) virus (17, 22). Treatment of Caco2 cells with recombinant human IFN- α (1000 IU/ml) for 48 hrs induced the expression of a panel of ISGs and exerted potent anti-rotavirus (SA11 strain) activity ($87 \pm 3\%$ inhibition, Mean \pm SEM, $n = 5$, $P < 0.05$, Figure 6A and 6B). Similarly, treatment with human IFN- α (1000 IU/ml) for 48 hrs in human organoids led to induction of ISG expression with anti-rotavirus activity ($73 \pm 8\%$ inhibition, Mean \pm SEM, $n = 5$, $P < 0.05$, Figure 6C and 6D).

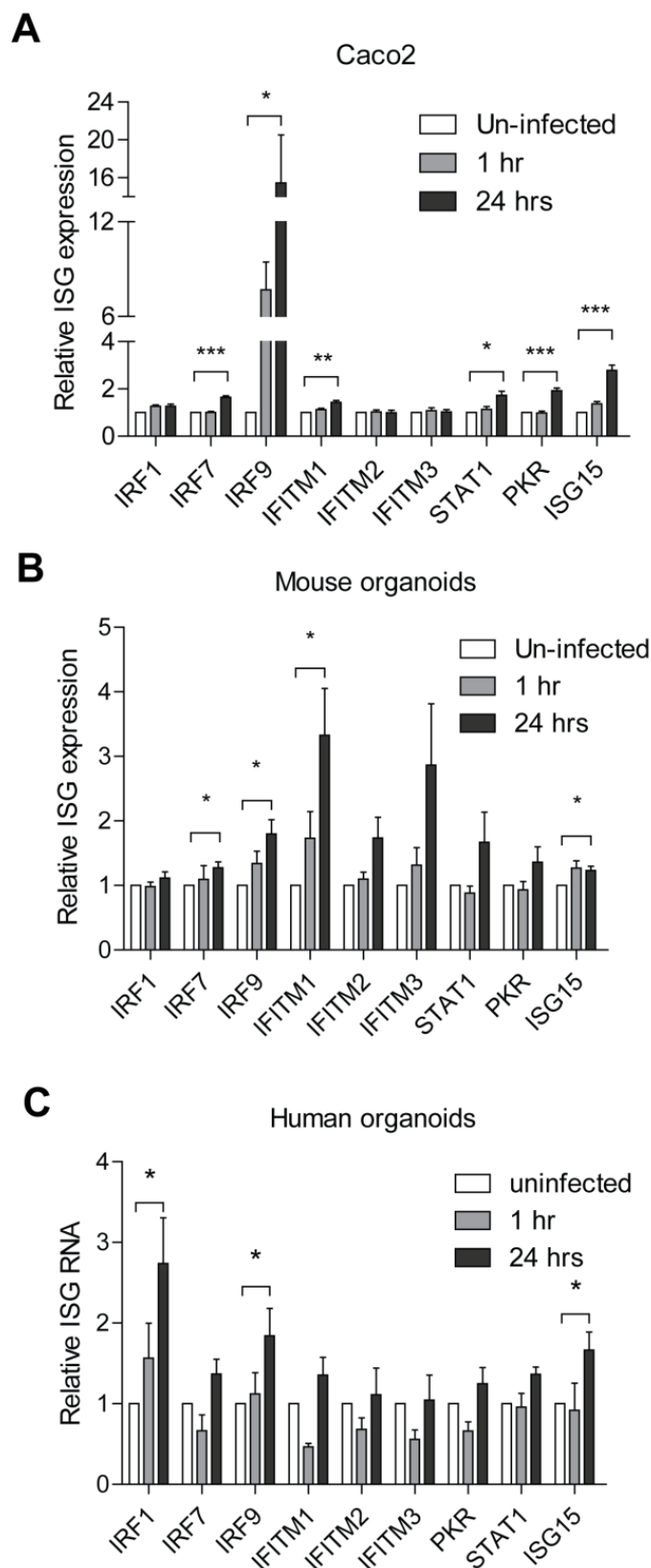


Figure 5. Rotavirus infection induced the expression of Interferon-stimulated genes (ISGs). Upon inoculation of SA11 rotavirus in the Caco2 cell line ($n \geq 4$) (A), mouse organoids ($n \geq 5$) (B) and human organoids ($n \geq 5$) (C), the relative expression of ISGs, including IRF1, IRF7, IRF9, IFITM1, IFITM2, IFITM3, STAT1, PKR, and ISG15, was quantified by qRT-PCR and normalized to the reference gene GAPDH. Statistic comparison was performed between uninfected and 24 hr post-infection groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, t test.

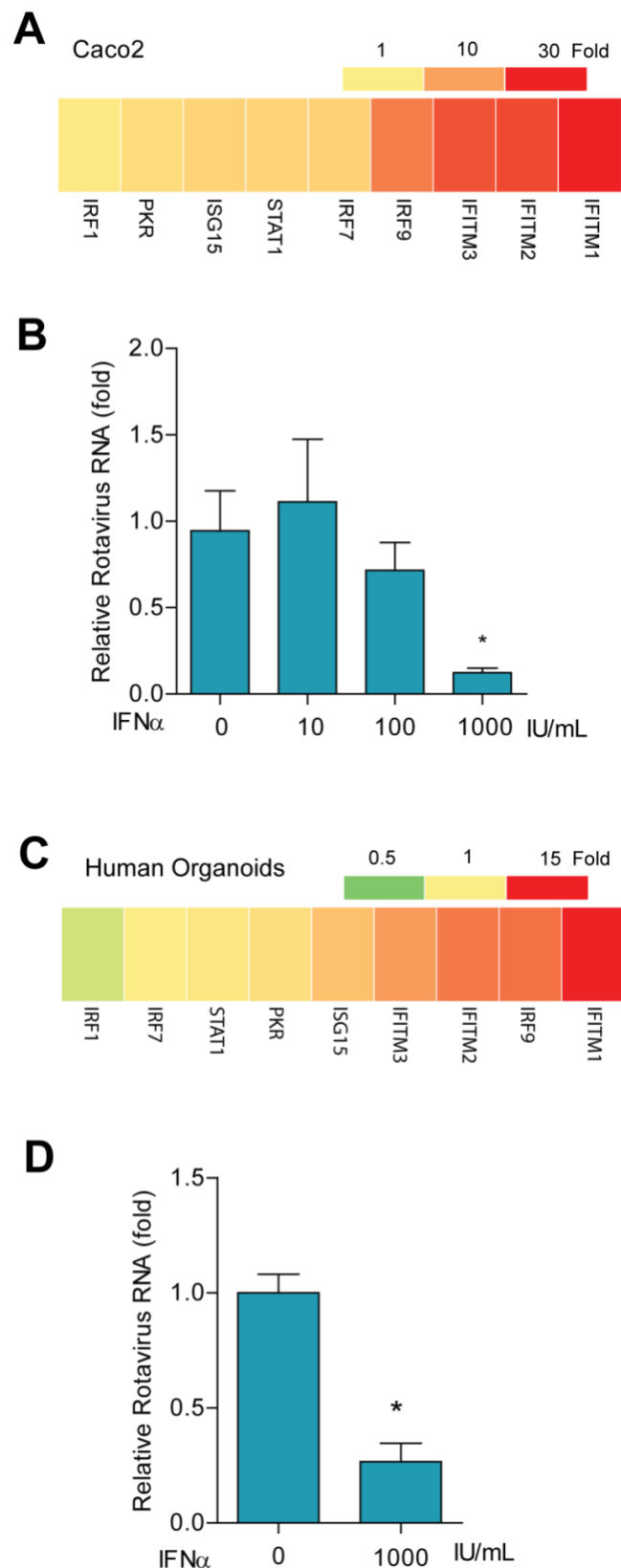


Figure 6. Anti-rotaviral effects of IFN- α . Treatment IFN- α (1000 IU/ml) induced the expression of ISGs (A) in Caco2 cells and inhibited SA11 rotavirus infection (B). * $P < 0.05$, Mann–Whitney test; $n = 5$. IFN- α (1000 IU/ml) induced the expression of ISGs (C) in human organoids and inhibited SA11 rotavirus infection (D) * $P < 0.05$, Mann–Whitney test; $n = 5$. The expression of ISGs and the level rotavirus RNA were quantified by qRT-PCR and normalized to reference gene GAPDH.

Ribavirin has a broad antiviral activity against a spectrum of viruses (23), and is used for treating chronic HCV in combination with IFN- α (18, 24). Treatment with ribavirin (1, 5, 10 $\mu\text{g}/\text{mL}$) for 48 hrs on Caco2 cells infected with SA11 rotavirus dose-dependently inhibited viral RNA. At concentrations of 5 and 10 $\mu\text{g}/\text{mL}$, ribavirin significantly decreased viral RNA by $81 \pm 7\%$ (Mean \pm SEM, $n = 6$, $P < 0.01$) and $85 \pm 7\%$ (Mean \pm SEM, $n = 6$, $P < 0.01$), respectively (Figure 7A). In mouse and human organoids, ribavirin (10 $\mu\text{g}/\text{mL}$) reduced rotavirus RNA levels by $48 \pm 17\%$ (Mean \pm SEM, $n = 5$, $P < 0.05$, Figure 7B) and $44 \pm 8\%$ (Mean \pm SEM, $n = 4$, $P < 0.05$, Figure 7C), respectively. Thus, 3D organoid cultures appear to display a different sensitivity compared with conventional 2D immortalized cell line model.

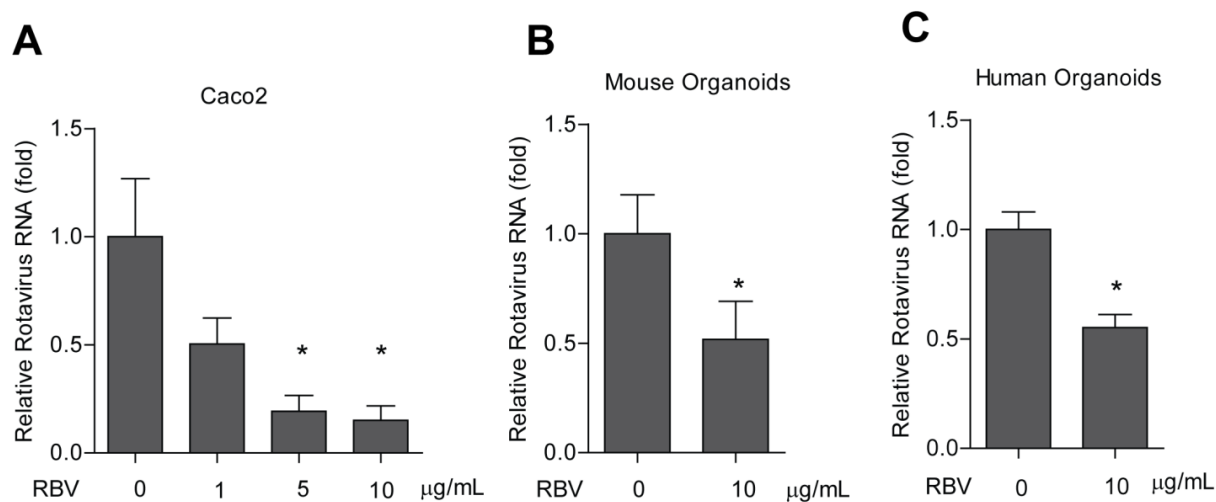


Figure 7. Antiviral effects of ribavirin against SA11 rotavirus. (A) Relative levels of rotavirus RNA in the Caco2 cells treated with different concentrations (0, 1, 5, 10 $\mu\text{g}/\text{mL}$). ** $P < 0.01$, Mann-Whitney test; $n = 6$. (B) Relative levels of rotavirus RNA in mouse organoids treated with 10 $\mu\text{g}/\text{mL}$ of ribavirin. * $P < 0.05$, Mann-Whitney test; $n = 5$. (C) Relative levels of rotavirus RNA in human organoids treated with 10 $\mu\text{g}/\text{mL}$ of ribavirin. * $P < 0.05$, Mann-Whitney test; $n = 4$. Rotavirus RNA was quantified by qRT-PCR and normalized to reference gene GAPDH.

3.7. Modeling infection and antiviral therapy using patient-derived rotavirus strains

Since SA11 is a well-adapted laboratory strain that has high infectivity, we further explored whether our system can model patient-derived rotavirus infection. In total, fecal samples from 9 infected individuals were used to infect human organoids. All samples were positive for group A rotavirus (Table 1). Patient 7 was also sapovirus positive, and patient 1 was also adenovirus positive (Table 1). Seven of these inoculation provoked sustained infection of rotavirus as detected using our qRT-PCR

assay (Table 1, Figure 8 and Supplementary Figure 2). The increase of viral RNA after 24 hrs inoculation differed substantially among patients (Figure 8). These results were similar to the infection of Caco2 cell line (Supplementary Figure 2).

Next, we examined whether it is feasible to evaluate the responsiveness of each sample to antiviral therapy of IFN- α and ribavirin. Experiments using 6 out of 7 infectious fecal samples succeeded with respect to these antiviral tests (Figure 8). Interestingly, patient 2 did neither respond to IFN- α nor ribavirin. Patient 3 and 4 were sensitive to IFN- α but not to ribavirin. Patient 5, 6 and 7 responded to both IFN- α and ribavirin (Figure 8). Among all the responders, the sensitivity to IFN- α or ribavirin substantially varied (Figure 8 and Table 1). These data demonstrated that intestinal organoids are capable of individualized modeling rotavirus infection and antiviral therapy.

Table 1
Patient characteristics and responsiveness to antiviral treatment in primary human intestinal organoids.

Patient	Age (yrs)	Gender	Symptoms	Virus Detection					Infection of organoids	Response to RBV	Response to IFN α			
				Enterovirus	Parechovirus	Norovirus gene groups I	Norovirus gene groups II	Adenovirus				Astrovirus	Sapovirus	Rotavirus
1	28	Female	Nausea, stomach ache, watery diarrhea, fever, headache, vomiting	No	No	No	No	Yes	No	No	Yes	Yes	-	N/T
2	27	Female	Fever, diarrhea, nausea, vomiting	No	No	No	No	No	No	No	Yes	Yes	-	-
3	3.5	Female	Fever	No	No	No	No	No	No	No	Yes	Yes	-	+++
4	67	Male	Fever, stomach ache, watery diarrhea (Kidney transplant)	No	No	No	No	No	No	No	Yes	Yes	-	++
5	4	Male	Pneumococcal Sepsis	No	No	No	No	No	No	No	Yes	Yes	++	+++
6	74	Female	Congestive heart failure, myocarditis	No	No	No	No	No	No	No	Yes	Yes	+++	++
7	1.5	Female	Vomiting, watery diarrhea	No	No	No	No	No	No	No	Yes	Yes	+++	+++
8	37	Female	Diarrhea	No	No	No	No	No	No	No	Yes	No	N/T	N/T
9	5	Male	Vomiting, diarrhea, stomach ache, dehydration, fever	No	No	No	No	No	No	No	Yes	No	N/T	N/T

Note: Inhibition of rotavirus RNA: 0-20% (-); 20-40% (+); 40-70% (++) and 70-100% (+++). N/T: Not Tested; IFN- α : interferon-alpha; RBV: ribavirin. Also See Figure 7 and supplementary Figure 2.

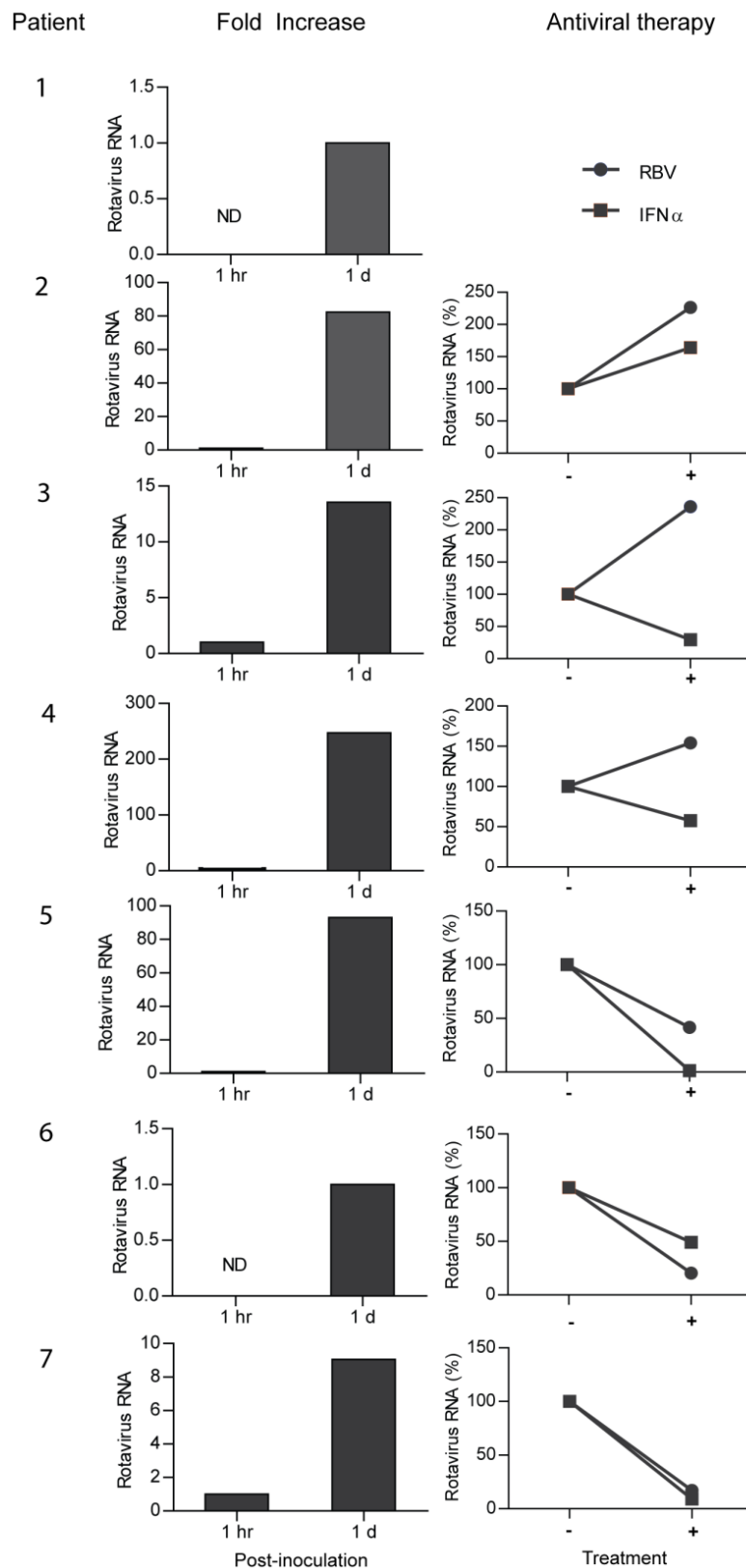


Figure 8. Human organoids support the infection of patient-derived rotavirus and might be utilized for individualized assessment of the antiviral activities of IFN- α and ribavirin. All samples of seven patients successfully infected human organoids. 6 out those 7 samples were able to model the antiviral activity of IFN- α and ribavirin. Distinct responsiveness was observed among these samples. The level rotavirus RNA was quantified by qRT-PCR and normalized to reference gene GAPDH. ND, not detectable. (Also see Table 1).

4. Discussion

The successful introduction of oral vaccines has dramatically reduced the burden of rotavirus gastroenteritis in some developed countries. However, in many developing countries from one third to one half of all vaccinated children failed to prevent infection, probably due to high prevalence and diversity of the virus (25). Thus, there is a clear need of developing specific antiviral therapy, considering the over 2 million severe ill and 450,000 deaths yearly of children under five years of age (1). Given that the burden predominantly exists in developing countries and vaccines are available, pharmaceutical companies appear to have less interest in developing specific anti-rotavirus therapies. A cost-effective scenario is thus to properly evaluate whether currently available antivirals, such as IFN- α and ribavirin, will be effective for treating rotavirus.

Although some early studies have explored the potential anti-rotavirus efficacy of IFN- α or ribavirin in experimental models (19, 23, 26-28), the results remain inconclusive. Limitations of these classical rotavirus models, which produced inconsistent results, hampered further clinical evaluation. We now report the application of a 3D culture model of primary intestinal organoids for modeling infection of patient-derived rotavirus strains and assessing antiviral therapy. Compared to the conventional 2D culture system of immortalized (intestinal carcinoma) cell lines and even primary intestinal epithelial cells (25), primary organoids are better in recapitulating the essential features of the *in vivo* intestinal tissue architecture. They maintain multiple cell types with similar functions as in the organ/tissue of origin (12, 29-31). Although human embryonic stem cell lines were also shown to be able to differentiate into intestinal organoids-like structure, it often requires 1-2 months to generate “mature” organoids that can be used for experimentation (7). In contrast, culturing mouse or human mature intestinal organoids from tissue or biopsy only requires a few days (12). Although there is no functional comparison yet available, primary cultures when compared to stem cell line-derived organoids would conceivably replicate and resemble *in vivo* intestinal tissue more accurately.

We demonstrated that both mouse and human intestinal organoids readily support infection of the laboratory-adapted SA11 rotavirus strain. As expected, human organoids are much more permissive to infection resulting in over 10,000-fold

increase of genomic RNA at 24 hrs of viral replication than at 1 hr of viral replication, and are capable of producing infectious virus particles, although it seems that the replication of rotavirus in human organoids is somewhat slower and less efficient compared with Caco2 cells. Interestingly, active virus-host interactions occurred as demonstrated by ISG induction. ISGs are key antiviral effectors induced by the activation of IFN signaling (32) and are crucial in regulating rotavirus infection (33). Treatment of IFN- α significantly inhibited rotavirus infection in both cell line and organoids models. Similarly, antiviral activity was also observed in both models upon treatment of ribavirin. However, organoids models are less sensitive to antivirals compared to the cell line model, which appears to be more consistent with experiences in *in vivo* models (18, 34).

More importantly, human organoids are even permissive for patient-derived rotavirus and the virus can efficiently replicate. The organoids model therefore circumvents one of the major limitations of using laboratory-adaptive strains that are not representative for the circulating 110,000 strains (10). However, two out of nine samples were not successful with inoculation in human intestinal organoids, which was probably due to low viral titers in these two stool samples (data not shown). Therefore, a possible solution is to increase the virus input, for instance, by concentrating viruses from fecal samples. Interestingly, virus samples derived from different patients have distinct sensitivities to IFN- α or ribavirin. A subset of patient samples are very sensitive to IFN- α , ribavirin or both, suggesting the potential of clinical application of these existing antiviral medications, at least for a selective population of rotavirus-infected patients. Given that primary organoids can be easily frozen and re-established in culture, results of antiviral assays in organoids can be obtained within 24-48 hrs after obtaining a fecal sample from the patient, giving clinicians sufficient time for decision making. Of note, our organoids models do not make the conventional models superfluous. In fact, these cell line and laboratory strain-based 2D culture models are still useful, in terms of pilot exploration or rough assessment. Thus, the combinatory use of both types of models will be necessary for particular projects.

In summary, we have reported the establishment and application of 3D cultures of primary intestinal organoids for modeling the infection by laboratory-adapted and patient-derived rotavirus strains. This system is robust and applicable in hospital, academic or industry laboratories. It bears broad implications for studying rotavirus-

host interactions and antiviral drug development, as well as potential for patient stratification and personalized medicine.

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Supplementary Table & Figures

Supplementary Table 1 Primers used in the study

Sequence of rotavirus primers

	SA11 Rotavirus	Human Patient Rotavirus
Sense	TGGTTAAACGCAGGATCGGA	ACCATCTACACATGACCCTC
Anti-sense	AACCTTTCCGCGTCTGGTAG	CACATAACGCCCTATAGCC

Primer sequences of GAPDH and ISGs

Primers	Human	Mouse
GAPDH-F	GTCTCCTCTGACTTCAACAGCG	TTCCAGTATGACTCCACTCACGG
GAPDH-R	ACCACCCTGTTGCTGTAGTAGCCAA	TGAAGACACCAGTAGACTCCACGAC
IRF1-F	GAGGAGGTGAAAGACCAGAGCA	TCCAAGTCCAGCCGAGACACTA
IRF1-R	TAGCATCTCGGCTGGACTTCGA	ACTGCTGTGGTCATCAGGTAGG
IRF7-F	CCACGCTATACCATCTACCTGG	CCTCTGCTTTCTAGTGATGCCG
IRF7-R	GCTGCTATCCAGGGAAGACACA	CGTAAACACGGTCTTGCTCCTG
IRF9-F	CCACCGAAGTTCCAGGTAACAC	CAACATAGGCGGTGGTGGCAAT
IRF9-R	GTCTGCTCCAGCAAGTATCGG	GTTGATGCTCCAGGAACACTGG
IFITM1-F	GCTTCATAGCATTTCGCCTACTC	GCCACCACAATCAACATGCCTG
IFITM1-R	AGATGTTTCAGGCACTTGGCGGT	ACCCACCATCTTCCTGTCCCTA
IFITM2-F	GGCTTCATAGCATTTCGCCTACTC	CACTCTTCTTCAACGCCTGCTG
IFITM2-R	AGATGTTTCAGGCACTTGGCGGT	GGAGCTGATATTCAGGCACTTGG
IFITM3-F	CTGGGCTTCATAGCATTTCGCCT	TTCTGCTGCCTGGGCTTCATAG
IFITM3-R	AGATGTTTCAGGCACTTGGCGGT	ACCAAGGTGCTGATGTTTCAGGC
STAT1-F	ATGGCAGTCTGGCGGCTGAATT	GCCTCTCATTGTCACCGAAGAAC
STAT1-R	CCAAACCAGGCTGGCACAATTG	TGGCTGACGTTGGAGATCACCA
PKR-F	GAAGTGGACCTCTACGCTTTGG	AGGTGTCACCAAACAGGAGGCA
PKR-R	TGATGCCATCCCGTAGGTCTGT	GCTGCTGGAAAAGCCACTGAATG
ISG15-F	CTCTGAGCATCCTGGTGAGGAA	CATCCTGGTGAGGAACGAAAGG
ISG15-R	AAGGTCAGCCAGAACAGGTCGT	CTCAGCCAGAACTGGTCTTCGT

Supplementary Figures

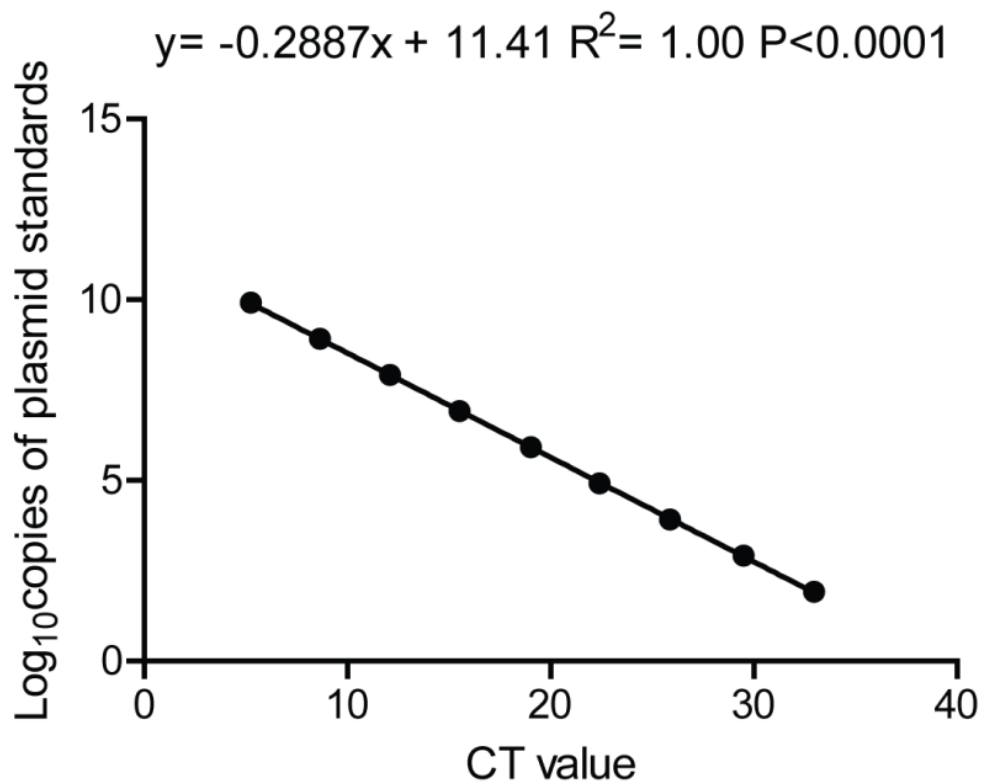


Figure S1. Standard curve for quantifying rotavirus genome copy numbers. An amplicon of the SA11 was cloned into the pCR2.1-TOPO vector. The plasmid was extracted, followed by a series of dilutions, from 10^{-2} to 10^{-10} , were prepared and then were amplified and quantified by qRT-PCR. Standard curve was generated by plotting the cycle threshold (CT) value with regard to the log copy number.

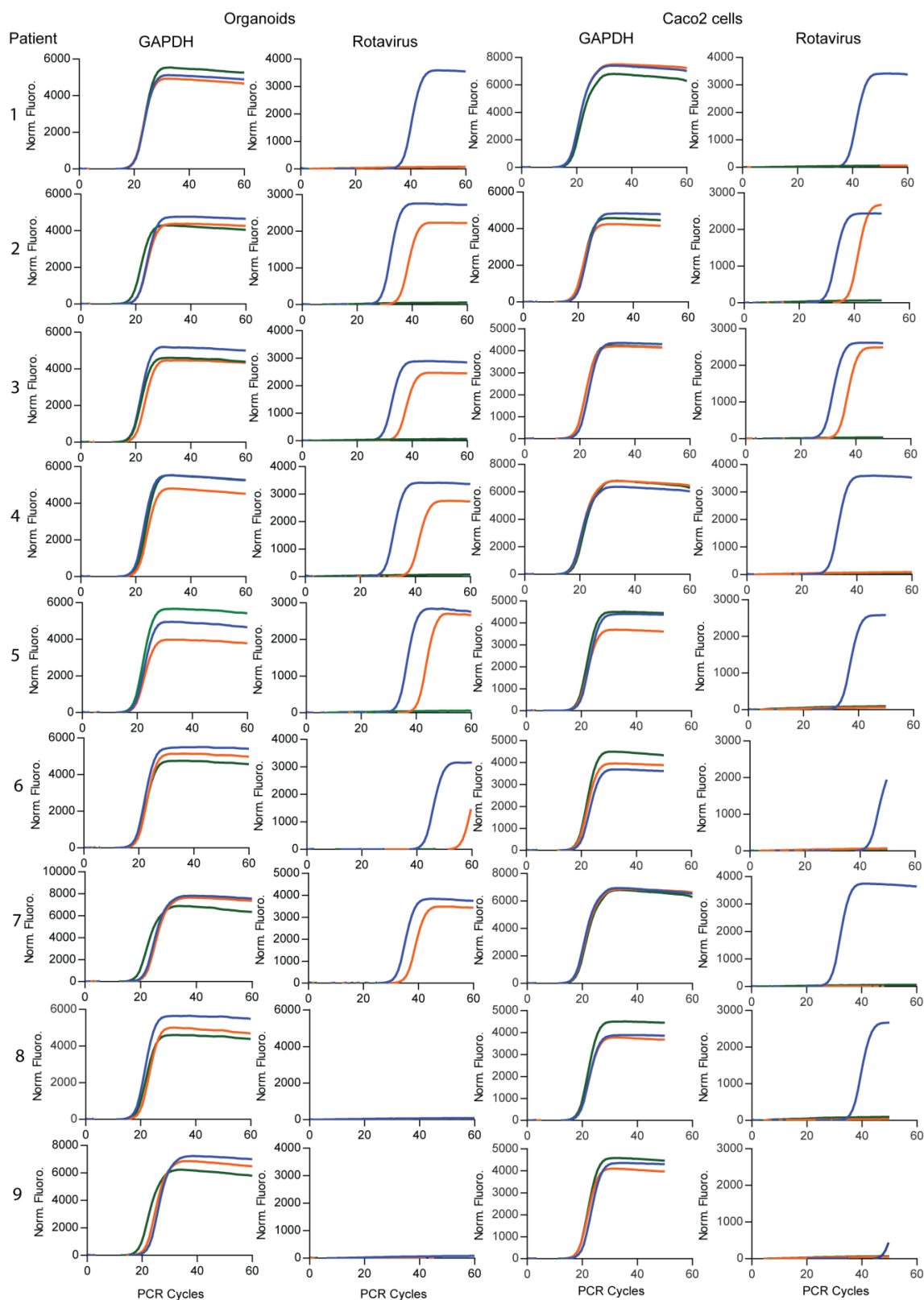


Figure S2. Human organoids and Caco2 cells support the infection of patient-derived rotavirus. Fecal samples from 9 infected individuals were used to infect human organoids and Caco2 cells. qRT-PCR was performed to quantify the infection (Norm. Fluoro.). The left and right two columns indicate infection in human organoids and Caco2 cells, respectively. Green line, uninfected; orange line, 1 hr post-infection; blue line, 1 day post-infection.

CHAPTER 4

Mycophenolic Acid Potently Inhibits Rotavirus Infection with a High Barrier to Resistance Development

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Abstract

Rotavirus infection has emerged as an important cause of complications in organ transplantation recipients. Immunosuppressants used to prevent alloreactivity can also interfere with virus infection, but the direct effects of the specific type of immunosuppressants on rotavirus infection are still unclear. Here we profiled the effects of different immunosuppressants on rotavirus using a 2D culture model of Caco2 human intestinal cell line and a 3D model of human primary intestinal organoids inoculated with laboratory and patient-derived rotavirus strains. We found that the responsiveness of rotavirus to Cyclosporine A treatment was moderate and strictly regulated in an opposite direction by its cellular targets cyclophilin A and B. Treatment with mycophenolic acid (MPA) resulted in a 99% inhibition of viral RNA production at the clinically relevant concentration (10 µg/mL) in Caco2 cells. This effect was further confirmed in organoids. Importantly, continuous treatment with MPA for 30 passages did not attenuate its antiviral potency, indicating a high barrier to drug resistance development. Mechanistically, the antiviral effects of MPA act via inhibiting the IMPDH enzyme and resulting in guanosine nucleotide depletion. Thus for transplantation patients at risk for rotavirus infection, the choice of MPA as an immunosuppressive agent appears rational.

Keywords: Rotavirus, Immunosuppressants, Mycophenolic acid, Intestinal organoids

1. Introduction

Rotavirus infects the enterocytes of the small intestine and is one of the major causative agents of gastroenteritis. Although rotavirus infection is usually asymptomatic or mild in adults (1), it yearly causes 1.4 billion diarrhea episodes and over 450,000 deaths of children younger than five years old (2). However, emerging evidence from organ transplantation patients indicate that both pediatric and adult organ recipients receiving immunosuppressants are at risk of rotavirus infection. Severe gastroenteritis with considerable morbidity and mortality has been reported in these organ transplantation recipients (3-5).

Immunosuppressants are universally used after organ transplantation to prevent graft rejection. It is not surprising that chronic use of these medications can increase the susceptibility to rotavirus infection due to inhibition of the host immune system that plays an important role in surveillance against infection (3, 4). Clinical evidence however appears to support this notion that early reduction of immunosuppression resulted in diminishing prolonged diarrheal illness caused by rotavirus (6). Apart from the general immunosuppressive property that favors infection, some immunosuppressants could also directly modulate viral infections (7, 8). By using experimental models, a previous study has reported that CsA, a calcineurin inhibitor, can inhibit rotavirus replication associated with restoration of the host antiviral signaling (9). However, to our knowledge, the direct effect of other types of immunosuppressants on rotavirus infection has not been documented.

Understanding such direct effects on rotavirus by different immunosuppressive agents is certainly relevant to the management of infected transplantation patients, as well as to the development of potential new antivirals. Therefore, we aimed to comprehensively profile the effects and mode-of-action of different types of immunosuppressants on rotavirus including corticosteroids (prednisolone (Pred) and dexamethasone (Dex)), calcineurin inhibitors (CsA and tacrolimus (FK506)) and selective antiproliferative agent (e.g. MPA). In vitro cell culture systems that do not contain immune cells are the ideal models for studying such direct effects of immunosuppressants on viral replication. Thus, we first employed models of 2D cell culture of immortalized cell lines. Secondly, we exploited 3D cultures of primary human intestinal organoids developed by us recently for modeling rotavirus infection (10). These organoids recapitulate most if not all aspects of in vivo tissue architecture

of intestinal epithelium (11). Therefore, key findings obtained from the 2D system were subsequently validated in these primary organoids either infected with laboratory or patient-derived rotavirus strains.

2. Materials and methods

2.1. *Immunosuppressants, IMPDH inhibitors, uridine and guanosine*

CsA and FK506 were purchased from Abcam (Cambridge, MA). Dex, Pred and MPA were purchased from Sigma (St Louis, MO). All reagents were dissolved in dimethylsulfoxide (DMSO) and clinically achievable concentrations were used (12-15). Uridine and guanosine were purchased from Sigma (St Louis, MO). 23 IMPDH inhibitors were synthesized at the Center for Drug Design, University of Minnesota, Minneapolis, USA, and their IMPDH1/2 inhibitory constant (K_i) were indicated in Table S1.

2.2. *Viruses*

Simian rotavirus SA11 was used and prepared as described previously (1). Five stool samples were obtained from the Erasmus MC biobank, Department of Viroscience, Erasmus Medical Center Rotterdam. These samples were taken from rotavirus-infected patients during diarrhea period and examined for parechovirus, enterovirus, norovirus genogroups I and II, rotavirus, astrovirus, adenovirus and sapovirus by qRT-PCR described in previous studies (16-18).

2.3. *Cell and human primary intestinal organoid culture*

Caco2 cells and human primary intestinal organoid culture were performed as described previously (10). Briefly, Caco2 cells were grown in DMEM (Lonza, Verviers, Belgium), containing 20% FCS (Sigma-Aldrich, St. Louis USA) and 100 P/S (Gibco, Grand Island, USA) solution at 37°C in a humidified 5% CO₂ incubator. For organoids, tissues were vigorously shaken in 8 mM EDTA for 15 min at 4°C, followed by removing the EDTA solution. Subsequently loosened crypts were collected by pipetting the solution up and down through a 10 ml pipette for 8-10 times. The crypt suspension was transferred into a 50 ml centrifuge tube (Greiner bio-one, the

Netherlands) and biopsies were re-used for repeated collection of crypts (2-3 times). Crypt suspensions were pooled and centrifuged at 300 g for 5 min. Pelleted crypts were re-suspended in 2 ml complete medium with growth factors CMGF-: advanced DMEM/F12 supplemented with 1% (vol/vol) GlutaMAX™ Supplement (Gibco, Grand island, USA), 10 mM HEPES, and collected by centrifugation at 130 g for 5 min at 4°C. Crypts were finally suspended in Matrigel (Corning, Bedford, USA), and placed in the center of a well of a 24-well plate (40 µL per well). After the Matrigel had solidified (15 min at 37°C), organoids were cultured in culture medium at 37°C, 5% CO₂. Culture medium was refreshed every 2-3 days, and organoids were passaged every 6-7 days.

2.4. Inoculation of SA11 and patient rotavirus and treatment of drugs

The protocol of inoculation of SA11 and patient rotavirus was described previously (10). Briefly, cell monolayers of Caco2 cells were incubated with SA11 rotavirus at 37°C with 5% CO₂ for 60 min for infection, followed by removing free virus. Then, cells were added with culture medium (FCS free) containing 5 µg/ml of trypsin and indicative drugs, followed by incubation at 37°C in a humidified 5% CO₂ incubator.

For organoids, which were inoculated to activated virus (5,000 genome copies) for 60 min at 37°C with 5% CO₂, followed by removing free virus. Then, organoids were aliquoted in wells of a 48-well plate coated with 20% (vol/vol) Collagen R Solution (SERVA, Heidelberg, Germany) and culture medium containing drugs of interest was added. Subsequently, the 48-well plate containing organoids was spin down at 500 g for 5 minutes to promote the adherence on the bottom of the wells, followed by maintaining them at 37°C with 5% CO₂.

Preparation of patient-derived rotavirus was performed as described earlier (10), and protocols of viral inoculation and treatment of drugs are similar to SA11 rotavirus.

2.5. RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was extracted using a NucleoSpin® RNA kit (MACHEREY-NAGEL, Düren, Germany), and the concentration was quantified with a Nanodrop ND-1000 (Wilmington, DE, USA). 500 ng of RNA was used as template for cDNA preparation with the reverse transcription system from TAKARA (TAKARA BIO INC). Real-time PCR reactions were performed with SYBR Green (Applied Biosystems®, Austin,

USA) according to the manufacturer's instruction. Quantitative PCR was performed in an IQ5 cycler PCR machine (Bio-Rad). The levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were used as an endogenous reference to normalize the quantities of target mRNA by using the formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{control}}$). All primer sequences were indicated in Table S2.

2.6. Gene silencing assays

Lentiviral shRNA vectors, targeting CypA, CypB, the type II isoform of inosine monophosphate dehydrogenase (IMPDH2) or non-targeted control lentivirus were produced in 293T cells as described previously (19, 20).

Caco2 cells were transduced with lentiviral vectors in order to generate stable gene knockdown cells. Transduced cells were subsequently selected with 5 $\mu\text{g/ml}$ puromycin (Sigma). After pilot study, the shRNA vectors exerting optimal gene knockdown were selected. Knockdown and control Caco2 cells were incubated with rotavirus as described in the foregoing.

2.7. Western blot assay

Cell lysates were subjected to SDS-PAGE, and proteins were transferred to PVDF membrane (Immobilon-FL). IMPDH2 (1:1000, rabbit polyclonal; Abcam), CypA (1:5000, rabbit polyclonal; Abcam), CypB (1:7500, rabbit polyclonal; Abcam) and SA11 rotavirus VP4 (1:1000, HS-2, mouse monoclonal; provided by professor Harry Greenberg, Stanford University School of Medicine, USA) were detected by Western analysis and Detection of β -actin served as loading control (1:1000, mouse monoclonal; Santa Cruz). The intensity of the immunoreactive bands of blotted protein was quantified by the Odyssey V3.0 software.

2.8. MTT assay

Caco2 cells were seeded 1×10^4 cells per well of a 96-well plate and viable cells were detected at indicated time points through adding 10 μl 5 mg/ml MTT per well, 3 h incubation at 37⁰C and replacement of the medium by 100 μl dimethyl sulfoxide (DMSO) (Sigma). Absorbance (490 nm) was analyzed. The effects of immunosuppressants on host cell viability were determined by MTT assay (Figure S1).

2.9. *Serial passaging of rotavirus with MPA treatment*

Caco2 cells were infected with SA11 strain rotavirus in three wells of 24-well plate in the presence of MPA at concentration of 0.5 µg/mL (two wells) or control (one well). One of infected cultures with MPA treatment and control were analyzed viral RNA by qRT-PCR for evaluating antiviral effect of MPA 48 h post-incubation. Another infected culture with MPA treatment were frozen, thawed once, and centrifuged. The supernatant containing passaged virus were stored at -80°C. Virus was serially passaged by using 1 aliquot of viral stock from the preceding passage to infect fresh Caco2 cells.

2.10. *IC₅₀ and CC₅₀ calculation*

50% inhibition concentration (IC₅₀) value and 50% cytotoxic (CC₅₀) were calculated based on model $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}$ by using GraphPad Prism 5 software (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA).

2.11. *Study approval*

Human intestinal tissues were obtained during surgical resection. The volunteers or patients agreed to participate by written informed consent, and the study was approved by the Medical Ethical Committee of the Erasmus Medical Center (Medisch Ethische Toetsings Commissie Erasmus MC).

2.12. *Statistics*

All numerical results are reported as Mean ± SEM. The statistical significance of differences between means was assessed with the Mann-Whitney test (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA). The threshold for statistical significance was defined as $P \leq 0.05$.

3. Results

3.1. *Glucocorticosteroids and FK506 did not affect rotavirus replication*

As the first generation of immunosuppressants, Pred and its close analogue Dex remain widely used in organ transplantation recipients to prevent graft rejection (21). To examine their possible effects on rotavirus, the human intestinal Caco2 cell line was infected with SA11 rotavirus and treated with Pred or Dex for 48 h. As shown in Figure 1, Pred and Dex had no significant effect on rotavirus replication.

We next tested FK506, a calcineurin inhibitor, which inhibits calcineurin by binding to FK506-binding protein (FKBP) (22). Similar to steroids, FK506 had no significant effect on rotavirus infection in Caco2 cell line (Figure 2).

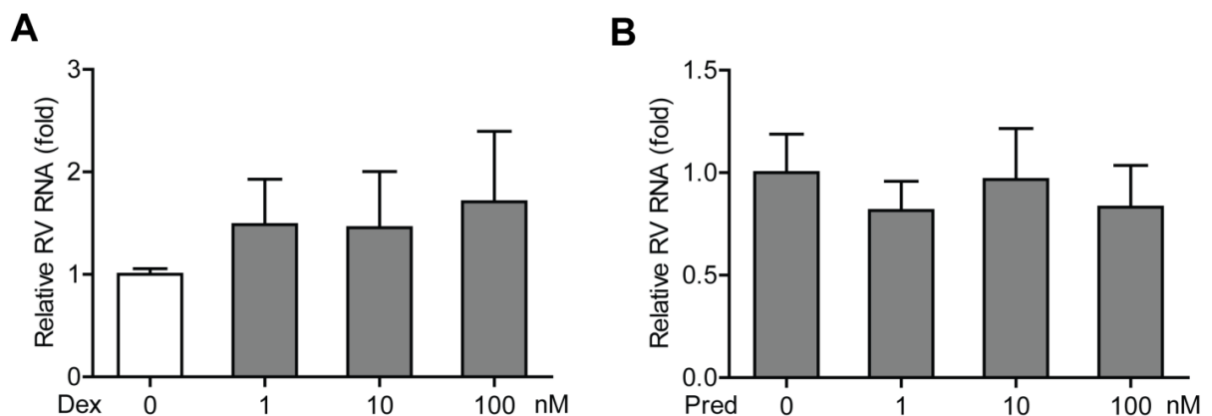


Figure 1. Effect of steroids on SA11 rotavirus in Caco2 cells. Dex (A) and Pred (B) do not influence SA11 rotavirus replication in Caco2 cells (48 h, $n = 4-8$, means \pm SEM, Mann-Whitney test). The rotavirus RNA was quantified by qRT-PCR and normalized to reference gene GAPDH.

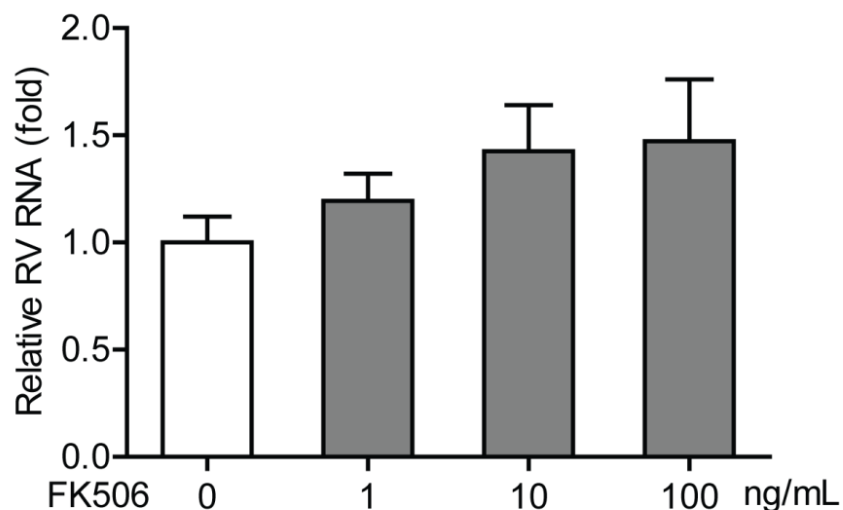


Figure 2. Effect of FK506 on SA11 rotavirus in Caco2 cells. FK506 (48 h treatment) does not affect SA11 rotavirus replication in Caco2 cells. The data represent medians and SEM from three independent experiments in triplicates. The rotavirus RNA were quantified by qRT-PCR and normalized to reference gene GAPDH.

3.2. *The cellular targets of CsA, CypA inhibits but CypB promotes rotavirus replication*

CsA is another type of calcineurin inhibitor, which binds to the cytosolic proteins cyclophilin A and B to inhibit calcineurin and, consequently, T cell proliferation (23). Treatment of SA11 rotavirus infected Caco2 cells with 1, 5, 10 $\mu\text{g/ml}$ of CsA resulted in moderate inhibition of rotavirus replication without a clear dose dependence. Significant inhibition was only observed at 5 $\mu\text{g/ml}$, resulting in a $58 \pm 9\%$ ($n = 7$; $P < 0.05$) reduction of viral RNA (Figure 3A).

The variability of rotavirus response to CsA inspired us to gain further its mechanistic insights. In an effort to characterize these, Caco2 cells were transduced with integrating lentiviral vectors expressing both shRNA and puromycin gene to silence CypA and CypB, the cellular targets of CsA. After puromycin selection, the effects of gene knockdown were quantified by western blot. Both of the shRNA clones targeting CypA (Figure 3B) and CypB (Figure 3D) showed potent gene knockdown. Caco2 cells with CypA and CypB knockdown or scrambled shRNA (as a control) were inoculated with SA11 rotavirus. The viral genomic RNA was quantified by qRT-PCR assay 24 h post inoculation. Surprisingly, silencing of CypA led to 7.35 ± 2.38 -fold ($n = 8$; $P < 0.001$) increase of rotavirus RNA (Figure 3C). In contrast, silencing of CypB resulted in $63 \pm 15\%$ ($n = 4$; $P < 0.05$) inhibition of viral genomic RNA (Figure 3E). Interestingly, the expression of CypA or CypB critically regulates the responsiveness of rotavirus to CsA treatment (Figure 3F). Taken together, the cellular targets of CsA, CypA and CypB have opposing effects on rotavirus infection, which together determine the responsiveness to CsA.

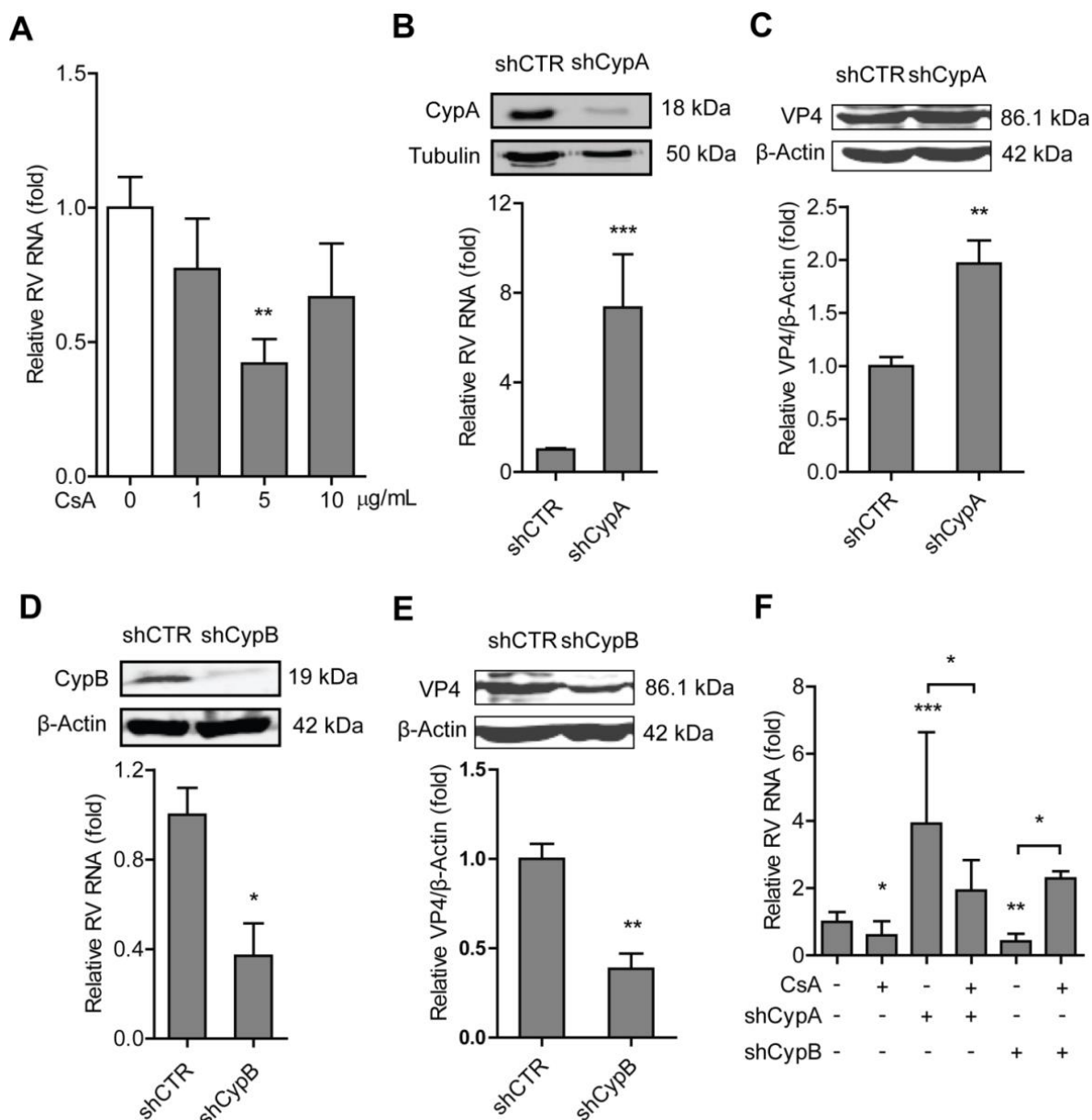


Figure 3. CypA inhibits, and CypB promotes rotavirus replication, which tightly regulates responsiveness of rotavirus to CsA treatment. (A) CsA moderately inhibits rotavirus replication in Caco2 cells without a clear dose dependence (24 h, $n = 7$, means \pm SEM, $**P < 0.01$, Mann-Whitney test). (B) Western blot analysis revealed potent knockdown of CypA in Caco2 cells. Silencing of CypA remarkably promoted rotavirus replication ($n = 10$, means \pm SEM, $***P < 0.001$, Mann-Whitney test). Viral genomic RNA was quantified by qRT-PCR. (C) Silence of CypA significantly increased the level of rotavirus protein VP4 ($n = 4$, means \pm SEM, $**P < 0.01$, Mann-Whitney test). (D) Western blot analysis indicates potent knockdown of CypB in Caco2 cells. Silencing of CypB potently inhibited rotavirus replication in Caco2 cells ($n = 4$, means \pm SEM, $*P < 0.05$, Mann-Whitney test). Viral genomic RNA was quantified by qRT-PCR. (E) Silence of CypB significantly decreased the level of rotavirus protein VP4 ($n = 4$, means \pm SEM, $**P < 0.01$, Mann-Whitney test). (F) The expression of CypA or CypB critically regulates the responsiveness of rotavirus to CsA treatment ($n = 3$, means \pm SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Mann-Whitney test).

3.3. *MPA potently inhibited the multiplication of SA11 and patient-derived rotavirus strains in both cell line and organoids models*

MPA and its prodrug, mycophenolate mofetil (MMF), are widely used in solid organ transplantation and are often administered in combination with other immunosuppressive agents (24). SA11 rotavirus infected Caco2 cells were incubated with different concentrations of MPA (0, 0.1, 0.3, 0.5, 1, 5, 10, 20 and 50 $\mu\text{g/ml}$), indicating that MPA could potently inhibit rotavirus replication (Figure 4A). 50% inhibition concentration (IC₅₀) value and 50% cytotoxic (CC₅₀) were calculated based on model $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}$ by using GraphPad Prism 5 software. IC₅₀ value of MPA against SA11 rotavirus was 0.885 μM (Figure 4A), CC₅₀ of MPA to Caco2 cells was 438.07 μM (Figure S2A) and selectivity index (SI, CC₅₀/IC₅₀) was 495.05. Western blot analysis also indicated that treatment with 1 and 10 $\mu\text{g/ml}$ of MPA (24 and 48 h) potently inhibited rotavirus infection (Figure 4B). To further validate these effects in untransformed cultures more close resembling the physiological situation, we employed a 3D culture model of human primary intestinal organoids infected with SA11 rotavirus (Figure 4C). Treatment of organoids for 24 h with 1 and 10 $\mu\text{g/ml}$ of MPA reduced cellular viral RNA by $40 \pm 6\%$ ($n = 5$; $P < 0.001$) and $63 \pm 3\%$ ($n = 5$; $P < 0.001$), respectively (Figure 4D). The Consistently, MPA also significantly inhibited virus production by organoids (Figure 4E), and the viral titer was determined by standard curve approach that was described previously (10).

Next, we examined whether MPA could also inhibit the infection of patient-derived rotavirus strains in these two models. Hereto, Caco2 cells or human intestinal organoids were incubated with stool samples from five rotavirus-infected patients (Table S3). Treatment of infected Caco2 cells with 1 or 10 $\mu\text{g/ml}$ of MPA for 24 h profoundly inhibited virus replication in all five instances (Figure 4F). The IC₅₀ value and selectivity index of MPA against patient derived rotavirus in Caco2 cells are 0.583 μM (Figure S2B) and 751.41, respectively. This inhibitory effect was further confirmed in the organoid model, although the level of inhibition attained was smaller than for Caco2 cells (Figure 4G), since the 3D organoids system is a more stringent model as we have described previously (10). Thus, we have demonstrated that MPA could effectively inhibit infection by the SA11 and patient-derived rotavirus stains in both a cell line and in the organoid model.

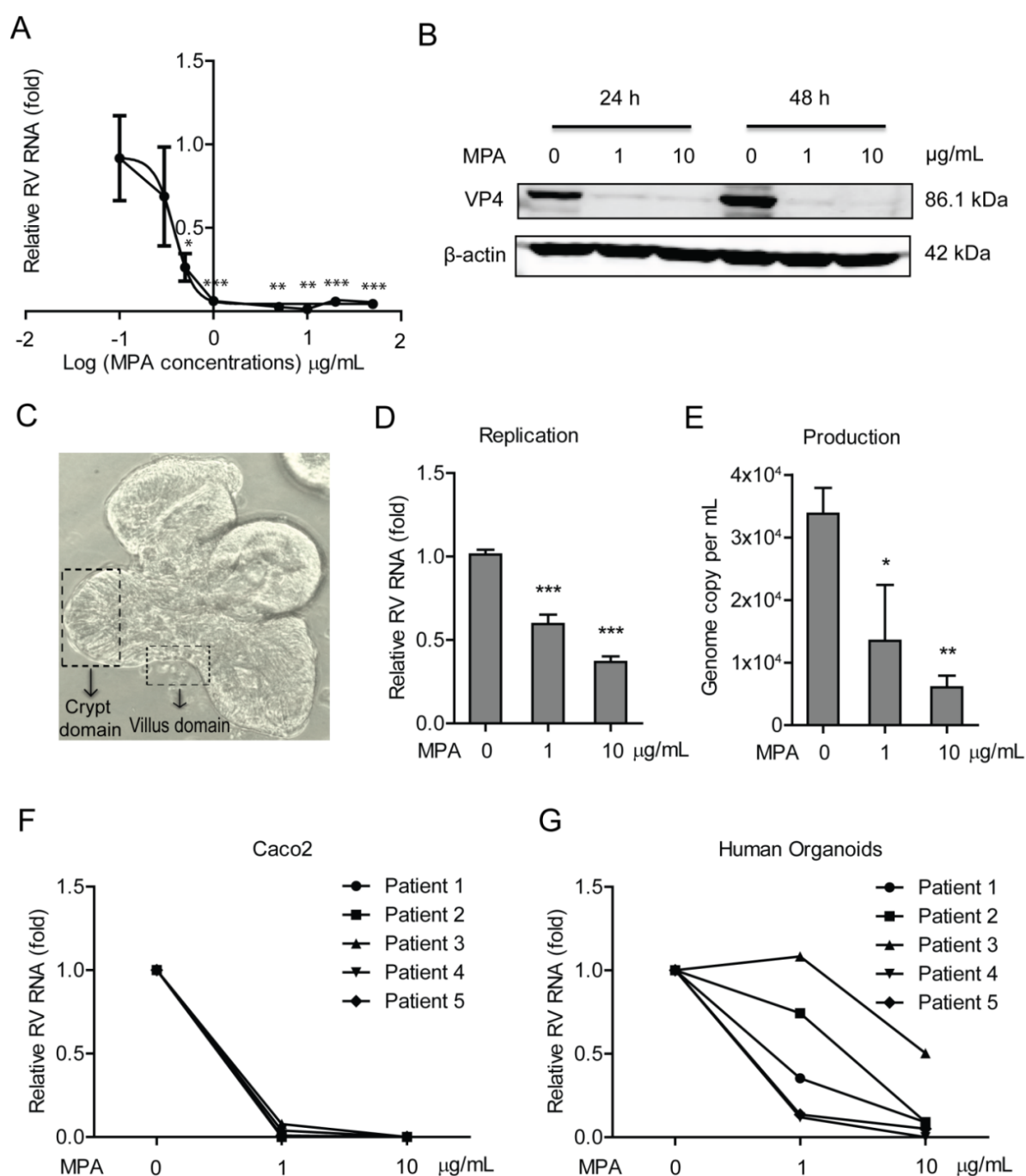


Figure 4. MPA effectively inhibits the replication of SA11 and patient-derived rotavirus strains in both Caco2 cell line and organoid models. (A) MPA potently inhibits SA11 rotavirus replication in Caco2 cells (24 h, $n = 3-12$, means \pm SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Mann-Whitney test). Viral genomic RNA was quantified by qRT-PCR. IC₅₀ of MPA against SA11 rotavirus in Caco2 cells was calculated by using GraphPad Prism 5 software. (B) Western blot analysis indicates potent inhibitory effect of 1 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$ of MPA against rotavirus infection. (C) Morphology of human small intestinal organoid on day 4 post embedding in Matrigel, which shows clear crypt domain and villus domain. (D) MPA significantly inhibits SA11 rotavirus genomic RNA in organoids (24 h, $n = 4-5$, means \pm SEM, $*P < 0.001$, Mann-Whitney test). (E) MPA significantly inhibits SA11 rotavirus production in organoids (24 h, $n = 4-5$, means \pm SEM, $*P < 0.05$, $**P < 0.01$, Mann-Whitney test). (F) MPA

inhibits patient-derived rotavirus replication in Caco2 cells. (G) MPA inhibits patient-derived rotavirus replication in human primary intestinal organoids.

3.4. *MPA, an IMPDH inhibitor, inhibits rotavirus replication through depletion of the cellular guanosine nucleotide pool*

An important mechanism of MPA to inhibit T cell proliferation is to deplete the guanine nucleotides (GTP and dGTP) pools by targeting IMPDH enzymes, in particular IMPDH2, which catalyze the biosynthesis of GTP/dGTP (25). To understand whether it exerts a similar mode-of-action against rotavirus infection, we first used an RNAi-based loss-of-function assay to study the role of IMPDH2. Caco2 cells were transduced with integrating lentiviral vectors expressing a shRNA targeting IMPDH2 and puromycin resistance gene. The shRNA clone with the highest knockdown of IMPDH2 was selected (Figure 5A). Silencing of IMPDH2 resulted in $60 \pm 9\%$ ($n = 6$; $P < 0.01$) inhibition of rotavirus genomic RNA (Figure 5B). To further verify the effect of IMPDH2 on rotavirus, IMPDH2 was overexpressed by transducing an integrating lentiviral vector expressing both GFP and IMPDH2 in Caco2 cells, and the expression of the IMPDH2-GFP fusion protein was detected by flow cytometric analysis of green fluorescence (Figure 5C). Consistently, forced expression of IMPDH2 increased viral genomic RNA by 3.62 ± 0.93 -fold ($n = 7$; $P < .05$) (Figure 5D). To further explore the potential of targeting IMPDH enzymes, we have developed a panel of new IMPDH inhibitors. 22 out of 23 tested compounds showed significant inhibitory effects against rotavirus (Figure 5E).

To further validate whether the anti-rotavirus effect of MPA is via depleting guanosine nucleotides pool, exogenous guanosine (100 $\mu\text{g/ml}$) was supplemented. Indeed, supplementation of guanosine almost completely abolished the anti-rotavirus effects of MPA in both Caco2 (Figure 5F) and human intestinal organoids (Figure 5G) models. In addition, supplementation of exogenous guanosine also remarkably restored the inhibitory effect of MPA on rotavirus production in the organoids model (Figure S3). Taken together, these data indicate that MPA exerts anti-rotavirus effect by targeting IMPDH enzymes to deplete the cellular nucleotide pool.

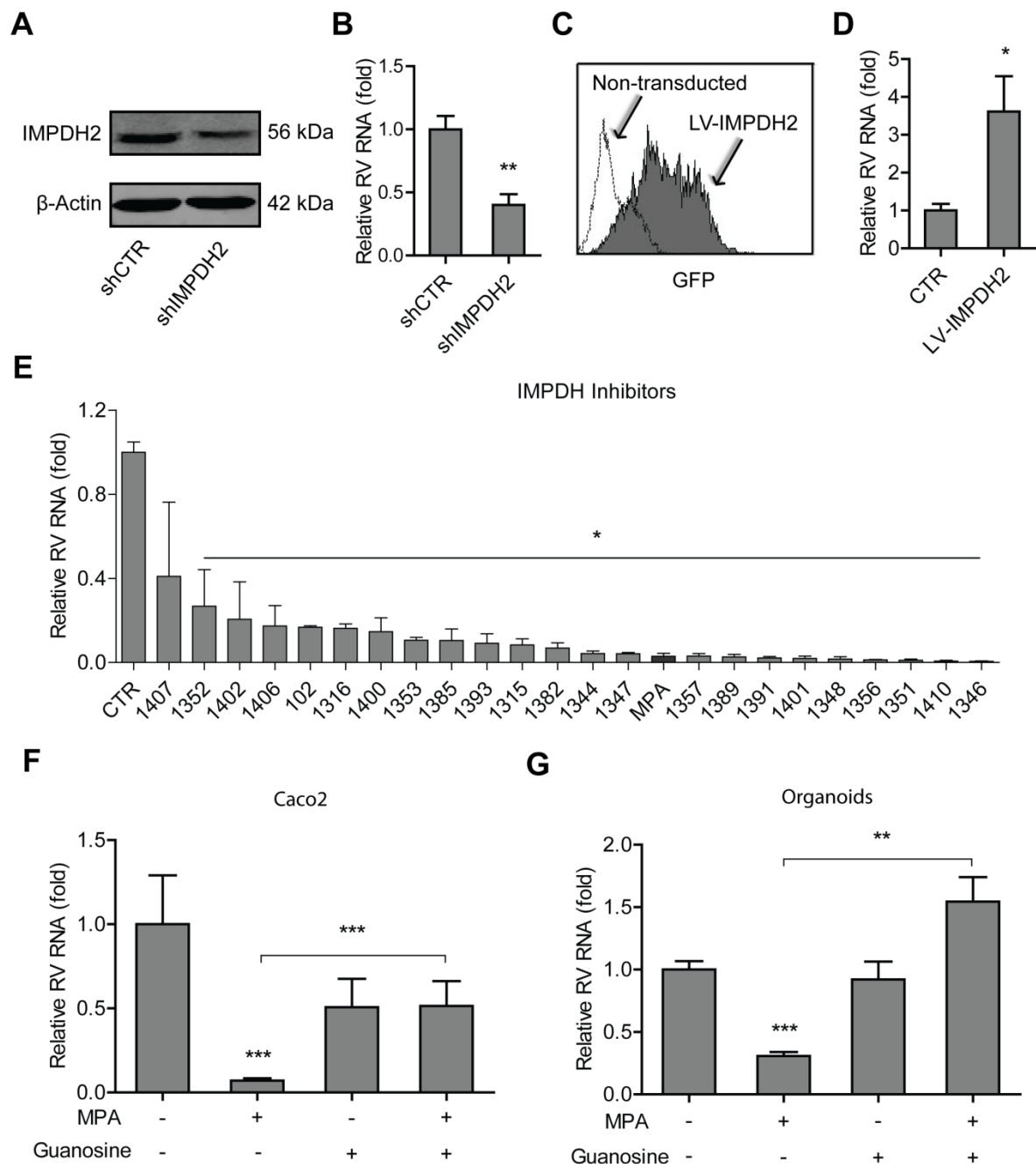


Figure 5. Inhibition of MPA on rotavirus is via depletion of cellular guanosine nucleotide pool by targeting IMPDH enzyme. (A) Western blot analysis shows knockdown of IMPDH2 in Caco2 cells. (B) Knockdown of IMPDH2 significantly inhibits SA11 rotavirus replication in Caco2 cells ($n = 6$, means \pm SEM, $**P < 0.01$, Mann-Whitney test). (C) Expression of the IMPDH2-GFP fusion protein in LV-IMPDH2 transduced Caco2 cells could be detected by flow cytometric analysis of green fluorescence. (D) Over-expression of IMPDH2 facilitates SA11 rotavirus replication in Caco2 cells ($n = 7$, means \pm SEM, $*P < 0.05$, Mann-Whitney test). (E) 22 out of 23 tested IMPDH inhibitors show significant inhibitory effects against rotavirus (24 h, $n = 3$, means \pm SEM, $*P < 0.05$, Mann-Whitney test) (F) Supplementation of exogenous guanosine attenuates the anti-rotavirus effect of MPA in either Caco2 cells (24 h, $n = 8-12$, means \pm SEM, $***P < 0.001$, Mann-Whitney test) or (G) in the human primary intestinal organoids (24 h, $n = 4-9$, means \pm SEM, $**P < 0.01$, $***P < 0.001$, Mann-Whitney test).

3.5. MPA has a high barrier to drug resistance development

For optimal antiviral efficacy, a medication should display a high barrier to resistance development, since viruses are prone to mutagenesis and thus drug resistance easily emerges. To characterize MPA in this respect, we employed a well-established multi-passaging experiment in which SA11 rotavirus was constantly kept under selection pressure by exposure to MPA. Interestingly, MPA retained its anti-rotavirus activity even after 30 passages (Figure 6A). Furthermore, the viruses of passage 0, 5, 10, 15, 20, 25 and 30 remain sensitive and dose-dependent in responding to MPA treatment (0.3, 0.5, 1, 5 and 10 $\mu\text{g/ml}$), suggesting that MPA has a high barrier to the development of drug resistance (Figure 6B).

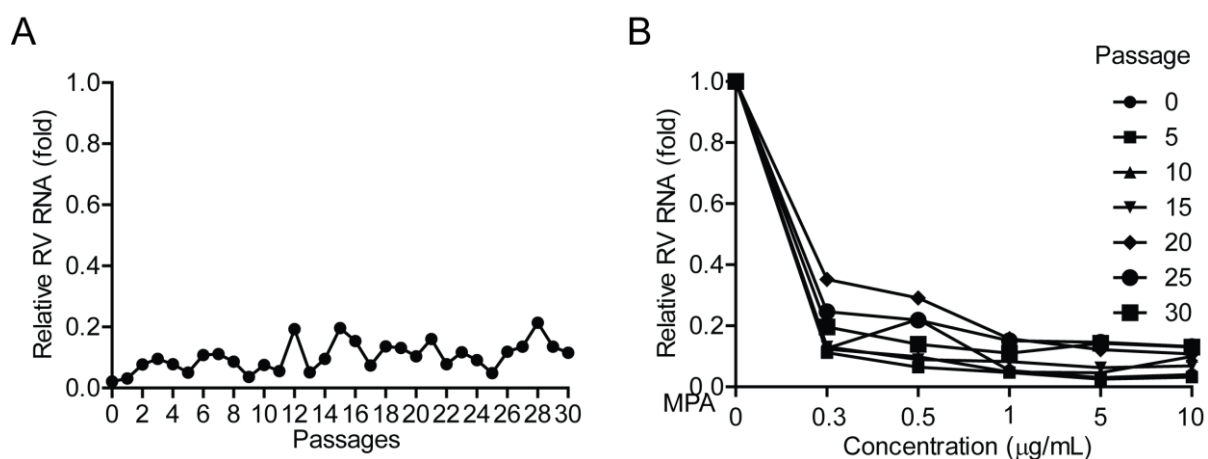


Figure 6. MPA has a high barrier to drug resistance development. (A) MPA retains its anti-rotavirus activity even with continuous treatment for 30 passages. (B) The viruses of passage 0, 5, 10, 15, 20, 25 and 30 remain sensitive and dose-dependent in responding to MPA treatment (0.3, 0.5, 1, 5 and 10 $\mu\text{g/ml}$).

4. Discussion

Rotavirus has been recently recognized as an emerging cause of complications in organ transplantation patients, which was generally thought to be associated with the use of immunosuppressants (5, 26). However, apart from clinically effective in preventing graft reaction, immunosuppressants could also directly affect viral infection (7). By profiling different types of immunosuppressants, this study identified MPA as a potent inhibitor of rotavirus infection.

To dissect such direct effects of immunosuppressants on viruses, *in vitro* models that do not contain immune cells are useful, as they avoid troublesome interpretation as a consequence of diminished anti-viral immunity *per se*. Since rotavirus infects the intestinal epithelium, 2D culture systems of intestinal carcinoma cell lines are widely used to study rotavirus infection. Although these are convenient and valid model systems, they do not capture the dynamics and architectures of the intestinal epithelium. Thus, we have recently developed 3D culture of human primary intestinal organoids for modeling rotavirus infection (10), to circumvent some intrinsic limitations of the classical models (11). Of note, primary organoids represent more stringent models and thus are less sensitive to antivirals compared to cell line systems (10), and thus can serve as a valuable tool for validation of results obtained from conventional models. Secondly, only a limited number of well-adapted laboratory rotavirus strains are used for experimental research, which clearly do not fully recapitulate the diversity of the over 110,000 strains that are circulating over the world (17). Another strength of the present study is the use of both laboratory and patient-derived strains. Hence, we feel our setup appropriate for characterizing the direct effects of immunosuppressive medication on rotavirus infection.

The debate on the possible differential impact of immunosuppressants on viral infection post-transplantation was sparked by the observation of a direct antiviral effect of CsA in HCV cell culture models (27). Further mechanistic studies revealed that CsA acts via inhibition of cyclophilins, including CypA and CypB, which are required for efficient replication of HCV (28, 29). In contrast, we recently have demonstrated that CsA can promote HEV replication, as basal CypA and CypB are suppressing HEV (8). Although previous studies have reported anti-rotavirus effect of CsA (9, 30), we found only moderate inhibitory effects in Caco2 cells and no clear effect in the intestinal organoid model (Figure S4). Interestingly, we found opposing effects of CypA and CypB on rotavirus, inhibiting and promoting infection respectively. In our model system, as in many other cell types examined (31), CypA and CypB are concurrently and abundantly expressed, which may explain the moderate effect of CsA on rotavirus infection. More interestingly, the expression levels of CypA and CypB tightly regulate the responsiveness to CsA (Figure 3F). Gene silencing of CypA facilitated rotavirus infection but resulted in better response to CsA. In contrast, silencing of CypB inhibited rotavirus but led to further enhancement by CsA treatment (Figure 3F). If translating these results into clinical practice, it will be critical to first

determine the expression levels of CypA and CypB in the intestinal tissue of the patients, before applying CsA for these rotavirus infected organ recipients.

MPA has also been reported to have a broad antiviral activity against including dengue virus (32), West Nile virus (33), yellow fever virus (34) Chikungunya virus (35), HEV (8) and HCV (33, 36, 37), although studies also reported its pro-viral effect for hepatitis B virus (38, 39). In the present study, we have demonstrated that clinically achievable concentrations of MPA could profoundly inhibit the infection of both laboratory and patient-derived rotavirus stains in Caco2 cells. These inhibitory effects on both virus replication and production were further confirmed in the organoid model. Importantly, we found a high barrier to drug resistance development, which potentially enables a long-term treatment of MPA for rotavirus infected organ recipients without big concerns with respect to the emergence of innate resistance in viral strains.

As an uncompetitive inhibitor of the IMPDH enzymes, MPA preferentially binds to the IMPDH2 isoform to suppress purine nucleotide synthesis, resulting in depletion of intracellular guanosine nucleotide pools (36). This is thought to be the general mechanism of immunosuppressive and antiviral actions of MPA (7), although there are exceptions, such as the anti-HCV effect that is largely independent of nucleotide depletion (7). Using gain- and loss-of-function assays, we have demonstrated that IMPDH2 plays an important role in supporting rotavirus infection. Targeting IMPDH by MPA or a panel of other IMPDH inhibitors have resulted in potent anti-rotavirus effects. Consistently, supplementation of exogenous guanosine could almost completely abrogate the anti-rotavirus activity of MPA. Thus, the anti-rotavirus action of MPA occurs via the classical cascade of targeting IMPDH enzymes to deplete nucleotides.

In summary, by profiling different immunosuppressants, we have found that the responsiveness of rotavirus to CsA treatment is largely determined by the relative expression levels of its cellular targets, CypA and CypB. Importantly, we have identified MPA as a potent inhibitor of rotavirus infection with a high barrier to resistance development. It acts via the inhibition of the IMPDH enzymes to deplete cellular nucleotide to restrict rotavirus infection. Thus, understanding the distinct effects and their mode-of-actions of different immunosuppressants on rotavirus infection bears important implications for transplant clinicians to design optimal protocols of immunosuppression for infected organ recipients.

Acknowledgments

The authors are grateful to Professor Harry Greenberg (Stanford University School of Medicine, USA) for providing the mouse monoclonal antibody against rotavirus VP4 protein. We also thank the general support of the Center for Drug Design (University of Minnesota, USA) for developing the IMPDH inhibitors. This work was supported by the Dutch Digestive Foundation (MLDS) for a career development grant (No. CDG 1304 to Q. P.), the Netherlands Organization for Scientific Research (NWO/ZonMw) for a VENI grant (No. 916-13-032 to Q. P.), the Erasmus MC Mrace grant (360525 to Q. P.), and the China Scholarship Council for funding PhD fellowship (201307720045 to Y. Y.), (201207720007 to Y. W.), (201406180072 to W. D.), (201306300027 to L. X.), (No. 201206150075 to X. Z), (201303250056 to W. W.).

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Supplementary Tables & Figures

Table S1. The IMPDH1/2 inhibitory constant (Ki) of 23 IMPDH inhibitors.

Compound ID	102	1315	1316	1344	1346	1347	1348	1351	1352	1353	1356	1357
IMPDH1 Ki in nM	330	0,6	82,1	162,8	859,1	703,4	1725	618,4	136,2	1255	70,12	1813
IMPDH2 Ki in nM	250	13,9	55,7	102,4	243,9	230,1	365,7	185,9	93,01	364,7	65,69	552,5
Compound ID	1382	1385	1389	1391	1393	1400	1401	1402	1406	1407	1410	
IMPDH1 Ki in nM	1815	521,8	1127,4	4221	5556	9138	2553	2203	ND	ND	ND	ND
IMPDH2 Ki in nM	2154	487,4	297,9	2160	2398	367,7	231,1	146,7	ND	ND	ND	ND

Table S2. Primers used in the study

Sequence of rotavirus primers

	SA11 Rotavirus	Human Patient Rotavirus
Sense	TGGTTAAACGCAGGATCGGA	ACCATCTACACATGACCCTC
Anti-sense	AACCTTCCGGTCTGGTAG	CACATAACGCCCTATAGCC

Primer sequences of GAPDH

Primers	Human	Mouse
GAPDH-F	GTCTCCTCTGACTTCAACAGCG	TTCCAGTATGACTCCACTCACGG
GAPDH-R	ACCACCCTGTTGCTGTAGTACC	TGAAGACACCAGTAGACTCCACCAG

Table S3. Patient characteristics.

Patient	Age (yrs)	Gender	Symptoms	Virus Detection								
				Enterovirus	Parechovirus	Norovirus genogroups I	Norovirus genogroups II	Adenovirus	Astrovirus	Sapovirus	Rotavirus	
1	3.5	Female	Fever	No	No	No	No	No	No	No	No	Yes
2	74	Female	Congestive heart failure, myocarditis	No	No	No	No	No	No	No	No	Yes
3	27	Female	Fever, diarrhea, nausea, vomiting	No	No	No	No	No	No	No	No	Yes
4	67	Male	Fever, stomach ache, watery diarrhea (Kidney transplant)	No	No	No	No	No	No	No	No	Yes
5	28	Female	Nausea, stomach ache, watery diarrhea, fever, headache, vomiting	No	No	No	No	Yes	No	No	No	Yes

Supplementary Figures

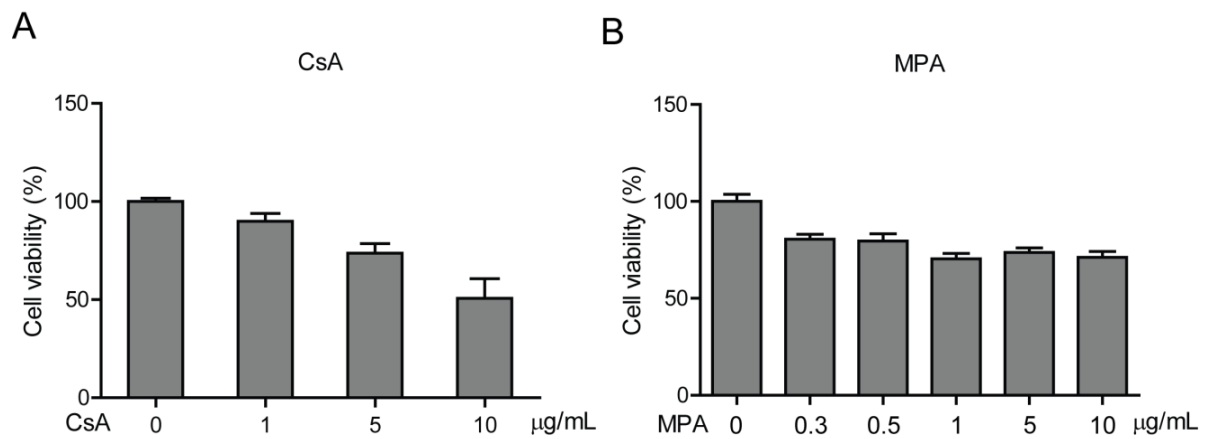


Figure S1. Effect of immunosuppressants on host cell viability determined by MTT assays. (A) Effect of CsA on viability of Caco2 cells (24 h). (B) Effect of MPA on viability of Caco2 cells (24 h).

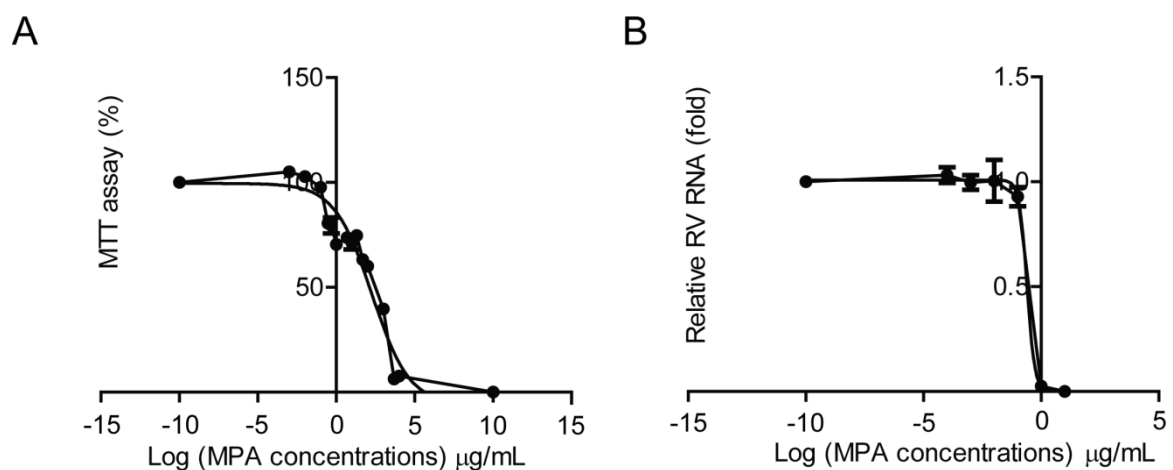


Figure S2. 50% cytotoxic (CC_{50}) of MPA against Caco2 cells and 50% inhibition concentration (IC_{50}) of MPA against patient-derived rotavirus in Caco2 cells. (A) CC_{50} of MPA against Caco2 cells. Caco2 cells were incubated with indicated doses of MPA (0, 0.001, 0.01, 0.1, 0.3, 0.5, 1, 5, 10, 20, 50, 100, 1000, 5000 and 10000 μg/mL) for 24 h, and effect of MPA on host cell viability determined by MTT assay. (B) IC_{50} of MPA against patient-derived rotavirus in Caco2 cells. Five patient rotavirus isolates infected Caco2 cells were incubated with indicated doses of MPA (0, 0.001, 0.01, 0.1, 1 and 10 μg/mL) for 24 h, and viral RNA was quantified by qRT-PCR.

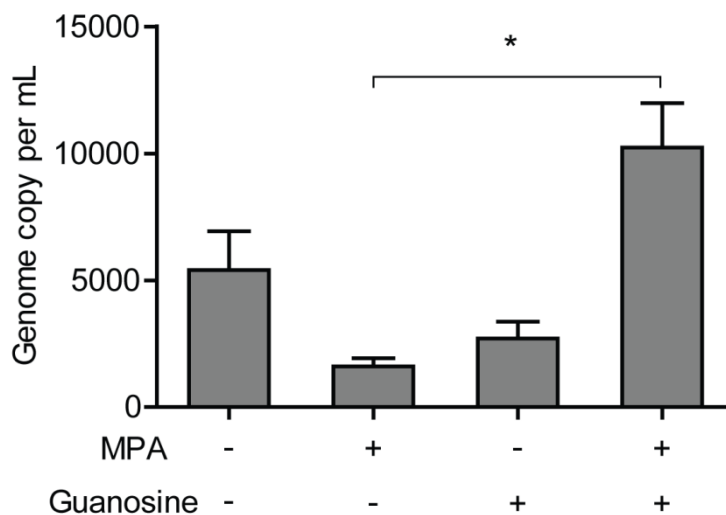


Figure S3. Supplementation of exogenous guanosine restores anti-rotavirus effects of MPA in the organoids. Rotavirus infected human primary intestinal organoids were treated with MPA (10 $\mu\text{g}/\text{mL}$), guanosine (100 $\mu\text{g}/\text{mL}$) and both MPA and guanosine (10 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$, respectively). Rotavirus genome copies were determined by qRT-PCR referring to a plasmid template using a standard curve calculation method described previously (1) ($n = 4$, means \pm SEM, * $P < 0.05$, Mann-Whitney test).

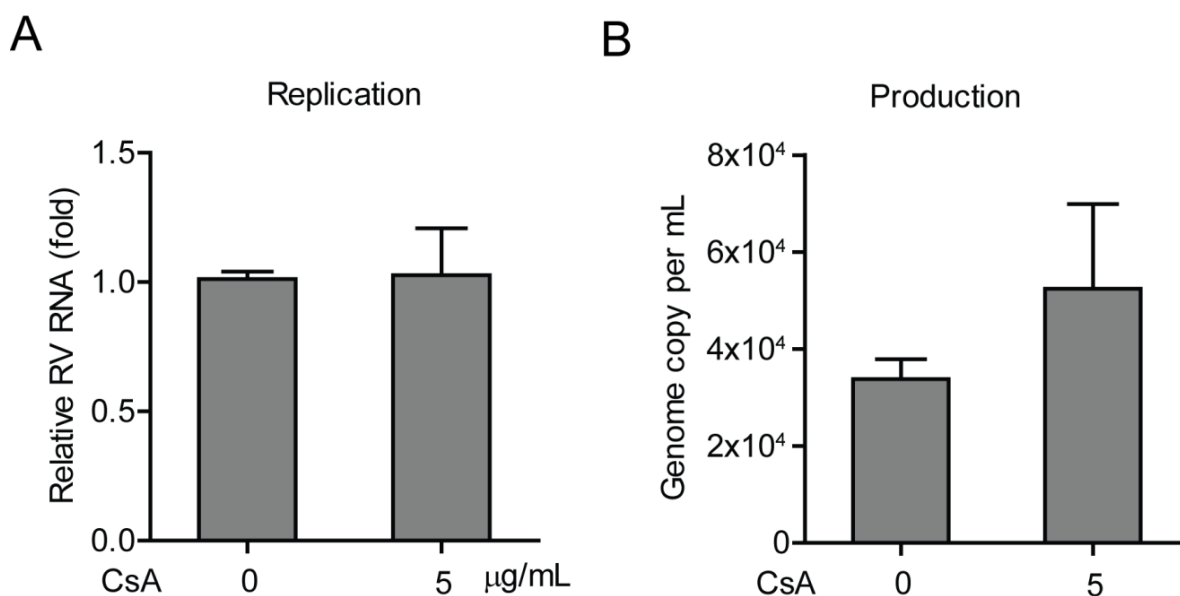


Figure S4. Effect of CsA on SA11 rotavirus replication and production in organoids. CsA dose not influence SA11 rotavirus replication (A) ($n = 4-10$, means \pm SEM, Mann-Whitney test) and rotavirus particles production (B) ($n = 4-10$, means \pm SEM, Mann-Whitney test) in human primary intestinal organoids.

Supplementary Reference

1. Yin Y, Bijvelds M, Dang W, Xu L, van der Eijk AA, Knipping K, Tuysuz N, Dekkers JF, Wang Y, de Jonge J, Sprengers D, van der Laan LJ, Beekman JM, Ten Berge D, Metselaar HJ, de Jonge H, Koopmans MP, Peppelenbosch MP, Pan Q. 2015. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. *Antiviral Res* **123**:120-131.

CHAPTER 5

6-thioguanine potently inhibits rotavirus infection through suppression of Rac1 activation

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Under Review

Abstract

Rotavirus has emerged as an important cause of complications in organ transplantation recipients, and might be associated with inflammatory bowel disease (IBD). 6-thioguanine (6-TG) has been used as an immunosuppressive drug for organ recipients and treatment of IBD in the clinic. 6-TG has been reported to exert antiviral effects on particular viruses, but its effect on rotavirus infection remains unclear. To investigate the effects and mode-of-action of 6-TG on rotavirus infection. Caco2 cell line, 3D model of human primary intestinal organoids, laboratory rotavirus strain (SA11) and patient-derived rotavirus isolates were used. 6-TG potently inhibits rotavirus RNA replication and VP4 protein synthesis in Caco2 cell model. This effect was further confirmed in primary organoids model with both laboratory and patient-derived isolates. Importantly, continuous treatment with 6-TG for 20 passages did not attenuate its antiviral potency, indicating a high barrier to drug resistance development. Mechanistically, we find that Rac1, the cellular target of 6-TG, is essential in supporting rotavirus infection that gene knockdown or knockout of Rac1 significantly inhibited its infection. We further demonstrated that 6-TG can effectively inhibit the active form of Rac1 (GTP-Rac1). Consistently, ectopic over-expression of GTP-Rac1 facilitates but an inactive Rac1 (N17) or a specific Rac1 inhibitor (NSC23766) inhibits rotavirus infection. We have identified 6-TG as a potent inhibitor of rotavirus infection via the inhibition of Rac1 activation. Thus for transplantation patients or IBD patients infected or at risk of rotavirus infection, the choice of 6-TG as a treatment appears rational.

Introduction

Rotavirus, a member of family *Reoviridae*, is a double stranded RNA virus mainly infecting infants younger than 5 years old, resulting in severe gastroenteritis disorders. This virus causes 114 million diarrhea episodes, 2.4 million hospitalizations, and an estimated half million deaths annually (1). Although rotavirus infection mainly occurs in developing countries, it has been reported to result in over 200 deaths and more than 87,000 hospital admissions in infants in European Union (2). Besides young children, organ transplantation patients were found to be susceptible to rotavirus infection irrespective to ages, which caused long-term diarrhea even death due to graft failure (3). Nowadays, two licensed rotavirus vaccines including RotaTeq (Merck and Co, PA, USA) and Rotarix (GSK Biologicals, Rixensart, Belgium) have been confirmed to be useful to reduce rotavirus epidemic (4). But for most middle-income and low-income countries, the protective effectiveness of vaccines is still suboptimal (5). No specific antiviral medicines are currently available to treat rotavirus infection, except for supportive cares including rehydration and maintenance of fluid and electrolyte balance (6). Hereto, antiviral therapy represents an important complement to combat rotavirus infection that cannot be substituted by vaccines. For those resource-limited regions, it is more accessible and cost-effective to identify effective treatment from the existing approved medications.

6-thioguanine (6-TG) is a thio analogue of the naturally occurring purine base guanine, and it has been used in the clinic since the early 1950s (7). 6-TG was initially developed to treat cancer; whereas currently it is widely used as an immunosuppressive agent in organ transplantations and as treatment for acute lymphoblastic leukemia in children and for rheumatic diseases (8). In particular, 6-TG is more often used to treat inflammatory bowel disease (IBD) (9). IBD including Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis (IC) represents a heavy burden in western countries (10). Although the causes of exacerbations of IBD remain poorly characterized, gastrointestinal infections including rotavirus might induce flares in IBD (11).

Upon entry into the host, 6-TG is metabolized into 6-thioguanosine monophosphate (6-TGMP), followed by being metabolized into 6-thioguanosine diphosphate (6-TGDP), and subsequently 6-thioguanosine triphosphate (6-TGTP)

(12). Among these metabolites, 6-TGDP and 6-TGTP are able to competitively bind to Rac1 to form 6-TGNP•Rac1 complex to inhibit the activation of Rac1 (13). As a member of the Ras superfamily of Rho GTPases, Rac1 involves in various cellular signaling and regulates a spectrum of cellular processes including actin reorganization and gene transcription. The cycling between the inactive GDP-bound (GDP-Rac1) and active GTP-bound states (GTP-Rac1) has been implicated in regulating viral infections (13). Silence of the active form of Rac1 (GTP-Rac1) has been reported to inhibit hepatitis C virus (HCV) replication, viral protein synthesis and production of infectious viral particles (14). Rac1 also regulates viral entry of many viruses including African swine fever virus, dengue virus, adenovirus and Ebola virus (15). The inhibitors of Rac1, 6-TG and NSC23766, were reported to inhibit middle east respiratory syndrome coronavirus (16) and influenza virus (15), respectively. These accumulating evidence indicate that Rac1 could be an potential target for developing antiviral drugs against a broad spectrum of viruses.

Given the clinical relevance of rotavirus infection in organ transplantation and IBD patients and the common use of 6-TG in these patient, we aim to investigate the effects and mechanism-of-action of 6-TG on rotavirus infection. We used rotavirus infection models based on the conventional 2D cell culture and 3D culture of human primary intestinal organoids, which are more resemble of the *in vivo* architecture of intestine. We have demonstrated that 6-TG potently inhibits the infection of both experimental strain and patient-derived rotavirus isolates with a high barrier to drug resistance development. We further dissect that the anti-rotavirus effect of 6-TG is via the inhibition of the activation of Rac1, an essential host factor supporting rotavirus infection.

Materials and methods

Viruses and reagents

Simian rotavirus SA11 and patient-derived rotavirus isolates were prepared as described previously (17, 18).

Stocks (0.1 mg/mL) of 6-TG (Sigma-Aldrich) was dissolved in alkali solution (1M NaOH, 50 mg/ml), and NSC23766 (Merck Millipore) was dissolved in H₂O (2 mM). All chemicals were stored in 25 μ L aliquots and frozen at -80 °C.

Cells and human primary intestinal organoids culture

Human colon cancer cell line Caco2 and human embryonic kidney cell line 293T (HEK 293T) cells were grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, The Netherlands) supplemented with 20% (vol/vol) heat-inactivated fetal calve serum (FCS) (Hyclone, Lonan, Utah) and penicillin (100 IU/mL)/streptomycin (100 mg/mL) (Invitrogen-Gibco) at 37 °C in a humidified 5% CO₂ incubator. Rac1 knockout mouse embryonic fibroblast (MEF) cells were cultured in DMEM supplemented with 10% FCS, Penicillin (100 IU/mL)/streptomycin (100 mg/mL), L-Glutamine (Gibco® by Life Technologies), non-essential amino acids (Gibco® by Life Technologies) and sodium-pyruvate (Gibco® by Life Technologies). Caco2 Cells with stable knockdown of Rac1 were generated by transduction of lentiviral shRNA (produced in HEK293T cells) targeting Rac1, and selected with puromycin (6 µg/mL) as described previously (18). The shRNA targeting sequences used in this work were listed in supplementary table 1.

3D model of human intestinal organoids were generated as previously described (17).

Rotavirus inoculation and drug treatments

Inoculation of Caco2 cells and human intestinal organoids with SA11 and patient-derived rotavirus were performed as previously described ¹⁸.

Viability assay of cells or organoids

The viability of cells or organoids was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, Caco2 cells (1×10^4 cells/well) or organoids were seeded into a 96-well culture plate and incubated with various concentrations of 6-TG or NSC23766 for 48 hrs, followed by adding 500 µg/mL of MTT solution to each well and incubation at 37 °C for 3 hrs. Subsequently, the medium was removed, and replaced with 100 µL DMSO and incubated at 37 °C for 50 min. Then, the absorbance was measured at 490 nm in an enzyme-linked immunosorbent assay reader (BIO-RAD).

Transfection of plasmids

A constitutively active Rac1 (V12) and a dominant inactive Rac1 (N17) plasmids were prepared as previously described (19). HEK 293 cells were seeded into 6-well plates for Rac activation assay or 48-well plates for cell infection assay. When the confluence reached around 70%, the cells were washed with PBS, followed by adding 500 μ L of Opti-MEM[®] reduced serum medium (Thermo Fisher Scientific) with 2 μ g Rac1 (V12) or Rac1 (N17) plasmids and 10 μ g polyethylenimine (PEI) (Sigma-Aldrich) per well of a 6-well plate, or 100 μ L of Opti-MEM[®] reduced serum medium with 0.25 μ g Rac1 (V12) or Rac1 (N17) plasmids and 1.25 μ g PEI per well of a 48-well plate. After 4 to 5 hrs of incubation, 2 mL or 0.5 mL of DMEM containing 10% FCS was added to each well of 6-well plate or 48-well plate. After 24 hrs transfection, cells were infected with rotavirus for 48 hrs, following by being performed Rac activation assay or qRT-PCR.

Quantitative real-time PCR (qRT-PCR)

Total cellular RNA was isolated using a NucleoSpin[®] RNA kit (MACHEREY-NAGEL, Düren, Germany) following the manufacturer's protocol, and quantified with a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was synthesized using the reverse transcription system from TAKARA according to manufacturer's instructions (TAKARA BIO INC). The resulting cDNA was diluted 1:10, and 2 μ L of the diluted cDNA was used for qRT-PCR with primers listed in Supplementary Table 1. All qRT-PCR experiments were performed by SYBR-Green-based (Applied Biosystems SYBR Green PCR Master Mix; Thermo Fisher Scientific Life Science) real-time PCR with the StepOnePlus System (Thermo Fisher Scientific Life Sciences). The expression of target mRNAs was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Gene expression analysis was performed by the $\Delta\Delta C_T$ method (17, 18).

Rac activation assay

The levels of Rac1-bound GTP were detected using Rac interactive binding domain of PAK (aa56-272) as described previously (20). In brief, GST-PAKcrib protein was pre-coupled to glutathione-Sepharose beads (Sigma-Aldrich) for 45 min at 4 °C. Caco2 cells treated with various concentrations of 6-TG or NSC23766 (48 hrs, seeded in 6-well-plate) were lysed for 10 min in lysis buffer (50 mM Tris, pH 7.4, 10%

glycerol, 200mM NaCl, 1% NP-40, 2mM MgCl₂, 2 mM sodium orthovanadate, and protease inhibitors). Cell lysates were incubated with pre-coupled beads for 45 min. Then, agarose beads were washed 3 times with 1x lysis buffer, followed by boiling in Laemmli buffer, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15%) analysis.

Western blotting

Cell lysates were subjected to SDS-PAGE, and proteins were transferred to PVDF membrane (Immobilon-FL). Rac1 monoclonal antibody (1:1000, rabbit; cell signaling), SA11 rotavirus VP4 (1:1000, HS-2, a mouse monoclonal antibody; provided by professor Harry Greenberg, Stanford University School of Medicine, USA) were detected by western blot analysis and detection of β -actin served as loading control (1:1000, mouse monoclonal; Santa Cruz).

Serial passaging of rotavirus with 6-TG treatment

To investigate whether rotavirus can develop resistant to 6-TG treatment, viruses were passaged in Caco2 cells in the absence of drug (as control) or in the presence of gradually increasing levels of the drug (1000 ng/mL of 6-TG for passage 1-10 and 2000 ng/mL of 6-TG for passage 11-20). In brief, Caco2 cells in 24-well plate were inoculated 200 μ L virus at 37°C for 1 hr, followed by adding 6-TG or without drug (as control). After 48 hrs, both cells and supernatant were harvested, followed by being frozen, thawed once, and centrifuged. The supernatant containing passaged viruses were stored at -80°C until used for the next passage. Viruses were serially passaged by using 1 aliquot of viral stock from the preceding passage to infect fresh Caco2 cells. Effect of each passage of virus (same titer) was quantified by qRT-PCR. After harvesting all 20 passages of virus (control and 6-TG treated), the effect of 6-TG on each passage of virus was determined by qRT-PCR.

IC50 and CC50 calculation

IC50 and CC50 calculation were described as previously (18).

Statistics

Data are presented as means, and statistical comparison between different groups was performed by Mann-Whitney test using GraphPad Prism 5. Error bars represent the SEM, and P value <0.05 was considered statistically significant.

Results

6-TG potently inhibits rotavirus

To test the effect of 6-TG on rotavirus infection, we treated SA11 rotavirus infected Caco2 cells with various concentrations (0.001 – 10000 ng/mL) of 6-TG for 48 hrs. This resulted in significant reduction of rotavirus RNA in a dose-dependent manner (Figure 1A, $n = 6-17$, $*P<0.05$, $***P<0.001$). The IC_{50} value of 6-TG against SA11 rotavirus was 3.0×10^{-4} nM (Figure S2A), CC_{50} of 6-TG to Caco2 cells was 9847.6 nM (Figure S3A) and selectivity index (SI, CC_{50}/IC_{50}) was 3.3×10^7 . It was further confirmed that 6-TG vigorously inhibited rotavirus VP4 protein in a dose-dependent manner (Figure 1B). The effect of this drug was further validated in human primary intestinal organoids, which contain villus domain and crypt domain (Figure 1C), and have been considered to be more resemble the *in vivo* architecture of intestine (21). 6-TG significantly inhibited SA11 rotavirus replication and virus production in these organoids (Figure 1D,E $n = 6$, $*P<0.05$, $**P<0.01$). This was further validated in five patient-derived rotavirus isolates by inoculation of Caco2 cells or human intestinal organoids with stool samples from the rotavirus-infected patient (Supplementary Table S2). Treatment with 100 ng/mL 6-TG inhibited patient-derived isolates in both Caco2 cells (Figure 1F) and human intestinal organoids (Figure 1G). Thus, we have demonstrated that 6-TG is a potent antiviral drug against rotavirus.

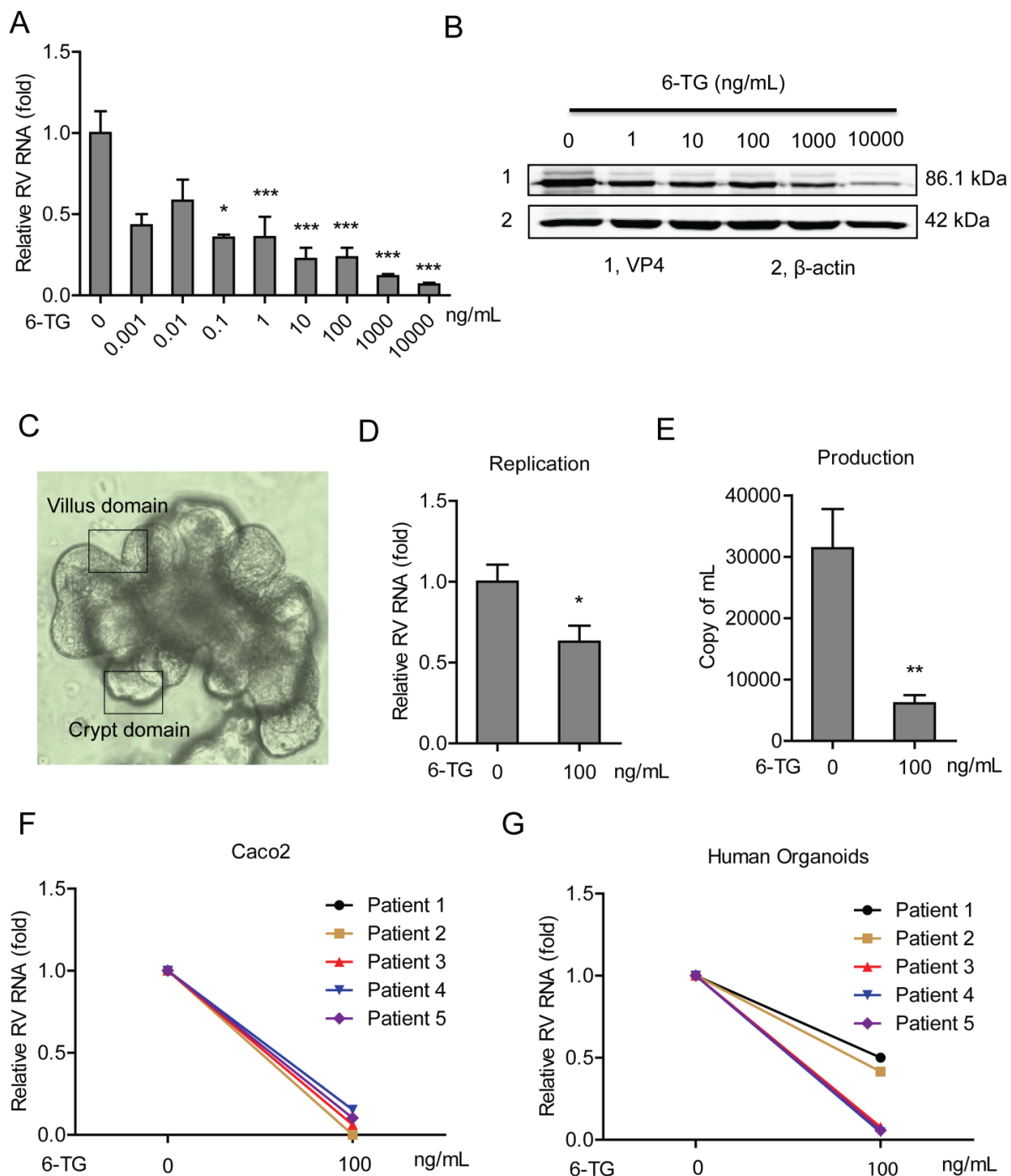


Figure 1. 6-TG potently inhibits rotavirus infection. (A) Treatment with 6-TG (48 hrs) potently inhibited viral genomic RNA in SA11 rotavirus infected Caco2 cells in a dose-dependent manner ($n = 6-17$, means \pm SEM, $*P < 0.05$, $***P < 0.001$, Mann-Whitney test). (B) Treatment with 6-TG (48 hrs) potently inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells in a dose-dependent manner. (C) The morphology of human primary small intestinal organoids with clear villus domain and crypt domain. (D) Treatment with 6-TG (48 hrs) significantly inhibited genomic RNA in SA11 rotavirus infected human intestinal organoids ($n = 6$, means \pm SEM, $*P < 0.05$, Mann-Whitney test). (E) Treatment with 6-TG (48 hrs) significantly inhibited viral production in SA11 rotavirus infected human intestinal organoids ($n = 6$, means \pm SEM, $**P < 0.01$, Mann-Whitney test). (F) Treatment with 100 ng/mL of 6-TG (48 hrs) inhibited viral RNA of patient-derived rotavirus isolates in Caco2 cells. (G) Treatment with 100 ng/mL of 6-TG (48 hrs) inhibited viral RNA of patient-derived rotavirus isolates in human intestinal organoids.

6-TG has a high barrier of drug resistance in inhibiting rotavirus

Rotavirus was serially passaged in the presence of escalating concentrations of 6-TG to assess the barrier of drug resistance development towards of 6-TG. As a control, wild-type virus was passaged in the absence of the drug. During initial ten passages, virus was exposure to 1000 ng/mL of 6-TG, while the drug concentration was increased to 2000 ng/mL during the course of the selection of later passages. After 20 passages, no reduction of the antiviral effect of 6-TG was found (Figure 2). Therefore, it is clearly indicated that 6-TG can potently inhibit rotavirus infection and has a high barrier of drug resistance development.

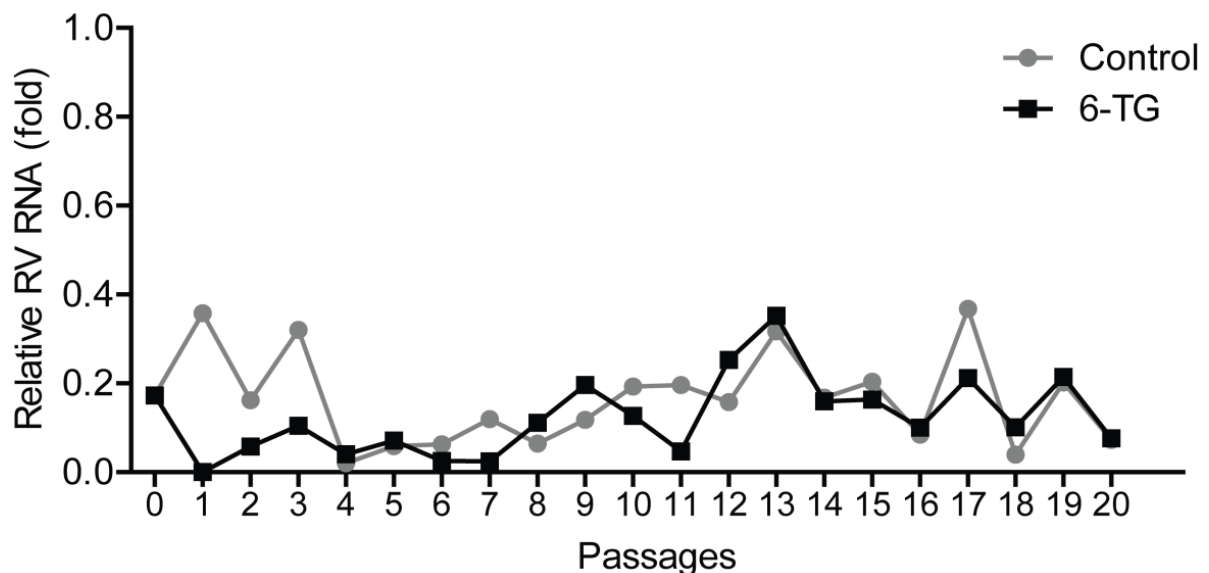


Figure 2. 6-TG has a high barrier to drug resistance development. 6-TG retains its anti-rotavirus activity even with continuous exposure to 6-TG for 20 passages.

Rac1, the drug target of 6-TG, sustains rotavirus infection

We next explored the underlying mechanism-of-action of the effect of 6-TG on rotavirus. We investigated the role of Rac1, an important cellular target of 6-TG, in regulating rotavirus infection. Firstly, we performed lentiviral RNAi-mediated loss-of-function assay to silence Rac1 gene. Two (no. 9689 and 9691) out of five RNAi vectors showed potent knockdown (Figure 3A). Importantly, these two RNAi vectors resulted in $65.0 \pm 8.7\%$ ($n = 10$, $P < 0.001$) and $61.8 \pm 15.6\%$ ($n = 6$, $P < 0.05$) reduction of SA11 rotavirus viral RNA, respectively (Figure 3B). To further confirm, we obtained Rac1 knockout mouse embryonic fibroblasts cells (MEFs), and a *bona*

fide knockout was confirmed by western blot assay (Figure 3C). Consistently, rotavirus infection in Rac1 knockout MEFs (-/-) is significantly less efficient ($79.8 \pm 9.0\%$; $n = 10$; $P < 0.001$), compared with the infection in wild type MEF (fl/fl) (Figure 3D).

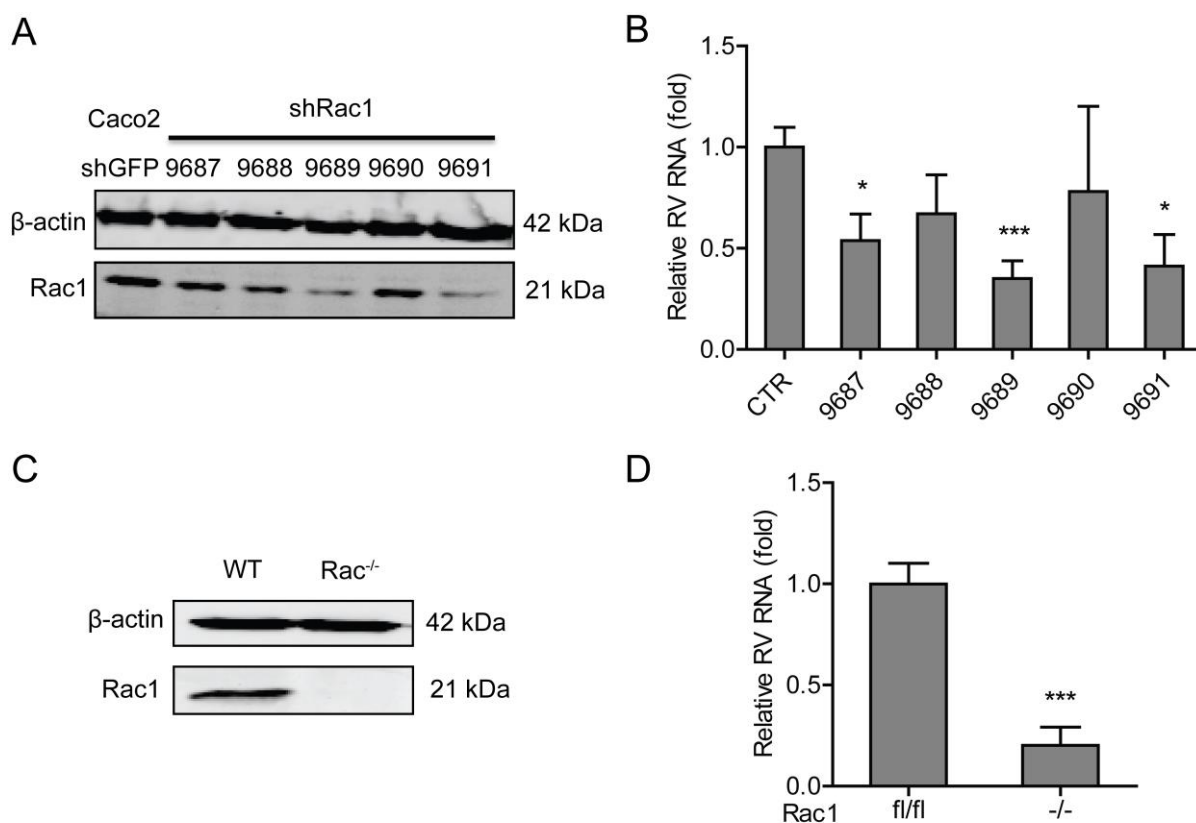


Figure 3. Rac1, the drug target of 6-TG, sustains rotavirus infection. (A) Western blot assay detected Rac1 in transduced Caco2 cells transduced lentiviral RNAi vectors against Rac1. (B) Three (No. 9687, 9689 and 9690) out of five lentiviral shRNA vectors inhibited rotavirus genomic RNA ($n = 6-10$, means \pm SEM, * $P < 0.05$, *** $P < 0.001$, Mann-Whitney test). (C) Western blot assay confirmed knockout of Rac1 in Rac1 knockout (-/-) MEF cells. (D) SA11 rotavirus replication was significantly attenuated in Rac1 knockout (-/-) MEF cells ($n = 10$, means \pm SEM, *** $P < 0.001$, Mann-Whitney test).

The activation form of Rac1 is required for supporting rotavirus infection

Rac1 regulates various cellular signaling mainly through the active GTP-bound form (GTP-Rac1) (13). NSC23766, a specific GTP-Rac1 inhibitor, was able to effectively inhibit GTP-Rac1 in Caco2 cells shown by a Rac activation pull-down assay (Figure 4A, B). Importantly, treatment with 5 and 25 μM NSC23766 for 48 hrs resulted in $58.8 \pm 11.6\%$ ($n = 8$; $P < 0.05$) and $77.48 \pm 6.2\%$ ($n = 10$; $P < 0.001$) reduction of viral RNA, respectively (Figure 4C). The IC_{50} value of NSC23766 against SA11 rotavirus was $1.1 \times 10^{-2} \mu\text{M}$ (Figure S2B), CC_{50} of NSC23766 to Caco2 cells was $312.8 \mu\text{M}$ (Figure S3B) and SI was 2.8×10^4 . The inhibitory effect of NSC23766 on rotavirus was further verified by western blot assay, as showing that treatment with 1, 5 and 25 μM NSC23766 could potently inhibit rotavirus VP4 protein synthesis in Caco2 cells (Figure 4D). The effect of this drug was further validated in human primary intestinal organoids, indicating $91.1 \pm 0.1\%$ ($n = 6$; $P < 0.05$) reduction of viral RNA in the organoids (Figure 4E).

To further verify, we obtained two Rac1 mutant forms including a constitutively Rac1 active Rac1 (V12) and a Rac1 dominant inactive Rac1 (N17) plasmid. We have demonstrated the successful transfection of Rac1 (V12) and Rac1 (N17) plasmids into cells by flow cytometry (Figure 4F). Rac1 (V12) showed higher level of GTP-Rac1 while Rac1 (N17) showed lower level of GTP-Rac1 determined by Rac activation pull-down assay (Figure 4G). Importantly, Rac1 (V12) promotes rotavirus infection, but Rac1 (N2) inhibits rotavirus infection (Figure 4H). Taken together, these data have demonstrated that the active Rac1 (GTP-Rac1) has a supportive role in rotavirus infection.

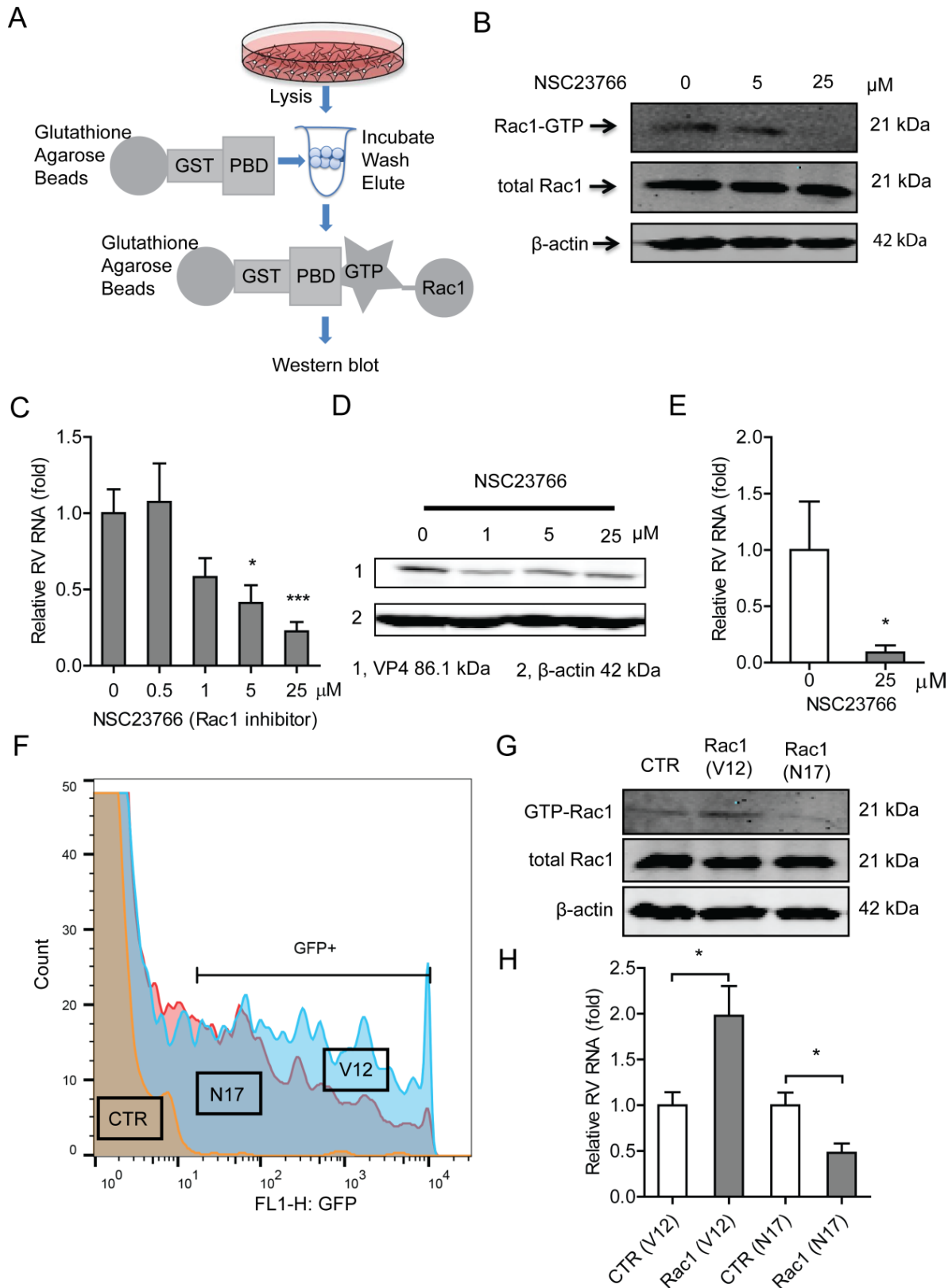


Figure 4. The activation form of Rac1 is required for supporting rotavirus infection. (A) Schematic depicting the pull-down assay. Caco2 cells were treated with NSC23766 for 48 hrs. (B) Activated Rac1 was precipitated using a pull-down assay and visualized by Western blotting with Rac1 antibody, total Rac1 and β -actin (as loading control) were detected by Western blotting. (C) Treatment with NSC23766 (48 hrs) potently inhibited viral genomic

RNA in SA11 rotavirus infected Caco2 cells in a dose-dependent manner ($n = 8-10$, means \pm SEM, $*P < 0.05$, $***P < 0.001$, Mann-Whitney test). (D) Treatment with NSC23766 (48 h) potently inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells in a dose-dependent manner. (E) Treatment with NSC23766 (48 hrs) significantly inhibited viral RNA in SA11 rotavirus infected human intestinal organoids ($n = 6$, means \pm SEM, $*P < 0.05$, Mann-Whitney test). (F) Flow cytometric analysis of green fluorescence indicated successful transduction of Rac1 (V12) and Rac1 (N17) plasmids. (G) Pull-down and western blot assays showed higher level of GTP-Rac1 with transduction of Rac1 (V12) but lower level of GTP-Rac1 with Rac1 (N17) plasmids. (H) Rac1 (V12) transduction facilitates but Rac1 (N17) inhibits rotavirus infection ($n = 10$, means \pm SEM, $*P < 0.05$, Mann-Whitney test).

6-TG inhibits rotavirus via suppression of Rac1 activation

6-TG was reported to inhibit the activation of Rac1 protein in many cell types (13, 20, 22). By the Rac activation pull-down assay (Figure 4A), we have demonstrated that 6-TG effectively inhibits GTP-Rac1 (the active form) but not the total Rac1 level in Caco2 cells (Figure 5A). Functionally, we verified that 6-TG or NSC23766 has lost their anti-rotavirus effects in Rac1 knockdown Caco2 cells (Figure 5B and 5C). Similar results were observed in Rac1 knockout MEF cells (Figure 5D). Herein, we confirm that 6-TG inhibits rotavirus via suppression of Rac1 activation.

6-TG has additive effect with IFN α , but moderate antagonistic effect with ribavirin on rotavirus infection

IFN α and ribavirin are widely used general antivirals, being confirmed to potently inhibit rotavirus infection *in vitro* (17). Consistently, we again demonstrated the inhibitory effects of IFN α and ribavirin on rotavirus RNA (Figure 6A, E) and viral protein (Figure 6B, F) in a dose-dependent manner. The IC₅₀ value of IFN α against SA11 rotavirus was 3.1×10^{-5} IU (Figure S2C), CC₅₀ of IFN α to Caco2 cells was 18706 IU (Figure S3C) and SI was 6.0×10^8 . The IC₅₀ value of ribavirin against SA11 rotavirus was 1.6×10^{-4} μ M (Figure S2D), CC₅₀ of ribavirin to Caco2 cells was 30.2 μ M (Figure S3D) and SI was 1.9×10^5 . Next, we assessed the combinatory antiviral effects of 6-TG and IFN α , as well as 6-TG and ribavirin in Caco2 cells infected with rotavirus of the SA11 strain. The combination of 6-TG and IFN α resulted in an additive antiviral effect, with a synergy volume of $-2.8 \mu\text{M}^2\%$ (Figure 6C, D). However, the combination of 6-TG and ribavirin resulted in moderately synergistic antiviral effect, with a synergy volume of $-26.02 \mu\text{M}^2\%$ (Figure 6G, H).

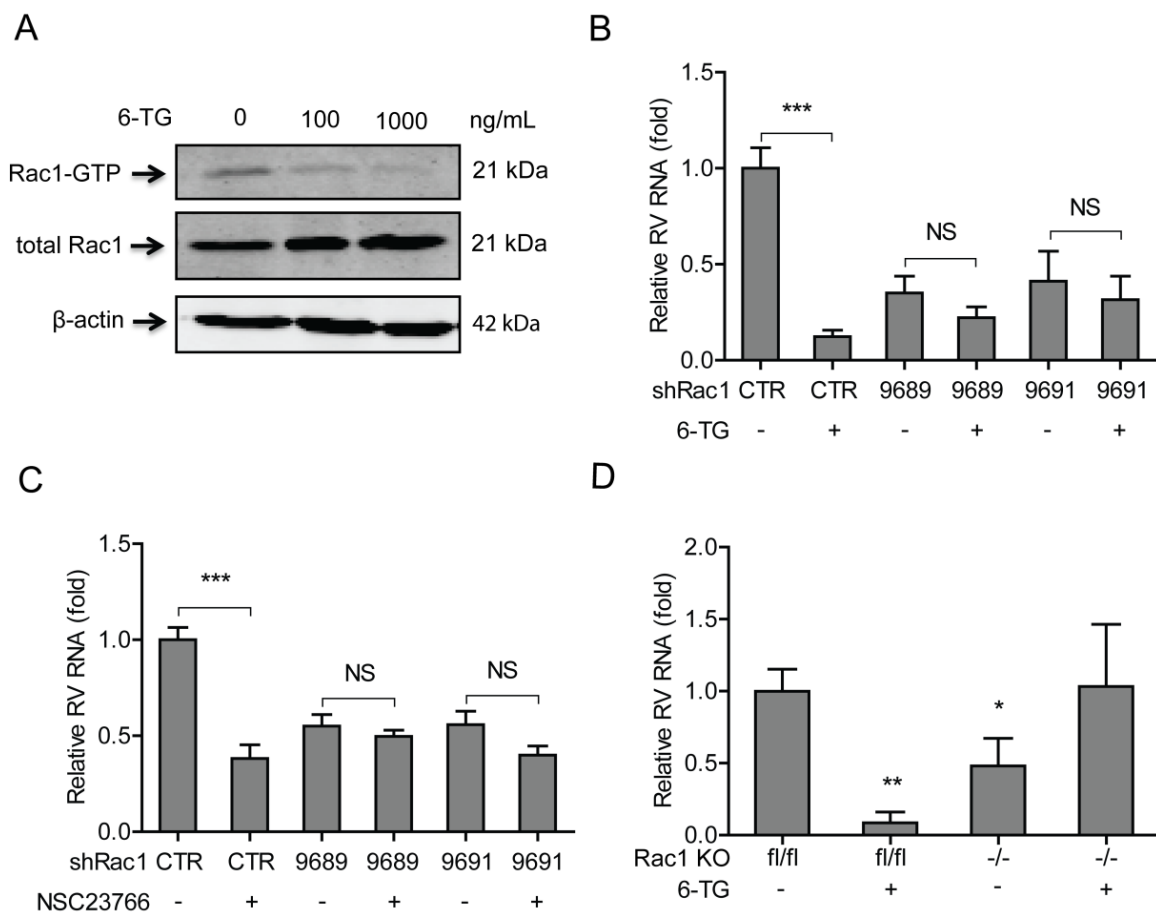


Figure 5. 6-TG inhibits rotavirus via suppression of Rac1 activation. (A) 6-TG inhibits GTP-Rac1 detected by pull-down assay. Activated Rac1 was precipitated using a pull-down assay and visualized by Western blotting with Rac1 antibody, total Rac1 and β -actin (as loading control) were detected by Western blotting. (B) Anti-rotavirus effect of 6-TG (100 ng/mL) was attenuated in Rac1 knockdown Caco2 cells. (C) Anti-rotavirus effect of NSC23766 (25 μ g/mL) was attenuated in Rac1 knockdown Caco2 cells. (D) The anti-rotavirus effect of 6-TG (100 ng/mL) was attenuated in Rac1 knockout (-/-) MEF cells (n = 6-8, means \pm SEM, *P < 0.05, **P < 0.01, Mann-Whitney test).

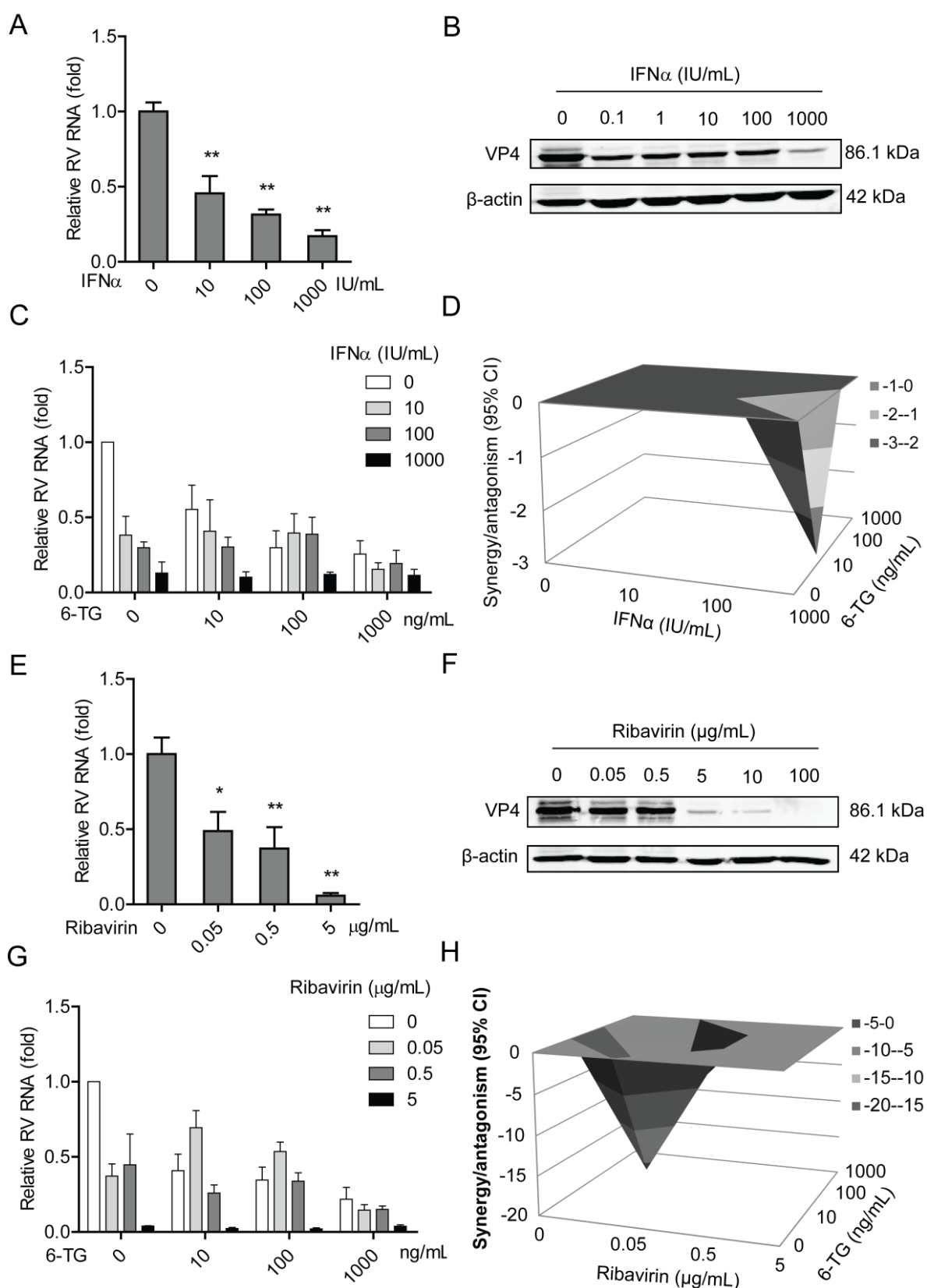


Figure 6. The effects of the combination of 6-TG with IFN α , or ribavirin on rotavirus infection. (A) Treatment with IFN α (48 hrs) potently inhibited viral genomic RNA in SA11 rotavirus infected Caco2 cells in a dose-dependent manner ($n = 4$, means \pm SEM, ** $P < 0.01$, Mann-Whitney test). (B) Treatment with IFN α (48 hrs) potently inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells in a dose-dependent manner. (C) Effect of the combination of

various concentrations of 6-TG and IFN α on rotavirus infection in Caco2 cells. (D) Synergy plot representing the percentage of antiviral activity above/below the expected activity for the 6-TG-IFN α combination based on the data shown in left panel. (E) Treatment with ribavirin (48 hrs) potently inhibited viral genomic RNA in SA11 rotavirus infected Caco2 cells in a dose-dependent manner ($n = 4-7$, means \pm SEM, * $P < 0.05$, ** $P < 0.01$, Mann-Whitney test). (F) Treatment with ribavirin (48 hrs) potently inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells in a dose-dependent manner. (G) Effect of the combination of various concentrations of 6-TG and ribavirin on rotavirus infection in Caco2 cells. (H) Synergy plot representing the percentage of antiviral activity above/below the expected activity for the 6-TG-ribavirin combination based on the data shown in left panel.

Discussion

Besides young children, accumulating evidence indicate that organ transplantation patients irrelevant to their ages are vulnerable to rotavirus infection (3). Rotavirus infection might also be one of the causative agents of IBD (9). This study has demonstrated the potent anti-rotavirus effects of 6-TG and dissected its underlying mechanism-of-action. These results, highlight the potential of using 6-TG to treat or prevent rotavirus infection, in particular organ recipients or IBD patients who are infected or at risk of rotavirus infection.

6-TG has been used in the clinic since the 1950's (16). It has been reported to treat various diseases including acute lymphoblastic leukaemia (23), and IBD (12). It is also used as immunosuppressive drug in organ transplantation patients to prevent graft rejection (24). Interestingly, increasing evidence have indicated an antiviral effect of 6-TG. It has been reported that 6-TG can potently inhibit the papain-like protease of the middle east respiratory syndrome coronavirus, which is vital for viral maturation and antagonizing interferon response of the host (16). It was also reported to inhibit viral DNA synthesis of Simian virus 40 (25). Consistently, we demonstrate that 6-TG is capable of vigorously inhibiting both laboratory adapted and patient-derived rotavirus isolates in Caco2 cells and human primary intestinal organoids (Figure 1).

As a 6-thiopurine (6-TP) prodrug, 6-TG is converted into pharmacologically active deoxy-6-thioguanosine phosphate (also called 6-thioguanine nucleotide) and 6-thioguanosine phosphate (6-TGNP). 6-TGNP can bind to Rac1 to form the 6-TGNP•Rac1 complex to inactivate Rac1 activity (13). We confirmed that 6-TG could inhibit GTP-Rac1 in Caco2 cells (Figure 1A). As a major player of the Rho family of small GTPases, Rac1 plays a vital role in various cellular signaling pathways to

regulate a wide variety of cell functions including gene transcription, cell proliferation, apoptosis, motility, and redox signaling (26). The expression of Rac1 is ubiquitous, but it has two conformational states including an inactive GDP-bound form and an active GTP-bound form (27). It exerts biological functions mainly through activation of Rac1 (i.e. GTP-bound form) (27). Many viruses interfere with or employ the conformational states of Rac1 to regulate their infection. At early stages of African swine fever virus (ASFV) infection, Rac1 is activated, and inhibition of Rac1 is able to suppress production of this virus (28). Rac1 is found to be activated during intracellular mature virus (MV) of Vaccinia virus entry (29).

Although rotavirus infection *per se* does not affect the activation of Rac1 (Figure S4), it is an important host factor in regulating its infection. We have demonstrated that loss-of-function of Rac1 by gene knockdown or knockout significantly impairs rotavirus infection. More specifically, the activation of Rac1 is required as shown by the opposing effects of ectopic over-expression of the active or inactive forms of Rac1 on rotavirus infection. This mechanistically supports the potent anti-rotavirus effects of the GTP-Rac1 inhibitors, 6-TG and NSC23766. Of note, NSC23766 has been shown to inhibit the replication of several influenza viruses including a human virus strain from the 2009 pandemic and the highly pathogenic avian virus strains (15).

Despite the absence of approved medications for treating rotavirus, the widely used general antivirals including ribavirin and IFN α have been studied on rotavirus in experimental models (17). Here, we have evaluated the combinatory effects of 6-TG with IFN α or ribavirin. Consistently, we confirmed that ribavirin and IFN α could inhibit rotavirus replication at both RNA and protein levels (Figure 6A, B, E, F). We further demonstrated that 6-TG had additive effect with IFN α while moderate antagonistic effect with ribavirin (Figure 6C, D, G, H).

In conclusion, this study has demonstrated that 6-TG could potentially inhibit rotavirus infection with a high barrier to drug resistance development. We further identified the active form Rac1 as an important host factor supporting rotavirus infection. 6-TG exerts its anti-rotavirus effects via the specific inhibition of Rac1 activation. Herein, this study provided important references for clinicians to optimize medications for organ recipients and IBD patients who are infected or at risk of rotavirus infection. These results may also help the development of new anti-rotavirus therapies.

Acknowledgements

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Supplementary tables and figures

Table S1. Primers and shRNA sequences used in the study

Sequence of rotavirus primers

	SA11 Rotavirus	Human Patient Rotavirus
Sense	TGGTTAAACGCAGGATCGGA	ACCATCTACACATGACCCTC
Anti-sense	AACCTTTCCGCGTCTGGTAG	CACATAACGCCCTATAGCC

Primer sequences of GAPDH

Primers	Human	Mouse
GAPDH- F	GTCTCCTCTGACTTCAACAGCG	TTCCAGTATGACTCCACTCACGG
GAPDH- R	ACCACCCTGTTGCTGTAGTAGCCAA	TGAAGACACCAGTAGACTCCACGAC

shRNA targeting sequences of Rac1

No.	Sequence
9687	CCTTCTTAACATCACTGTCTT
9688	GCTAAGGAGATTGGTGCTGTA
9689	CGCAAACAGATGTGTTCTTAA
9690	CGTGAAGAAGAGGAAGAGAAA
9691	CCCTACTGTCTTTGACAATTA

Table S2. Patient characteristics.

Patient	Age (yrs)	Gender	Symptoms	Virus Detection								
				Enterovirus	Parechovirus	Norovirus genogroups I	Norovirus genogroups II	Adenovirus	Astrovirus	Sapovirus	Rotavirus	
1	3.5	Female	Fever	No	No	No	No	No	No	No	No	Yes
2	74	Female	Congestive heart failure, myocarditis	No	No	No	No	No	No	No	No	Yes
3	27	Female	Fever, diarrhea, nausea, vomiting	No	No	No	No	No	No	No	No	Yes
4	67	Male	Fever, stomach ache, watery diarrhea	No	No	No	No	No	No	No	No	Yes
5	28	Female	(Kidney transplant) Nausea, stomach ache, watery diarrhea, fever, headache, vomiting	No	No	No	No	Yes	No	No	No	Yes

Supplementary Figures

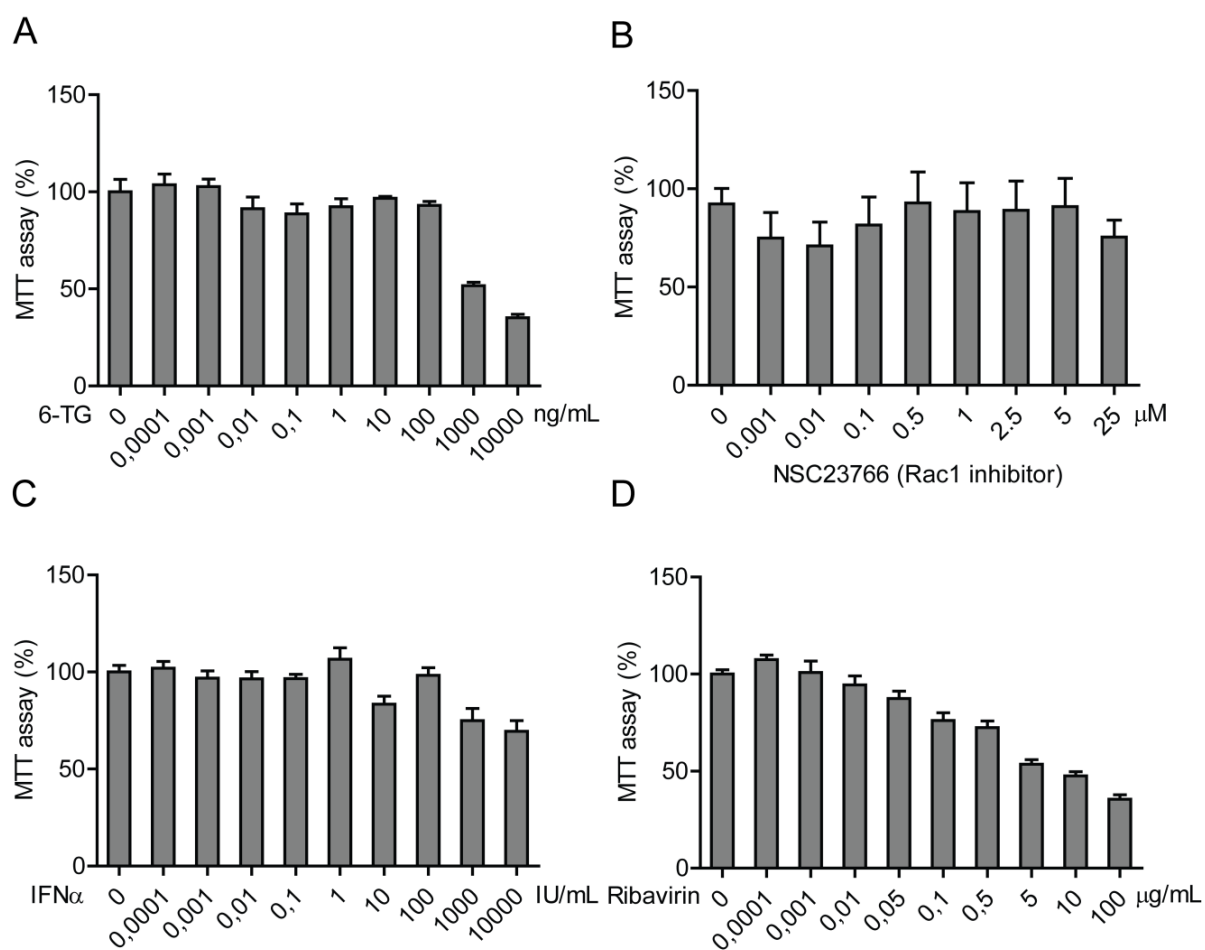


Figure S1. Effect of 6-TG (a), NSC294002 (b), IFN α (c) and ribavirin (d) on host cell viability determined by MTT assays. Caco2 cells treated with all four drugs for 48 h.

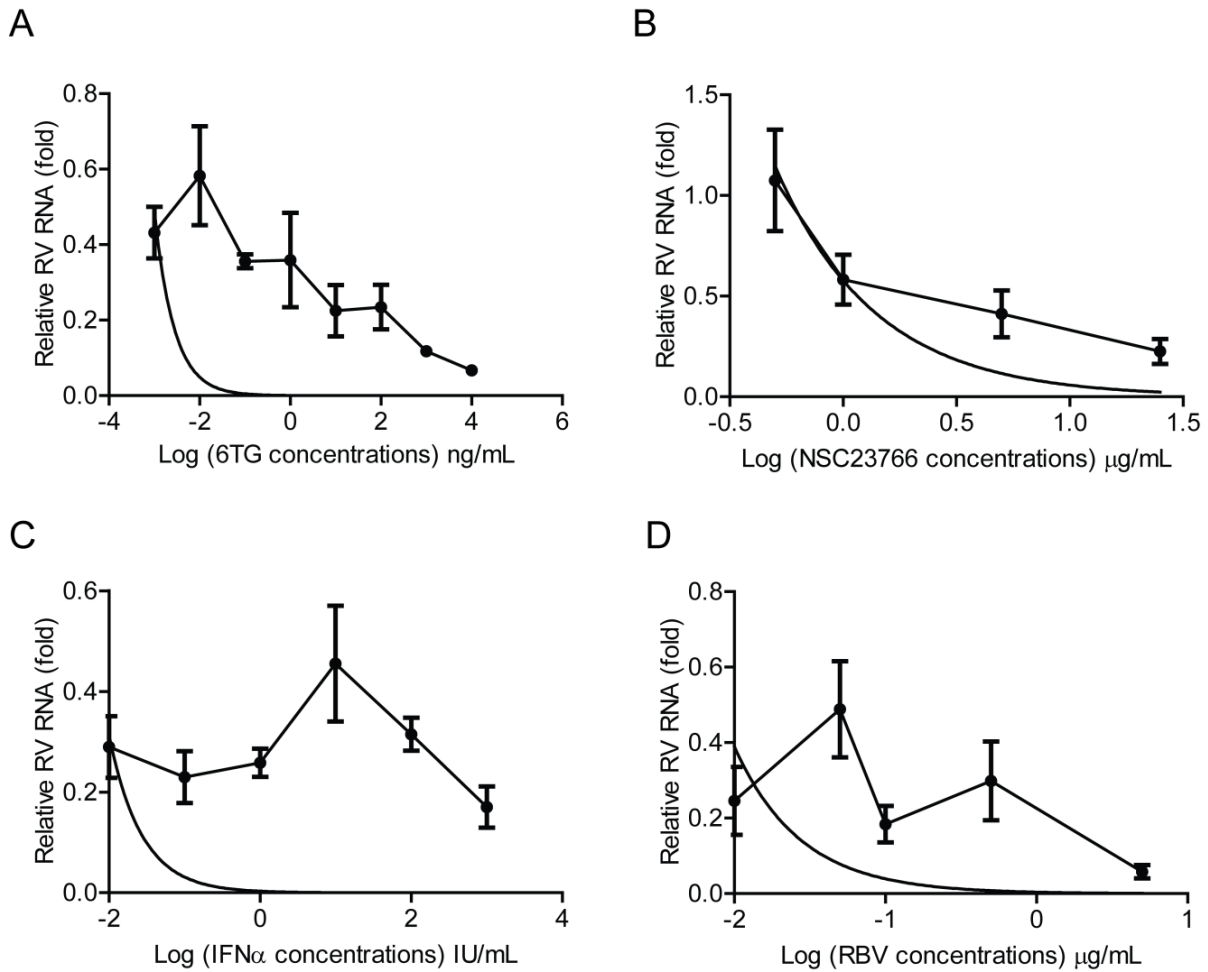


Figure S2. 50% inhibition concentration (IC_{50}) of 6-TG (a), NSC23766 (b), IFN α (c) and ribavirin (d) against patient-derived rotavirus in Caco2 cells.

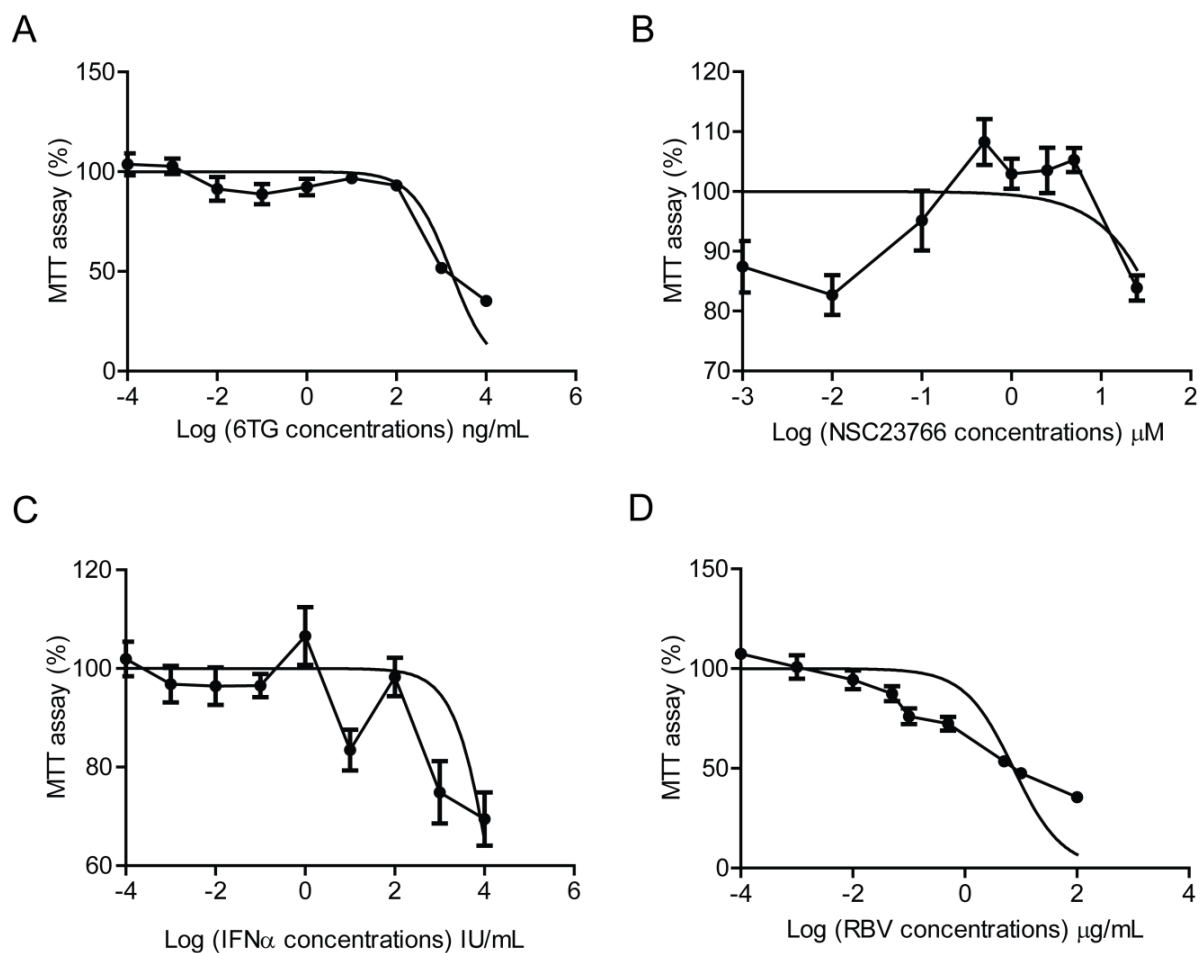


Figure S3. 50% cytotoxic (CC_{50}) of MPA against Caco2 cells 6-TG (a), NSC23766 (b), IFN α (c) and ribavirin (d) in Caco2 cells.

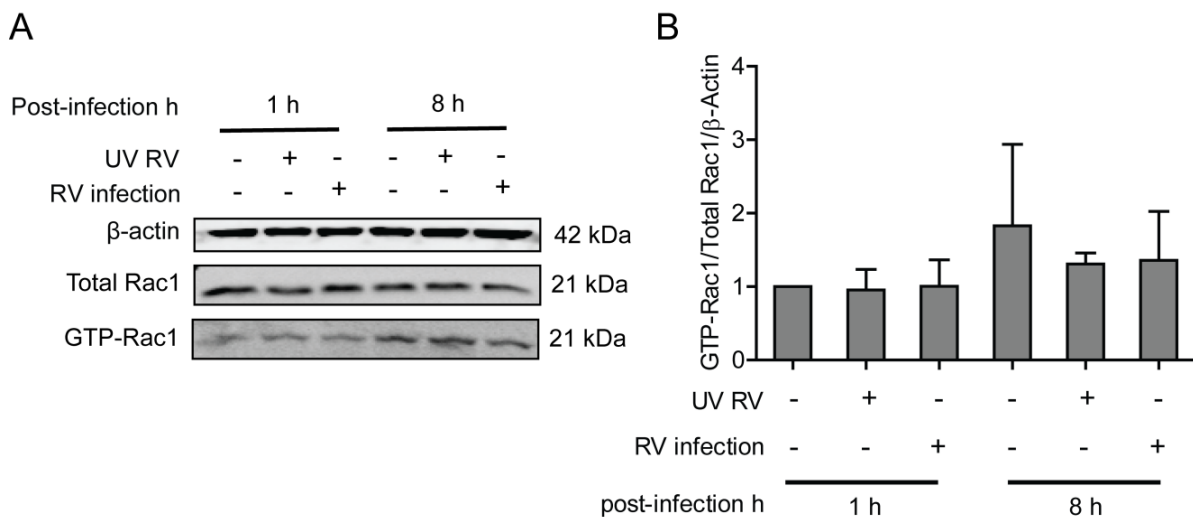


Figure S4. The effect of inactive rotavirus by UV treatment, rotavirus and mock infection (1 and 8 h) on the expression of GTP-Rac1. (a) Western blot assay detected the expression of GTP-Rac1 after 1 and 8 h post-infection by inactive rotavirus (UV treatment), rotavirus and mock infection in Caco2 cells. (b) Quantification of the intensity of the immunoreactive bands of Rac1 ($n = 4$, means \pm SEM, Mann-Whitney test) using Odyssey V 3.0 software.

CHAPTER 6

PI3K-Akt-mTOR-4E-BP1 axis sustains rotavirus infection via the 4E-BP1 mediated autophagy pathway and represents an antiviral target

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Abstract

Rotavirus infection is a major cause of severe dehydrating diarrhea in infants younger than five years old and in particular cases of immunocompromised patients irrespective to the age of the patients. Although vaccines have been developed, antiviral therapy is an important complement that cannot be substituted. Because of the lack of specific approved treatment, it is urgent to facilitate the cascade of further understanding of the infection biology, identification of druggable targets and the final development of effective antiviral therapies. PI3K-Akt-mTOR signaling pathway plays a vital role in regulating the infection course of many viruses. In this study, we have dissected the effects of PI3K-Akt-mTOR signaling pathway on rotavirus infection using both conventional cell culture models and a 3D model of human primary intestinal organoids. We found that PI3K-Akt-mTOR signaling is essential in sustaining rotavirus infection. Thus, blocking the key elements of this pathway, including PI3K, mTOR and 4E-BP1, has resulted in potent anti-rotavirus activity. Importantly, a clinically used mTOR inhibitor, rapamycin, potently inhibited both experimental and patient-derived rotavirus strains. This effect involves 4E-BP1 mediated induction of autophagy, which in turn exerts anti-rotavirus effects. These results revealed new insights on rotavirus-host interactions and provided new avenues for antiviral drug development.

Introduction

Rotavirus is a member of the Reoviridae family (1). As the leading cause of severe dehydrating diarrhea, it mainly attacks children younger than five years old, resulting in approximate 114 million diarrheal episodes and 453,000 infant deaths annually (2, 3). Besides children, accumulating evidences indicate that rotavirus could also cause chronic infection in organ transplantation patients, irrespective to the age of the patients (4-6). Although two safe licensed vaccines named RotaTeq (Merck and Co., PA, USA) and Rotarix (GSK Biologicals, Rixensart, Belgium) are available (1), the implementation of these vaccines in many developing countries remains challenge due to high cost and logistic issues (7). Since no approved medication is available, the development of effective antiviral therapies is indispensable to combat this pathogen.

Numerous signaling pathways play important roles in regulating virus infection either by supporting or defending the infection of virus (8). Phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt)-mammalian target of rapamycin (mTOR) axis takes a vital part in regulating various cellular functions and biological processes, including protein synthesis, cell cycle progression, cell survival, apoptosis, angiogenesis and drug resistance (9). In this signaling, PI3K serves as a key node, which is capable of monitoring panels of biological processes via phosphoryl transfer (10). PI3K could be activated by many types of cellular stimuli, which subsequently phosphorylates the inositol PIP₂ to PIP₃ that recruits Akt to the plasma membrane by binding to its N-terminal pleckstrin homology (PH) domain in which Akt gets activation via phosphorylation (11). As a serine/threonine kinase, phosphorylated Akt is able to stimulate mTOR which belongs to a member of the serine/threonine phosphatidyl inositol 3' kinase-related kinase family (PIKK) (10).

mTOR is an evolutionarily conserved Serine/Threonine kinase playing indispensable roles in regulating mRNA translation, autophagy machinery and cell proliferation (12). Furthermore, mTOR can directly activate a variety of cellular effectors such as p70S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) to control cell growth through integrating nutritional information and receptor tyrosine kinase signaling (13). For instance, mTOR controls capped-dependent translation of both cells and viruses via inducing phosphorylation of 4E-BP1 to promote the formation of a functional eukaryotic initiation factor 4F (eIF-

4F) complex (12, 14). The eIF-4F complex comprises three subunits including a RNA helicase eIF4A, a cap-binding protein eIF4E and a scaffolding protein eIF4G (15). Most of the 4E-BPs share a canonical eIF4E-binding motif (4E-BM) of sequence YX4LΦ with eIF4G (where Y denotes Tyr, X denotes any amino acid, L denotes Leu, and Φ denotes a hydrophobic residue). But some 4E-BPs contain non-canonical 4E-BMs connected by a linker (15-30 residues), and the motifs are not conserved and not required for eIF4G binding (15). Autophagy machinery, as another important downstream element of the mTOR signaling, is a cellular non-specific, bulk degradation process involved in removing damaged macromolecules and organelles to defend nutrient and environmental stress (16, 17), which is also involved in regulating virus infection (18).

PI3K-Akt-mTOR pathway as a potential antiviral target is involved in the infection of a broad spectrum of viruses including lymphocytic choriomeningitis virus (LCMV) (19), Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (20), HIV (21) and human cytomegalovirus (22). In this study, we comprehensively studied the role of PI3K-Akt-mTOR signaling in rotavirus infection and explored the avenues of therapeutic targeting. Besides conventional two dimension (2D) culture based immortalized cells, we also employed three dimension (3D) cultures of primary human intestinal organoids for modeling rotavirus infection (23). These organoids can recapitulate most if not all aspects of in vivo tissue architecture of intestinal epithelium (24). We found that PI3K-Akt-mTOR pathway is crucial in sustaining rotavirus infection via its downstream effector 4E-BP1 and the induction of autophagy. Importantly, targeting this pathway by pharmacological inhibitors/FDA approved drugs potently inhibited rotavirus infection, providing novel therapeutic approaches for combating this virus.

Results

Inhibition of PI3K signaling potently inhibits rotavirus.

Since PI3K serves as a key node in the PI3K-Akt-mTOR pathway, its effect on rotavirus was tested. LY294002 is a potent inhibitor of PI3K. In human Caco2 cells, treatment with 5 μ M LY294002 potently inhibited phosphor-Ser-240/244 S6 and phosphor-4E-BP1 (T70) but not the corresponding total proteins (as controls) (Figure 1A and Figure S1A). Importantly, treatment with 1 and 5 μ M LY294002 for 48 h resulted in $93.5 \pm 1.7\%$ ($n = 4$; $P < 0.05$) and $81.0 \pm 6.8\%$ ($n = 4$; $P < 0.05$) reduction of viral RNA, respectively (Figure 1B). LY294002 could also significantly suppress rotavirus RNA secretion (Figure S2B). The inhibitory effect of this compound was further verified by TCID50 method, demonstrating that it could remarkably inhibit the production of infectious viral particles (Figure 1C). Western blot assay further confirmed that treatment with 0.1, 1 and 5 μ M LY294002 could vigorously inhibit rotavirus protein synthesis in Caco2 cells (Figure 1D). The effect of this drug was also validated in human primary intestinal organoids. Immunofluorescent staining showed that phosphor-Ser-240/244 S6 was potently inhibited by treatment of 5 μ M LY294002 (Figure 1E), supporting that human intestinal organoid is also a PI3K signaling proficient model. Consistently, LY294002 inhibited rotavirus infection in organoid model. For instance, 5 μ M LY294002 resulted in $86.0 \pm 6.4\%$ ($n = 4$; $P < 0.05$) reduction of viral RNA in human intestinal organoids (Figure 1F). Thus, blocking PI3K by LY294002 potently inhibits rotavirus infection in both Caco2 cells and human intestinal organoids.

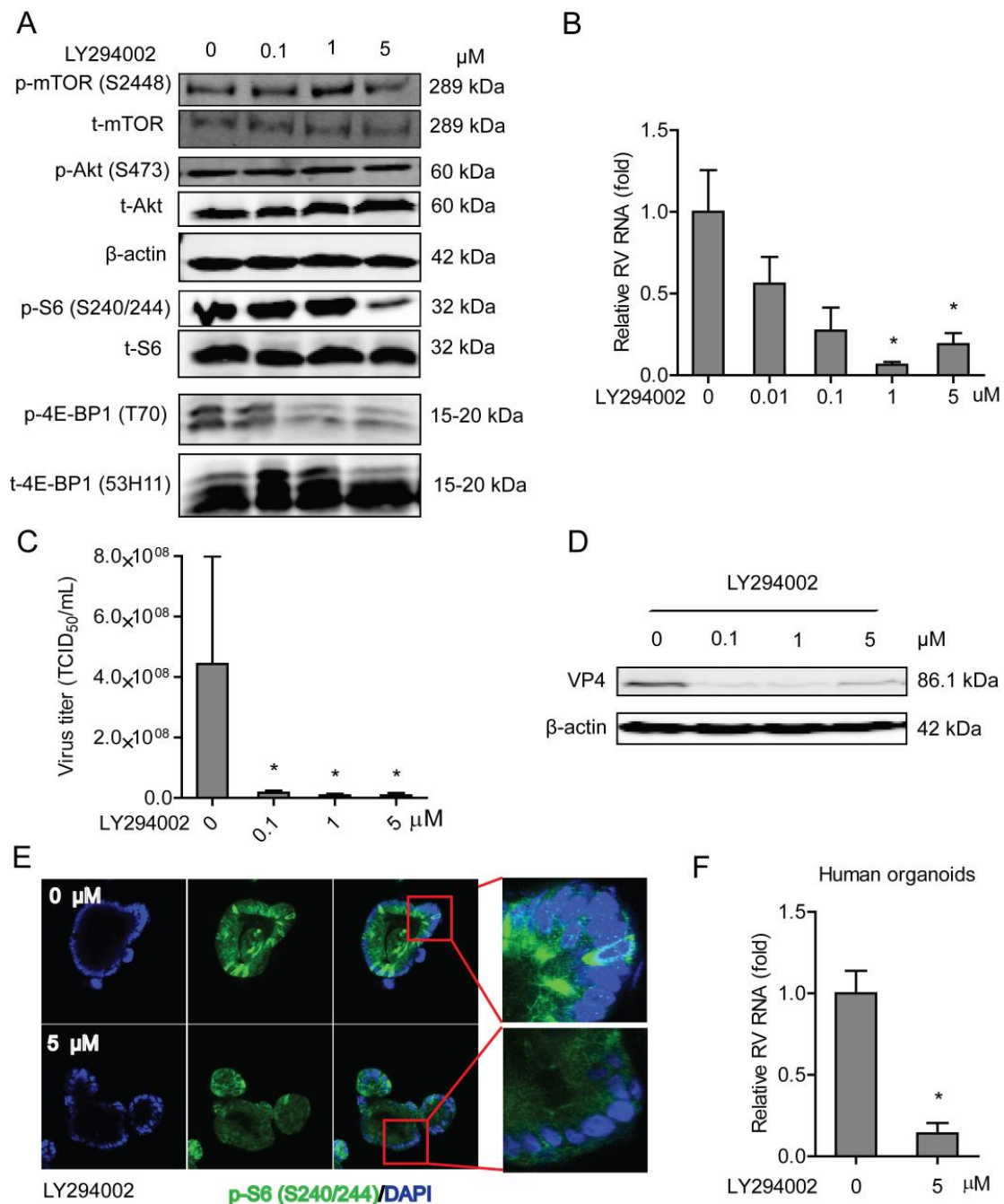


Figure 1. Inhibition of PI3K signaling potently inhibits rotavirus infection. (A) Western blot analysis of p-mTOR (S2448), t-mTOR, p-Akt (S473), p-S6 (S240/244) and p-4E-BP1 (T70) protein levels and the corresponding total protein levels under the treatment of indicated concentrations of LY294002 (48 h) in Caco2 cells. (B) Treatment with LY294002 (48 h) potently inhibited viral genomic RNA in SA11 rotavirus infected Caco2 cells measured by qRT-PCR (n = 4, mean ± SEM, *P < 0.05, Mann-Whitney test). (C) Effects of LY294002 on the production of infectious viral particles determined by TCID₅₀ method. Each bar represents the TCID₅₀/mL (mean ± SEM) (n = 5, *P < 0.05, **P < 0.01, Mann-Whitney test). (D) Treatment with LY294002 (48 h) potently inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells determined by western blot assay. (E) Representative confocal immunostainings of p-S6 (S240/244) (Green) after 48 h incubation with 0 (as control) and 5 μM LY294002 in human intestinal organoids. Nuclei were visualized by DAPI (blue). (F) Treatment with LY294002 (48 h) significantly inhibited viral genomic RNA in SA11 rotavirus infected human intestinal organoids examined by qRT-PCR (n = 4, mean ± SEM, *P < 0.05, Mann-Whitney test).

mTOR sustains rotavirus infection and the mTOR inhibitor rapamycin inhibits its infection.

mTOR is a central element of the PI3K-Akt-mTOR signaling pathway, we thus investigated its effect on rotavirus infection. To address this, Caco2 cells were transduced with integrating lentiviral vectors expressing shRNA to silence mTOR. One (No 8198) out of three clones showed potent knockdown based on western blot results (Figure 2A), which was also confirmed by qRT-PCR (Figure 2B). Caco2 cells with mTOR knockdown or scrambled shRNA (as control) were inoculated with SA11 rotavirus and viral genomic RNA was quantified by qRT-PCR 48 h post inoculation. Silencing of mTOR led to a significant reduction of rotavirus RNA (Figure 2C), suggesting a supportive role of mTOR for rotavirus infection. Rapamycin is a clinically used mTOR inhibitor, and it was approved by FDA to treat/prevent transplant allograft rejection (25). Phosphorylation of Ser-2448 mTOR, Ser-240/244 S6 and 4E-BP1 (T70) but not for the corresponding total proteins (as controls) were inhibited by treatment with 1, 5 and 10 nM rapamycin for 48 h in Caco2 cells (Figure 3A and Figure S1B), except for phosphorylation of Ser-473 Akt. mTOR/S6K inhibition by rapamycin was reported to trigger a negative feedback loop to activate Akt signaling (26), which might be the reason that rapamycin had minor inhibitory effect on phosphorylated Akt. Furthermore, treatment of 5 and 10 nM rapamycin significantly inhibited viral genomic RNA by $80.0 \pm 5.5\%$ ($n = 8$; $P < 0.01$) and $79.9 \pm 5.8\%$ ($n = 9$; $P < 0.01$) in Caco2 cells, respectively (Figure 3B). Rotavirus RNA secretion (Figure S2C) and infectious virus production (Figure 3C) were also significantly inhibited by treatment of rapamycin. Consistently, western blot assay indicated that rotavirus VP4 protein synthesis was potently inhibited by rapamycin treatment (Figure 3D). In contrast, rapamycin lost its anti-rotavirus activity in mTOR silenced Caco2 cells (Figure 3E), supporting its specificity in inhibiting its biological target to exert the antiviral action.

To verify the anti-rotavirus effect of rapamycin, human intestinal organoids infected with SA11 rotavirus were treated with 10 or 100 nM rapamycin for 48 h, which led to vigorous inhibition of phosphor-Ser-240/244 S6 (Figure 3F). Importantly, 10 nM rapamycin resulted in a significant reduction ($75.9 \pm 5\%$, $n = 5$; $P < 0.01$) of viral RNA replication (Figure 3G). This was further validated in five patient-derived strains by inoculation of Caco2 cells or human intestinal organoids with stool samples

from rotavirus-infected patients (Table S2). Treatment with 10 nM rapamycin inhibited patient-derived isolates in both Caco2 cells (Figure 3H) and human intestinal organoids (Figure 3I). These results have demonstrated that efficient infection of rotavirus requires mTOR, but the infection can be effectively inhibited by rapamycin.

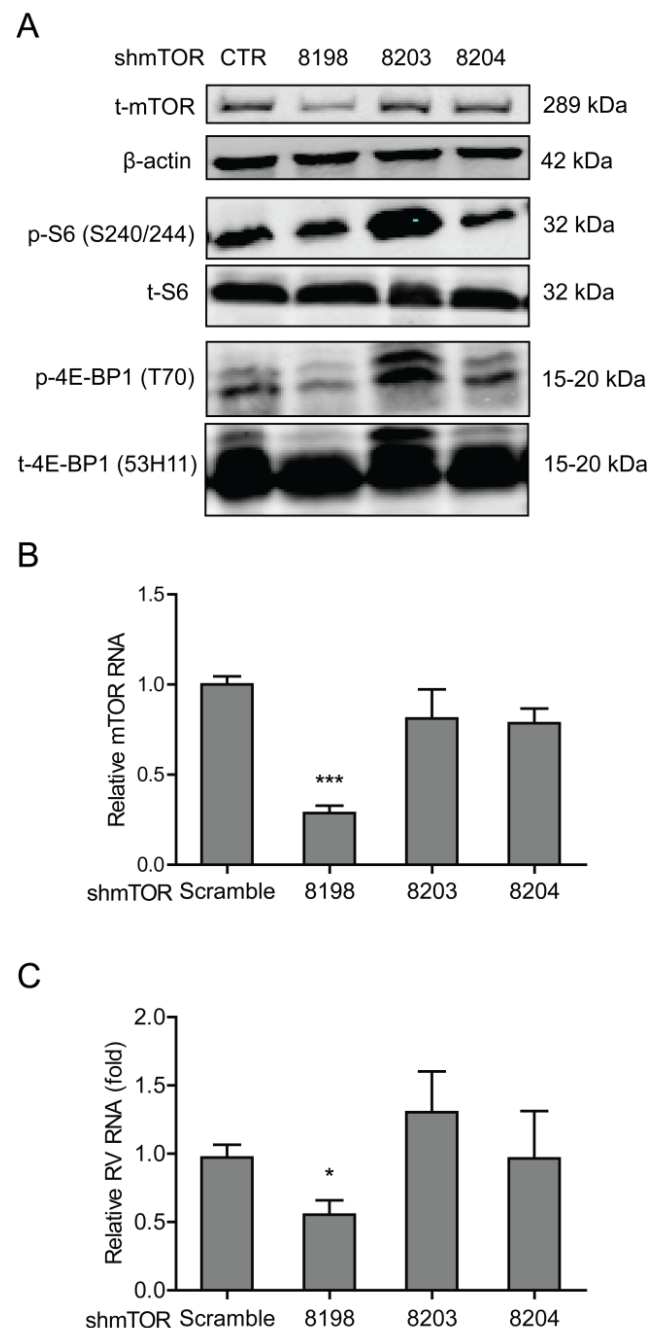


Figure 2. Silence of mTOR inhibits rotavirus replication. (A) Western blot analysis of t-mTOR, p-S6 (S240/244), t-S6, p-4E-BP1 (T70) and t-4E-BP1 (53H11) protein in lentiviral RNAi against mTOR transduced Caco2 cells. (B) One (No. 8198) of three lentiviral shRNA vectors showed successful knockdown determined by qRT-PCR (n = 9, means ± SEM, ***P < 0.001, Mann-Whitney test). (C) mTOR knockdown inhibited rotavirus genomic RNA determined by qRT-PCR (n = 7, means ± SEM, *P < 0.05, Mann-Whitney test).

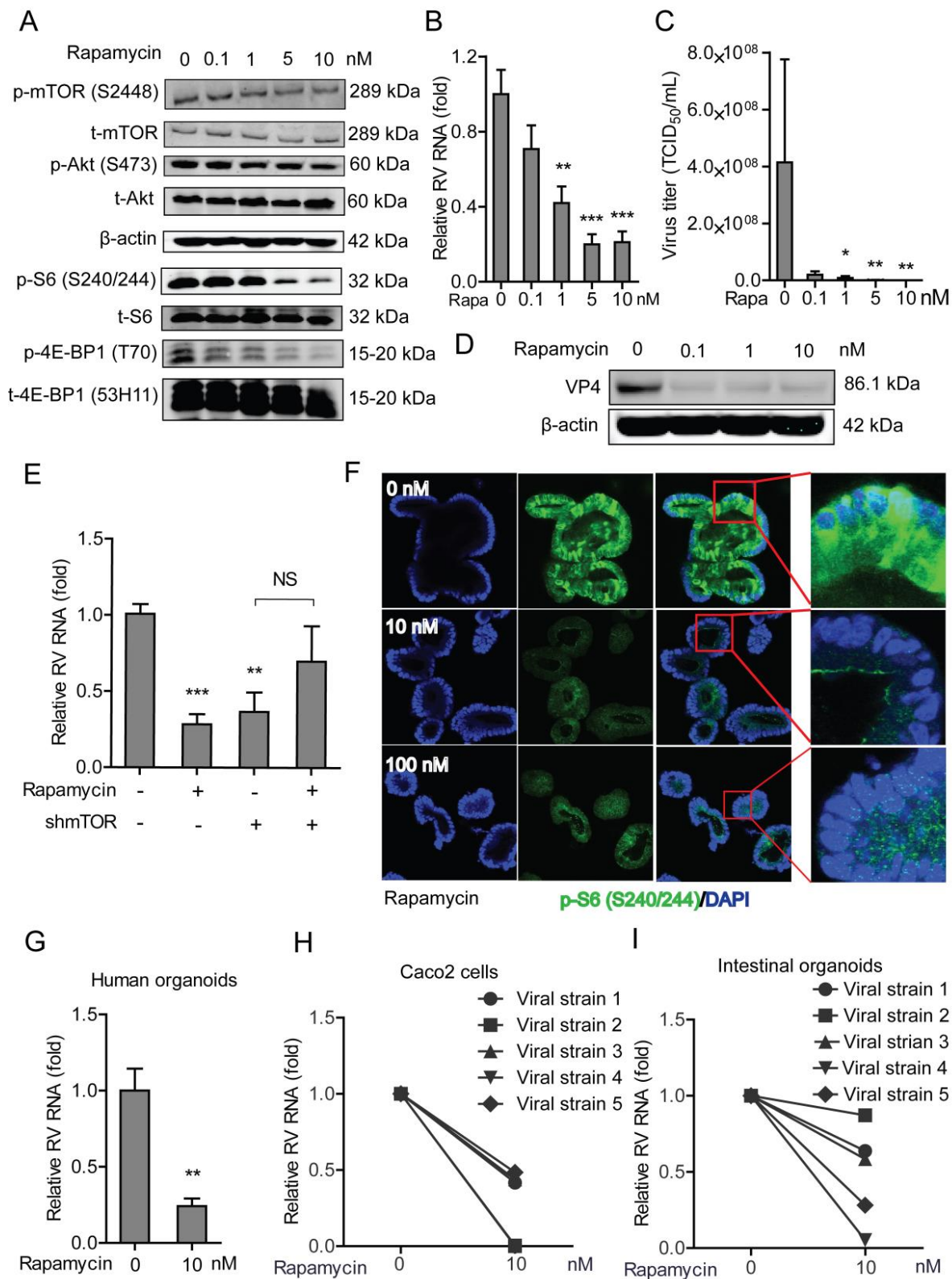


Figure 3. Rapamycin inhibits replication of SA11 and patient-derived rotavirus replication. (A) Western blot analysis of p-mTOR (S2448), t-mTOR, p-Akt (S473), t-Akt, p-S6 (S240/244), t-S6, p-4E-BP1 (T70) and t-4E-BP1 (53H11) protein levels with the treatment of indicated concentrations of rapamycin in Caco2 cells. (B) Treatment with rapamycin (48 h) significantly inhibited SA11 rotavirus replication in Caco2 cells ($n = 7-9$, mean \pm SEM, $^{**}P < 0.01$, Mann-Whitney test). (C) Effects of rapamycin on the production of infectious viral particles determined by TCID₅₀ method. Each bar represents the TCID₅₀/mL (mean \pm SEM) ($n = 5$,

*P < 0.05, **P < 0.01, Mann-Whitney test). (D) Treatment with rapamycin (48 h) inhibited viral VP4 protein determined by western blot assay in Caco2 cells. (E) Anti-rotavirus effect of rapamycin was abolished in mTOR knockdown Caco2 cells (n = 10, mean ± SEM, *P < 0.05, Mann-Whitney test). (F) Representative confocal immunostainings of p-S6 (S240/244) (Green) after 48 h treatment with 10 and 100 nM rapamycin in human intestinal organoids. Nuclei were visualized by DAPI (blue). (G) Treatment with rapamycin (48 h) inhibited SA11 rotavirus genomic RNA in human intestinal organoids determined by qRT-PCR (n = 5, mean ± SEM, *P < 0.01, Mann-Whitney test). (H) Treatment with 10 nM rapamycin (48 h) inhibited patient-derived rotavirus (isolate 1-5) genomic RNA in Caco2 cells determined by qRT-PCR. (I) Treatment with rapamycin (48 h) inhibited patient-derived rotavirus (isolate 1-5) genomic RNA in human intestinal organoids determined by qRT-PCR.

Dual inhibition of PI3K and mTOR inhibits rotavirus infection.

BEZ235 is a pharmacological dual inhibitor of PI3K and mTOR. Western blot confirmed that this compound could potently inhibit phosphorylation of Ser2448 mTOR, 70S6K (T389), Ser473 Akt, Ser240/244 S6 and 4E-BP1 (T70) but not for the corresponding total proteins (as controls) in Caco2 cells (Figure 4A and Figure S1C). Importantly, BEZ235 dose-dependently inhibited viral RNA (Figure 4B) and RNA secretion in Caco2 cells (Figure S2D). Using the TCID50 method, we have demonstrated that it remarkably inhibits the production of infectious viral particles (Figure 4C). BEZ235 was also capable of inhibiting synthesis of rotavirus VP4 protein (Figure 4D). This effect was also confirmed in human intestinal organoids of inhibiting phosphorylation of Ser240/244 S6 (Figure 4E) and rotavirus infection (Figure 4F). Although rotavirus infection did not directly affect the expression levels of the key components the PI3K-Akt-mTOR pathway (Figure S3), this pathway itself intrinsically sustained rotavirus infection.

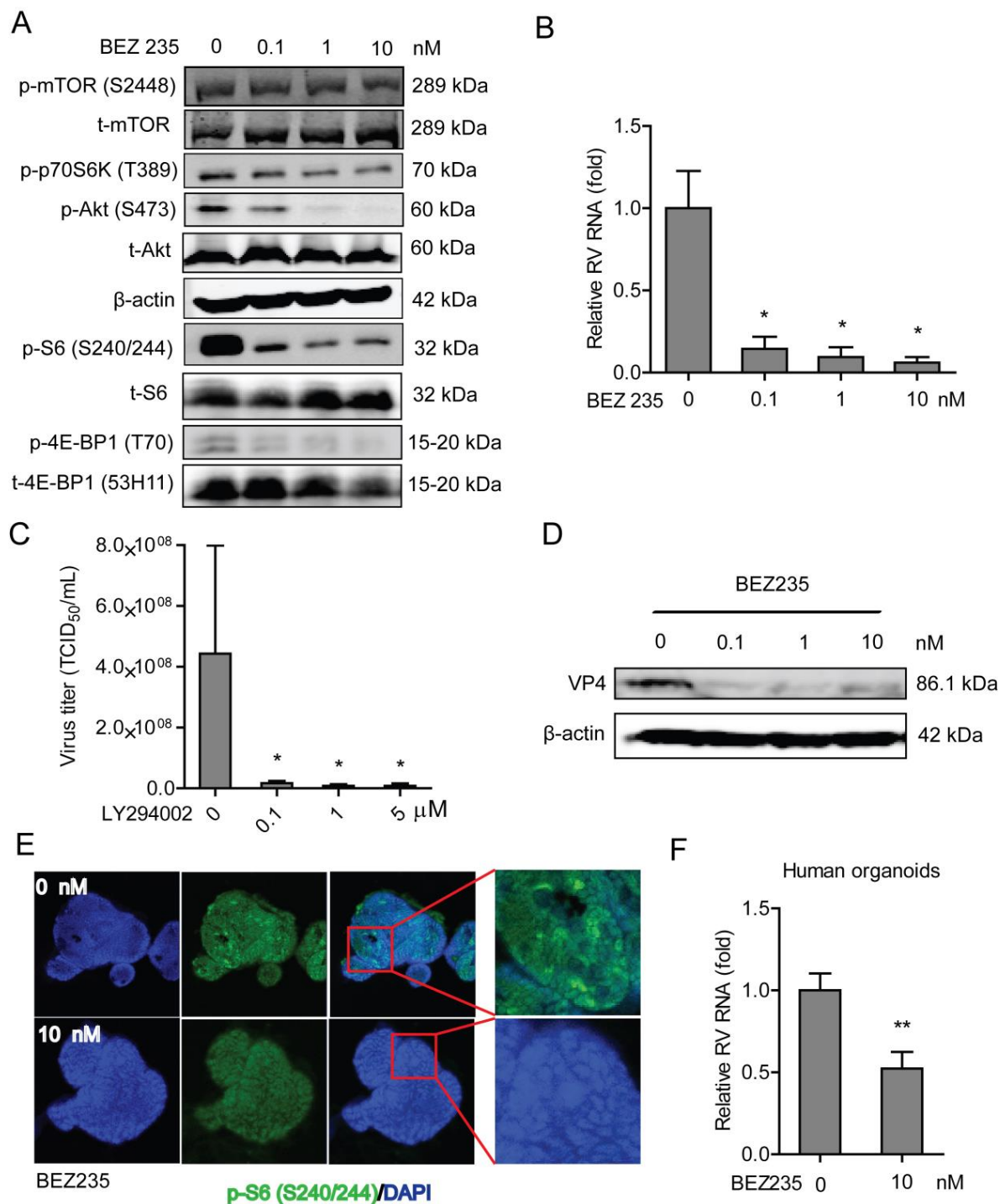


Figure 4. Dual inhibition of PI3K and mTOR inhibits rotavirus infection. (A) Western blot assay detected p-mTOR (S2448), p-p70S6K (T389), p-Akt (S473) and p-S6 (S240/244) and the corresponding total proteins after 48 h incubation with indicated concentrations of BEZ235 in Caco2 cells. (B) Treatment with BEZ235 (48 h) significantly inhibited SA11 rotavirus genomic RNA in dose-dependent-manner determined by qRT-PCR in Caco2 cells ($n = 5$, mean \pm SEM, * $P < 0.05$, Mann-Whitney test). (C) Effects of BEZ235 on the production of infectious viral particles determined by TCID₅₀ method. Each bar represents the TCID₅₀/mL (mean \pm SEM) ($n = 5$, * $P < 0.05$, ** $P < 0.01$, Mann-Whitney test). (D) Western blot showed that treatment with BEZ235 (48 h) significantly inhibited SA11 rotavirus VP4 protein in Caco2 cells. (E) Representative confocal immunostainings of p-S6 (S240/244) (Green) after 48 h incubation with 0 (as a control) and 10 nM BEZ235 in human intestinal organoids. Nuclei were visualized by DAPI (blue). (F) Treatment with BEZ235 (48 h) significantly

inhibited SA11 rotavirus genomic RNA in human intestinal organoids quantified by qRT-PCR (n = 11, mean \pm SEM, **P < 0.01, Mann-Whitney test).

4E-BP1 is a downstream effector of mTOR in sustaining rotavirus infection.

4E-BP1 is one of the important downstream elements of PI3K-Akt-mTOR signaling. We first studied its potential involvement by lentiviral RNAi mediated loss-of-function assay (Figure 5A and Figure S1D). Caco2 cells with the best knockdown of 4E-BP1 (no 6463) resulted in $62.4 \pm 6.1\%$ (n = 5, P < 0.01) reduction of SA11 rotavirus viral RNA 48 h post-inoculation (Figure 5B). Consistently, the anti-rotavirus effect of rapamycin was abolished in 4E-BP1 knockdown cells confirmed by both qRT-PCR assay (Figure 5C) and western blot assay (Figure 5D). To further verify, we obtained 4E-BP1 knockout mouse embryonic fibroblasts (MEFs), and bona fide knockout was confirmed by western blot assay (Figure 5E). Consistently, rotavirus infection in 4E-BP1 knockout MEFs (-/-) is significantly less efficient ($75.1 \pm 17.2\%$; n = 4; P < 0.05), compared with the infection in wild type MEFs (+/+) (Figure 5F). Furthermore, the anti-rotavirus effect of rapamycin was attenuated in 4E-BP1 knockout MEFs (Figure 5G). Consistently, rapamycin could inhibit rotavirus replication in 4E-BP1 KO MEFs transfected with 4E-BP1 wild-type (4E-BP1-WT) plasmids; while this antiviral effect was abolished in 4E-BP1 KO MEFs transfected with 4E-BP1 plasmids having five mutations of the phosphorylation sites (4E-BP1-5A) (Figure 5H). Of note, the inhibitor of S6K did not affect rotavirus infection (Figure S4). Collectively, these data suggest that 4E-BP1 but not S6K is a downstream effector of the PI3K-Akt-mTOR signaling in sustaining rotavirus infection.

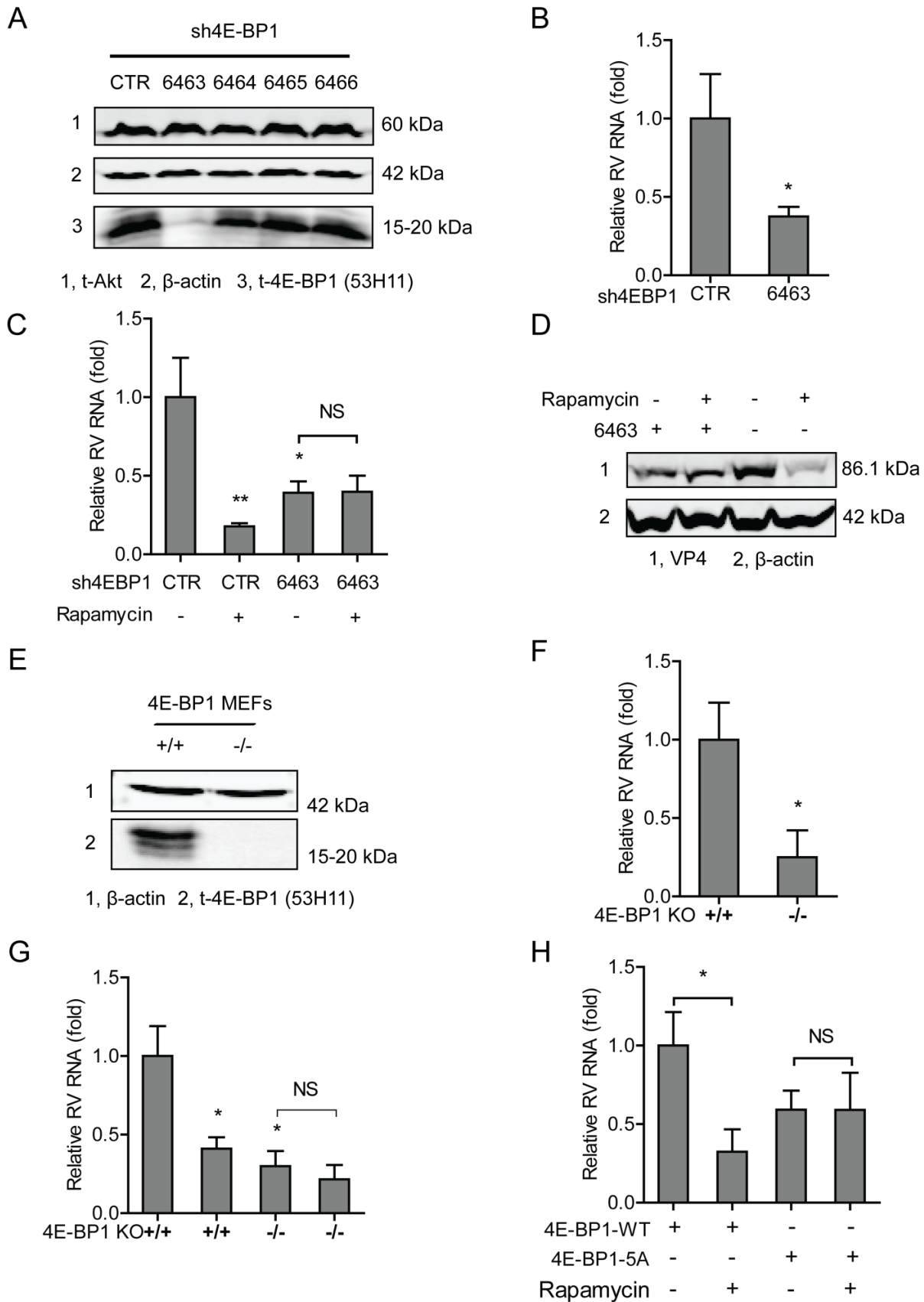


Figure 5. 4E-BP1 is a downstream effector of PI3K-Akt-mTOR signaling in sustaining rotavirus infection. (A) Western blot assay detected t-Akt and t-4E-BP1 (53H11) in lentiviral RNAi against 4E-BP1 transduced Caco2 cells. (B) Knockdown of 4E-BP1 significantly inhibited SA11 rotavirus genomic RNA quantified by qRT-PCR (n = 5, mean \pm SEM, *P <

0.05, Mann-Whitney test). (C) Anti-rotavirus effect of rapamycin (10 nM) was abolished in 4E-BP1 knockdown Caco2 cells (n = 5, mean \pm SEM, *P < 0.05, **P < 0.01, Mann-Whitney test). (D) Western blot assay confirmed that anti-rotavirus effect of rapamycin (10 nM) was abolished in 4E-BP1 knockdown Caco2 cells. (E) Western blot indicated bona fide knockdown of 4E-BP1 in knockout (-/-) MEF cells. (F) SA11 rotavirus replication was potently restricted in 4E-BP1 knockout (-/-) MEF cells (n = 4, mean \pm SEM, *P < 0.05, Mann-Whitney test). (G) Anti-rotavirus effect of rapamycin (10 nM) was attenuated in 4E-BP1 knockout (-/-) MEF cells (n = 8, mean \pm SEM, *P < 0.05, Mann-Whitney test). (H) Rapamycin inhibited rotavirus replication in 4E-BP1 KO MEFs transfected with 4E-BP1-WT plasmids; while this antiviral effect was abolished in 4E-BP1 KO MEFs transfected with 4E-BP1-5A plasmids (n = 5, mean \pm SEM, *P < 0.05, Mann-Whitney test).

4E-BP1 mediates rapamycin-induced autophagy that inhibits rotavirus infection.

Previous studies have reported that rapamycin or 4E-BP1 may affect type I IFN signaling (27-29), in particular the downstream antiviral proteins including interferon regulatory factor 1 (IRF1) and IRF7. Furthermore, we previously have reported the anti-rotavirus effect of IFN α (23). We thus investigated whether these antiviral proteins may play a role in this setting. We found that rapamycin treatment did not affect the protein expression of IRF1 or IRF7 in Caco2 cells and MEFs (Figure S5A and B). Although the deficiency of 4E-BP1 appears to slightly increase IRF7 but not IRF1 protein synthesis (Figure S5B). Thus, these results indicate that these antiviral proteins are likely not essential in the anti-rotavirus action of the PI3K-Akt-mTOR signaling.

We therefore redirected our attention to autophagy, a cellular metabolism process functioning in orderly degradation and recycling of cellular components, which has been implicated in regulating many types of virus infections (30). mTOR is the main inhibitor of autophagy. Rapamycin, as a specific inhibitor of mTOR, is a potent inducer of autophagy formation (31). We firstly confirmed that starvation (EBSS medium containing 1mM pepstatin A and E-64-d solution, as a positive control) stimulated the autophagy process in Caco2 cells (Figure 6A). Consistently, the ratio of LC3-II to LC3-I (hallmark of autophagy induction) was up-regulated by rapamycin treatment (Figure 6B and Figure S1E) or treatment with starvation (Figure 6C and Figure S1F). Similar to rapamycin treatment (Figure 3B and D), starvation also significantly inhibited rotavirus mRNA (Figure 6D) and viral protein synthesis (Figure 6E and Figure S1G). Interestingly, induction of autophagy by rapamycin and starvation was confirmed to be via 4E-BP1. Because the autophagy is equally

increased by rapamycin treatment, in which 4E-BP1 is not phosphorylated at all in the mutant (Figure 6F). Consistently, the up-regulation of LC3-II/LC3-I ratio by rapamycin or starvation was blocked in 4E-BP1 knockout MEFs cells (-/-) (Figure 6G and H).

To demonstrate the role of autophagy in rotavirus infection, we applied RNAi knockdown of key components of the autophagy machinery. To this aim, LC3 gene that is associated with the autophagosome membrane was silenced with 10 integrating lentiviral RNAi vectors. Five (no 10177, 10178, 10180, 10182 and 10184) out of them showed potent knockdown (Figure 7A). Significant increase of rotavirus genomic RNA was found in all of the five knockdown Caco2 cell lines as quantified by qRT-PCR (Figure 7B). It was further confirmed that viral protein synthesis was increased in these five knockdown Caco2 cell lines (Figure 7C). Beclin1 plays an important role in localization of autophagic proteins to a pre-autophagosomal structure (32). Similarly, knockdown of Beclin1 also favored rotavirus infection (Figure 7D, E and F). Of note, the three optimal knockdown (no 7865, 7866 and 7867) clones were found to increase rotavirus genomic RNA by 27.3 ± 11.9 ($n = 11$; $P < 0.01$), 12.8 ± 3.0 ($n = 13$; $P < 0.001$) and 8.2 ± 2.1 ($n = 17$; $P < 0.001$) fold, respectively (Figure 7E). The viral protein synthesis was further confirmed to be increased in Beclin1 knockdown Caco2 cells (Figure 7F). Thus, we conclude that 4E-BP1 mediates rapamycin-induced autophagy machinery in inhibiting rotavirus infection.

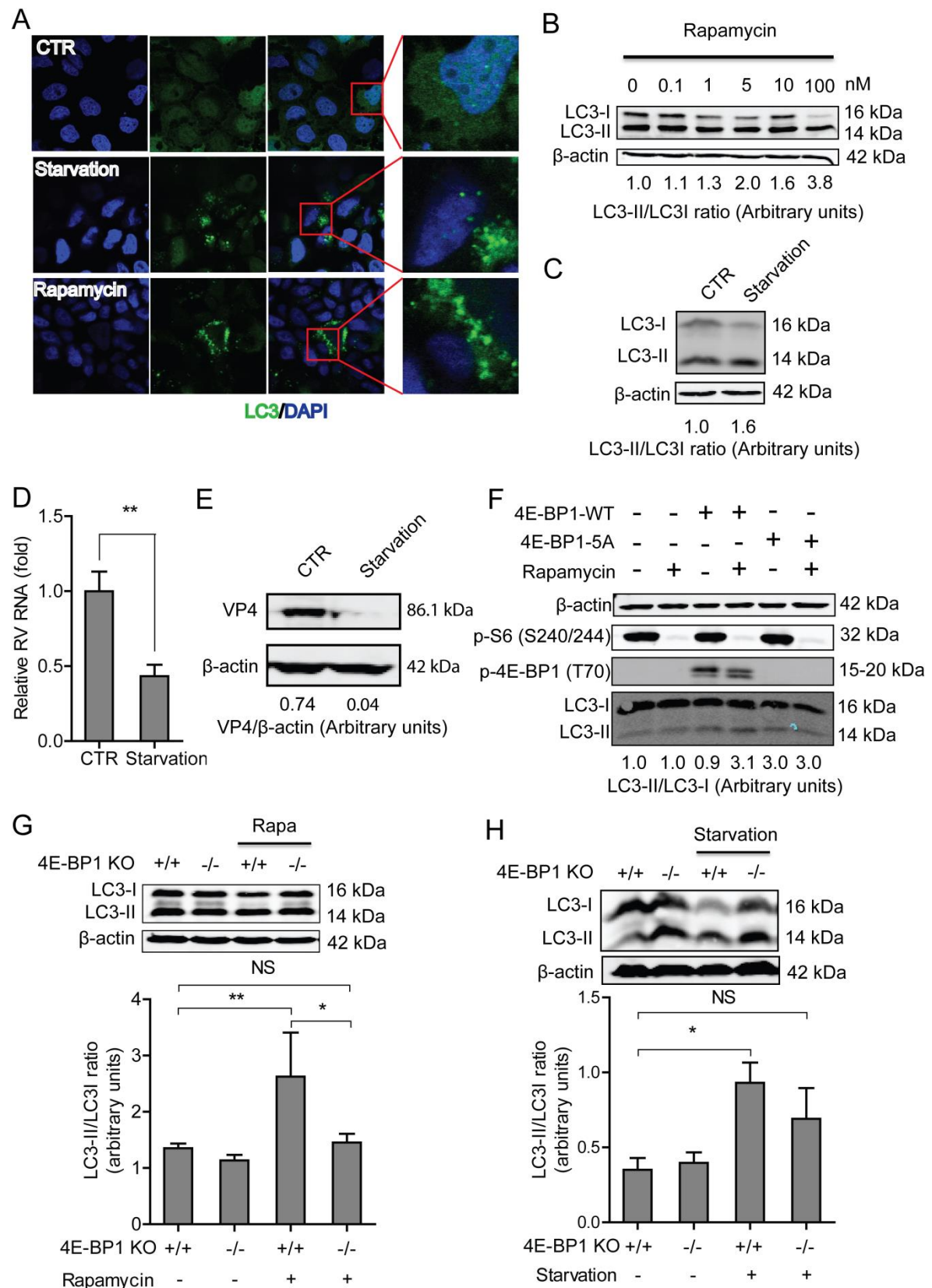


Figure 6. 4E-BP1 mediates rapamycin-induced autophagy that inhibits rotavirus infection. (A) Caco2 cells transduced with lentiviral particles carrying a construct of TagGFP2-LC3 driven by the elongation factor-1 promoter were cultured at 37 °C for 48 h in DMEM medium containing EBSS medium containing 1mM pepstatin A and E-64-d solution (for starvation) and 10 nM rapamycin. LC3-positive puncta was observed by confocal laser microscopy. (B)

LC3-I and LC3-II protein levels were examined by western blot analysis. Protein samples were extracted from Caco2 cells treated with indicated concentrations of rapamycin (48 h). Quantification of the intensity of the immunoreactive bands of both LC3-I and LC3-II was carried out using Odyssey V3.0 software. Densitometric analysis of immunoblots of LC3 was expressed as the ratio of LC3-II to LC3-I, and the ratio of LC3II/LC3I was expressed in arbitrary units. (C) Western blot visualized LC3-I and LC3-II protein levels in starvation (EBSS medium containing 1mM pepstatin A and E-64-d solution) treated Caco2 cells. The ratio of LC3II/LC3I was expressed in arbitrary units. (D) Starvation significantly inhibited rotavirus RNA in Caco2 cells ($n = 6$, mean \pm SEM, $*P < 0.05$, Mann-Whitney test). (E) Starvation dramatically inhibited rotavirus protein VP4 synthesis in Caco2 cells. (F) Rapamycin induced autophagy in 4E-BP1 KO MEFs transfected with 4E-BP1-WT plasmids; while the induction was abolished in 4E-BP1 KO MEFs transfected with 4E-BP1-5A plasmids. (G) Western blot demonstrated that up-regulated ratio of LC3-II to LC3-I by rapamycin was abolished in 4E-BP1 knockout (-/-) MEFs ($n = 6$, mean \pm SEM, $*P < 0.05$, $**P < 0.01$, Mann-Whitney test). (H) Western blot detected that up-regulated ratio of LC3-II to LC3-I by starvation (EBSS medium containing 1mM pepstatin A and E-64-d solution) was abolished in 4E-BP1 knockout (-/-) MEFs ($n = 5$, mean \pm SEM, $*P < 0.05$, Mann-Whitney test).

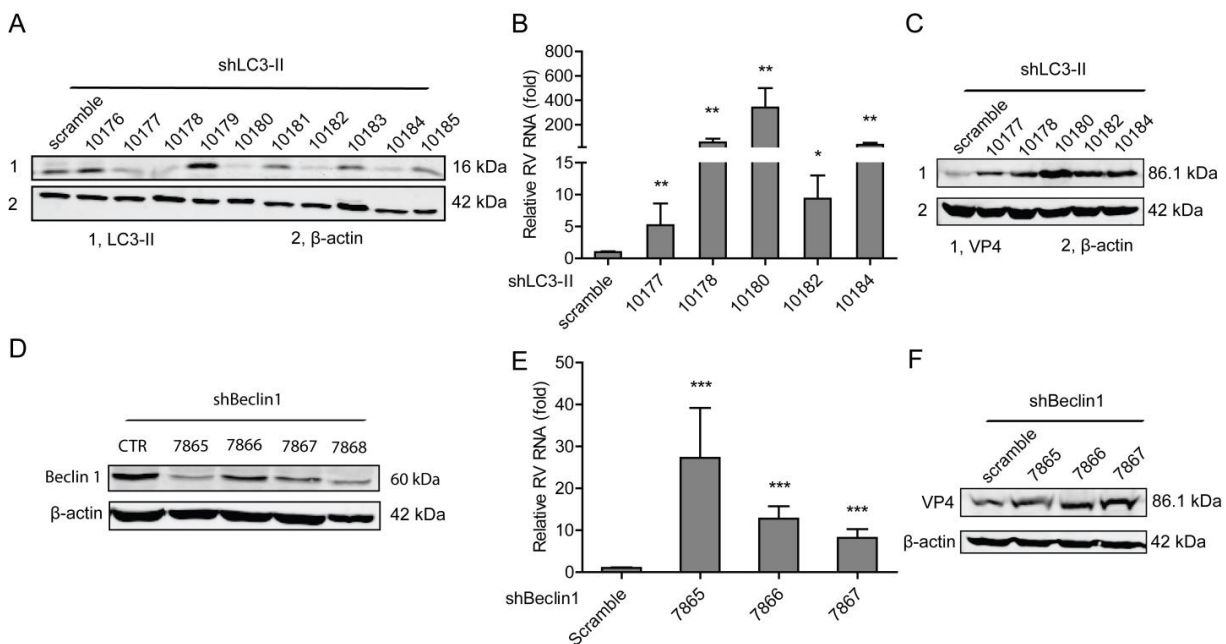


Figure 7. Silence of autophagy related genes, LC3-II and beclin1, inhibits rotavirus replication. (A) Western blot detected LC3-II protein in lentiviral RNAi against LC3-II transduced Caco2 cells. (B) LC3-II knockdown increased rotavirus genomic RNA determined by qRT-PCR ($n = 4-10$, mean \pm SEM, $*P < 0.05$, $**P < 0.01$, Mann-Whitney test). (C) LC3-II knockdown increased rotavirus VP4 protein synthesis detected by western blot assay. (D) Western blot detected beclin1 protein in lentiviral RNAi against beclin1 transduced Caco2 cells. (E) Beclin1 knockdown increased rotavirus genomic RNA determined by qRT-PCR ($n = 11-17$, mean \pm SEM, $***P < 0.001$, Mann-Whitney test). (F) Beclin1 knockdown increased rotavirus VP4 protein synthesis detected by western blot assay.

Discussion

Many viruses interfere with or employ PI3K-Akt-mTOR pathway to regulate their infection (19). Vesicular stomatitis virus (VSV) infection and replication have been reported to lead to dephosphorylation and inactivation of Akt (33). In contrast, influenza A virus replication activates Akt phosphorylation by the viral NS1 protein. It directly binds to the PI3K regulatory subunit (p85 β), allowing the catalytic domain (p110) to phosphorylate Akt (34). Recent structural study has provided insights into the mechanism by which NS1 activates the PI3K (P85 β :P110) holoenzyme (35). White spot syndrome virus (WSSV) stimulates phosphorylation of 4E-BP1, the downstream element of PI3K-Akt-mTOR pathway (36). Semliki Forest virus (SFV) and human papillomavirus type 16 (HPV16) have also been reported to phosphorylate the mTOR targets S6 kinase and 4E-BP1 (37, 38). In fact, PI3K-Akt-mTOR pathway serves as a pro- or antiviral double-edged sword depending on the types of viruses. The pathway itself has been demonstrated to exert inhibitory effect on hepatitis E virus (HEV) replication via 4E-BP1 (13). In contrast, it is also involved in promoting the infection of a broad-spectrum of viruses. Thus, blocking PI3K by its inhibitor could vigorously inhibit infection of numerous viruses including LCMV (19) and coxsackievirus (39). Over-expression of p70S6K or Akt stimulates mRNA expression of coxsackievirus B3 (CVB3) (40). Rotavirus infection has been reported to activate phosphorylation of p70S6K in piglets (41). However, we did not find evidence that rotavirus infection induced phosphorylation of 4E-BP1, S6, Akt or p70S6K in our system (cells of human origin). The underlying discrepancy might derive from the differences of the species and distinct physiological situation, since animal models in some cases are of limited relevance to human physiology (42). However, this pathway is essential in sustaining rotavirus infection, and inhibition of PI3K-Akt-mTOR signaling resulted in potent anti-rotavirus activity (Figure 1, 2, 3, 4 and 5).

PI3K-Akt-mTOR pathway functions by phosphorylation of its downstream effectors including ribosomal protein S6 Kinase 1 (S6K1) and 4E-BP1 to upregulate cap-dependent mRNA translation (43). Merkel cell polyomavirus (MCV) stimulates phosphorylation of 4E-BP1. Subsequently, it disassociates from eIF4E to form the eIF4F complex to induce mRNA translation (44). Rift valley fever virus (RVFV) induces decay of 5'-TOP mRNAs via 4E-BP1 to restrict virus replication (45). We

have demonstrated that the anti-rotavirus effect by inhibition of PI3K-Akt-mTOR pathway is predominantly via 4E-BP1 but not S6K. It has been reported that 4E-BP1 mediates the inhibitory effect of mTOR on autophagy formation (46). In this study, we confirmed that autophagy was induced by inhibiting mTOR with treatment of rapamycin in our system, and this process is mediated by 4E-BP1.

The process of autophagy induction has been implicated in regulating many types of virus infection with various mechanism-of-action (31, 47). We have functionally demonstrated that autophagy machinery exerts antiviral effect against rotavirus (Figure 6D and E). Some viruses including human cytomegalovirus (48), hepatitis B virus-X (HBx) (49) and chikungunya virus (50) have been reported to lure autophagy machinery in host. Rotavirus was also reported to induce autophagy in piglet intestine (51). However, we did not observe changed ratio of LC3-II to LC3-I upon rotavirus infection. The key autophagy related genes have been well studied in regulating virus infections with different model-of-actions. During Sindbis virus infection, beclin 1 expression was upregulated that may result in xenophagy (a selective autophagy) of Sindbis virus to defend the infection (52). Several autophagy related genes can promote both innate and adaptive immune response to combat viral infection (53). We demonstrated that silence of LC3-II and beclin1 led to potent increase of rotavirus infection (Figure 7).

In the clinic, organ transplantation patients irrespective to their age are very sensitive to rotavirus infection. Long-term use of immunosuppressants is an important risk factor causing persistent infection in these patients (4). Rapamycin as a potent mTOR inhibitor has been used as immunosuppressant in transplant patients for decades (54). It has been well documented that different types of immunosuppressants may differentially affect virus infection (4, 55). Antiviral effects of rapamycin have been demonstrated in models of herpes simplex virus (56), coxsackievirus (39) and HIV-1 infections (21). Our data demonstrating potent anti-rotavirus effect of rapamycin favor the clinical application of this immunosuppressant in rotavirus-infected organ recipients.

In summary (Figure 8), we have dissected the effects of PI3K-Akt-mTOR signaling on rotavirus infection, and demonstrated that blocking this pathway can potently inhibit rotavirus replication. Importantly, the mTOR inhibitor, rapamycin, induced autophagy machinery to inhibit rotavirus infection via 4E-BP1. Thus, this study revealed new insights on rotavirus-host interactions and provided new avenues for

antiviral drug development. Furthermore, it provides important references for transplant clinicians to design optimal immunosuppression protocols for rotavirus infected organ transplant patients.

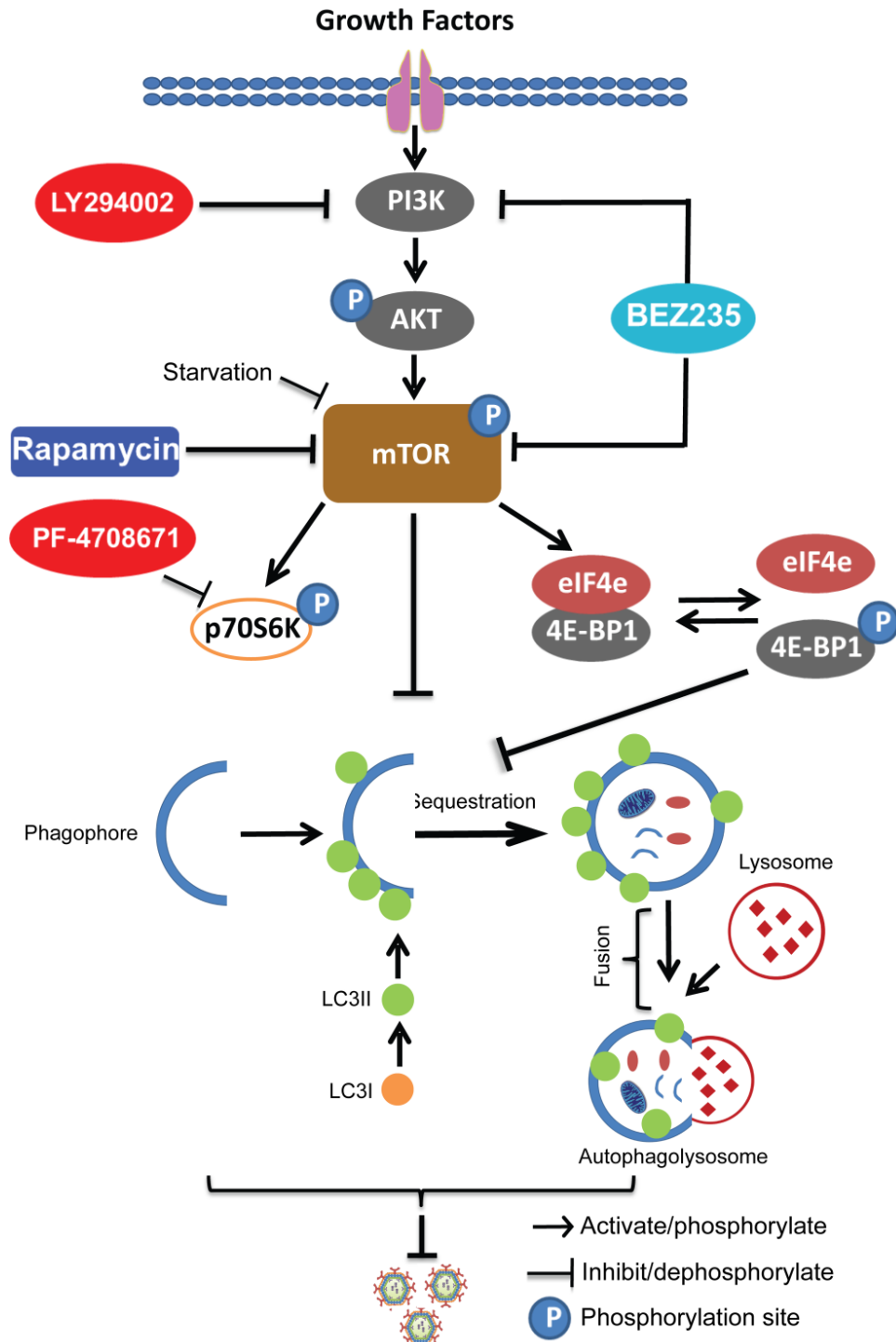


Figure 8. Schematic depicting the PI3K-Akt-mTOR signaling involved in regulating rotavirus replication. Pharmaceutical blocking PI3K-Akt-mTOR pathway inhibits phosphorylation and activation of its downstream targets including p70S6K and 4E-BP1. 4E-BP1 mediates inhibition of mTOR on autophagy machinery via 4E-BP1. Autophagy itself exerts anti-rotavirus effect.

Materials and methods

Reagents.

Stocks of LY294002, an inhibitor of PI3K-Akt (Sigma-Aldrich), BEZ235, a dual inhibitor of PI3K-Akt and mTOR (Selleck Chemicals), rapamycin, a mTOR inhibitor (Merk, Schiphol-Rijk, The Netherlands) and PF-4708671, an inhibitor of p70S6K (Selleck Chemicals) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO). All reagents were stored in 25 μ L aliquots and frozen at -80 °C. Other reagents including Earle's Balanced Salt Solution (EBSS) (Lonza), E-64-d (Santa Cruz Biotech, Santa Cruz, CA) and pepstatin A (Santa Cruz Biotech, Santa Cruz, CA) were also used.

Viruses.

Simian rotavirus SA11 was used and prepared as described previously.(22) Patient-derived rotavirus isolates were isolated from stool samples of rotavirus-infected patients during diarrhea period as described previously (22).

Cell and human primary intestinal organoid culture.

Caco2 (Caucasian colon adenocarcinoma) cells are the heterogeneous human epithelial colorectal adenocarcinoma cells, representing numerous characteristics of enterocytes. They are commonly used for in vitro modelling rotavirus infection. Caco2 cells were maintained in culture in T25/T75 flask with Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, The Netherlands) as previously described (23). Immortalized mouse embryonic fibroblasts (MEFs) derived from 4E-BP1 wild-type (+/+) and 4E-BP1 knock-out (-/-) mice (gifts from E.N. Fish's lab) were cultured in DMEM (Invitrogen-Gibco, Breda, The Netherlands) containing 10% vol/vol fetal calf serum (FCS) (Hyclone, Lonan, Utah), 100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine (Invitrogen-Gibco).

3D cultures of human intestinal organoids were performed as previously described (23). Briefly, intestinal tissues were vigorously shaken in 8 mM EDTA for 15 min at 4 °C, followed by removing the EDTA solution. Subsequently loosened crypts were collected by pipetting the solution up and down through a 10 mL pipette for 8-10 times. The crypt suspension was transferred into a 50 mL centrifuge tube

(Greiner bio-one, the Netherlands) and biopsies were re-used for repeated collection of crypts (2-3 times). Crypt suspensions were pooled and centrifuged at 300 g for 5 min. Pelleted crypts were re-suspended in 2 mL complete medium with growth factors CMGF-: advanced DMEM/F12 supplemented with 1% (vol/vol) GlutaMAX™ Supplement (Gibco, Grand island, USA), 10 mM HEPES, and collected by centrifugation at 130 g for 5 min at 4 °C. Crypts were finally suspended in Matrigel (Corning, Bedford, USA), and placed in the center of a well of a 24-well plate (40 µL per well). After the Matrigel had solidified (15 min at 37 °C), organoids were cultured in culture medium at 37 °C, 5% CO₂. Culture medium was refreshed every 2-3 days, and organoids were passaged every 6-7 days.

TCID₅₀ assay.

Reed and Muench method was applied to determine infectious virus titration with a standard TCID₅₀ protocol by means of MA104 cells (57).

Virus infections and treatment of drugs.

The protocols of inoculation of Caco2 cells with SA11 and patient-derived rotavirus were described previously (23). Briefly, cell monolayers of Caco2 cells were incubated with SA11 rotavirus at 37 °C with 5% CO₂ for 60 min for infection, followed by removing free virus particles. Subsequently, cells were added with culture medium (FCS free) containing 5 µg/mL of trypsin and indicative drugs, followed by incubation at 37 °C in a humidified 5% CO₂ incubator.

Organoids were inoculated with activated virus (5,000 genome copies) for 60 min at 37 °C with 5% CO₂, followed by removing free viral particles. Then, organoids were aliquoted in wells of a 48-well plate coated with 20% (vol/vol) Collagen R Solution (SERVA, Heidelberg, Germany) and culture medium containing drugs of interest was added. Subsequently, the 48-well plate containing organoids was spin down at 500 g for 5 minutes to promote the adherence on the bottom of the wells, followed by maintaining them at 37 °C with 5% CO₂.

Preparation of patient-derived rotavirus was performed as described earlier (23), and protocols of viral inoculation and treatment of drugs are similar to SA11 rotavirus. 48 h post-infection was used to determine viral replication levels, since it is the optimal time point for viral replication without lysis of the cells.

RNA isolation, cDNA synthesis and qRT-PCR.

Total RNA was extracted using a NucleoSpin[®] RNA kit (MACHEREY-NAGEL, Düren, Germany) and quantified with a Nanodrop ND-1000 (Wilmington, DE, USA). 500 ng of RNA was used as template for cDNA preparation with the reverse transcription system from TAKARA (TAKARA BIO INC). Quantitative real-time PCR (qRT-PCR) reactions were performed with SYBR Green (Applied Biosystems[®], Austin, USA) according to the manufacturer's instruction. qRT-PCR was performed in an IQ5 cycler PCR machine (Bio-Rad). The levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were used as an endogenous reference to normalize the quantities of target mRNA by using the formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{control}}$). All primer sequences were indicated in Table S1.

Gene silencing assays.

Lentiviral pLKO knockdown vectors (Sigma-Aldrich) targeting mTOR, 4E-BP1 and non-targeted control lentivirus were obtained from the Erasmus Biomics Center and produced in human embryonic kidney cell-line 293T (HEK293T) cells.

Caco2 cells were transduced with lentiviral vectors in order to generate stable gene knockdown cells. Transduced cells were subsequently selected with 6 $\mu\text{g/mL}$ puromycin (Sigma). After pilot study, the shRNA vectors exerting optimal gene knockdown were selected. Knockdown and control Caco2 cells were incubated with rotavirus as described in the foregoing.

Transient transfection

The plasmids including 4E-BP1-WT and 4E-BP1 5A were transfected in 4E-BP1 knockout MEFs. Cells were seeded in 6-well plates till ~70% confluence, followed by adding 200 μL of Opti-MEM[®] reduced serum medium (Thermo Fisher Scientific) with 2 μg 4E-BP1-WT and 4E-BP1-5A plasmids and 6 μL Fu-GENE 6 transfection reagent (Roche Applied Science, Inc.). Transfected cells were incubated in complete medium for 24 h, followed by refreshing with 100 nM rapamycin or control medium for 48 h. The cells were analyzed by western blot after treatment. The experiments were executed in triplicate.

Cell lysis, SDS-PAGE and western blotting.

After treatment and washing with PBS, cells in six-well plates were lysed with 400 μ L of Laemmli buffer (20% [vol/vol] glycerol, 4% [wt/vol] SDS and 120 mM Tris-HCl, pH 6.8) containing 100 mM DTT, and boiled for 10 min at 95 °C. Then, cell lysates were subjected to SDS-PAGE, and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-FL). Subsequently, the membrane was blocked with 2.5 mL blocking buffer and 2.5 mL PBS containing 0.05% Tween 20 (PBS-T) for 1 hour at room temperature. It was followed by incubation with p-mTOR (1:1000, rabbit; cell signaling), t-mTOR (1:1000, rabbit; cell signaling), p-Akt (1:1000, rabbit; cell signaling), t-Akt (1:1000, rabbit; cell signaling), p-S6 (1:1000, rabbit; cell signaling), t-S6 (1:1000, rabbit; cell signaling), p-4E-BP1 (1:1000, rabbit; cell signaling), t-4E-BP1 (1:1000, rabbit; cell signaling), LC3-I/II (1:1000, rabbit; cell signaling) and SA11 rotavirus VP4 (1:1000, HS-2, mouse monoclonal (provided by professor Harry Greenberg, Stanford University School of Medicine, USA) overnight in 5 mL of blocking buffer/PBST (1:1) cocktail at 4°C. After three more washes with PBST, the immunoreactive bands were detected by western blot analysis and detection of β -actin served as loading control (1:1000, mouse monoclonal; Santa Cruz).

Cytospin preparations and Confocal Laser Scanning Microscopy (CLSM) for organoids.

Organoids were harvested from Matrigel using cold PBS, followed by fixing in 4% paraformaldehyde in PBS at 4 °C for 10 min. Fixed organoids were added into the appropriate wells of the CytoSpin II Cytocentrifuge (Shandon Scientific Ltd, Runcorn, England), and spin down at 1000 rpm for 2 min. The slides containing organoids were rinsed in PBS-baths 3x5 min, followed by treatment with 0.1% (vol/vol) Triton-x100 for 4 min. Then, the slides were rinsed in PBS-baths 2x5 min, followed by being incubated with milk-tween-glycine medium (0.05% tween, 0.5% skim milk and 0.15% glycine) to block background staining for 30 min. The slides were incubated in a humidity chamber with p-S6 antibodies (1:100, rabbit; cell signaling) diluted in milk-tween-glycine medium at 4 °C overnight. The slides were washed 3x5 min in PBS-baths prior to 1 h incubation with 1:1000 dilutions of the anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor® 488 Conjugate) secondary antibody. Nuclei were stained with DAPI (4, 6-diamidino-2-phenylindole; Invitrogen). Images were detected using confocal electroscope.

Lentiviral expression of green fluorescence protein bound to LC3 and live imaging.

Caco2 cells were cultured on cover slide on the bottom of wells of a 6-well-plate with culture medium and transduced with lentiviral particles carrying a construct of TagGFP2-LC3 driven by the elongation factor-1 promoter (Millipore LentiBrite) at a multiplicity of infection (MOI) of 50 for 48 h. Then, the medium was changed into the pharmacological treatment for indicated time period. Live images were obtained by using confocal electroscope.

Cytotoxicity assays in cells and organoids.

The effects of chemical reagents on cell viability were determined by MTT assay (see Figure S1). Briefly, Caco2 cells were seeded 1×10^4 cells per well of a 96-well plate and viable cells were detected at indicated time points by adding 10 μ L 5 mg/mL MTT per well, 3 h incubation at 37 °C and replacement of the medium by 100 μ L DMSO (Sigma). Absorbance (490 nm) was analyzed.

Intestinal organoids were embedded in Matrigel and cultured in wells of 24-well-plate with organoids culture medium containing relevant chemical reagents. The effects of related chemical reagents on intestinal organoids viability were determined by morphology of organoids observed by light microscope (see Figure S2)

Statistical analyses.

All numerical results are expressed as Mean \pm SEM. Statistical comparisons were analyzed by Mann-Whitney test for the data without normal distribution or t test for the data with normal distribution. P-values less than 0.05 were considered to be statistically significant. Analysis was performed using GraphPad Prism Version 5 (GraphPad Software Inc., La Jolla, CA).

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Supplementary Tables & Figures

Table S1. Primers used in the study

Sequence of rotavirus primers

	SA11 Rotavirus	Human Patient Rotavirus
Sense	TGGTTAAACGCAGGATCGGA	ACCATCTACACATGACCCTC
Anti-sense	AACCTTTCCGCGTCTGGTAG	CACATAACGCCCTATAGCC

Primer sequences of other genes

Primers	Human	Mouse
GAPDH-F	GTCTCCTCTGACTTCAACAGCG	TTCCAGTATGACTCCACTCACGG
GAPDH-R	ACCACCCTGTTGCTGTAGTAGCCAA	TGAAGACACCAGTAGACTCCACGAC
mTOR-F	AGCATCGGATGCTTAGGAGTGG	
mTOR-R	CAGCCAGTCATCTTTGGAGACC	
4E-BP1-F	CACCAGCCCTTCCAGTGATGAG	
4E-BP1-R	CCTTGGTAGTGCTCCACACGAT	

Table S2. Patient characteristics.

Patient	Age (yrs)	Gender	Symptoms	Virus Detection								
				Enterovirus	Parechovirus	Norovirus genogroups I	Norovirus genogroups II	Adenovirus	Astrovirus	Sapovirus	Rotavirus	
1	3.5	Female	Fever	No	No	No	No	No	No	No	No	Yes
2	74	Female	Congestive heart failure, myocarditis	No	No	No	No	No	No	No	No	Yes
3	27	Female	Fever, diarrhea, nausea, vomiting	No	No	No	No	No	No	No	No	Yes
4	67	Male	Fever, stomach ache, watery diarrhea	No	No	No	No	No	No	No	No	Yes
5	28	Female	(Kidney transplant) Nausea, stomach ache, watery diarrhea, fever, headache, vomiting	No	No	No	No	Yes	No	No	No	Yes

Supplementary Figures

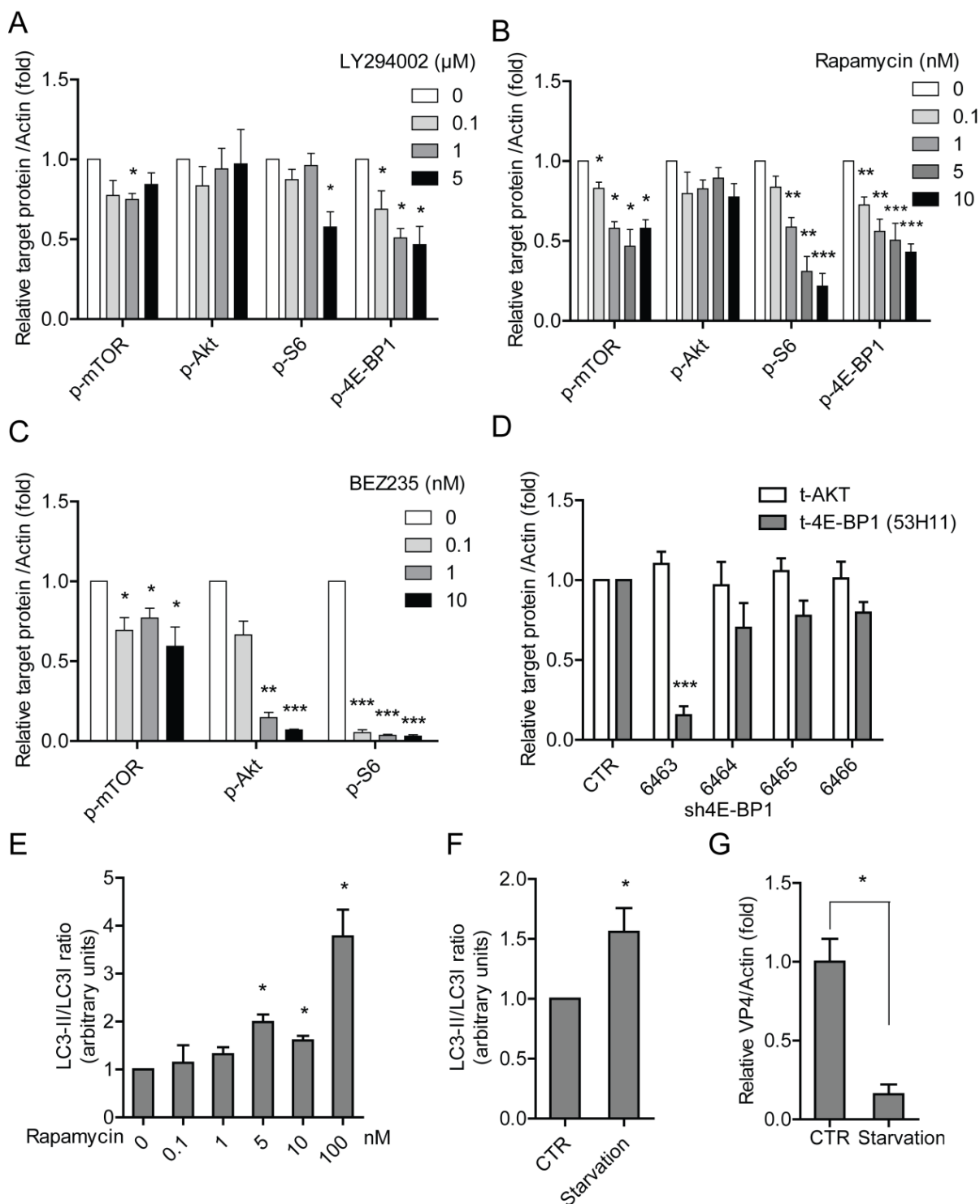


Figure S1. Quantification of the intensity of the immunoreactive bands of p-mTOR, p-Akt, p-S6 and p-4E-BP1 with treatments of (A) LY294002 ($n = 4$, mean \pm SEM, $*P < 0.05$, t test), (B) rapamycin ($n = 4-7$, mean \pm SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, t test) or (C) BEZ235 ($n = 4$, mean \pm SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, t test) at indicated concentrations using Odyssey V3.0 software. (D) Quantification of the intensity of the immunoreactive bands of t-Akt and t-4E-BP1 (53H11) in 4E-BP1 knockdown Caco2 cells using Odyssey V3.0

software ($n = 4$, mean \pm SEM, $*P < 0.001$, t test) (E) Effect of rapamycin on autophagy ($n = 3$, means \pm SEM, $*P < 0.05$, t test). Quantification of the intensity of the immunoreactive bands of both LC3-I and LC3-II was carried out using Odyssey V3.0 software. Densitometric analysis of immunoblots of LC3 was expressed as the ratio of LC3-II to LC3-I, and the ratio of LC3II/LC3I was expressed in arbitrary units. (F) Effect of starvation on autophagy ($n = 3$, means \pm SEM, $*P < 0.05$, t test). Quantification of the intensity of the immunoreactive bands of both LC3-I and LC3-II was carried out using Odyssey V3.0 software. The ratio of LC3II/LC3I was expressed in arbitrary units. (G) Quantification of the intensity of the immunoreactive bands of VP4 with treatment of starvation using Odyssey. Data were presented as means \pm SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

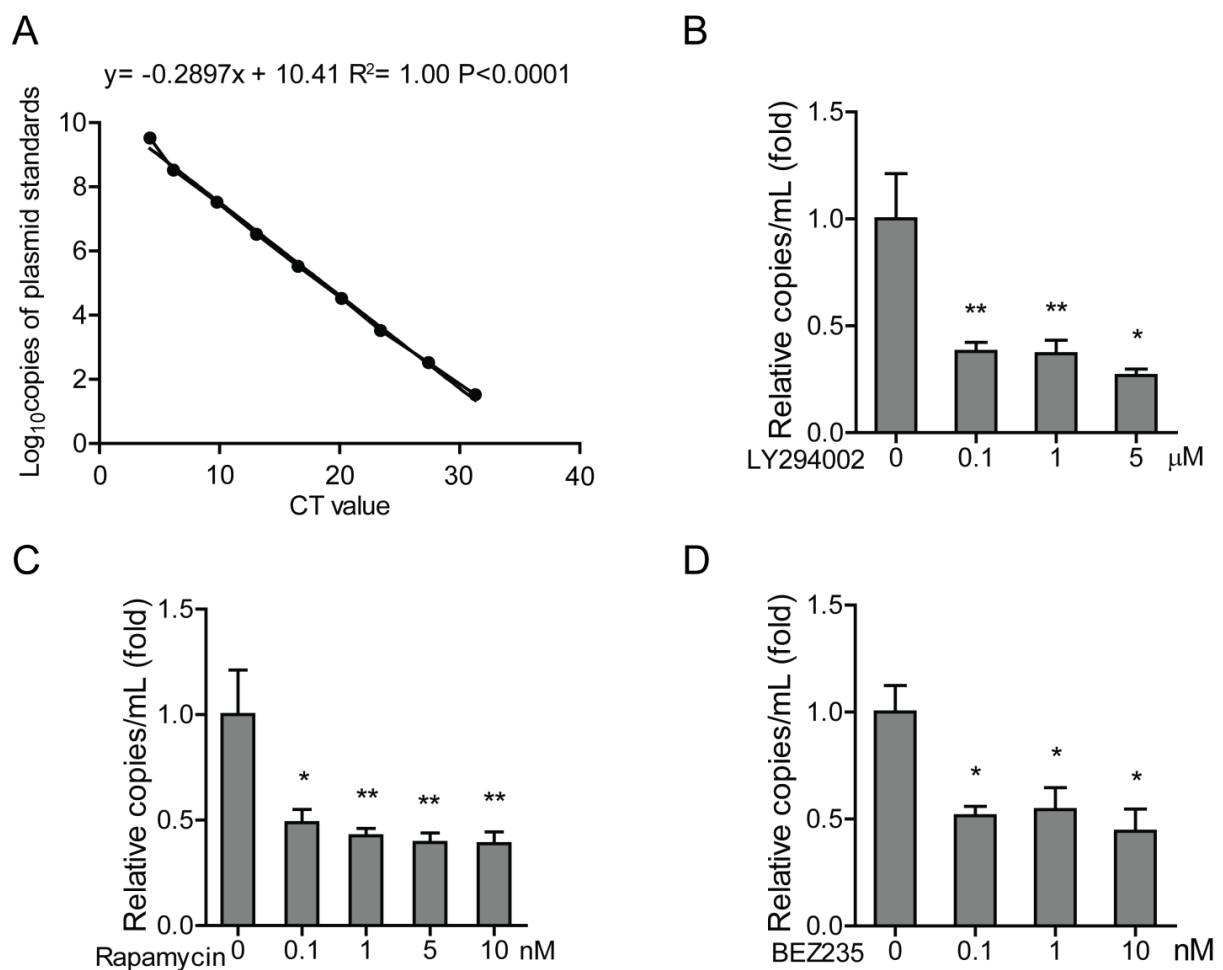


Figure S2. Effects of (B) LY294002 ($n = 6$), (C) rapamycin ($n = 6$) and (D) BEZ235 ($n = 5$) on rotavirus secretion calculated by (A) a standard curves described previously (1). Caco2 cell monolayers were infected by trypsin activated rotavirus for 1 h, followed by removing free viral particles by washing with PBS for four times. Then, culture medium with indicative drugs was added for 48 h incubation, followed by that secreted viral particles in the medium were detected by qRT-PCR. Data were presented as means \pm SEM, $*P < 0.05$, $**P < 0.01$.

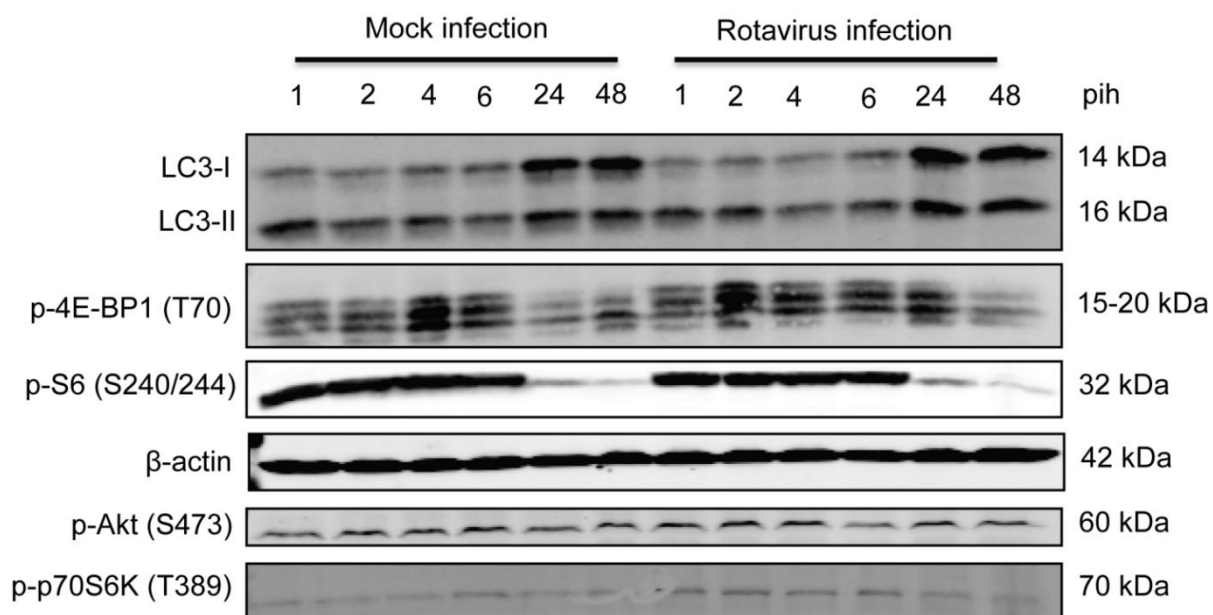


Figure S3. Rotavirus infection did not affect the key elements of PI3K-Akt-mTOR pathway. Western blot detects phosphorylated 4E-BP1 (T70), S6 (S240/244), Akt (S473), p70S6K (T389) and LC3-I/II proteins in mock and rotavirus infected Caco2 cells at indicated time points.

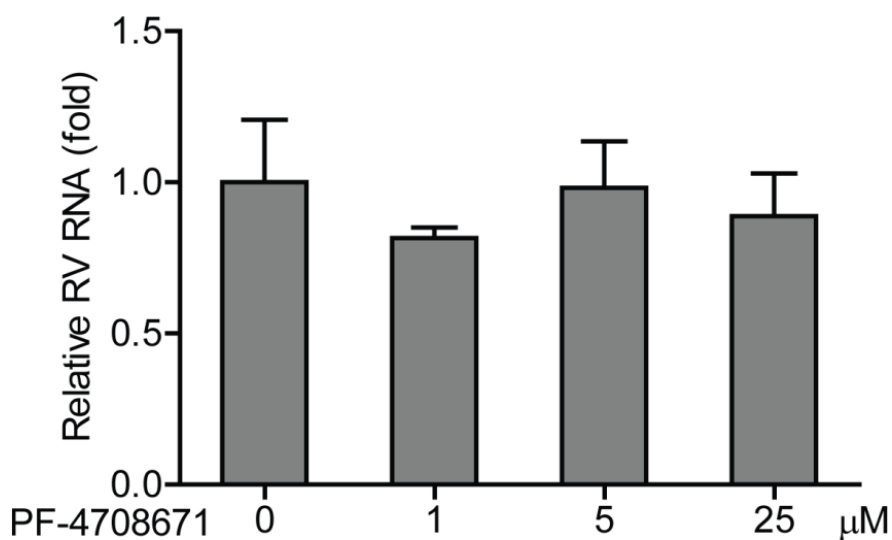


Figure S4. Effect of PF-4708671 (p70 ribosomal S6 kinase inhibitor) on rotavirus replication in Caco2 cells. Rotavirus infected Caco2 cells were treated with 1, 5 and 25 μM PF-4708671 for 48 h. Viral genomic RNA was quantified by qRT-PCR.

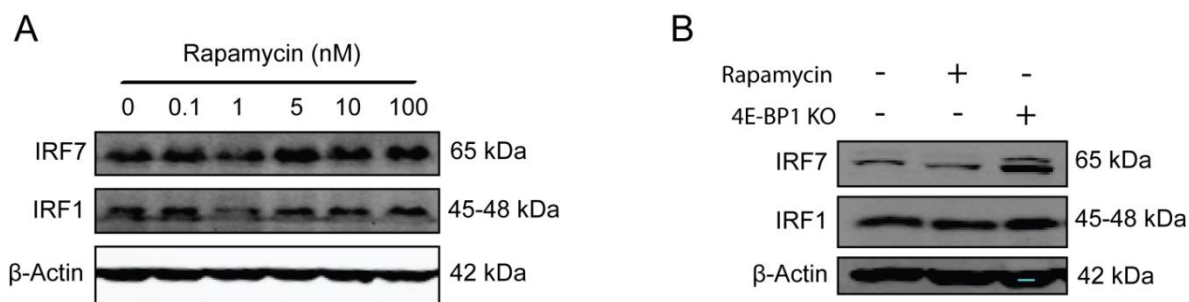


Figure S5. Effects of rapamycin (A) and 4E-BP1 knock out (B) on IRF1 and IRF7 protein synthesis detected by western blot assay.

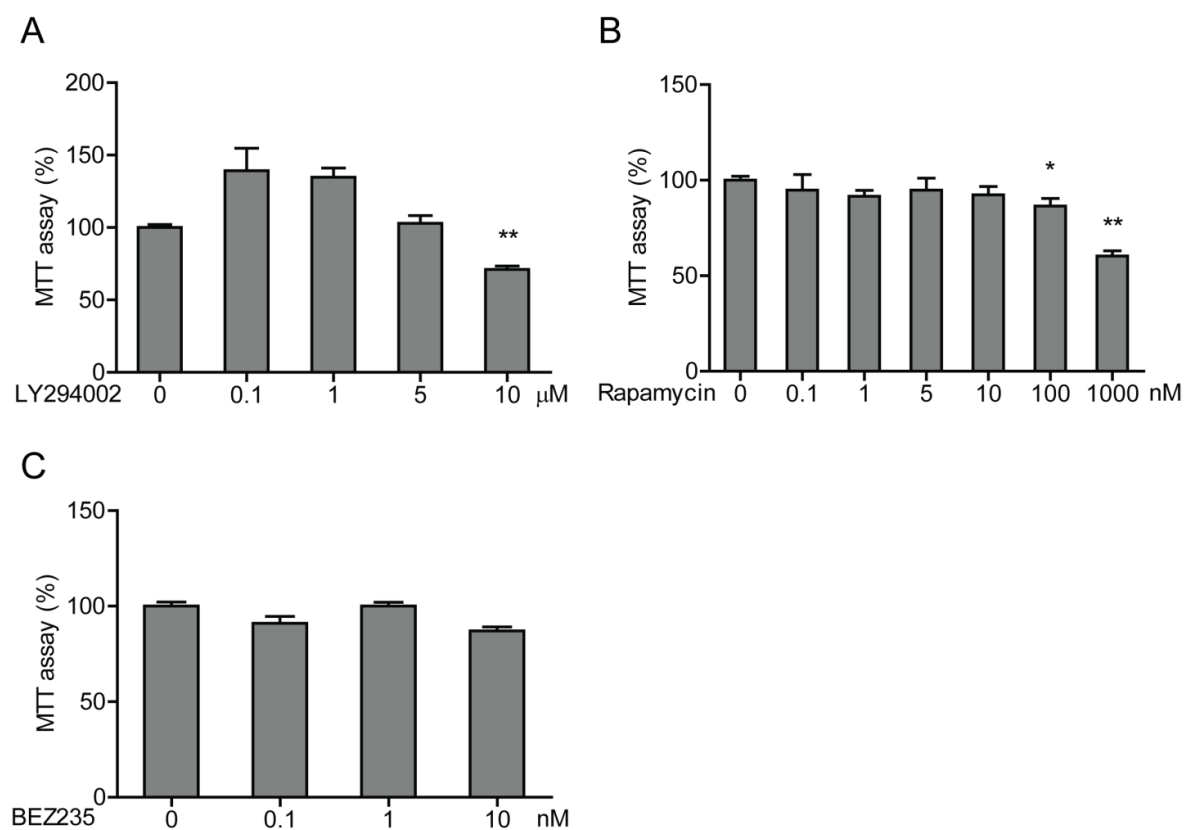


Figure S6. Effects of LY294002, BEZ235 and rapamycin on host cell viability determined by MTT assays. (A) Effect of LY294002 on viability of Caco2 cells (48 h) ($n = 6$, means \pm SEM, ** $P < 0.01$, Mann-Whitney test). (B) Effect of rapamycin on viability of Caco2 cells (48 h) ($n = 6$, means \pm SEM, * $P < 0.05$, ** $P < 0.01$, Mann-Whitney test). (C) Effect of BEZ235 on viability of Caco2 cells (48 h).

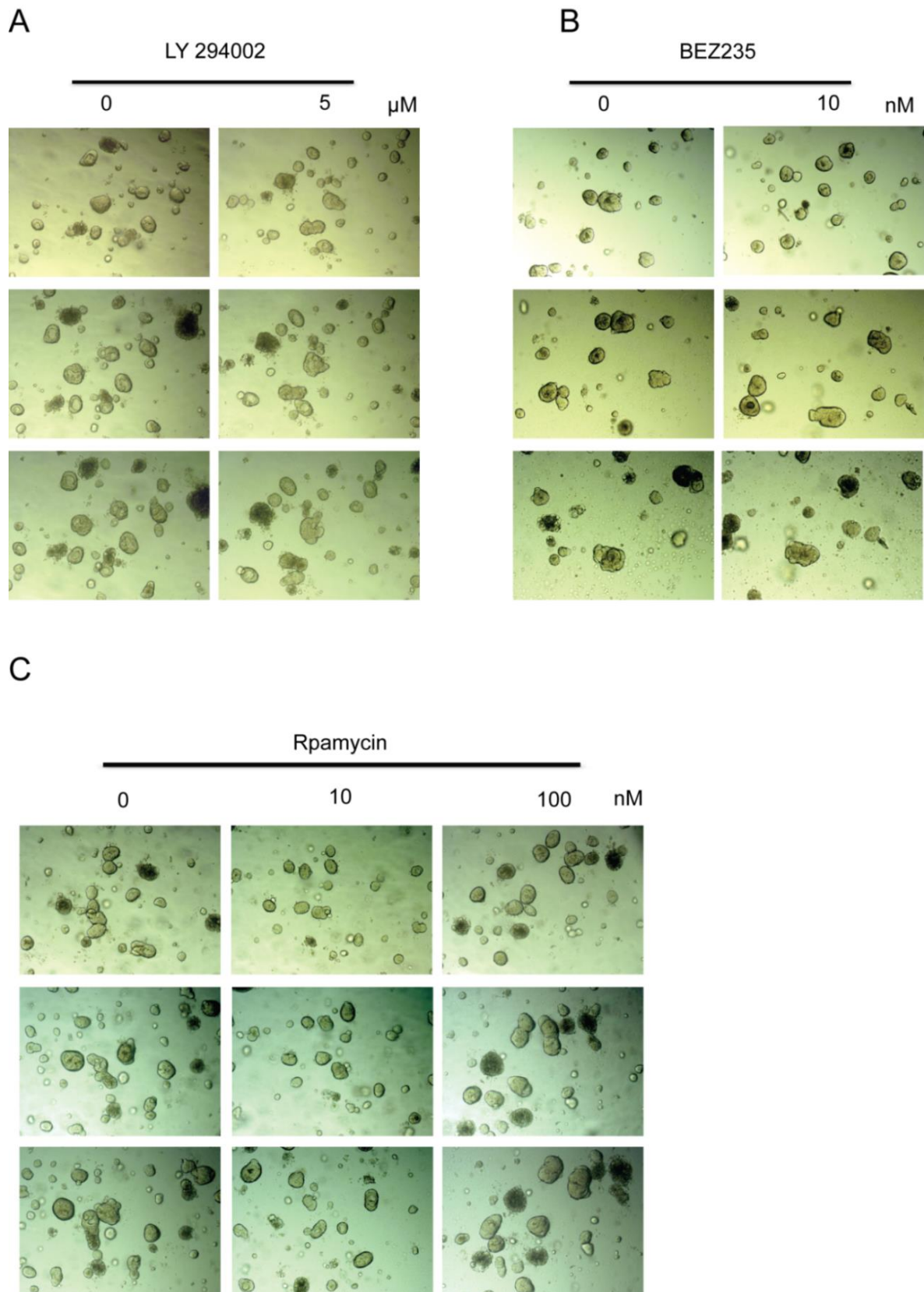


Figure S7. Effects of LY294002, BEZ235 and rapamycin on growth of human intestinal organoids. (A) Effect of LY294002 on growth of organoids (48 h). (B) Effect of BEZ235 on organoids growth (48 h). (C) Effect of rapamycin on organoids growth (48 h).

Supplementary reference

1. **Yin Y, Bijvelds M, Dang W, Xu L, van der Eijk AA, Knipping K, Tuysuz N, Dekkers JF, Wang Y, de Jonge J, Sprengers D, van der Laan LJ, Beekman JM, Ten Berge D, Metselaar HJ, de Jonge H, Koopmans MP, Peppelenbosch MP, Pan Q. 2015. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. Antiviral Res 123:120-131.**

CHAPTER 7

The eukaryotic translation initiation factor 4F complex inhibits rotavirus infection

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In Preparation

Abstract

Viral protein synthesis often completely relies on the translational machinery of the host due to the lack of the machinery for mRNA translation from the virus. eIF4F complex is a tripartite complex composed of three components including a cap-binding subunit (eIF4E), an RNA helicase (eIF4A) anchored to a large molecular scaffold (eIF4G) being able to associate with eIF3-bound 40S ribosome subunits. eIF4F closely monitors translation in response to a multitude of environmental conditions including viral infection. Rotavirus infection is a leading causative agent of severe diarrhea in infants younger than five years old, which has also emerged as important complications in organ transplant patients irrespective to their age. How translation initiation factors regulate rotavirus infection remains obscure. In this study, we have demonstrated that blocking the three components of eIF4F including eIF4A, eIF4E and eIF4G remarkably promote rotavirus infection, although rotavirus infection *per se* does not affect the expression level of the eIF4F complex. Consistently, the negative regulator of eIF4F, PDCD4, supports rotavirus infection. These results have demonstrated that the eIF4F complex is an essential host factor in defending rotavirus infection, thus revealing new insight on rotavirus-host interactions.

Keywords: Rotavirus; eIF4F complex; knockdown; crispr/cas9

Introduction

There are three phases in protein synthesis including initiation, elongation, and termination (1); whereas initiation determines translation rates (2). Most of the eukaryotic mRNAs are characterized by the m⁷GpppX structure (where X is any nucleotide), termed as a cap, at their 5' ends or the poly (A) tail at the 3' end (3). These mRNA are translated in a cap-dependent manner (2). The cap structure is bound by the eukaryotic translation initiation factor 4F (eIF4F) that is a protein complex containing three constituent proteins: an eukaryotic translation initiation factor 4A (eIF4A), an eukaryotic translation initiation factor 4E (eIF4E), and an eukaryotic translation initiation factor 4G (eIF4G) (4). The eIF4F complex plays a vital role in cap-dependent mRNA protein translation initiation by recognizing an RNA helicase that unwinds the secondary structure of the 5' region (eIF4A), the mRNA 5' cap structure (eIF4E), and bridging of the mRNA and the ribosome (eIF4G), and circularization of the mRNA via interaction with poly (A)-binding protein (eIF4G) (3). The end-independent binding of ribosomes to an internal ribosome entry site (IRES) in the 5' untranslated region is able to initiate the translation of a subset of cellular and viral mRNAs (5). The ribosome binding is facilitated by that eIF4F is directed to the 5' end of the mRNA via eIF4E, which is achieved via eIF4A along with eIF4B to unwind the mRNA 5' secondary structure (2).

Viruses require components from the host cell to propagate, replicate and assemble viral components and release their progeny virions (4). The viral protein synthesis completely relies on the translational machinery of the host due to the lack of the machinery for mRNA translation from the viruses (6). Viruses are able to exploit the translational machinery including eIF4F to support the translation of their transcripts (4). Particular mammalian viruses are involved in targeting eIF4F complex to regulate both viral and host mRNA translation; while many RNA viruses inhibit host protein synthesis to initiate the translation of their own mRNAs by inhibition of eIF4F (7). Feline calicivirus (FCV) and mouse norovirus (MNV) were reported to be capable of directly interacting with the initiation factors eIF4E, and viral RNA translation required the RNA helicase component of the eIF4F complex (8). Blocking eIF4E-eIF4G interaction has been demonstrated to result in impairing Coronavirus replication (9). Interestingly, certain genes from the eIF4G family were thought to be

resistance to *Rice yellow mottle virus* (RYMV) (10). Collectively, eIF4F complex has diverse effects on various viruses.

Rotavirus infection is considered to be the leading causative agent of severe diarrhea in infants younger than five years old (11). It causes an estimated 611 000 death of children each year globally (12). Although rotavirus infection mainly occurs in low-income countries (13), it also bears heavy burden in industrialized countries. In European Union, rotavirus causes more than 200 deaths, over 87 000 admissions, and almost 700 000 outpatient visits in children younger than 5 years of age annually (14). Increasing evidence indicate that rotavirus infection causes severe complications in organ transplant patients irrespective to their ages (15). Although vaccines have been developed, no approved antiviral treatment is available. Furthermore, the biology of rotavirus infection remains to be further explored. In the study, we dissect effects of the cellular translation machinery on rotavirus infection. We have demonstrated that the eIF4F complex is an essential host factor in counteracting rotavirus infection.

Materials and Methods

Cell culture and Virus

Caucasian colon adenocarcinoma cell line (Caco2) and human embryonic kidney cells 293 (HEK293) were grown in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Verviers, Belgium) containing 20% (vol/vol) heat-inactivated fetal calve serum (FCS) (Hyclone, Lonan, Utah) and penicillin (100 IU/mL)/streptomycin (100 mg/mL) (P/S) Invitrogen-Gibco) at 37 °C in a humidified 5% CO₂ incubator.

Simian rotavirus SA11 was used and prepared as described previously (16).

Inoculation of SA11 rotavirus in cells

The protocol of inoculation of SA11 rotavirus was described previously (16). Briefly, cell monolayers of Caco2 cells were incubated with SA11 rotavirus at 37⁰C with 5% CO₂ for 60 min for infection, followed by removing free viral particles. Then, cells were added with culture medium (FCS free) containing 5 µg/ml of trypsin and indicative drugs, followed by incubation at 37⁰C in a humidified 5% CO₂ incubator. Viral titer was detected by qRT-PCR after 48 hrs inoculation.

Gene knockdown by shRNA

Lentiviral shRNA vectors, targeting eIF4A, eIF4E, eIF4B and PDCD4 or non-targeted control lentivirus were produced in HEK293 cells. Briefly, the target vector was co-transfected with packaging plasmids including pVSV-G, pMD, and pREV into HEK293 cells and viral vectors were harvested.

Caco2 cells were incubated with lentiviral vectors for three days, followed by replacing virus-containing medium by medium containing puromycin (8 µg/mL), since the vectors also express a puromycin resistance gene. After selection, stable clones were established in 2-3 weeks. The knockdown effect was detected by western blot.

Gene knockdown by crispr/cas9

sgRNAs against eIF4A (forward: 5'-CACCGCCCCCGATACAGGCGTGAC-3'; reverse: 5'-CGGGGGCTATGTCCGCACTGCAAA-3'), eIF4E (forward: 5'-CACCGGGACGTCCCCACTTGTCCG-3'; reverse: 5'-CCCTGCAGGGGTGAACAGGCCAAA-3'), and eIF4G (forward: 5'-CACCGCTATCCAGTCGAACACCCGC-3'; reverse: 5'-CGATAGGTCAGCTTGTGGGCGCAA-3') were designed manually or using freely available online tools. Oligos were annealed according to the manufacturer's protocol (37 °C for 30 min; 95 °C for 5 min and then ramp down to 25 °C at 5 °C/min). Subsequently, annealed oligos were ligated into pX330, followed by transforming 10 µl of electrocomp™ cells (ThermoFisher SCIENTIFIC) with 5 µl of annealed oligos, and then incubated at 37 °C for overnight. Pick 2-3 colonies and inoculate into LB medium, followed by performing a sequence-verification. Inoculate verified colony into a mini-prep culture and isolate DNA by using DNA extraction kit (ThermoFisher SCIENTIFIC) according to manufacturer's protocol. CRISPR DNA was co-transfected with packaging plasmids including pVSV-G, pMD, and pREV into HEK293 cells and lentiviral vectors were harvested.

Caco2 cells were incubated with lentiviral vectors for three days, followed by replacing virus-containing medium by medium containing puromycin (8 µg/mL), since the vectors also express a puromycin resistance gene. After selection, stable clones were established in 2-3 weeks. The knockdown effect was detected by western blot.

qRT-PCR analyses

Total RNA was isolated using NucleoSpin[®] RNA kit (MACHEREY-NAGEL, Düren, Germany), and the RNA concentration was measured by a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was synthesized using the reverse transcription system from TAKARA according to manufacturer's instructions (TAKARA BIO INC). The resulting cDNA was diluted 1:10, and 2 μ L of the diluted cDNA was used for qRT-PCR with primers listed in Supplementary Table 1. All qRT-PCR experiments were performed by SYBR-Green-based (Applied Biosystems SYBR Green PCR Master Mix; Thermo Fisher Scientific Life Science) real-time PCR with the StepOnePlus System (Thermo Fisher Scientific Life Sciences). The expression of target mRNAs was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Gene expression analysis was performed by the $\Delta\Delta C_T$ method.

MTT assay

Caco2 cells were seeded 1×10^4 cells per well of a 96-well plate and viable cells were detected at indicated time points through adding 10 μ l 5 mg/ml MTT per well, 3 hrs incubation at 37^oC. Then, medium containing MTT was replaced by 100 μ l dimethyl sulfoxide (DMSO) (Sigma), followed by measuring the absorbance (490 nm) after 50 mins inoculation. The effects of shRNA vectors on host cell viability were determined by MTT assay.

Western blot assay

Cells were washed three times with PBS and lysed in Laemmli buffer (4% (W/V) SDS, 20% glycerol, 120 mM Tris-Cl (PH 6.8), and 0.002% (W/V) bromophenol blue) containing 10% dithiothreitol (DTT, Thermo Fisher Scientific), following by heating for 5 mins at 95 ^oC. Proteins in the lysates were separated by SDS-PAGE. Subsequently, they were transferred onto PVDF membrane (Immobilon-FL), and then probed with the indicated antibodies including eIF4A (1:1000, rabbit polyclonal; cell signaling), eIF4E (1:1000, rabbit polyclonal; cell signaling), eIF4G (1:1000, rabbit polyclonal; cell signaling), PDCD4 (1:1000, rabbit polyclonal; cell signaling) and SA11 rotavirus VP4 (1:1000, HS-2, mouse monoclonal; provided by professor Harry Greenberg, Stanford University School of Medicine, USA). β -actin was used as a loading control (1:1000, mouse monoclonal; Santa Cruz).

Statistics

All numerical results are reported as Mean \pm SEM. The statistical significance of differences between means was assessed with the Mann-Whitney test (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA). The statistical significance was defined as $P \leq 0.05$.

Results

eIF4A inhibits rotavirus infection

We first examined the effects of rotavirus infection on the expression of the components of the eIF4F complex, including eIF4A and eIF4E at various time points (1, 2, 3, 4, 6, 24 and 48 hr) (Figure 1).

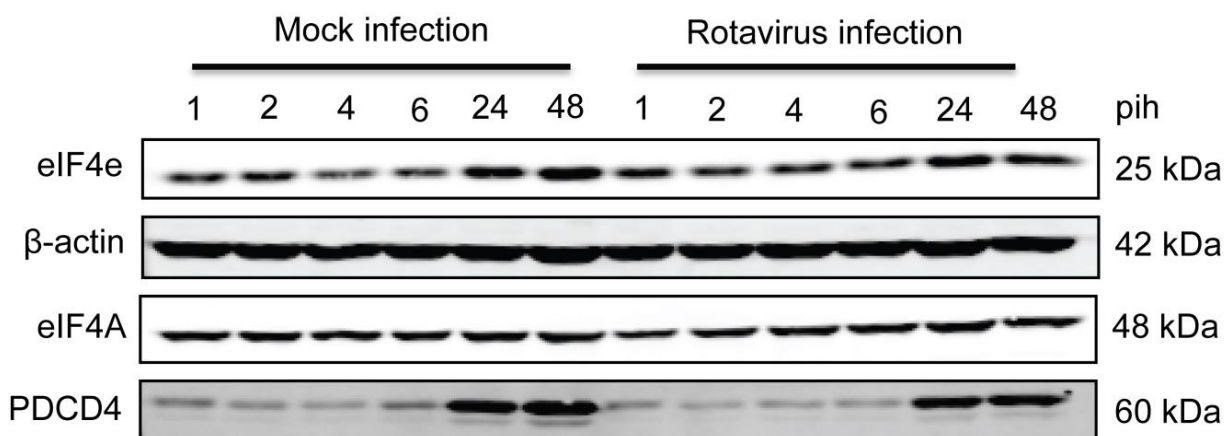


Figure 1. The effect of rotavirus and mock infection (1, 2, 4, 6, 24 and 48 hr) on the expression level of eIF4E, eIF4A and PDCD4. Western blot assay detected the expression of eIF4E, eIF4A and PDCD4 after 1, 2, 4, 6, 24 and 48 hrs post-infection by rotavirus and mock infection in Caco2 cells.

Although rotavirus infection did not affect the expression level of eIF4F complex (Figure 1), it is interesting to investigate whether the subunits of the eIF4F complex can regulate the course of rotavirus infection. To this aim, we first detected the effects of eIF4A, by performing lentiviral RNAi-mediated loss-of-function assay to silence eIF4A gene. All five shRNA vectors showed successful knockdown (Figure 3A and 3B). Importantly, two shRNA vectors (No 1 and 2) resulted in 121.7 ± 60.6 ($n = 6$, $P < 0.01$) and 56.7 ± 29.9 ($n = 6$, $P < 0.01$) fold increase of SA11 rotavirus viral

RNA, respectively (Figure 3C). All shRNA vectors showed no clear cytotoxicity determined by MTT assay (Figure 2A). Western blot assay indicated that all five shRNA vectors promoted rotavirus VP4 protein synthesis (Figure 3D). To further verify, we used crispr/cas9 to knockdown eIF4A gene in Caco2 cells (Figure 3E). After knockdown, viral RNA and protein synthesis were decreased (Figure 3F, G).

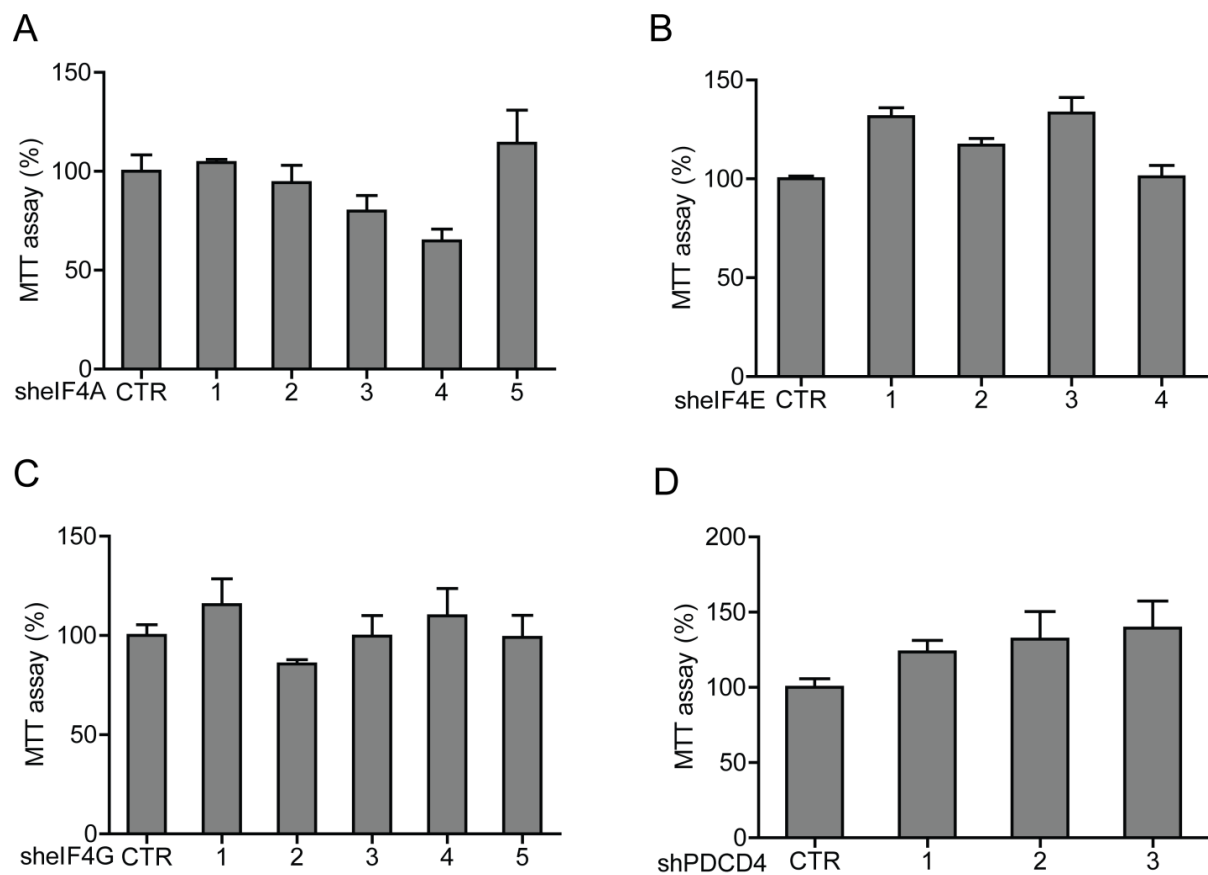


Figure 2. Effect of shRNA against eIF4A (A), eIF4E (B), eIF4G (C) and PDCD4 (D) on host cell viability determined by MTT assays.

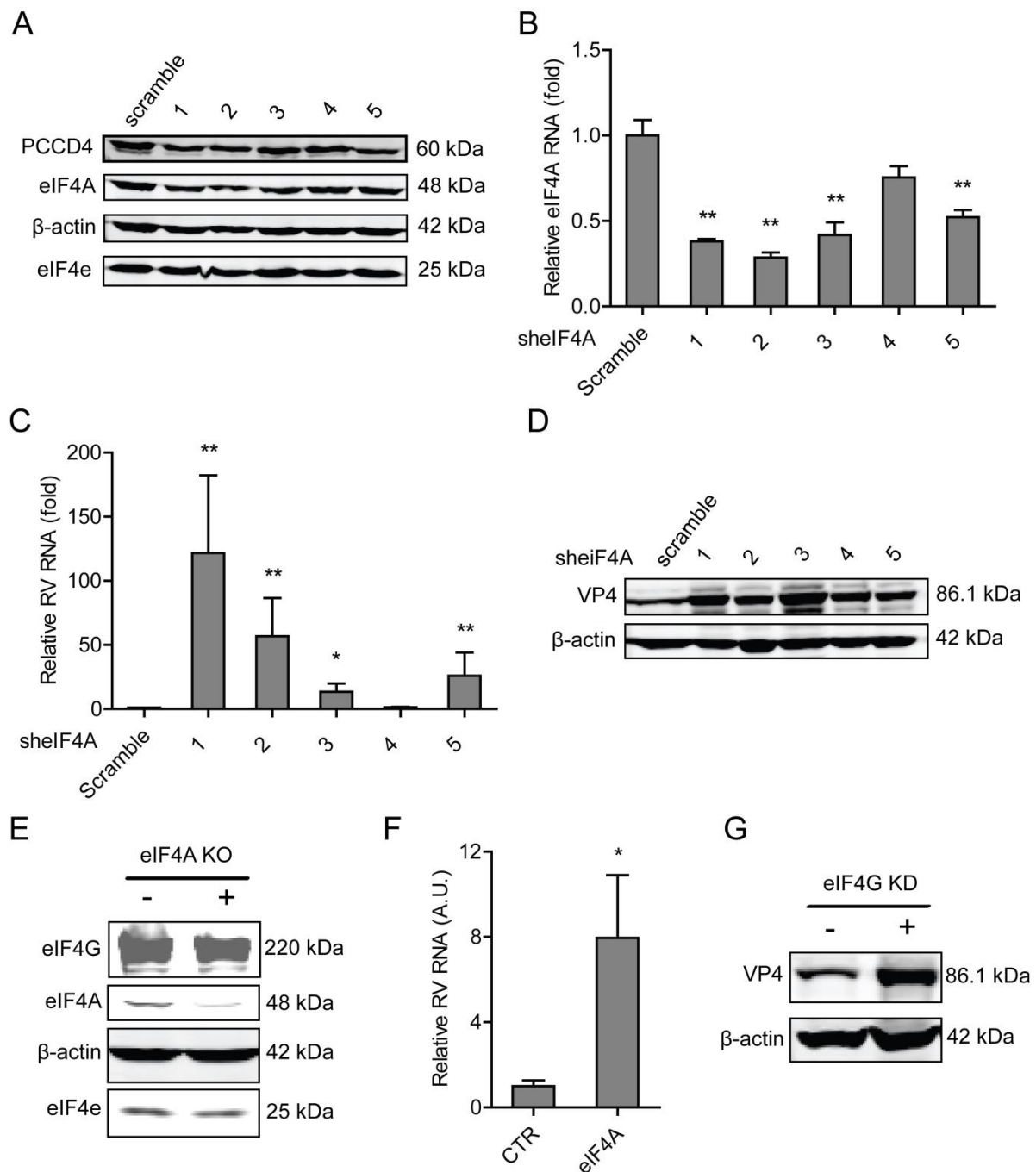


Figure 3. Knockdown of eIF4A supports rotavirus infection. (A) Western blot assay detected eIF4A in Caco2 cells transduced with lentiviral RNAi vectors against eIF4A. (B) Successful knockdown by shRNA vectors measured by qRT-PCR ($n = 6$, $**P < 0.01$, Mann-Whitney test). (C) Silence of eIF4A increased rotavirus genomic RNA ($n = 6$, $*P < 0.05$, $**P < 0.01$, Mann-Whitney test). (D) Silence of eIF4A increased viral VP4 protein in SA11 rotavirus infected Caco2 cells. (E) Western blot assay detected eIF4A in Caco2 cells transduced with CRISPRs against eIF4A. (F) Silencing of eIF4A by crispr/cas9 increased rotavirus genomic RNA ($n = 6$, $*P < 0.05$, Mann-Whitney test). (G) Silence of eIF4A by crispr/cas9 increased viral VP4 protein in SA11 rotavirus infected Caco2 cells.

Blocking eIF4E promotes rotavirus infection

Another member of the eIF4F complex named eIF4E is responsible for binding to the cap of mRNA. Similarly, a lentiviral RNAi-mediated loss-of-function assay was performed to silence eIF4E gene to detect its effects on rotavirus infection. One out of the four (No 8723) shRNAs exerted potent silence of eIF4E gene (Figure 4A). Consistently, the No 8723 shRNA vector resulted in 14.5 ± 5.8 ($n = 6$, $P < 0.01$) fold increase of SA11 rotavirus viral RNA (Figure 4B). All shRNA vectors showed no clear cytotoxicity detected by MTT assay (Figure 2B). The No 8723 shRNA vector resulted in the increased of SA11 rotavirus viral protein VP4 synthesis (Figure 3C). To further verify, we used crispr/cas9 to knockdown eIF4E gene in Caco2 cells (Figure 4D). After knockdown, viral RNA and protein synthesis were decreased (Figure 3E, F).

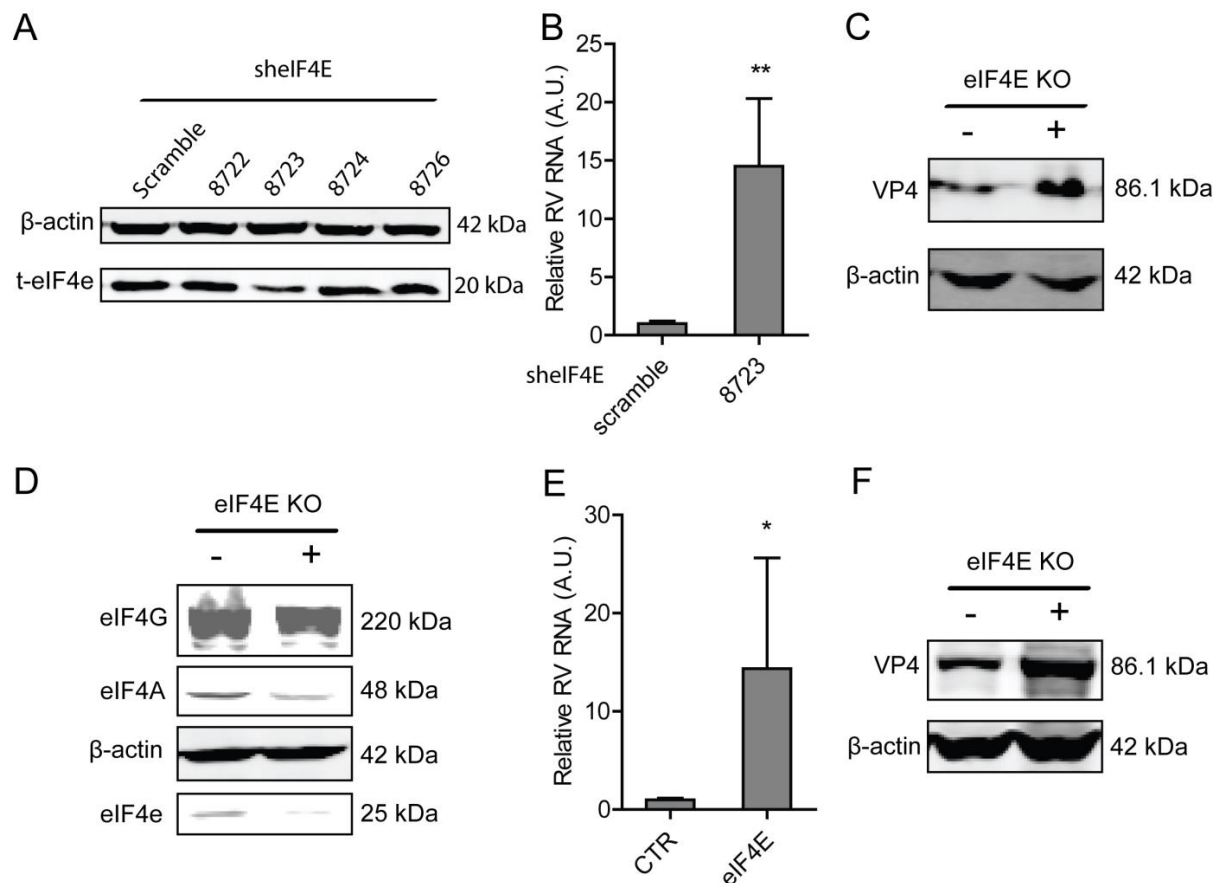


Figure 4. eIF4E suppresses rotavirus infection. (A) Western blot assay detected eIF4E in Caco2 cells transduced lentiviral RNAi vectors against eIF4E. (B) One (No 8723) out of three shRNA vectors showed potent knockdown, and the vector significantly promoted rotavirus genomic RNA ($n = 6$, $*P < 0.05$, Mann-Whitney test). (C) Western blot assay confirmed eIF4E knockdown increased rotavirus protein synthesis. (D) eIF4E was knockdown by crispr/cas9 technique. (E) Silence of eIF4E by crispr/cas9 increase rotavirus RNA ($n = 6$, $*P < 0.05$, Mann-Whitney test). (F) Silence of eIF4E by crispr/cas9 increase rotavirus protein VP4 synthesis.

eIF4G suppresses rotavirus infection

As a scaffolding protein, eIF4G protein is able to physically link the mRNA and the small ribosomal subunit via several protein-protein interactions. To investigate the effects of eIF4G protein on rotavirus infection, a lentiviral RNAi-mediated loss-of-function assay was performed, which resulted in the significant silence of eIF4G by all five vectors (Figure 5A and 5B). Importantly, all these shRNA vectors resulted in potent increase of viral RNA and viral protein synthesis (Figure 5C and 5D). The vitality of cells was not altered by these shRNA vectors (Figure 2C). To further verify, we used crispr/cas9 to knockdown eIF4G gene in Caco2 cells (Figure 5E). After knockdown, viral RNA and protein synthesis were decreased (Figure 5F, G).

Silence eIF4A, eIF4E, and eIF4G together promotes rotavirus infection

To further verify the effect of eIF4A, eIF4E, and eIF4G on rotavirus infection. CRIPRs against eIF4A, eIF4E, and eIF4G were co-transfected in Caco2 cells. After puromycin selection, the knockdown of eIF4A, eIF4E, and eIF4G was detected by western blot, indicating a successful knockdown (Figure 6A). The effect of silence of eIF4A, eIF4E, and eIF4G on rotavirus infection was further detected by qPCR assay and western assay, indicating both increased viral genomic RNA and viral protein VP4 synthesis (Figure 6B, C).

Programmed cell death 4 (PDCD4) inhibits rotavirus infection

PDCD4 functions inhibition of translation by directly interacting with and inhibiting the helicase activity of eIF4A to competitively bind to the scaffold protein eIF4G. To further verify the effects of eIF4F complex, the effects of PDCD4 on rotavirus infection were investigated. To this aim, a lentiviral RNAi-mediated loss-of-function assay was performed to silence PDCD4 gene, which indicated that one out of three shRNA vectors (No 1) exerted potent knockdown (Figure 7A and 7B). Consistently, the shRNA vector (No 1) resulted in $50 \pm 12 \%$ (n= 8, P< 0.05) reduction of SA11 rotavirus viral RNA (Figure 7C), and remarkable reduction of viral VP4 protein synthesis (Figure 7D). All shRNA vectors have no clear cytotoxicity (Figure 2D).

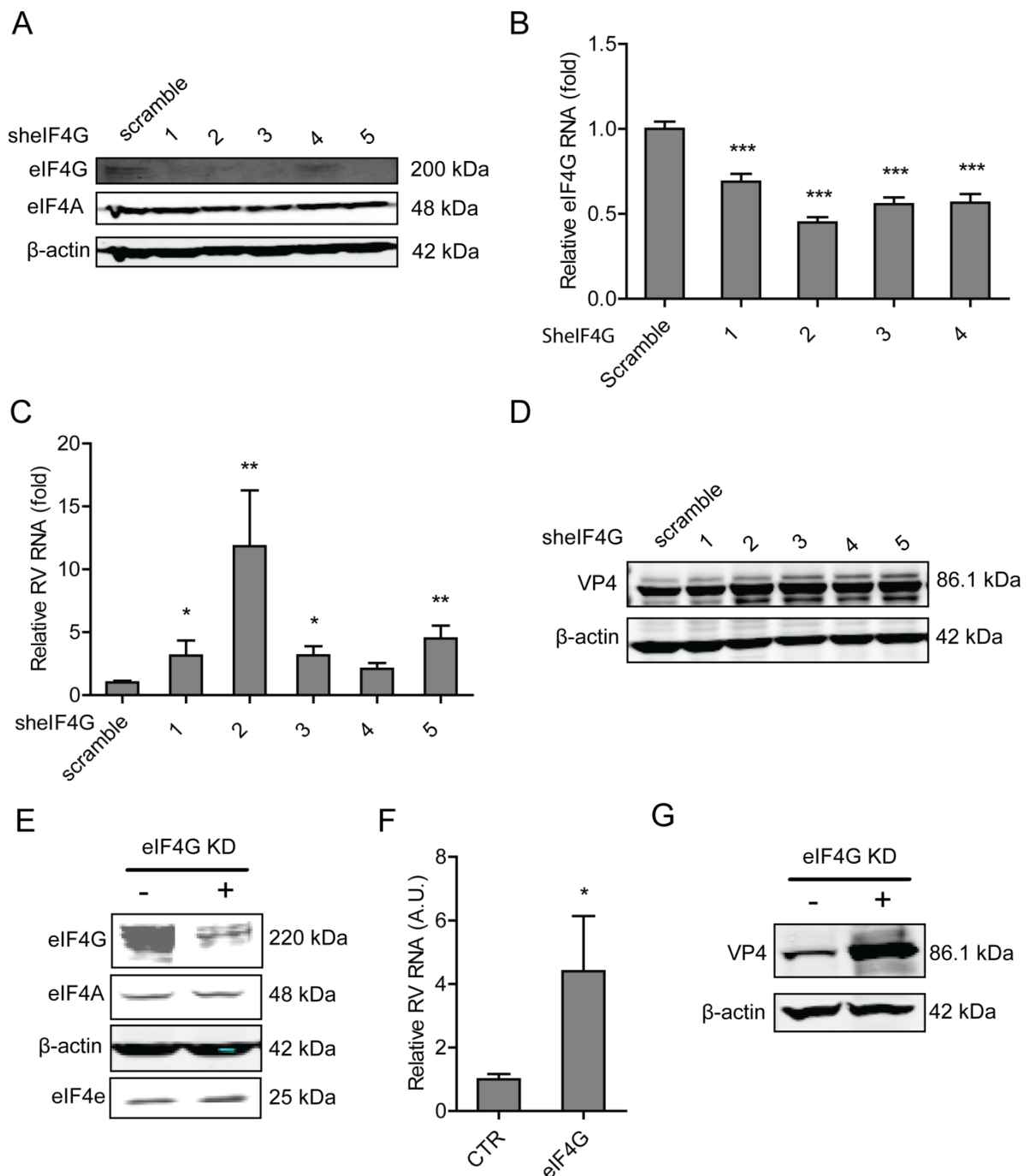


Figure 5. Silence of eIF4G promotes rotavirus infection. (A) Western blot assay detected eIF4G in Caco2 cells transduced lentiviral RNAi vectors against eIF4G. (B) Successful knockdown by shRNA vectors measured by qRT-PCR ($n = 6$, $**P < 0.01$, Mann-Whitney test). (C) Silence of eIF4G showed increased rotavirus genomic RNA ($n = 6$, $*P < 0.05$, $**P < 0.01$, Mann-Whitney test). (D) Silence of eIF4G promoted viral VP4 protein in SA11 rotavirus infected Caco2 cells. (E) Western blot assay detected eIF4G in Caco2 cells transduced with CRISPRs against eIF4G. (F) Silencing of eIF4A by crispr/cas9 increased rotavirus genomic RNA ($n = 6$, $*P < 0.05$, Mann-Whitney test). (G) Silence of eIF4G by crispr/cas9 increased viral VP4 protein in SA11 rotavirus infected Caco2 cells.

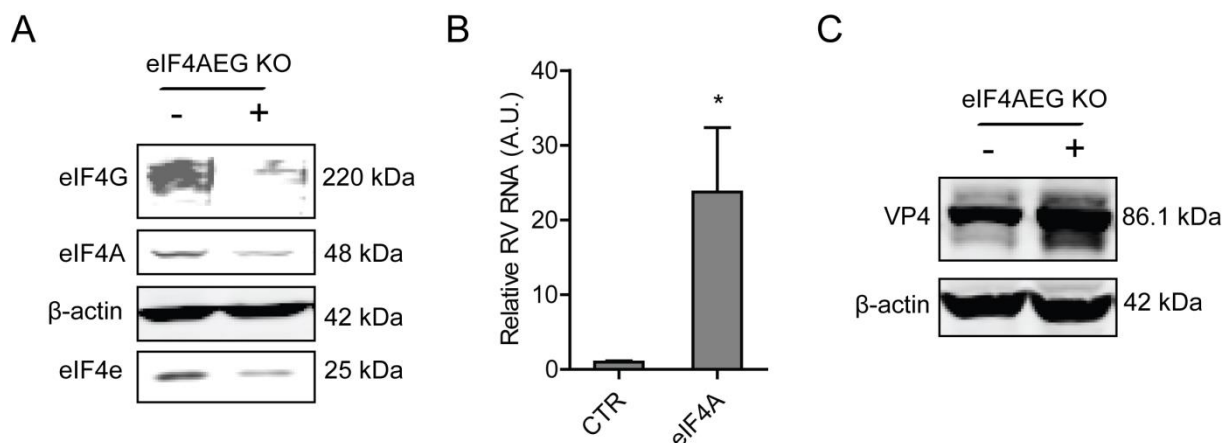


Figure 6. Silencing of eIF4A/E/G promotes rotavirus infection. (A) Western blot assay detected eIF4 A/E/G in Caco2 cells transduced CRIPRs against eIF4A, eIF4E and eIF4G. (B) Silencing of eIF4A/E/G promoted rotavirus genomic RNA detected by qPCR assay ($n = 6$, $**P < 0.01$, Mann-Whitney test). (C) Silencing of eIF4A/E/G showed increased rotavirus protein VP4.

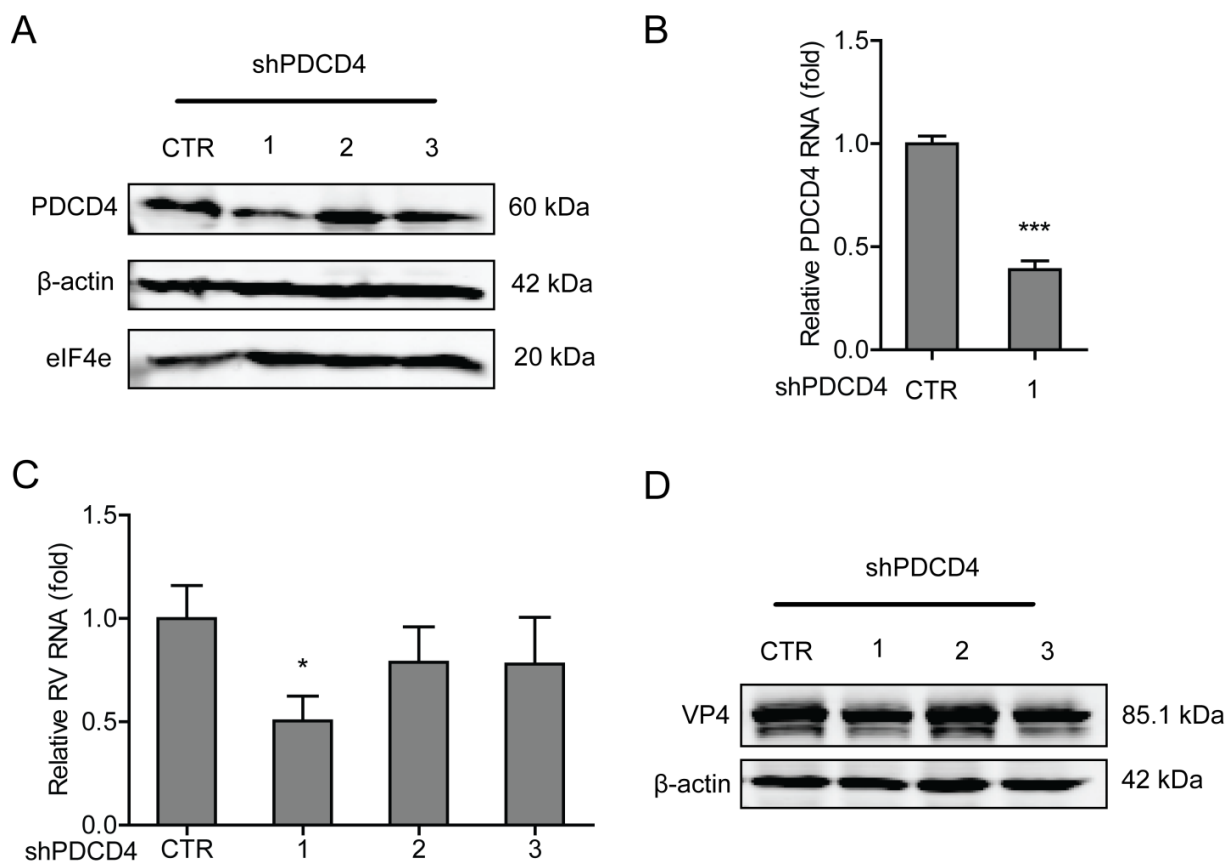


Figure 7. PDCD4 sustains rotavirus infection. (A) Western blot assay detected PDCD4 in Caco2 cells transduced lentiviral RNAi vectors against PDCD4. (B) One (No 1) out of three shRNA vectors showed successful knockdown detected by qRT-PCR ($n = 6$, $**P < 0.01$, Mann-Whitney test). (C) Silencing of PDCD4 showed inhibition of rotavirus genomic RNA ($n = 8$, $*P < 0.05$, Mann-Whitney test). (D) Silencing of PDCD4 inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells.

Discussion

Many viral RNAs have acquired a variety of sophisticated mechanisms to compete with cellular mRNA that are already present in the cytoplasm. This allows the selective translation of viral mRNAs, since they do not possess the required components to initiate mRNA translation (8, 17). The eIF4F initiation factor complex plays a vital role in initiating translation by recognizing the 5' cap structure in a cap-dependent manner (8). As the target of numerous cellular signaling pathways, eIF4F closely monitors translation in response to a multitude of environmental conditions including viral infection (18). In contrast, viral infections often cause dysregulation of eIF4F expression to benefit its replication. Vesicular stomatitis virus (VSV) was reported to cause modifications of the eIF4F complex to inhibit host protein synthesis (19). Human Cytomegalovirus (HCMV) replication was demonstrated to increase the overall abundance of the eIF4F components and promote assembly of the eIF4F complexes (20). The cellular cap-dependent protein synthesis was reported to be rapidly inhibited by foot-and-mouth disease virus (FMDV) via inducing the cleavage of eIF4G (21). But we did not find altered expression level of eIF4F upon rotavirus infection (Figure 1).

eIF4F complex is a tripartite complex composed of three components including a cap-binding subunit (eIF4E), an RNA helicase (eIF4A) anchored to a large molecular scaffold (eIF4G) being able to associate with eIF3-bound 40S ribosome subunits (18). These three components of the eIF4F complex were confirmed to closely regulate the life cycle of various viruses. A functional eIF4A factor was confirmed to fully determine the translation of influenza virus mRNAs (22). Hepatitis E virus (HEV) requires all three subunits of eIF4F to replicate in the host (23). The protein synthesis of Junin virus (JUNV), Tacaribe virus (TCRV) and Pichinde virus (PICV) requires the participation of eIF4A and eIF4G but not eIF4E (24). The eIF4F complex is required for the translation efficiency of HCMV at the start of infection; while viral protein synthesis becomes increasingly insensitive to inhibition of eIF4F complex at late stage of virus infection (25). Rotavirus mRNAs are capped but not polyadenylated (26), and we find that blocking three components of eIF4F complex promotes rotavirus infection (Figure 3, 4, 5, and 6). PDCD4 is capable of directly interacting with and inhibiting the helicase activity of eIF4A by competing its binding to eIF4G to suppress activity of eIF4F complex (27). Consistently, we find that blocking PDCD4

can inhibit rotavirus infection (Figure 7). Our findings suggest that eIF4F complex exerts inhibitory effects on rotavirus infection instead of being required for rotavirus infection.

In summary, this study has demonstrated that rotavirus infection does not affect the expression level of the eIF4F complex; whereas the three components of the eIF4F complex can remarkably inhibit rotavirus infection. The negative regulator of the eIF4F complex, PDCD4, inhibits rotavirus infection. Hereto, this study revealed new insight in rotavirus-host interactions, and the eIF4F complex may represent as important targets for the development of new antivirals against rotavirus infection.

Acknowledgements

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CHAPTER 8

Inhibition of calcineurin or IMPDH exerts moderate to potent antiviral activity against norovirus replication

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Under Review

Abstract

Objective Norovirus is a major cause of acute gastroenteritis worldwide and has emerged as an important issue of chronic infection in transplantation patients. Since no approved antiviral is available, we evaluated the effects of different immunosuppressants and ribavirin on norovirus and explored their mechanism-of-actions.

Design Immunosuppressants and ribavirin were profiled in a human norovirus (HuNV) replicon-harboring model and a surrogate murine norovirus 1 (MNV-1) model. Roles of corresponding drug targets were investigated by gain- or loss-of-function approaches.

Results Calcineurin inhibitors cyclosporin A (CsA) and tacrolimus (FK506) moderately inhibited HuNV replication. Gene silencing of their cellular targets, cyclophilin A, FKBP12 and calcineurin, significantly inhibited HuNV replication. A therapeutically-speaking low concentration of mycophenolic acid (MPA), an uncompetitive inosine monophosphate dehydrogenase (IMPDH) inhibitor, potently and rapidly inhibited norovirus replication and ultimately cleared norovirus without inducible MPA resistance following long-term drug exposure. Knockdown of the MPA cellular targets, IMPDH1 and IMPDH2, suppressed HuNV replication. Consistent with the nucleotide synthesizing function of IMPDH, exogenous guanosine counteracted the anti-norovirus effects of MPA. Furthermore, the competitive IMPDH inhibitor, ribavirin efficiently inhibited norovirus and resulted in an additive effect when combined with immunosuppressants.

Conclusions We demonstrate that calcineurin phosphatase activity and IMPDH guanine synthase activity are crucial in sustaining norovirus infection, thus could be therapeutically targeted. Our results suggest that MPA shall be preferentially considered as immunosuppressive medication for transplantation patients at risk of norovirus infection; whereas ribavirin represents as a potential antiviral for both immunocompromised and immunocompetent patients with norovirus gastroenteritis.

Introduction

Following wide-spread implementation of rotavirus vaccination, norovirus infection is now becoming the major cause of acute gastroenteritis worldwide (1). As a single-strand positive RNA virus belonging to the family *Caliciviridae*, norovirus is classified into 6 distinct genogroups (GI-VI). Genogroup 2, genotype 4 (GII.4) is the most prevalent, accounting for 96% of all sporadic infections (2). Although norovirus infection is often self-limiting in adults, it results in up to 200,000 deaths in children below five years of age, mainly in developing countries (3).

Accumulating evidence indicates that transplant recipients are highly susceptible to norovirus infection, irrespective of their status as a pediatric or adult patient (4, 5). Norovirus infection has been described in many types of transplant recipients, including those receiving orthotopic transplantation of lung (6), kidney (7-9), liver (10), heart (11, 12), renal (13-15), intestinal (16-18), small bowel (19) or of hematopoietic stem cells (8, 20-23). Infection with norovirus in such patients often results in severe clinical pathology with prolonged illness (20, 24). Although the exact mechanism of norovirus susceptibility in orthotopic organ transplantation recipients remains unclear, the use of immunosuppressants for preventing organ rejection is conceivably an important risk factor (4, 25). It is plausible that an immunosuppressed status weakens host immunity defending virus invasion. Therefore, a cautious reduction and withdrawal of immunosuppressants has been taken into consideration for managing chronic norovirus gastroenteritis in transplant recipients (26). Intriguingly, immunosuppressants have been reported to directly modulate viral infection (27), but the direct effects on norovirus infection remain to be investigated.

Despite its significant impact on public health, no vaccination or specific antiviral treatment is available. Ribavirin has broad antiviral activities against many viruses *in vitro*, and has been approved for treating chronic hepatitis C virus (HCV) patients for decades. A recent study has described eight patients with common variable immunodeficiency (CVID) showing persistent norovirus infection. Viral clearance occurred in two patients with oral ribavirin therapy, leading to complete symptomatic and histological recovery (28), although the definitive effect of ribavirin on norovirus requires further evaluation.

In this study, we profiled the effects of different immunosuppressants on norovirus in cell culture models. We reveal that cellular calcineurin and IMPDH enzymes are

essential factors supporting norovirus replication, which could be targeted by corresponding immunosuppressants, calcineurin inhibitors and mycophenolic acid (MPA), to exert anti-norovirus effects. Furthermore, we demonstrate that ribavirin, a general antiviral drug of a non-immunosuppressive IMPDH inhibitor, has potent anti-norovirus effect and exerts additive inhibition of norovirus replication when combined with calcineurin inhibitors or MPA. These results provide an important reference for managing immunosuppression and antiviral treatment for transplant patients infected by norovirus or at risk of norovirus infection.

Materials and methods

Compounds

The immunosuppressants, including dexamethasone (DEX), prednisolone (PRED), rapamycin (RAP), leflunomide (LEF), brequinar (BQR) sodium salt hydrate and MPA were purchased from Sigma-Aldrich (St Louis, MO). Calcineurin inhibitors (CNIs), cyclosporin A (CsA) and tacrolimus (FK506) were obtained from Bio-Connect (Huizen, The Netherlands) and Abcam (Cambridge, MA), respectively. Two non-immunosuppressive cyclosporine A derivatives 431-32 and 440-02, together with a novel calcineurin inhibitor voclosporin (VCS), were provided by Isotechnika Pharma Incorporation (Edmonton, AB, Canada). All the compounds were dissolved in DMSO and stored in aliquots at -20°C before use. Cytotoxicity of the compounds on host cells were determined by MTT assay (supplementary figure 1).

Cell cultures and virus propagation

HG23 (Huh7 cells containing a stable subgenomic HuNV replicon), RAW 264.7 and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT, USA). A marker cassette containing the neomycin phosphotransferase gene was inserted between nts 5456 and 6753 of the ORF2 of HuNV, conferring HG23 resistance to neomycin. Gentamicin (G418; Gibco) was added to HG23 culture medium at 1.5 mg/ml for selection before experimentation.

The Murine norovirus 1 (MNV-1) was produced by consecutively inoculating MNV (kindly provided by Herbert Virgin IV, Department of pathology and immunology, Washington University School of Medicine) into RAW cells. After 4 consecutive passages, the P4 MNV cultures were purified, aliquoted and stored at -80°C for all subsequent experiments. The MNV stock was quantified by the 50% tissue culture infective dose (TCID₅₀) and copy number analysis (supplementary material; supplementary figure 2). P4 stocks were quantified three independent times prior to use.

Long-term treatment and rebound assay

HG23 cells were seeded into 48-well plates at 2.5×10^4 cells/well containing drugs but no G418. After 2 days treatment, the cells were trypsinized, (i) 2.5×10^4 cells were sub-cultured into 48-well plates containing the same concentrations of drugs for another 4 days of treatment; (ii) remaining cells were collected for qRT-PCR analysis. After 6 days treatment, the same treatment process was performed for the second time. After 10 days treatment, 2.5×10^4 cells were sub-cultured into 48-well plate with fresh medium containing G418 (1 mg/ml). With further 5 days of routine culture, the cell layers were stained with hematoxylin.

***In vitro* selection of MPA-resistant MNV**

MPA-resistant MNV was selected by culturing MNV for 20 passages under antiviral pressure. Briefly, MNV-infected RAW cells were treated with either medium or MPA (0.1 or 0.5 µg/ml). After 24 h incubation, the MNV cultures were purified and titrated by qRT-PCR for use in the next passage. After 10 passages, (i) MNV was continuously exposed to the same MPA concentrations for another 10 passages; (ii) MNV were exposed to increased MPA concentrations for another 10 passages. When MNV serial passaging with MPA was done, the inhibitory effect of MPA on MNV was quantified by qRT-PCR.

Statistics

Data are presented as Mean \pm SEM. Comparisons between groups were performed with Mann-Whitney test using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at a p value less than 0.05.

Results

Not all immunosuppressants directly affect norovirus replication

A major challenge in anti-norovirus drug development is the lack of an infectious human isolate that recapitulates the entire life-cycle of the virus in a cell culture model. Therefore, we used a HuNV subgenomic replicon model that closely mimicked viral replication in the absence of the production of infectious particles (29). In our study, we observe that glucocorticoids, including PRED (supplementary figure 3A) and DEX (supplementary figure 3B), rapamycin (supplementary figure 3C), brequinar (supplementary figure 3D) and leflunomide (supplementary figure 3E) show no effect on HuNV replication, demonstrating that modulation of norovirus infection is not a general property of immunosuppressants.

CsA and its immunosuppressive analogue voclosporin, inhibit HuNV replication through a CyPA-dependent mechanism

CsA is a calcineurin inhibitor targeting cellular cyclophilins. Treatment with CsA (5 μ g/ml) for 48 h inhibits HuNV replication by $82 \pm 3.3\%$ ($n=6$, $p<0.01$; figure 1A). To further confirm that this inhibitory effect relates to the compound targeting cyclophilins, a series of CsA derivatives were tested. Voclosporin, a novel immunosuppressive cyclophilin inhibitor, diminishes cellular HuNV RNA level by $49 \pm 10\%$ ($n=6$, $p<0.05$; figure 1B) after 48 h treatment, even at low concentration of 1 μ g/ml; whereas the parental compound CsA at this concentration has no effect on HuNV. This observation relates well with the notion that voclosporin is a more potent calcineurin inhibitor as compared to CsA (30). Two non-immunosuppressive CsA derivatives, 431-32 and 440-02 (figure 1C), which are chemically similar to CsA, if functionally distinct, have no effect on HuNV replication, suggesting that effects of CsA analogues on norovirus replication relate to calcineurin inhibition.

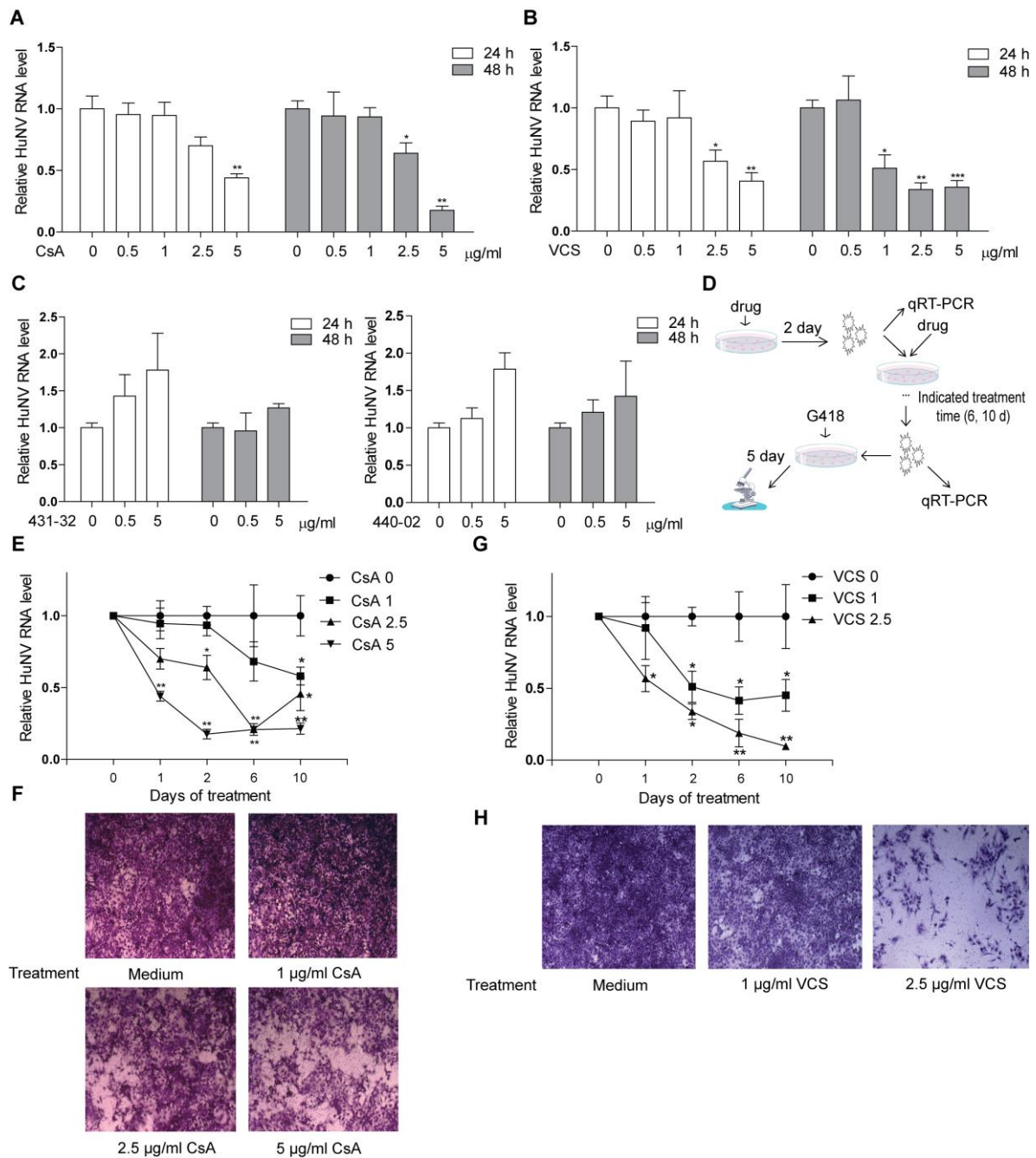


Figure 1. CsA and voclosporin suppress HuNV replication. CsA (A) and its immunosuppressive analogue voclosporin (B) inhibit HuNV replication in a time- and dose-dependent manner (n=6). Non-immunosuppressive cyclosporine derivatives 431-32 and 440-02 at high concentration fail to inhibit HuNV replication (n=4; C). (D) Design of long-term treatment and rebound assay. During clearance phase, HG23 cells were treated with immunosuppressants for 10 days without G418. During rebound phase, cells were subcultured in fresh medium with G418 (1 mg/ml) for 5 days. Pictures were taken under light microscope. (E) A moderate reduction of HuNV RNA over 10 days of treatment with CsA (n=6). (F) Rebound assay after long-term treatment with CsA. The graphs show the confluence of the cell monolayers after 5 days of culture with G418. (G) Voclosporin causes a drastic reduction of HuNV RNA level after long-term treatment (n=6). (H) Rebound assay after long-term treatment with voclosporin.

It is necessary to determine whether long-term exposure to these immunosuppressants completely eliminates HuNV replicons from host cells. For this purpose, a long-term treatment and rebound assay (figure 1D) was employed. If the replicons are completely cleared, the cells die in the presence of G418. If the cells still carry some replicons, they will survive and proliferate. CsA at 5 $\mu\text{g/ml}$ causes a reduction of HuNV RNA level by $79 \pm 3.8\%$ ($n=6$, $p<0.01$; figure 1E) after 10 days of treatment. Consistently, CsA-treated cells survive and proliferate in the presence of the selection marker (figure 1F), demonstrating that although CsA inhibits HuNV replication, it does not completely cause elimination of all replicons from cells. Voclosporin at 2.5 $\mu\text{g/ml}$ provokes potent inhibition of HuNV replication, as evident from a reduction of $91 \pm 1.5\%$ ($n=6$, $p<0.01$; figure 1G) in viral RNA. Following subsequent challenge with the selection marker, only a small portion of cells stays alive (figure 1H), showing that voclosporin treatment clears most HuNV replicons from cultures, but a replicon containing compartment remains.

Cellular cyclophilins, in particular the cyclophilin A (CyPA) and cyclophilin B (CyPB) isoforms, are the canonical drug targets of CsA. To study the potential involvement of CyPA and CyPB in CsA inhibition of HuNV replication, a RNAi-mediated loss-of-function assay was performed. Upon transduction of lentiviral vectors targeting CyPA (shCyPA) or CyPB (shCyPB), specific down-regulation of the target genes at mRNA level was observed at both day 6 and 10 post-transduction, when compared to a scrambled control (shCTR) (figure 2A,B). Down-regulation at the protein level was only observed at day 10 (figure 2C). Consistently, following knockdown no effect on HuNV was observed at day 6, but significant reduction of viral replication by $60 \pm 5.7\%$ ($n=8$, $p<0.001$; figure 2D) was observed by knockdown of CyPA, but not that of CyPB, at day 10 post-transduction. Notably, knockdown of CyPA also resulted in elevation of CyPB mRNA expression at day 10 post-transduction and *vice versa*, suggesting that a compensatory mechanism may exist between CyPA and CyPB (figure 2A, B). Concomitant with CyPA knockdown, CsA ($n=5$, $p<0.05$; figure 2E) and voclosporin ($n=5$, $p<0.05$; figure 2F) lost their capacity to inhibit HuNV after 48 h treatment. Collectively, these results show that CsA and voclosporin inhibit HuNV replication through targeting CyPA, but not CyPB.

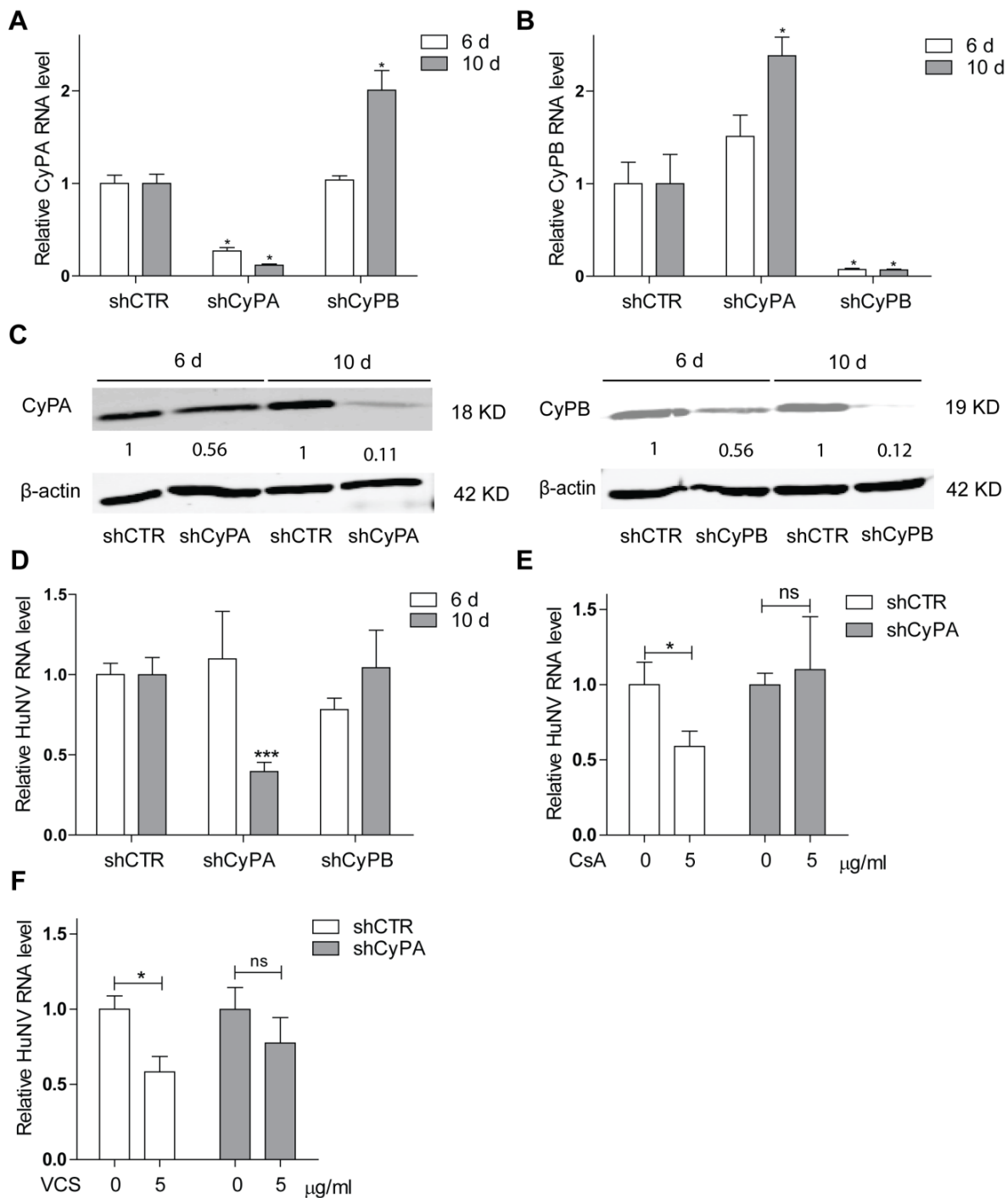


Figure 2. CyPA, but not CyPB, is involved in CsA-caused inhibition of HuNV replication. (A, B) Lentiviral shRNA vectors targeting CyPA, CyPB as well as the non-targeting scrambled vector were transduced into HG23 cells. The mRNA levels of cyclophilins were quantified at day 6 and day 10 post-transduction (n=4). (C) Western blot analysis of HG23 cells transduced with shRNAs. Transduction of CyPA or CyPB shRNAs results in a dramatic reduction in CyPA or CyPB proteins at day 10, but not at day 6 post-transduction. (D) HuNV RNA in replicon cells was determined by qRT-PCR at day 6 and day 10 post-transduction and was normalized to the non-targeting control shRNA. Knockdown of CyPA, but not CyPB results in a significant decrease in viral replication at day 10, but not at day 6 post-transduction (n=8). (E) Knockdown of CyPA abrogated CsA-mediated inhibition of HuNV replication (n=5). (F) Loss-of-function of CyPA abrogated voclosporin-caused inhibition of HuNV replication (n=5).

FK506 moderately inhibits HuNV replication via targeting FKBP12

FK506 (tacrolimus) is another calcineurin inhibitor. FK506 at 2.5 $\mu\text{g/ml}$ causes an reduction of HuNV replication by $77 \pm 5.0\%$ ($n=5$, $p<0.05$) and $70 \pm 8.3\%$ ($n=6$, $p<0.01$) after short-term (2 days; figure 3A) and long-term (10 days; figure 3B) treatment, respectively. Of note, FK506 at high concentrations quickly inhibits HuNV replication being evident from effects even after one day of treatment. Similar to CsA analogues, however, FK506 does not completely clear HuNV replicons from cultures (figure 3C).

FK506 suppresses the immune system by binding to FKBP12, resulting in inhibition of the phosphatase activity of calcineurin. To understand the role of FKBP12 in FK506 inhibition of HuNV, we used five different lentiviral shRNA vectors to knock down the expression of FKBP12. We demonstrate that knockdown of FKBP12 (figure 3D; supplementary figure 4), but not FKBP8 (as a control; supplementary figure 4), results in reduced HuNV replication (figure 3E). At the same time, FKBP12 knockdown partially abrogates FK506 inhibition of HuNV (figure 3F), suggesting that FK506 inhibits HuNV replication through a pathway involving FKBP12. Thus the calcineurin pathway appears essential for norovirus propagation and experiments were initiated to confirm this notion.

Calcineurin activation is indispensable for efficient replication of norovirus

Calcineurin inhibitors are effective against norovirus replication; whereas non-immunosuppressive CsA derivatives are ineffective, suggesting that calcineurin activity is required for CsA and FK506 inhibition of norovirus. To confirm this hypothesis, we used RNAi to knock down PPP3CA for inhibiting calcineurin phosphatase activity. As shown in figure 3G and H, knockdown of PPP3CA potently inhibited HuNV replication. Thus, calcineurin activation is vital for norovirus replication, supporting the idea that CsA and FK506 exert anti-norovirus activity through inhibition of calcineurin.

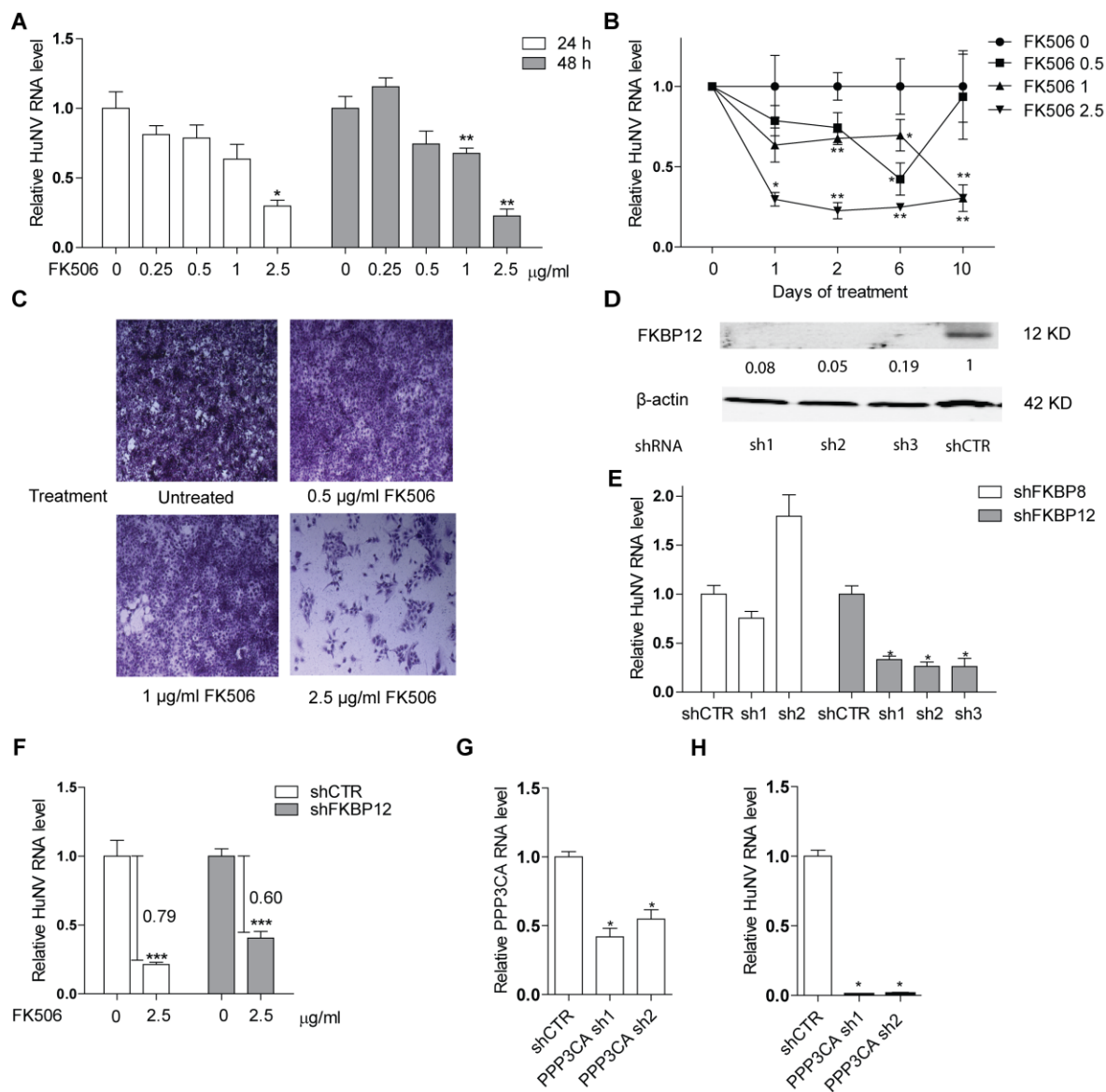


Figure 3. FK506 moderately inhibits HuNV through FKBP12 and calcineurin. (A) Time- and concentration-dependent antiviral effects of FK506 on HuNV replication (n=5-6). (B) Long-term exposure to FK506 results in a moderate reduction of HuNV replication (n=6). (C) Rebound assay after long-term treatment with FK506. A reduced confluence of cell layers was observed after long-term treatment with FK506. (D) Western blot analysis of HG23 cells transduced with shRNA targeting FKBP12 or non-targeting control. (E) qRT-PCR analysis of HuNV RNA level in HG23 cells transduced with lentiviral shRNA vectors shows that knockdown of FKBP12, but not FKBP8, causes a reduction of HuNV replication (n=5). (F) Knockdown of FKBP12 partially diminishes FK506-induced inhibition of HuNV (n=6). (G) Efficiency of calcineurin gene knockdown. The RNA level of calcineurin subunit PPP3CA was determined by qRT-PCR and presented as the relative values to the scrambled control (n=5). (H) qRT-PCR analysis of HuNV RNA level in PPP3CA knockdown cells (n=5).

MPA potently inhibits norovirus replication and completely clears HuNV replicons from host cells

Examining the effect of MPA on HuNV replication, we observe that treatment with only 0.25 µg/ml of MPA, which is even lower than blood concentration (approx. 1-10 µg/ml) in transplantation patients (31), potently inhibits HuNV replication by $79 \pm 2.7\%$ ($n=6$, $p<0.01$; figure 4A) after 48 h treatment. Likewise, following long-term treatment by MPA (0.5 µg/ml; 10 day; figure 4B), the HuNV replication is reduced by $94 \pm 1.7\%$ ($n=6$, $p<0.01$). Correspondingly, in rebound experiment (figure 4C), when challenged with G418, the cells treated by 0.5 µg/ml MPA completely lose the ability to proliferate and succumb to the selection marker, suggesting that MPA treatment totally clears replicons from the host cells. In apparent support, when we tested other 23 IMPDH inhibitors with differential inhibitory efficacy on IMPDH enzymatic activity (supplementary table 2), 17 out of these 23 IMPDH inhibitors exert significant inhibition of HuNV replication at a 1 µM concentration ($n=6$, $p<0.01$; figure 4D).

In face of the lack of a cell culture system for HuNV, we considered MNV as a suitable surrogate for studying HuNV biology and pathogenesis. Consistent with the results in the replicon model, MPA at 0.1 µg/ml reduces MNV cellular viral RNA by $83 \pm 8\%$ ($n=8$, $p<0.001$; figure 4E) and virus production by $91 \pm 6\%$ ($n=6$, $p<0.01$; figure 4F). The inhibitory effect is also confirmed in a CPE inhibition assay, demonstrating that MPA at 0.1 µg/ml protects RAW cells from norovirus-induced CPE formation by $57 \pm 14\%$ ($n=8$, $p<0.01$; figure 4G). Collectively, our results demonstrate that MPA potently inhibits norovirus replication and could even completely clear the virus.

High barrier to resistance development

In the HuNV replicon model, MPA completely clears the viral replicons, which obviously prevents the emergence of resistance and thus leaves to extent to which such resistance can emerge unanswered. To address this issue, MNV-infected RAW cultures were exposed to MPA in different setups for 20 passages. As shown in figure 4H, MNV retains its sensitivity to MPA, even at passage 20, with inhibitory efficacy varying from $98.5 \pm 0.07\%$ to $99.6 \pm 0.07\%$ following treatment with 0.5 µg/ml MPA, which is comparable to unchallenged control ($99.6 \pm 0.05\%$ inhibition). Thus, norovirus is not prone to developing resistance to MPA.

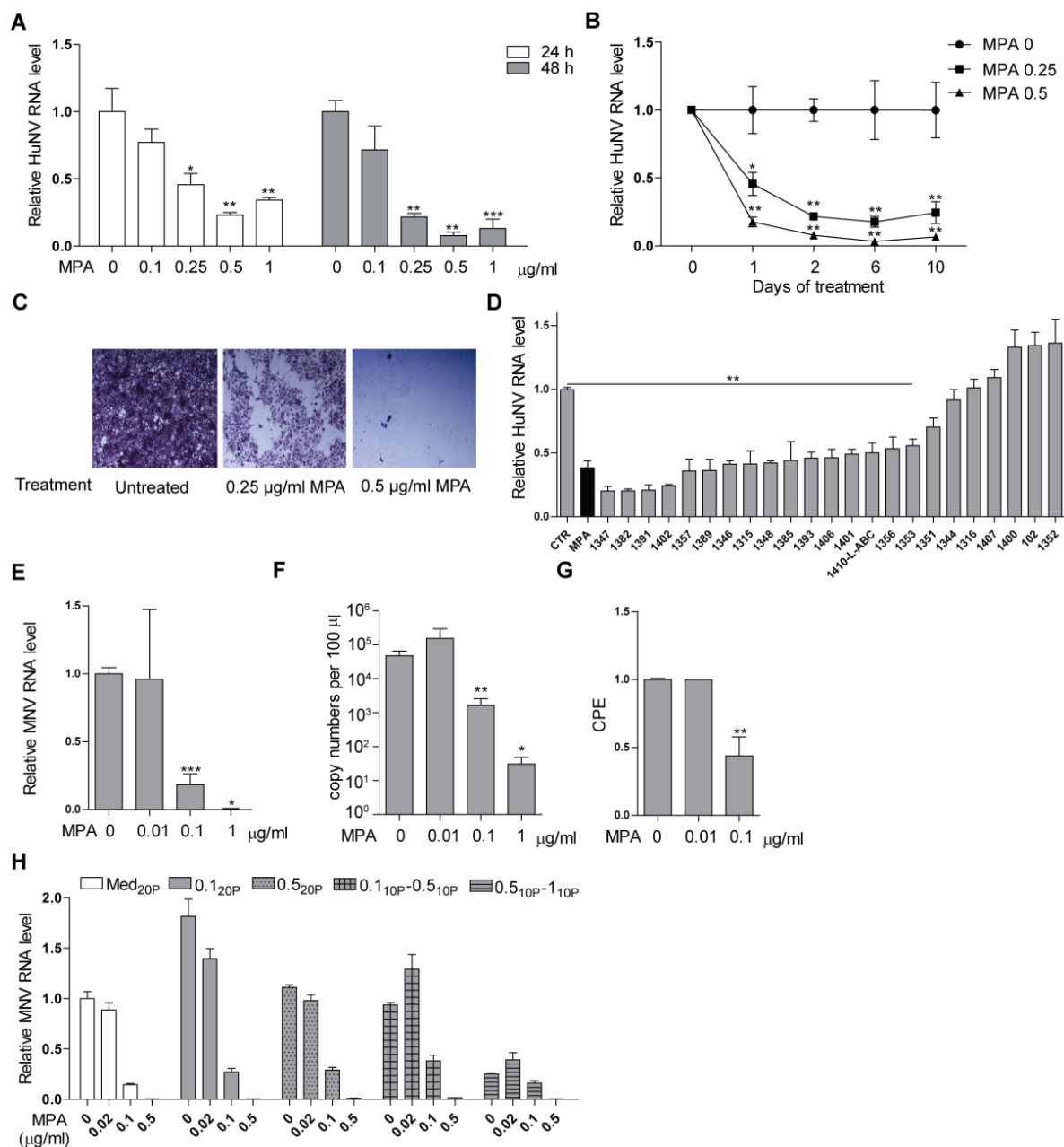


Figure 4. MPA potently inhibits norovirus replication and completely eliminates HuNV replicons from cells without concomitant drug resistance. (A, B) qRT-PCR analysis of HuNV RNA after short-term and long-term treatment with MPA (n=6). (C) MPA completely clears HuNV replicons from cells, and HG23 cells do not survive after treatment with the selection marker of G418. (D) The effects of 23 IMPDH inhibitors on HuNV were determined (n=6). The effects of MPA were validated on a murine norovirus model by quantifying the relative MNV intracellular RNA level (E) and copy numbers of MNV in supernatant (secreted viruses; F) after MPA treatment using qRT-PCR (n=5-6). (G) The inhibitory effect of MPA is also confirmed by a CPE inhibition assay. MNV induces CPE in RAW cells, but MPA protects RAW cells from CPE (n=8). (H) The inhibitory efficacy of MPA on MNV following 20 passages exposed to MPA or vehicle control. In the selection process, MNV was either directly cultured in the presence of a fixed MPA concentration (0.1 or 0.5 µg/ml) or in a lengthy stepwise selection concentration from 0.1 to 0.5 µg/ml or from 0.5 to 1 µg/ml. After selection, the effects of MPA on the 20th passage of MNV were determined by qRT-PCR.

MPA inhibits norovirus replication by simultaneously targeting IMPDH1 and IMPDH2

MPA acts through inhibition of IMPDH. There are two isoforms of IMPDH, IMPDH1 and IMPDH2, in mammals. MPA has a five-fold more potent inhibitory effect on IMPDH2 as compared to IMPDH1 (32). In view of the low concentrations of MPA required to counteract norovirus, IMPDH2 appears the more likely drug target with respect to its effect on the norovirus life cycle. However, knockdown of IMPDH2 has no significant effect on HuNV replication, even though such knockdown profoundly decreases IMPDH2 mRNA and protein level, as assessed by qRT-PCR and western blot (figure 5A, C; supplementary figure 5). Similarly, knockdown of IMPDH1 causes no significant change in HuNV replication (figure 5B, C). Surprisingly, simultaneous knockdown of both IMPDH1 and IMPDH2 suppresses HuNV replication by $64 \pm 5.3\%$ ($n=4$, $p<0.05$; figure 5D, E), showing the IMPDH1 and IMPDH2 interchangeable roles in norovirus life cycle.

Guanosine restored norovirus replication in MPA-treated and IMPDH1/2 knockdown cells

MPA inhibits *de novo* guanosine nucleotide biosynthesis by inhibiting IMPDH. To examine whether MPA exerts inhibitory effect on norovirus via guanosine depletion, we challenged norovirus cultures either with only MPA or with the combination of MPA and guanosine (the latter at 1, 10 or 100 $\mu\text{g/ml}$ concentrations) for 24 h. We observe that the inhibitory effect of MPA on norovirus replication is sensitive to supplementation with guanosine. A 100 $\mu\text{g/ml}$ guanosine concentration completely negates MPA effects in both HuNV replicon model ($n=5$; figure 5F) and MNV model ($n=8$; figure 5G). In apparent agreement exogenous supplementation of guanosine reverses the inhibitory effects of IMPDH1/2 knockdown on HuNV replication ($n=5$; figure 5H). Thus, norovirus biology requires a substantial intracellular guanosine levels and the inhibitory effects of MPA and IMPDH knockdown are mediated by depletion of this guanosine pool.

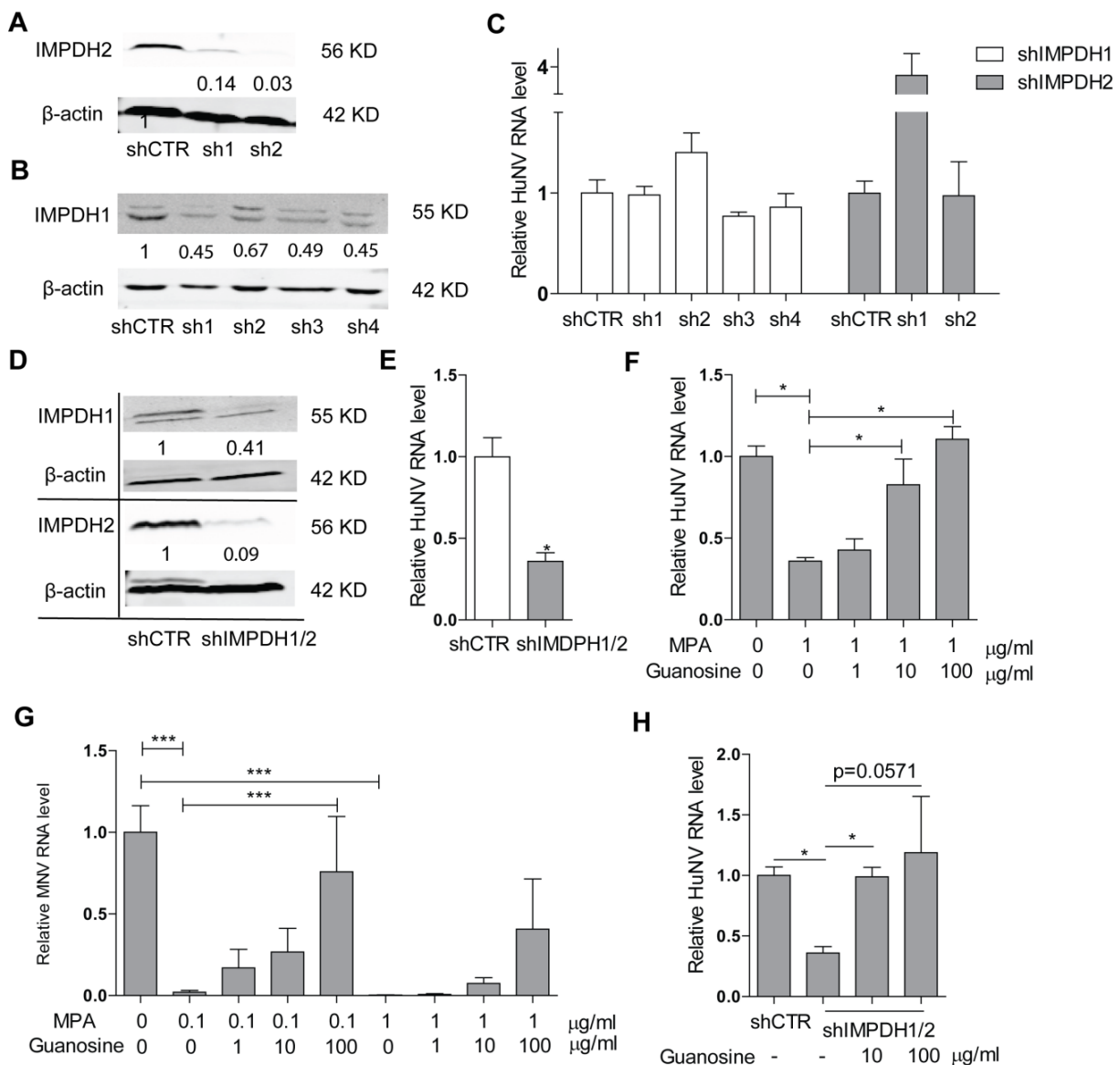


Figure 5. Simultaneous knockdown of IMPDH1/2 reduces HuNV replication, which is reversed by exogenous guanosine. (A, B) Western blot analysis of IMPDH2 and IMPDH1 knockdown. (C) qRT-PCR analysis of HuNV RNA level in knockdown cells. Knockdown of IMPDH1 or IMPDH2 alone has little effect on HuNV replication (n=4). (D) The efficacy of gene knockdown was quantified by western blot. Simultaneous knockdown of IMPDH1/2 decreases HuNV replication (n=4; E). Guanosine restores norovirus replication in MPA-treated and IMPDH1/2 knockdown cells. HuNV replicon (n=5; F) and MNV (n=7; G) cultures were treated with MPA alone or combined with guanosine (1, 10 or 100 μg/ml). After 24 h incubation, relative levels of norovirus RNA were quantified by qRT-PCR. (H) IMPDH1/2 knockdown cells were cultured with medium or increasing concentrations of guanosine. After 24 h incubation, the relative HuNV RNA level was analyzed by qRT-PCR (n=4).

Ribavirin efficiently inhibits norovirus

Ribavirin at 2.5 μg/ml concentration results in a reduction of HuNV replication by $64 \pm 6.1\%$ (n=5, $p < 0.01$; figure 6A) and $61 \pm 8.5\%$ (n=6, $p < 0.01$; figure 6B) after short-

term and long-term treatment, respectively. Additionally, ribavirin at higher concentrations (5 and 10 $\mu\text{g/ml}$) is capable of clearing cells of most replicons following 10 days of consecutive culture (figure 6C). The antiviral activity of ribavirin is confirmed in MNV model. Ribavirin at lower concentration (1 $\mu\text{g/ml}$) effectively decreases MNV cellular RNA (figure 6D) and extracellular viral particles in the supernatant (figure 6E). In the CPE inhibition experiment, treatment with ribavirin for 3 days dose-dependently increases cell viability and decreases CPE formation by inhibiting MNV ($n=8$, $p<0.001$; figure 6F), further validating the antiviral activity of ribavirin on MNV.

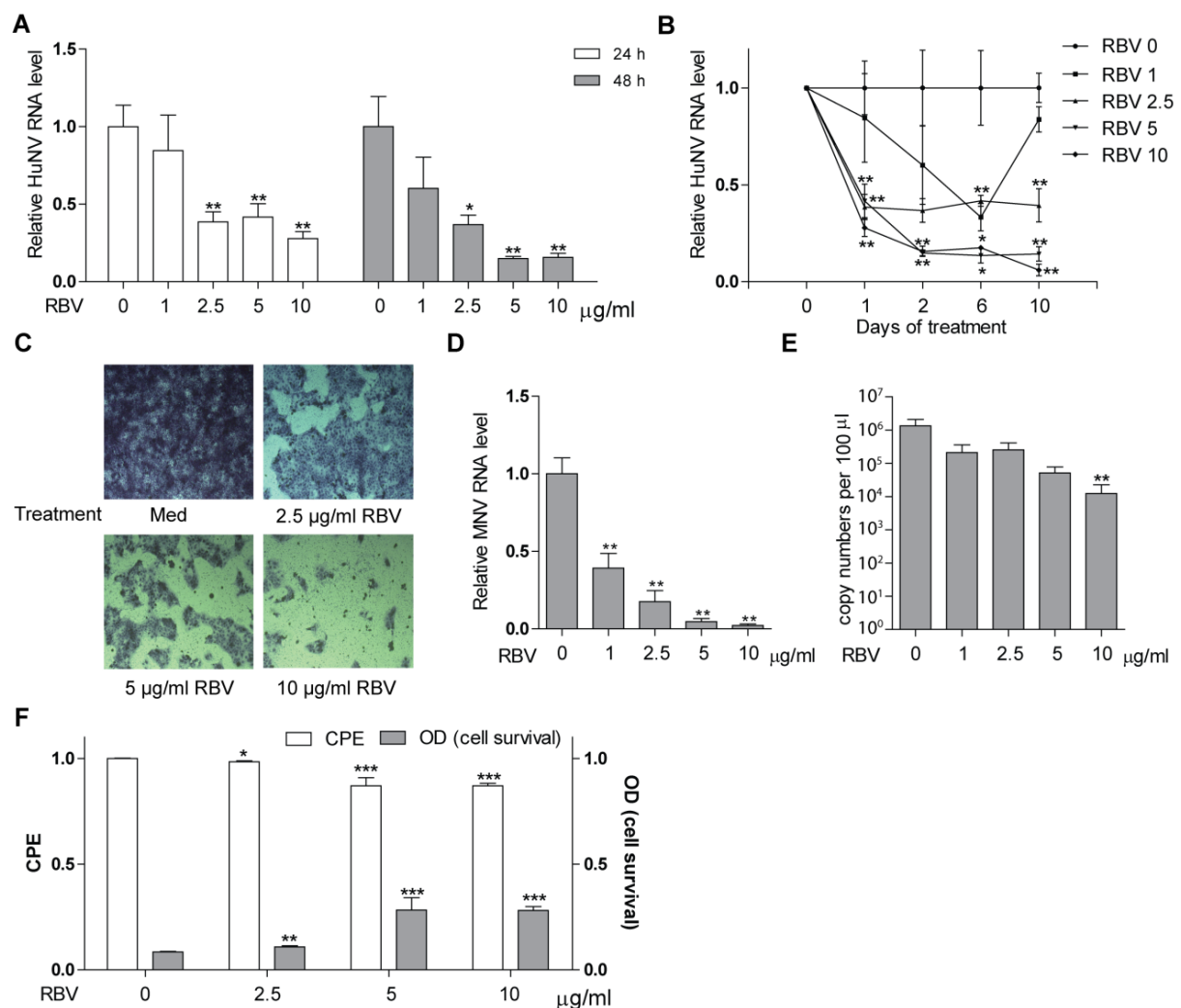


Figure 6. Ribavirin effectively inhibits norovirus replication. Short-term (2 days; A) and long-term (10 days; B) effects of ribavirin on norovirus RNA replication in HuNV model. Results are expressed as the percentage relative to the medium control ($n=6$). (C) In the rebound phase, ribavirin-treated cells were cultured under the selective pressure of G418. The graph shows the cell confluence of 48-well plates after 5 days culture. Effect of ribavirin on

norovirus replication in MNV model was determined by quantifying the intracellular MNV RNA (n=6; D) and extracellular MNV particles (n=6; E) using qRT-PCR after 24 h incubation. (F) The antiviral effect of ribavirin on MNV was confirmed by CPE inhibition assay using a colorimetric method (MTT) after 3 days incubation (n=8).

Combinatory effects of ribavirin and immunosuppressants on norovirus replication

Immunosuppressants are often used in combination in the clinic. First, we evaluated the interaction of immunosuppressants on norovirus using MacSynergy 2 combination analysis (supplementary material). As shown in figure 7A, the combined calcineurin inhibitors, FK506 and CsA, exert a moderately synergistic antiviral effect, with a log volumes of 5.55. The maximal degree of combined inhibition between FK506 and CsA was achieved at 1 $\mu\text{g/ml}$ CsA and 0.4 $\mu\text{g/ml}$ FK506, with a synergy volume of 35.06 $\mu\text{M}^2\%$ above the expected value. The combination of calcineurin inhibitors, FK506 (figure 7B) or CsA (figure 7C) with MPA achieved a synergy volume of -7.79 $\mu\text{M}^2\%$ (log volume of -0.93) and -14.58 $\mu\text{M}^2\%$ (log volume of -1.32), respectively, indicating that the combination effect of calcineurin inhibitors with MPA was additive. These results highlight the dependency of norovirus biology on both calcineurin phosphatase activity and the presence of an adequate intracellular guanosine pool.

Next, we assessed the combinatory antiviral effects of ribavirin and immunosuppressants. The combined effects of ribavirin and immunosuppressants, including CsA (n=5; figure 7D), FK506 (n=5; figure 7E), and MPA (n=5; figure 7F), is additive with a synergy volume of 0 $\mu\text{M}^2\%$ (log volume of 0), 0 $\mu\text{M}^2\%$ (log volume of 0) and -4.04 $\mu\text{M}^2\%$ (log volume of -0.37), respectively.

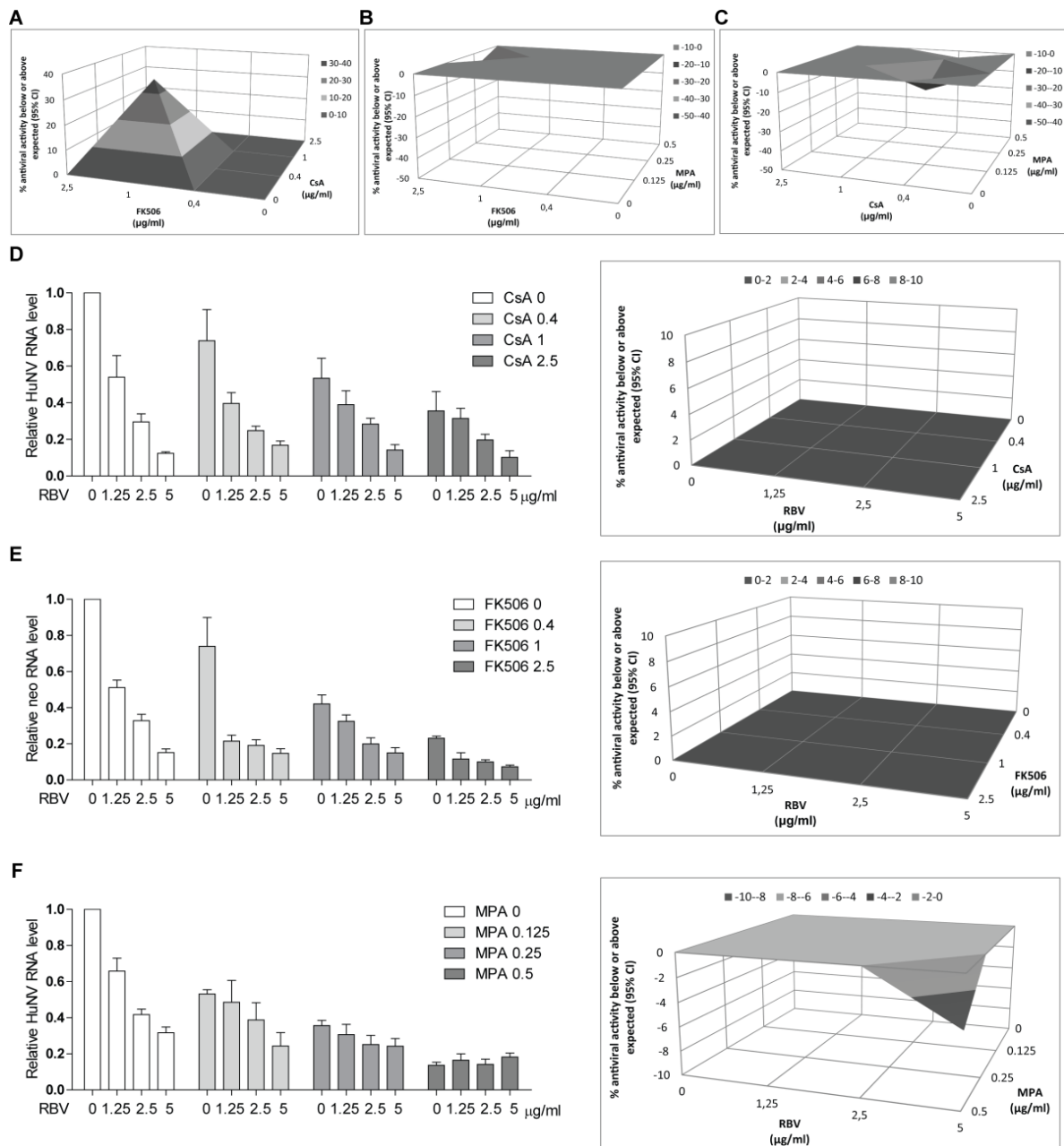


Figure 7. The combinatory effects of ribavirin and immunosuppressants on norovirus replication. The combinatory effects of two drugs in the 48 h antiviral assay with HuNV were analyzed using the mathematical model MacSynergy. The three-dimensional surface plot represents the differences (within 95% confidence interval) between actual experimental effects and theoretical additive effects of the combination at various concentrations of the two compounds ($n=5$). MacSynergy analysis of the antiviral effect of FK506 in combination with CsA (A), MPA (B) as well as CsA in combination with MPA (C), as measured on HuNV model after 48 h of treatment. Combinations of ribavirin with CsA (D), FK506 (E) or MPA (F) were expressed by the relative HuNV RNA level compared to the control and the three-dimensional surface plot.

Discussion

Immunosuppressants are universally used to deliberately induce immunosuppression after organ transplantation as to prevent allograft rejection. However, this results in increased susceptibility to opportunistic infections, and especially norovirus infection has emerged as a major concern in this respect (4). A recent study reported among 116 immunocompromised pediatric hematopoietic stem cell and solid organ transplant recipients, norovirus infection was identified in 22% of patients and that in these patients norovirus infection was associated with prolonged diarrhea and frequent intensive care unit (ICU) admission (4). Although new classes of immunosuppressive medications have been introduced to the transplant community during the last decades, T-lymphocytes remain the key targets of these agents as this cell type is a major effector in graft rejection. T-lymphocytes, however, also play an important role in the adaptive immune response following viral infection and thus reduced resistance to viral infection is an expected side-effect of graft tolerance-inducing medications. Intriguingly, immunosuppressive medications may also directly interfere with the viral life cycle and for various viruses, it is now clear that the choice of immunosuppressive medications relates to patient sensitivity to infection (33). Norovirus biology, however, is relatively poorly understood and its interaction with different immunosuppressive regimens remains obscure at best. Here we have profiled the commonly used immunosuppressants on norovirus infection and related the effects to their cellular effectors. Thus our data provide guidance as to host biochemical mechanisms essential for norovirus replication and may guide clinical management of transplantation patients at risk for norovirus infection. With respect to latter especially the remarkable sensitivity of norovirus to MPA is important, especially in view of the absence of norovirus-directed vaccines or antiviral medications.

Interestingly, our study reveals a hitherto unexpected role for calcineurin phosphatase activity in the norovirus life cycle. Calcineurin inhibitors include FK506 and CsA. FK506 is currently a widely-used immunosuppressive agent for managing transplantation patients. CsA binds to cyclophilins and FK506 binds to FK binding proteins (FKBPs). Both events result in a profound inhibition of the phosphatase activity of calcineurin which in turn suppresses T cell proliferation. In general, FK506 is thought to have no effect on viral infections (27). In contrast, CsA has been

demonstrated to interact with the biology of a broad range of viruses mainly through inhibiting cyclophilins. Of note, CsA inhibited HCV replication both *in vitro* and *in vivo* in an apparently specific manner. HCV RNA replication requires the non-structural HCV NS5A protein that serves as a direct ligand for CyPA. CsA could disrupt the CyPA-NS5A interaction, further reducing HCV RNA replication (34). In our study, FK506 and CsA exert a moderate inhibitory effect on norovirus replication through FKBP12 and CyPA, respectively. Furthermore non-immunosuppressive CsA analogues, which do not inhibit calcineurin, fail to suppress norovirus replication, which supports the hypothesis that calcineurin activation is required for norovirus life cycle.

Calcineurin is a heterodimeric Ca^{2+} - and calmodulin-dependent serine/threonine protein phosphatase composed of a 60-kDa catalytic subunit (PPP3CA) and a 19-kDa calcium binding regulatory subunit (PPP3R1) (35). Our results show that knockdown of PPP3CA results in a dramatic reduction of HuNV replication, demonstrating that calcineurin is required for HuNV replication. Although the exact mechanism as to how calcineurin facilitates norovirus replication is unknown, but may resemble its role in the life cycle of polyomavirus BK (36). The observation that CsA, but not NIM811 (a non-immunosuppressive CsA derivative) inhibits BKV replication by inhibiting CyPA and calcineurin activation is highly reminiscent of the results obtained in the present study, but concluding that effects are mechanistically identical requires further validation especially with respect to the role of the nuclear factor of activated T cells (NFAT).

Mycophenolate mofetil (MMF) has become the first-line immunosuppressive treatment in kidney, pancreas, liver and heart transplantation (37). MPA, the active form of MMF *in vivo*, is a potent inhibitor of IMPDH, a rate-limiting enzyme in the *de novo* synthesis of guanosine nucleotides. By inhibiting IMPDH, MPA results in the depletion of the intracellular GTP and dGTP pools and exerts potent immunosuppressive effects. MPA has been demonstrated to inhibit a variety of viruses, ranging from DNA virus, including HBV, to RNA viruses, including HCV, dengue virus, yellow fever virus and Chikungunya virus (27). Here, we demonstrate that MPA potently inhibits both MNV and HuNV replication through simultaneously targeting IMPDH1 and IMPDH2. Furthermore, MPA at levels below serum concentrations in patients completely eliminates HuNV replicons from cells after long-term treatment. In apparent agreement, 17 out of 23 IMPDH inhibitors also inhibit

HuNV replication in our experiments. Apparently, MPA has a high barrier against developing drug resistance with respect to its action on norovirus biology. In conjunction, our data provide compelling evidence that MPA should be the immunosuppressant of choice in transplantation patients at risk for norovirus infection.

Optimization of immunosuppression could potentially help the clearance of norovirus infection in a proportion of infected organ recipients. However, for the transplant or other types of patients who fail to clear the virus, effective antiviral therapy is urgently needed; whereas no proven antiviral medications are available. Ribavirin, as a general antiviral, has been used in the clinic for decades to treat various types of viral infections. A recent study reported that ribavirin cleared norovirus and resulted in complete symptomatic and histological recovery in two COVID patients, which raises the possibility of ribavirin as potential antiviral therapy for norovirus infection. Ribavirin is a guanosine analogue, which inhibits the replication of a wide range of RNA and DNA viruses. Ribavirin functions through a multiple mechanism-of-actions, including depleting GTP pool via IMPDH inhibition, inhibiting viral RNA polymerase activity and causing mutagenesis. In our study, ribavirin at concentrations comparable to serum concentrations in patients (38) inhibited norovirus replication. Ribavirin, combined with MPA or calcineurin inhibitors, additively inhibited norovirus replication, which support the potential of ribavirin as a therapy for norovirus infection in transplant patients.

In summary, we profiled the effects of clinically relevant immunosuppressants on norovirus replication and observe that norovirus is sensitively dependent on IMPDH guanine synthesizing activity but also requires calcineurin phosphatase activity. MPA represents a potent inhibitor of norovirus replication with a high barrier towards development of drug resistance. Ribavirin is a promising candidate in treating norovirus infection, though further evaluation particularly in patients is needed.

Materials and methods

Antibodies

Primary antibodies targeting cyclophilin a (CyPA; polyclonal rabbit, 1:1000, Abcam), cyclophilin b (CyPB; polyclonal rabbit, 1:1000, Abcam), IMPDH1 (polyclonal rabbit, ab33039, 1:1000, Abcam), IMPDH2 (monoclonal rabbit, ab131158, 1:1000, Abcam), FKBP12 (polyclonal rabbit, sc-28814, 1:500, Santa Cruz) and β -actin (monoclonal

mouse, 1:1000, Santa Cruz) were used. Secondary antibodies IRDye® 800CW Goat anti-Rabbit IgG (H + L) (1:10000; Li-Cor) and IRDye® 680RD Goat anti-Mouse IgG (H + L) (1:5000; Li-Cor) were used, as appropriate.

Quantification of MNV

The MNV was quantified by the 50% tissue culture infective dose (TCID₅₀). Briefly, RAW cells were cultured in 96-well plates at 1000 cells/well. After overnight incubation, 50 µl of 10-time serial dilutions of MNV were added. The plate was incubated at 37°C for further 5 days, followed by observing the cytopathic effect (CPE) of each well under light scope. The TCID₅₀ was measured by using the Reed-Muench method.

Another optimized qRT-PCR assay was developed to quantify MNV genomes as copy numbers. In brief, cDNA containing target sequence positioning between nts 4972 and 5064 in MNV were amplified, purified and 10 times serially-diluted. The dilutions were quantified by qRT-PCR to generate a standard curve, which was expressed by plotting the log copy numbers against the cycle threshold (C_t) value. Then 100 µl of MNV was lysed and C_t value was quantified by qRT-PCR assay. The viral genome copy numbers in the MNV culture were calculated by comparing the C_t with that of the standard curve.

Antiviral assay with HuNV replicon model

Before experiments, HG23 cells were cultured overnight without G418. Next day, HG23 cells were treated with drugs. At indicated time points, cells were lysed and HuNV subgenomic RNA was determined using SYBR Green-based quantitative real-time polymerase chain reaction (qRT-PCR) method. GAPDH was used as housekeeping gene and data were analyzed with the $2^{-\Delta\Delta CT}$ method. All primer combinations were listed in supplementary table 1.

Antiviral assay with MNV

The antiviral assay with MNV was initiated by inoculating MNV into RAW cells at a multiplicity of infection (MOI) of 0.1. After 1 h infection, MNV-infected cells were treated with drugs for 24 h. The supernatants and cell layers were separately collected for qRT-PCR analysis.

MNV could induce cytopathic effect (CPE) in RAW cells (1). The antiviral activity of drugs on MNV was further verified by using a MTT-based CPE inhibition assay. Briefly, MNV-infected RAW cells were seeded into 96-well plates at 10000 cells/well. After 72 h treatment with drugs, more than 95 percent of CPE was observed in medium control. Then MTT assay was initiated and the absorbance at 490 nm was recorded. CPE inhibition was calculated as $[(OD_{\text{treated}})_{\text{MNV}} - OD_{\text{VC}}] / [OD_{\text{CC}} - OD_{\text{VC}}]$, where $(OD_{\text{treated}})_{\text{MNV}}$ represented OD of virus-infected cells treated with drugs, while OD_{CC} and OD_{VC} represented the OD of cell control and virus control, respectively.

Short-hairpin RNA delivery by lentiviral transduction

Gene knockdown was performed as described previously (40). In brief, pLKO.1-based vectors containing the RNA interference sequences were obtained from the Biomics Center at the Erasmus Medical Center. The lentiviral vectors were generated and condensed by high-speed centrifugation (if indicated). Transduction was initiated by inoculating shRNA vectors into HG23 cells. Following 3 days of transduction, cells were cultured with 3 $\mu\text{g/ml}$ of puromycin. A validated nonsilencing scrambled vector (shCTR) was used as a control. All the shRNA sequences are listed in supplementary table 1. For simultaneous knockdown, HG23 cells were transduced with a first lentiviral vector. After selection with puromycin, HG23 cells were subsequently transduced with a second condensed lentiviral vector.

Western blotting

Cell samples were lysed and loaded onto a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). After electrophoresis, the proteins were electrotransferred to a polyvinylidene difluoride membrane (PVDF, Invitrogen). The membrane was probed with primary antibody plus secondary antibody and detected with Odyssey 3.0 Infrared Imaging System (LI-COR Biosciences).

Synergy analysis

To evaluate the interaction of ribavirin and immunosuppressants on norovirus replication, MacSynergy 2 (kindly provided by Mark Prichard) (41), a mathematical

model based on Bliss Independence theory, was employed to analyze the data from 48 h combined treatment of ribavirin and immunosuppressants on HuNV model.

In the MacSynergy model, the theoretical additive effect with two compounds could be calculated using the equation: $Z = X + Y(1-X)$, where X and Y represent the inhibition produced by the individual drugs and Z represents the theoretical effect produced by the combination of two drugs. The theoretical additive surface is subtracted from the actual experimental surface. When a combination is additive, data points of the calculated surface lies in the zero plane. A surface that lies > 20% above the zero plane indicates a synergistic effect of the combination; a surface > 20% below the zero plane indicates antagonism. The 95% confidence interval was considered to be statistically significant.

Acknowledgements

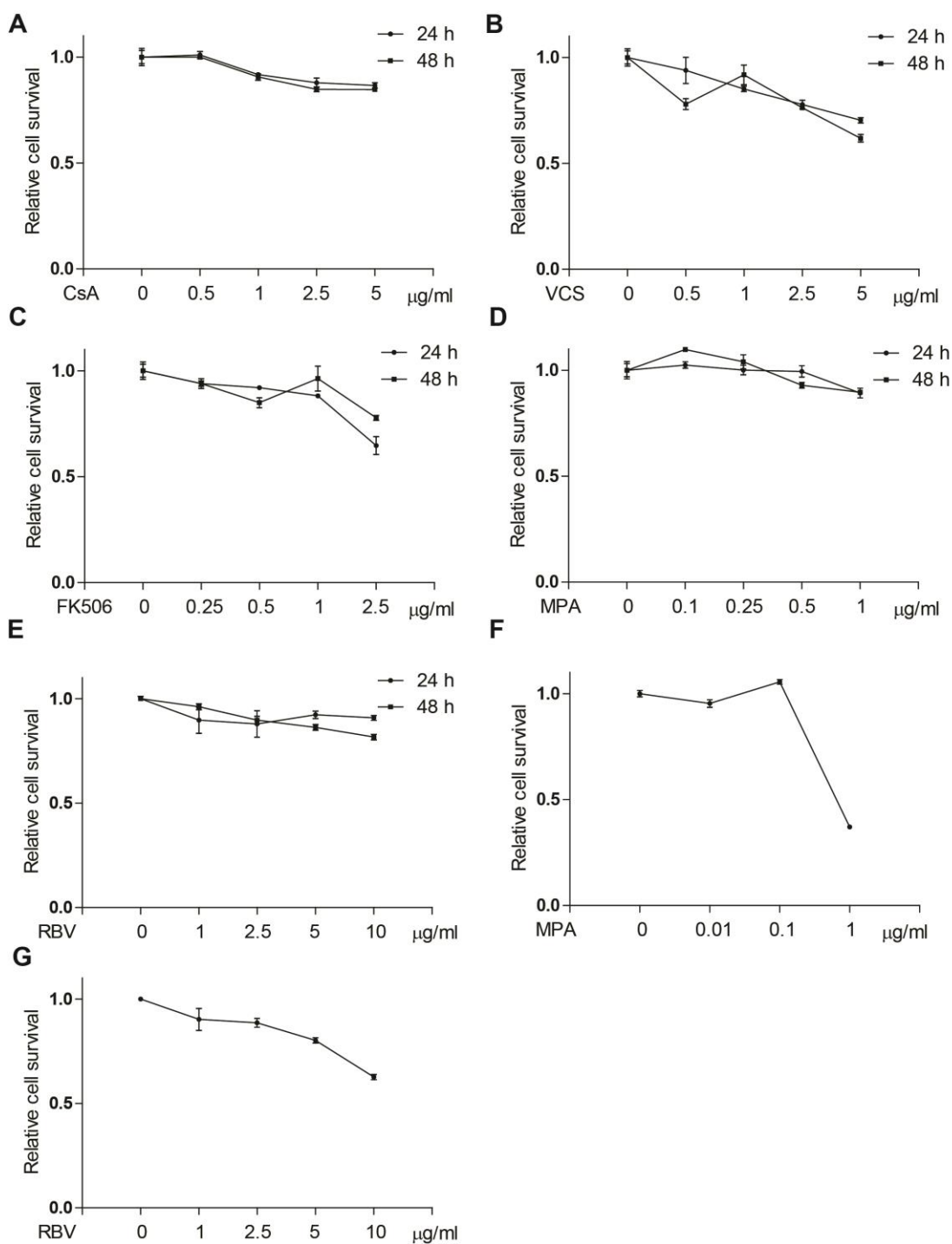
We gratefully acknowledge Professor Herbert W. Virgin (Washington University, St Louis, MO, USA) for providing the MNV-1 and RAW 264.7 cell. We thank the Center for Drug Design, University of Minnesota, for the financial support of developing new IMPDH inhibitors. We also thank Isotechnika Pharma Incorporation (Edmonton, AB, Canada) for providing two non-immunosuppressive cyclosporin A derivatives 431-32 and 440-02, and a novel calcineurin inhibitor, voclosporin.

Reference

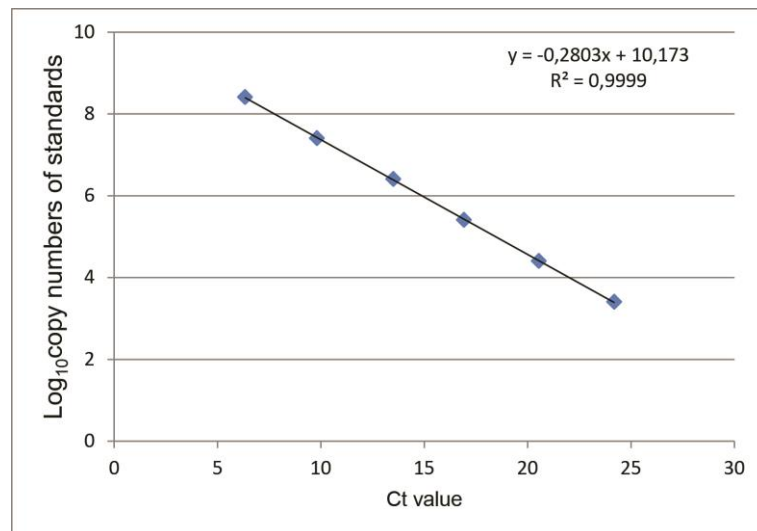
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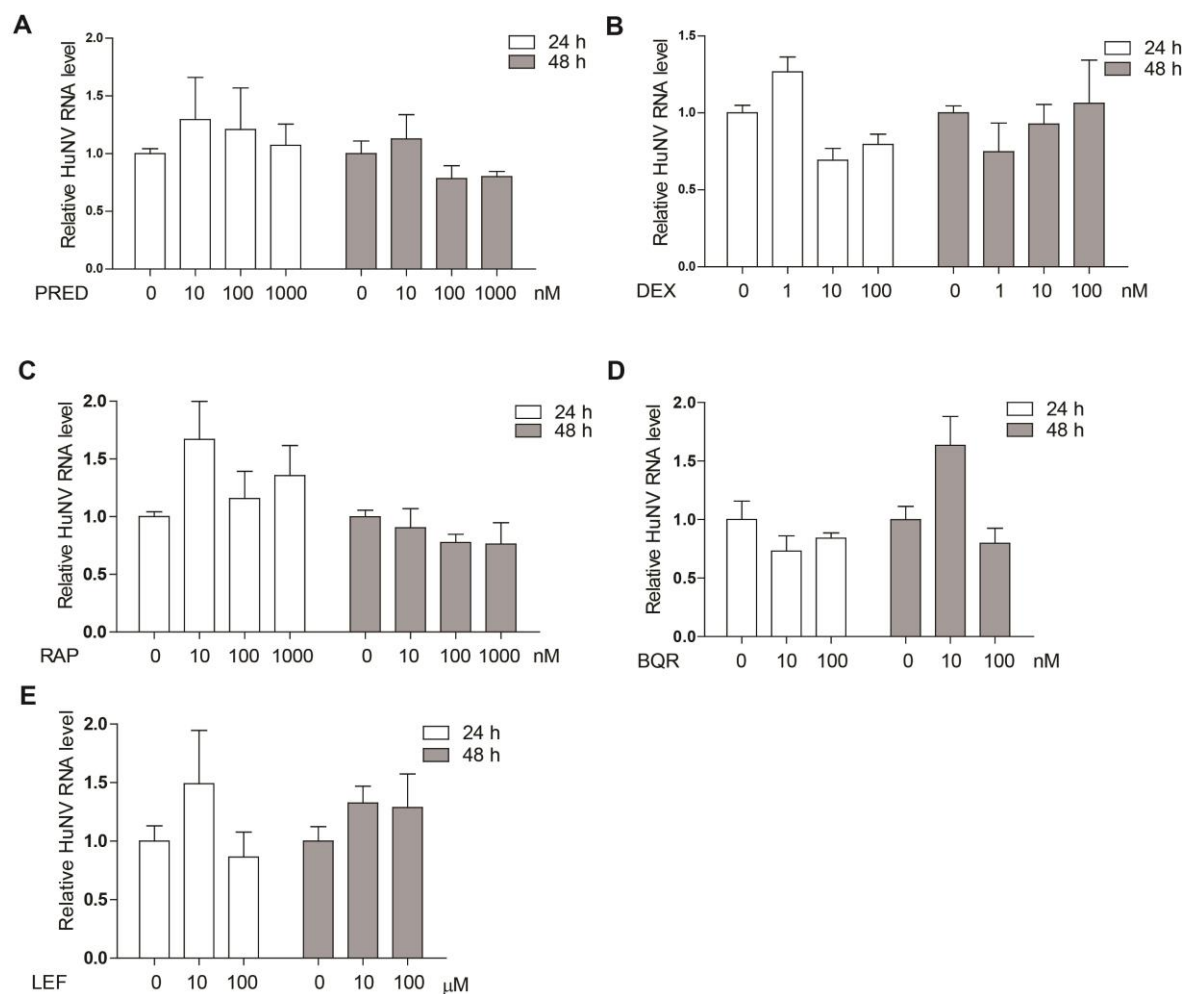
Supplementary Results



Supplementary Figure 1. Cytotoxicity of compounds determined by MTT assay. Effects of CsA (A), voclosporin (B), FK506 (C), MPA (D) and ribavirin (E) on viability of HG23 cells were determined after 24 h and 48 h treatment. Effects of MPA (F) and ribavirin (G) on RAW cell viability were detected after 24 h treatment (n=8).

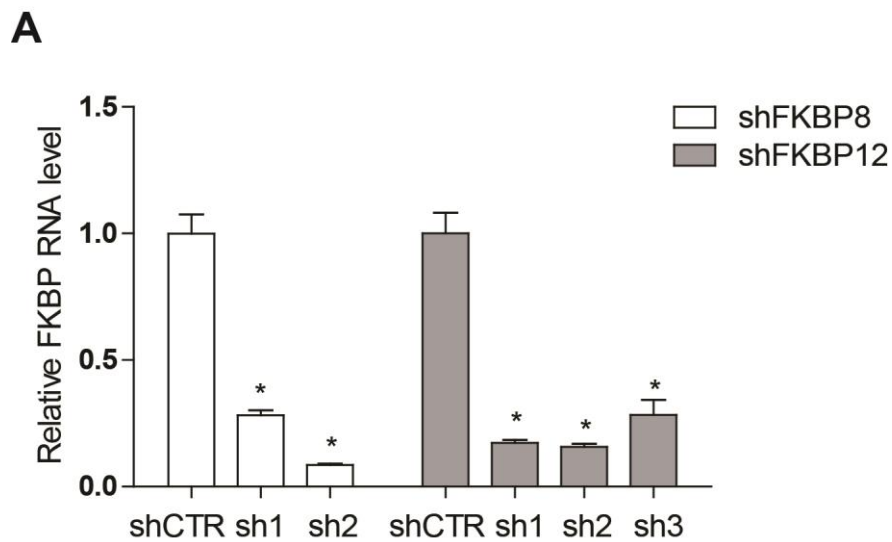


Supplementary Figure 2. Standard curve for quantifying MNV genome copy numbers.

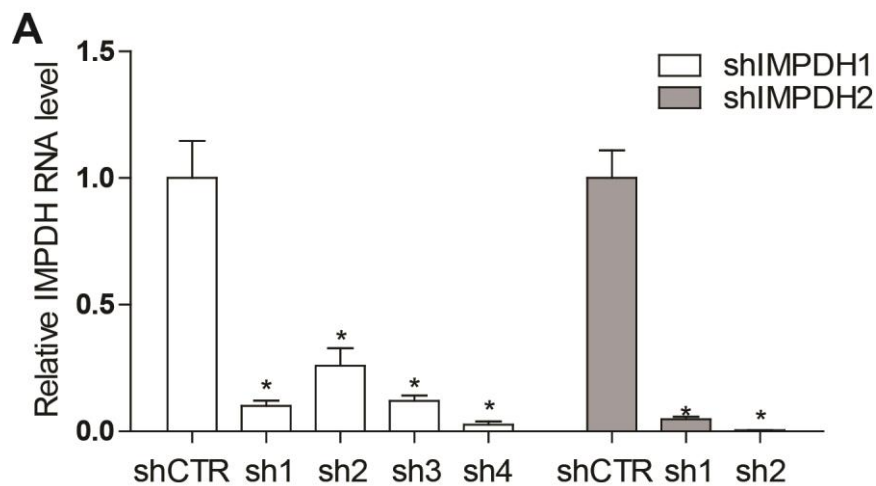


Supplementary Figure 3. Not all immunosuppressants directly affect norovirus replication. Glucocorticoids, including PRED (A) and DEX (B) show no effect on HuNV replication.

Likewise, rapamycin, a mTOR inhibitor and immunosuppressant with low nephrotoxicity (4) produces no significant changes in HuNV replication (C). Similarly, BQR (D) and LFM (E), which are in clinical development for use in transplantation medicine, show no significant effects on HuNV replication. Thus modulation of norovirus infection is not a general property of immunosuppressants



Supplementary Figure 4. qRT-PCR analysis of FKBP8 and FKBP12 RNA levels after shRNA-based knockdown.



Supplementary Figure 5. qRT-PCR analysis of IMPDH RNA levels in IMPDH1 or IMPDH2 knockdown cells.

Supplementary Table 1

PCR primer sequences and shRNA-mediated vectors sequences were listed.

Name	Sequences
Neomycin phosphotransferase MNV-1 ^a	F: 5'-CCGGCTACCTGCCCATTC-3' R: 5'-CCAGATCATCCTGATCGACAA G-3'
Human GAPDH	F: 5'-GTCTCCTCT GACTTCAACAGCG-3' R: 5'-ACCACCCTGTTGCTGTAGTAGCCAA-3'
Murine GAPDH	F: 5'-TTCCAGTATGACTCCACTCACGG-3' R: 5'-TGAAGACACCAGTAGACTCCACGAC-3'
CyPA primer	F: 5'-TAAAGCATACGGGTCCTGG-3' R: 5'-TCGAGTTGTCCACAGTCAG-3'
CyPB primer	F: 5'-ATGTAGGCCGGGTGATCTTT-3' R: 5'-TTTATCCCGGCTGTCTGTCT-3'
FKBP12 primer	F: 5'- TGCTAGGCAAGCAGGAGGTGAT-3' R: 5'- GTGGCACCATAGGCATAATCTGG-3'
FKBP8 primer	F: 5'- ACTCCTACGACCTCGCCATCAA-3' R: 5'- GGTAGTGGTTCGAGCTTCAGCTG-3'
PPP3CA primer	F: 5'-GCCCTGATGAACCAACAGTTC-3' R: 5'-GCAGGTGGTTCTTTGAATCGGTC-3'
IMPDH1 primer	F: 5'-GCACACTGTGGGCGAT-3' R: 5'-GAGCCACCACCAGTTCA-3'
IMPDH2 primer	F: 5'-TCTTCAACTGCGGAGAC-3' R: 5'-CTGTAAGCGCCATTGCT-3'
Scrambled vector	5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3'
CyPA vector	5'-CCGGTGGTGACTTCACACGCCATAACTCGAGTTATGGCGTGTGAA GTCACCATTTTTG-3'
CyPB vector	5'-CCGGGCCTTAGCTACAGGAGAGAAACTCGAGTTTCTCTCCTGTAGCTAAGGCTTTTTG-3'
FKBP8 sh1 vector	5'-CCGGACGTGCTGGAGAATGGCACACTCGAGTGTGCCATTCTC CAGCGACGTTTTTT-3'
FKBP8 sh2 vector	5'-CCGGAGTGGACATGACGTTGAGGACTCGAGTCCTCGAACGTC ATGTCCACTTTTTT-3'
FKBP12 sh1 vector	5'- CCGGAGAGAGCCAAACTGACTATATCTCGAGATATAGTCAGTTTG GCTCTCTTTTTT-3'
FKBP12 sh2 vector	5'-CCGGGCCAAACTGACTATATCTCCACTCGAGTGGAGATATAGTCA GTTTGGCTTTTTT-3'
FKBP12 sh3 vector	5'- CCGGGAGAGCCAAACTGACTATATCCTCGAGGATATAGTCAGTTTG GCTCTCTTTTTT-3'
PPP3CA sh1 vector	5'-CCGGCACCACAACATAAGATCACTACTCGAGTAGTGATCTTATGTT GTGGTGTTTTTT-3'
PPP3CA sh2 vector	5'- CCGGAATAATAACAGAGGGTGCATCTCGAGATGCACCCTCTGTTA TTATTCTTTTTT-3'
IMPDH1 sh1 vector	5'-CCGGCCAGGATTCATAGACTTCATACTCGAGTATGAAGTCTATGAA TCCTGGTTTTT-3'
IMPDH1 sh2 vector	5'-CCGGCGGAAGGTCAAGAAGTTTGAAGTTCGAGTTCAAACCTCTTG ACCTTCCGTTTTT-3'
IMPDH1 sh3 vector	5'-CCGGGTGACGTTGAAAGAGGCCAAATCTCGAGATTTGCCTCTTTCA ACGTCACTTTTTT-3'
IMPDH1 sh4 vector	5'-CCGGCCTGAAGAAGAACCGAGACTACTCGAGTAGTCTCGGTTCTT CTTCAGGTTTTT-3'
IMPDH2 sh1 vector	5'-CCGGCACCTACAATGACTTTCTCATCTCGAGATGAGAAAGTCATTG TAGGTGTTTTT-3'

IMPDH2 sh2 vector	5'-CCGGGACTGTTTCTTGGAAGAGATACTCGAGTATCTCTTCCAAGAA ACAGTCTTTTT-3'
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a, accession# NC008311

b, accession# M87661

Supplementary Table 2

The K_i values of 23 IMPDH inhibitors towards IMPDH1 and IMPDH2. Because of confidentiality, the structures are not shown.

#	IMPDH I K_i in nM	IMPDH in nM	MW
102	330	250	667.44
1316	82.10	55.70	749.56
1315	0.60	13.90	744.53
1401	2553	231.1	569.58
1344	162.80	102.40	569.58
1346	859.1	243.9	546.54
1347	703.4	230.1	562.60
1348	1725	365.7	433.51
1351	618.40	185.90	549.59
1352	136.20	93.01	646.66
1353	1255	364.70	790.35
1356	70.12	65.69	588.67
1401-L-ABC ^a	-	-	588.67
1357	1813	522.50	569.58
1389	11274	297.90	706.75
1391	4221	2160	526.55
1393	5556	2398	481.55
1400	9138	367.7	596.69
1402	2203	146.7	465.57
1406 ^b	-	-	269.32

1407 ^b	-	-	287.31
1382	1815	2154	568.59
1385	521.80	487.40	391.42

a, 1401-L-ABC inhibit IMPDH.

b, If 1406 and 1407 were converted in the cell into their corresponding NAD analogues, they should show some inhibition of the enzyme.

Supplementary Discussion

Glucocorticosteroids were widely used during the early years of organ transplantation. Nowadays clinicians generally attempt to avoid prescribing steroids in case of active infection because of their potential of facilitating the infectious process and concern about long-term complications associated with such treatment regimens (5). For instance, steroids boluses used to treat acute rejection in hepatitis C virus (HCV) infection-related liver transplantation are often associated with an increase in viral load and the severity of HCV recurrence (5, 6). In cell culture, steroids are reported to have no effect on HCV replication (7), but can specifically facilitate viral entry by enhancing the expression of the HCV co-receptors (8). In the present study, however, we observe no direct effect of steroids on norovirus replication in cell culture, although their impact on norovirus infection in patients remains to be addressed. Rapamycin when complexed to FKBP12 to form the FKBP-rapamycin complex that directly binds to mTOR complex 1 and thus inhibits the mTOR pathway, appears likewise safe but is associated with anti-norovirus activity *per se*. This contrasts the situation with HCV infection. Rapamycin potently inhibits HCV RNA replication, but does not influence the early replication cycle steps associated with its biology such as cell entry and viral RNA translation (9). Diametrically opposing the situation with HCV, another study showed that rapamycin facilitated hepatitis E virus (HEV) replication through inhibition of PI3K-PKB-mTOR pathway. The latter pathway limits HEV infection and acts as a gate-keeper with respect to HEV in human HEV target cells. In our study, rapamycin treatment results in no change on norovirus replication in cell culture models and thus effects of mTOR inhibitors on viral life cycle appear highly virus specific, hampering design of rational immunosuppressive therapies using this medication. Brequinar and leflunomide, two best-known inhibitors of dihydroorotate dehydrogenase (DHOD), interfere with cell proliferation by inhibiting

pyrimidine nucleotide biosynthesis (10). Leflunomide has been extensively studied and is approved for the treatment of psoriatic arthritis and rheumatoid arthritis (11). Leflunomide has been shown to inhibit HIV-1 replication mainly through pyrimidine nucleotide pool depletion (12). In our study, both leflunomide and brequinar showed no effect on HuNV replication and appear thus not specifically useful for the management of patients at risk for norovirus infection.

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Chapter 9

Summary and general discussion

How do the findings presented in this thesis advance our understandings on the importance of rotavirus infection in organ transplantation, the utilization of primary intestinal organoids model to study viral infection and antiviral therapy, the understanding of the effects of immunosuppressive medicines on rotavirus infection and identification of the cellular signaling pathways important for rotavirus infection? These are the question I set out to answer in this thesis and in this chapter, I aim to analyze my success in answering them and to relate the findings obtained in this respect to the contemporary body of biomedical literature with also a special aim in defining future directions of research I deem appropriate.

Enteric viruses including rotavirus and norovirus in organ transplantation

Diarrhea has been emerged as a common complication after solid organ transplantation, and is particularly the consequence of viral infections in these patients (1). Rotavirus, as one of most important causative agents of severe enterogastritis in infants younger than three years old, has become increasingly recognized as a pathogen in solid-organ transplantation (2). At the start of my research, motivated by the incompleteness of information about this subject I decided to embark on a literature study which resulted in the completion of a comprehensively review, on this issue (**Chapter 2**). The results show that rotavirus infection is major driver of severe gastroenteritis in organ recipients irrespective to their age (contrasting the situation in the population at large, where such gastroenteritis is mainly to small children). An average incidence of 3% of rotavirus infections in organ recipients was found by combining results of 6176 organ recipients, and eight deaths were found to be related to rotavirus infection. Thus rotavirus is a serious concern in this patient group (**Chapter 2**). Most of data were collected from industrialized countries, it is plausible to assume that rotavirus infect is not regularly diagnosed in organ recipients in developing countries, which might cause underestimate of the incidence in this disease in developing countries (**Chapter 2**). We also find that intestinal transplant patients (12.95%), liver transplant patients (4.44%) and HSCT patients (4.40%) are more susceptible to rotavirus infection as compared to other organ transplantation recipients, but the reason behind this discrepancy is not very clear, also in view of largely similar immunosuppressive regimens in different groups

of organ recipients, unravelling the factors that drive this difference is of utmost interest also in view of my finding that rotavirus might be able to cause acute cellular rejection (ACR) leading to graft loss in organ recipients. With respect to the latter, I suspect that the virus can stimulate the host immune system provoking graft loss (**Chapter 2**). For instance, there is cellular infiltration of gut-associated lymphoid tissue (GALT) in the rotavirus-infected intestine and it is rational to assume that these GALT areas represent local intestinal immunity stimulation. Particular viruses are reported to result in non-specific abnormal regulation of the immune system. Even though very limited information is available about as to whether rotavirus can cause ACR via promotion of GALT, I demonstrated that rotavirus infection can induce expression of a panel of interferon stimulated genes (ISGs) in both Caco2 cells and organoids models (3 and **Chapter 3**), which provides strong evidence for the immunostimulatory function of rotavirus infection. Apart from rotavirus, I discovered that another enteric virus, norovirus was also reported to occasionally infect organ-transplanted patients (4). I concluded that more attentions should be paid about rotavirus and norovirus infections in transplanted patients to reduce burden of these viruses, but also that more attention should be given to investigating the interaction of immunosuppressive medication and process of viral infection.

Immunosuppressive medicines including steroids (prednisolone and dexamethasone), cyclosporine A (CsA), tacrolimus (FK506), mycophenolic acid (MPA), mTOR inhibitor etc. have been extensively used to combat organ rejection in transplanted patients, albeit at the cost of opportunistic infections (5). As such medication usually shows extensive interactions with multiple aspects of intestinal cell biology it is possible that certain types of immunosuppressive medication can directly interfere with the viral life cycle and combat viral infection while simultaneously preventing graft rejection. Indeed this has already been shown for other viruses (6). With this possibility in mind I profiled the effects of commonly-used immunosuppressive drugs on rotavirus and norovirus infections. I find that glucocorticosteroids do not interact with the viral life cycle of these enteric viruses (**Chapter 4 and 8**), which contrasts earlier reports that dexamethasone and prednisolone inhibit HCV replication (7). Thus interaction with viral life cycle appears highly virus type specific. CsA has been reported to inhibit various virus infections including HCV (8) and HIV (9). As the calcineurin inhibitors, CsA and FK506 are often used as immunosuppressive medicine in organ transplantation recipients, and also in

view of the reported interaction with the viral life cycle for other viruses infecting endodermal derivatives. CsA was reported to inhibit HCV infection in the absence of effects on the rate of cell growth or viability (8), in addition CsA was also reported to suppress HIV (9) and Flavivirus (10). I investigated the effect of this medication on the life cycle of enteric viruses. However, I find that the calcineurin inhibitors including CsA only moderately inhibits rotavirus and norovirus, while FK506 has no effect on rotavirus but moderate inhibitory effect on norovirus. Thus usefulness of these drugs in organ transplantation patients at risk for enteric viral infection is questionable, while simultaneously these results uncover a mechanistic dichotomy between the effects of different calcineurin inhibitors on enterocyte physiology (**Chapter 4 and 8**). MPA is a nucleoside analogue, which non-competitively inhibits inosine monophosphate dehydrogenase (IMPDH), and is thus essential for biosynthesis of guanine nucleotides (11). The replication of RNA or DNA viruses evidently requires guanine nucleotides, and thus it is plausible that MPA is able to impair viral replication (11). In apparent agreement, I found that MPA report to exert potent antiviral effects on rotavirus and norovirus via inhibition of IMPDH. Importantly, MPA displays a high barrier toward the emergence of drug resistant viral variants, implying that this drug potentially combines immunosuppressive therapy with antiviral activity and should thus be considered as the drug of choice for organ transplantation recipients at risk for infection by enteric viruses (e.g. those patients in frequent contact with young children).

Increasing evidence indicate that rotavirus might be one of the causative agents in IBD (12). 6-TG has been increasingly used for the treatment in IBD patients. Interestingly, we demonstrated that 6-TG could potently inhibit rotavirus infection with a high barrier towards developing drug resistance (**Chapter 5**). Mechanically, this inhibitory effect is through inhibition of the active form of the Rac1 protein (GTP-Rac1) (**Chapter 5**). The biomedical literature indicates a remarkable low incidence of rotavirus infection in the setting of IBD (12, 13). Based on my findings and the relatively frequent use of 6-TG in the IBD patient group, I propose that the use of 6-TG in this setting mediates protection against rotavirus explaining the low incidence of rotavirus infecting in this particular case, but this notion obviously requires further substantiation. In conjunction, the findings presented in my thesis suggest that clinical management of organ transplant patients should be adapted as to incorporate the potential risk for enteric viruses and suggest that patients with a high risk for

contracting such viral infection should be managed with MPA. Similarly, also IBD patients at risk for rotavirus infection would benefit from aggressive implementation of 6-TG therapy.

Development of primary intestinal organoids as novel model for study rotavirus infection and antiviral therapy

As a double stranded RNA virus, rotavirus has eleven segments which are highly variable, which results in more than 110,000 strains from all over the world and a large number of distinctly-evolved rotavirus strains is continuously emerging (3). The current models for rotavirus research mainly include immunized cancer cells and animal models, which only recapitulate certain aspects of the rotavirus infection process, hampering development in the field and in particular the identification of effective novel antiviral medication. Primary intestinal organoids (also called mini-gut) attract increasing global attention, since the model retains the hallmarks of an *in vivo* epithelium (14). We confirmed the primary intestinal organoids can support infection with both laboratory rotavirus strains and patient-derived rotavirus isolates (**Chapter 3**). This model can also be used for modeling virus-host interaction and antivirals including IFN α and ribavirin (**Chapter 3**). Organoids can be passaged for a long term, long-term storage by freezing is also possible, and these organoids allow investigation by a host of approaches including various molecular biological experimental modalities like infection by retro- and lentiviruses, western blotting and immunohistochemistry. Thus, organoids are in general versatile experimental tools (14). Importantly, I find that the inhibition by antivirals on rotavirus biology is less potent in organoids as compared to conventional cell line models. Thus my results suggest that organoids model are closely resembling the experience in *in vivo* models (**Chapter 3**) but without the associated ethical concerns or the species restriction. I thus propose the organoid model to be an effective model for personalized evaluation of medicines with regard to rotavirus infection (**Chapter 3**).

The PI3K-Akt-mTOR-4E-BP1 axis sustains rotavirus infection and represents an antiviral target

The PI3K signaling axis regulates a variety of cardinal processes in cellular physiology including sustaining cell growth and providing an important survival signals in addition to stimulating protein synthesis, and angiogenesis while also mediating anti-cancer drug resistance in mammalian cells (15). The main elements of the pathway include PI3K, Akt and mTOR. PI3K consists of p110 catalytic subunit and a p85 regulatory subunit. Akt (also known as PKB) is a serine/threonine kinase and mTOR is a serine threonine protein kinase (16). Many viruses hijack the PI3K-Akt-mTOR pathway to further perform their infection (17). It was reported that the Old World alphavirus Semliki Forest virus strongly activates PI3K-Atk-mTOR pathway (18), while Vesicular stomatitis virus (VSV) negatively regulates PI3K-Akt-mTOR pathway (19) suggesting that modulation of activity of this pathway is a general characteristic of viral infection. We find, however, that rotavirus has no effects on this pathway (**Chapter 6**). Antagonism of a PI3K-Akt-mTOR pathway inhibits the life cycle of various viruses including influenza A virus (20), CCR5 (R5)-tropic HIV (21), and middle east respiratory syndrome coronavirus (22). I find that the suppression of PI3K-Akt-mTOR pathway is able to inhibit rotavirus infection (**Chapter 6**) as well. mTOR is well established to inhibit autophagy, a process in which cells initiate a lysosomal-dependent self-digestive process and that is particularly triggered by nutrient starvation (23). Autophagy was reported to constitute a protective mechanism against infection by Sindbis Virus (24), and induction of autophagy by trehalose through the inhibition of mTOR was demonstrated to inhibit human cytomegalovirus infection (25). Consistent with these observations, I demonstrate that the mTOR inhibitor, rapamycin is able to induce autophagy, and that the thus-evoked increase in autophagy indeed inhibits rotavirus infection (**Chapter 6**). In this sense my observations fit well with the contemporary body of biomedical literature.

The eIF4F complex exerts an important role in regulating the cap-dependent mRNA translation. eIF4F consists the eIF4E (as a cap-binding protein), the eIF4A (as a DEAD box RNA helicase) and the eIF4G (as a scaffolding protein) (**Figure 1**) (26). The eIF4F complex was reported to sustain HEV replication (27), West Nile Virus infection (28), and barley yellow dwarf virus replication (29). I find, however, that the eIF4F complex inhibits rotavirus infection (**Chapter 7**). We propose our findings as potential target for development of novel antiviral medicines against rotavirus infection.

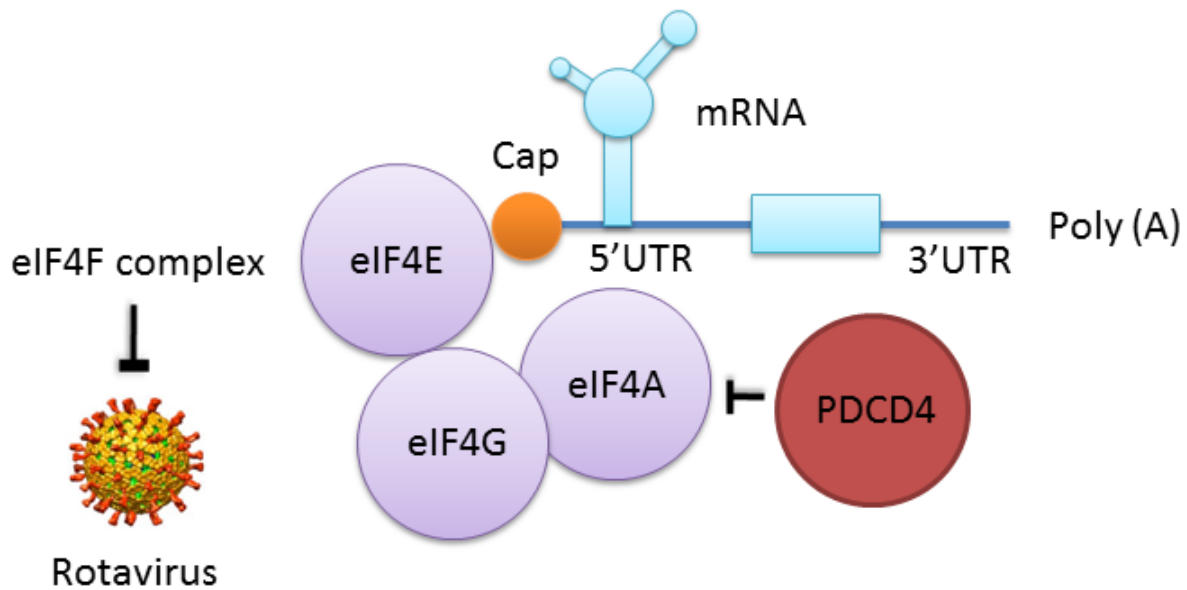


Figure 1. Schematic diagram of eIF4F which contains eIF4E, eIF4A and eIF4G. PDCD4 inhibits eIF4A to negatively regulate the complex. eIF4F complex inhibits rotavirus infection.

Conclusion and future perspective

In this thesis, we first highlighted important clinical complications associated with rotavirus infection in setting of orthotopic organ transplantation, I then analyzed the incidence of rotavirus infection, its diagnosis, its pathogenesis, how to rationally use of immunosuppressive agents in patients at risk for rotavirus infection, especially by studying the interactions of rotavirus with specific immunosuppressants, and also how to manage rotavirus infection in organ recipients. I also found 3% incidence of rotavirus infections among 6176 transplantations. I conclude that rotavirus infections occurring in transplantation patients remain clinically largely not diagnosed, and thus more attention should be paid to this pathogen.

The situation might be improved through the development of new superior model systems allowing new directions of research. I demonstrated that primary intestinal organoids can support infection with both laboratory rotavirus strains and with patient-derived rotavirus isolates. Thus, the organoid model might become exceedingly useful for obtaining new scientific insights but may also become important for individualized assessment of the efficacy of different antivirals in a particular patient, next to their potential for developing new and effective medicines against rotavirus. In support for this notion I found that the broadly used antivirals

including interferon α and ribavirin inhibit rotavirus replication utilizing the organoid model. I thus propose that organoids provide a promising novel avenue for investigating rotavirus-host interactions and the evaluation of medicines against rotavirus.

I profiled common used immunosuppressive medicines on enteric rotavirus and norovirus infections. I found that CsA moderately inhibits rotavirus and norovirus infections, and that MPA is very in this respect for both viruses and also with high barrier towards the development of drug resistance. Mechanistically, the antiviral effects of MPA are mediated through inhibition of its canonical cellular target IMPDH and depend on the resulting guanosine nucleotide depletion. I also found that 6-TG potently inhibits rotavirus via blocking the formation of the active form of Rac1 (GTP-Rac1), again with high barrier towards the development of drug resistance. I hope my findings will become an important reference for clinicians to design optimal immunosuppressive therapy for rotavirus infected transplantation patients and aid the development of novel antiviral medicines against rotavirus.

I also demonstrated that PI3K-Akt-mTOR signaling pathway sustains rotavirus infection and that the clinically used mTOR inhibitor rapamycin significantly inhibits rotavirus infection. Rapamycin induces autophagy via 4E-BP1, and induction of autophagy exerted antiviral effect on rotavirus, suggesting that this is the mechanistic explanation of this finding.

Although I perceive that the findings presented in this thesis contributed to a better understanding of the complex nature of enteric viruses including rotavirus and norovirus, I also have to acknowledge that more work is needed to reduce the burden of rotavirus and norovirus infection and hence there is plenty of opportunities for my successors either at the Erasmus MC or elsewhere to further contribute to such efforts.

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Chapter 10

Appendix

Dutch Summary

Curriculum Vitae

PhD Portfolio

Acknowledgements

List of Publications

Dutch Summary

Samenvatting voor niet ingewijden

Buikloop of diarree kenmerkt zich door een hoofdzakelijk dunne, brijige tot waterige, soms slijmerige, ontlasting die gepaard kan gaan met ernstige uitdroging of zelfs ondervoeding. Het woord diarree is afgeleid van het Grieks [$\delta\iota\alpha$ ["dia"] = door, $\rho\epsilon\omega$ ["reo"] = stromen]. De oorzaken van diarree zijn divers, zo zijn er toxische, bacteriologische en virale oorzaken. In dit proefschrift concentreer ik mij op virale oorzaken en met name op de zogenaamde rotavirussen en noravirussen.

Een rotavirus is vooral bekend van dat het bij jonge kinderen overgeven en diarree veroorzaakt. Het is een van de ernstigere diarreeverwekkers in deze leeftijdsgroep, omdat de diarree die het veroorzaakt zowel erg besmettelijk als hevig is, en vaak ook lang aanhoudend. In ontwikkelingslanden overlijden naar schatting 600.000 kinderen per jaar aan een rotavirus infectie. In Nederland is een rotavirus veel minder bedreigend door de betere uitgangstoestand (voeding, weerstand) van de patiëntjes en de mogelijkheid om spoedig in een ziekenhuis door middel van een infuus te worden gerehydrateerd, waardoor uitdroging wordt voorkomen. Toch is het ook bij ons een belangrijke oorzaak van ziekenhuisopnamen wegens diarree bij kinderen. Vrijwel ieder kind maakt echter in zijn of haar leven minstens één rotavirusinfectie door en ziekenhuisopname is gelukkig maar zelden noodzakelijk.

In een door mij uitgevoerde literatuurstudie (**Hoofdstuk 2**) ontdekte ik dat ook patiënten die door transplantatie een orgaan hebben ontvangen vatbaar zijn voor rotavirusinfectie en dat dergelijke virussen een belangrijke oorzaak zijn voor diarree in deze patiëntengroep. Opvallend is dat de leeftijd weinig uit lijkt te maken, jonge patiënten zijn niet meer gevoelig dan oudere patiënten. De immunosuppressieve medicatie die dergelijke patiënten krijgen maskeert waarschijnlijk de leeftijd afhankelijke bescherming tegen rotavirusinfectie die algemene populatie wel geniet. De notie dat virusinfectie interacteert met het immuunsysteem werd verder gesubstantieerd in **hoofdstuk 3**. Ik infecteerde intestinale cellen en darmorganoïden (een simpele miniatuur versie van de darm die in het laboratorium kan blijven leven en vermenigvuldigd kan worden) met rotavirussen en ontdekte dat deze darmcellen

na infectie allerlei inflammatoire producten gingen uitscheiden. Het werd dus belangrijk om te gaan onderzoeken hoe individuele soorten immuunsuppressieven interacteert met de virale levenscyclus. Immers, een medicijn dat naast afstoting ook met de virale levenscyclus interfereert, zou dus van grote waarde zijn. De resultaten van een dergelijk onderzoek beschrijf ik in **hoofdstuk 4**. Ik laat in deze studie zien dat corticosteroïden geen interactie met virale levenscyclus van darmvirussen vertoont. Sommige calcineurin inhibitoren hadden wel enig effect op darmvirussen, andere weer niet. Het bleek echter dat mycofenolzuur een sterk immuunsuppressief effect combineert met een potente antivirale werking met betrekking tot enterische virussen. Dit zou dan ook de aanbevolen immuunsuppressie moeten zijn voor die patiënten die verhoogd risico lopen op infectie met dergelijke virussen (bijvoorbeeld die patiënten die frequent in contact komen met kleine kinderen). Deze gedachtegang werd nog verder uitgebouwd in mijn studie (**hoofdstuk 5**) met 6-thioguanine (een Immuunsuppressivum dat wordt gebruikt na orgaantransplantatie maar vooral bij auto-immuunziekten zoals de ziekte van Crohn). Het bleek dat deze medicatie een sterk remmend effect had op het rotavirus en haar infectie van cellen. Ook bij deze patiënten zou men dus moeten kiezen voor een behandelstrategie waarin deze medicatie snel wordt ingezet bij die patiënten die een verhoogd risico lopen op rotavirusinfectie.

Een belangrijk aspect van mijn dissertatie is dat ook een belangrijk conceptueel manco probeer te repareren: de afwezigheid van een goed model systeem om virus infectie in te bestuderen en om nieuwe medicatie in te kunnen ontwikkelen. Het is met name deze situatie die rotavirus infectie zo'n groot probleem maakt. Met de introductie darmorganoïden (een simpele miniatuur versie van de darm die in het laboratorium kan blijven leven en vermenigvuldigd kan worden) in dit proefschrift als nieuw rotavirusinfectie model kan aan deze situatie wellicht iets gedaan worden.

Dergelijke technologie kan ook gebruikt worden om de cellulaire mechanismen te ontrafelen die het virus nodig heeft voor infectie en reproductie. Een belangrijke biochemische route betrokken bij de levenscyclus van vele virussen is de zogenaamde PI3-kinase-PKB-mTOR cascade, een cruciale groep van processen die bijvoorbeeld de hoeveelheid eiwitsynthese regelt maar ook de van cellen resistentie tegen geprogrammeerde celdood controleert. Vele virussen veranderen de activiteit van deze belangrijke cascade. In **hoofdstuk 6** stel ik echter vast dat dit niet geldt

voor rotavirussen. Wel kon ik vaststellen dat de achtergrondsactiviteit van deze signaalweg essentieel is voor succesvolle virusinfectie. Het remmen van deze signaalweg is dan ook een mogelijkheid om rotavirus medicamenteus te behandelen. In **hoofdstuk 7** beschrijf ik de ontdekking van nog een andere strategie om rotavirusinfectie te bestrijden. In dit hoofdstuk focus ik mij op het eukaryote initiatiecomplex 4F, wat belangrijk is bij de vertaling in eiwit en ik stel vast dat dit complex een remmende werking heeft op het doorlopen van de rotavirus levenscyclus. Het stimuleren van dit complex is dan ook een interessante mogelijkheid om slecht-controleerbare rotavirus infectie klinisch te gaan behandelen, al moet er nog wel veel werk gebeuren voordat zoiets echt in de zorg kan worden toegepast.

In **hoofdstuk 8** plaats al deze bevindingen in het kader van de reeds bestaande ideeën en beschikbare biomedische wetenschappelijke literatuur aangaande het rotavirus en daarmee geassocieerde ziektebeelden. Ook geef ik daar mijn ideeën over hoe we nu verder moeten met betrekking tot het ontwikkelen van behandelingen en de vragen waar verder onderzoek zich op zou moeten richten. Het mijn hoop dat ik met dit werk in ieder geval een eerste aanzet heb gegeven tot beter begrip en betere therapie van rotavirusinfectie.

Curriculum vitae

Yuebang Yin was born on 25th February 1989, in Shangqiu City, Henan province, China. He grew up and finished his primary school, middle school, and high school in his hometown.

In 2008, he entered a national key University (Chinese 985, 211 project) named Northwest Agriculture and Forest University for his bachelor in Yangling high-tech agricultural district, Shaanxi province. He studied animal and veterinary science in Innovative and Experimental College of the University. The college was specifically set up by the university for training students to master the state-of-the-art technologies in particular subject. In 2011, he got an opportunity to study in Wageningen University in Netherlands with scholarship of tuition waiver after selection by professors of Wageningen University. He focused on investigations about animal breeding & genetics and animal nutrition, and he achieved a minor degree with Agricultural Economics and Policy when he got Master of Science (MSc) degree in Wageningen University in 2013.

In 2013, he started his PhD program at the department of Gastroenterology and Hepatology, Erasmus medical Center Rotterdam, the Netherlands. Under supervision of Prof. Maikel .P Peppelenbosch and the sharp-mined, young-talented researcher Dr. Qiuwei Abdullah Pan, he focused on primary intestinal organoids culture and utilize the model for investigations of antiviral medicines and underlying mechanism-of-action.

PhD Portfolio

Name PhD student:	Yuebang Yin
Erasmus MC Department	Gastroenterology and Hepatology
PhD Period	October 2013 ~ June 2017
Promotor	Prof. Dr. Maikel P. Peppelenbosch
Co-promotor	Dr. Q Pan

General Courses

Biomedical Research Techniques XIII & XIV

Microscope Image Analysis: From Theory to Practice

Course in Virology

Laboratory Animal Science course (Art. 9)

Oral Presentations at national/international Conferences

2017, the 10th Dutch Experimental Gastroenterology and Hepatology (DEGH)

2016, the 29th International Conference on Antiviral Research (ICAR)

2016, the 9th Dutch Experimental Gastroenterology and Hepatology (DEGH)

Poster Presentation at national/international Conferences

2017, the 21th Annual Molmed day, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands

2016, the 20th Annual Molmed day, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands

2015, the 19th Annual Molmed day, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands

2015, the 8th Dutch Experimental Gastroenterology and Hepatology (DEGH)

Academic Award

Travel Merit Award of the 29th International Conference on Antiviral Research (ICAR)

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To Lei, we are very good friends. We start phd study in the same year. In addition, we both graduated same university in China. You gave me a lot supports for my experiments. He is very smart person, and very knowledgeable in virology. We spent very nice time in the lab, and fight together for the better career for the future. I believe you will have a bright career in the future. I hope we can be good friends forever!

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List of Publications

- 1, **Yuebang Yin**, Wen Dang, Xinying Zhou, Lei Xu, Wanlu Cao, Sunrui Chen, Junhong Su, Xuepeng Cao, Shaobo Xiao, Maikel P. Peppelenbosch and Qiuwei Pan,. PI3K-Akt-mTOR axis sustains rotavirus infection via the 4E-BP1 mediated autophagy pathway and represents an antiviral target. VIRULLENCE, 2017, *in press*.
- 2, **Yuebang Yin**, Wenshi Wang, Lei Xu, Wen Dang, Changbo Qu, Maikel P. Peppelenbosch and Qiuwei Pan,. 6-Thioguanine Potently Inhibits Rotavirus Infection and is antagonistic with alpha interferon and ribavirin. (Submitted)
- 3, W. Dang, **Y. Yin**, Y. Wang, J. Su, D. Sprengers, L. J. W. van der Laan, K. Felczak, K. W. Pankiewicz, K. Chang, M. P. G. Koopmans, H. J. Metselaar, M. P. Peppelenbosch, and Q. Pan,. Profiling immunosuppressants revealed that calcineurin inhibitors moderately and mycophenolic acid potently inhibited norovirus replication. (Submitted)
- 4, **Yuebang Yin**, Xinying Zhou, Ruisun Chen, Lei Xu, Wenshi Wang, Wen Dang, Changbo Qu, M. P. Peppelenbosch, and Q. Pan,. Inhibition of the eukaryotic translation initiation factor 4F complex on rotavirus infection. (In preparation)
- 5, **Yin Y.** Wang Y, Dang W, Xu L, Su J, Zhou X, Wang W, Felczak K, van der Laan LJ, Pankiewicz KW et al (2016) Mycophenolic acid potently inhibits rotavirus infection with a high barrier to resistance development. Antiviral Res 133: 41-49
- 6, **Yin, Y.** Bijvelds, M., Dang, W., Xu, L., van der Eijk, A.A., Knipping, K., Tuysuz, N., Dekkers, J.F., Wang, Y., de Jonge, J., Sprengers, D., van der Laan, L.J., Beekman, J.M., Ten Berge, D., Metselaar, H.J., de Jonge, H., Koopmans, M.P., Peppelenbosch, M.P., Pan, Q., 2015. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. Antiviral Res. 2015 Nov;123:120-131.
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- 10, Xu L, Wang W, Li Y, Zhou X, **Yin Y**, Wang Y, Man RA, Laan LJWV, Huang F, Kamar N, Peppelenbosch MP1, Pan Q. RIG-I is a key antiviral interferon-stimulated gene against hepatitis E virus regardless of interferon production. *Hepatology*. 2017 Feb 13. doi: 10.1002
- 11, L Xu, X Zhou, W Wang, Y Wang, **Y Yin**, LJW van der Laan, D Sprengers, IFN regulatory factor 1 restricts hepatitis E virus replication by activating STAT1 to induce antiviral IFN-stimulated genes. *The FASEB Journal* 30 (10), 3352-3367
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