The Role of Differential Host Glycan Interactions in Rotavirus Cell Entry and Replication

Thesis

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Abstract

Rotavirus A (RVA) is the primary cause of acute viral gastroenteritis in children and young animals globally; however, its replication and pathogenesis remain poorly understood. We have previously demonstrated contrasting modes of interactions with the host cell glycans for two prevalent porcine RVA strains: OSU G5P[7] (historically associated with severe disease in piglets) and G9P[13] (globally emerging variant in humans and swine). Specifically, OSU G5P[7] and G9P[13] strain replication was significantly decreased and significantly increased, respectively, following removal of terminal sialic acids (SA) by neuraminidase (NA) treatment. The latter contrasting effects coincided with the presence of distinct mutations found in the VP4 fusion region of these strains.

In our first study, to clarify cellular mechanisms associated with these differential mechanisms of cell attachment/entry we conducted transcriptome analysis of porcine small intestinal enteroids (PIEs) infected with the two RVA strains with and without NA treatment. NA treatment of porcine intestinal enteroids alone, before individual RVA G9P[13]/OSU G5P[7] infection resulted in altered expression of genes associated with biological regulation, transporter activity, protein binding, and multicellular organismal processes. This was shown with significant contradicting impacts, with G9P[13] being significantly enhanced, and OSU G5P[7] replication being significantly inhibited.

Cholesterol (a key component of the host plasma membranes) has been shown to play a critical role in RVA replication. To further improve our understanding of RVA pathogenesis taking into consideration RVA genotype-specific features, in our second study, we comparatively evaluated the effects of cholesterol and cholesterol-related additives [Methyl-β-cyclodextrin (MβCD), and diethylaminoethyl (DEAE), and bile acids (BAs)] on G9P[13] vs. OSU G5P[7] replication *in vitro*. Consistent with our previous findings, treatment with cholesterol and DEAE has increased replication of both strains. Further, our data demonstrated that depletion of cellular cholesterol levels by MβCD treatment resulted in decreased replication of RVA G9P[13] and OSU G5P[7]. Finally, in contrast to previous findings, we found that treatment of MA104 cells with bile acids led to enhanced replication of RVA strains G9P[13] and OSU G5P[7].

To further clarify the mechanisms behind the contrasting modes of RVA interaction with terminal SAs, in our third study, we have established a reverse genetics system (RGS) carrying 11 RVA OSU genes and demonstrated that replacement of VP4 in NA-sensitive OSU G5P[7] with the one from RVA G9P[13] led to the generation of a viable recombinant progeny virus.

Thus, our study has expanded our understanding of the mechanisms of RVA cell attachment and entry and suggested that the differential entry mechanisms utilized by OSU G5P[7] and G9P[13] strains could have altered mechanisms of the cholesterol-dependent intracellular replication of these strains. Additionally, our study generated a robust RGS platform to study RVA pathogenesis and gene function and confirmed the pivotal role of the VP4 in genotype-specific interactions with the host-cell glycans.

Dedication

To my parents, David and Tammy Raque, for their continued love, support, and sacrifices they made to make my education possible. Also, to my oldest sister, Melissa Raque Patterson, who has continuously encouraged and supported me.

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Chapter 1. Literature Review

1.1 Introduction to Rotavirus

Acute infectious diarrhea has been a major cause of mortality in young children and animals globally for centuries [1]. Rotaviruses (RVs) are a major viral cause of diarrhea in the young of many mammalian species, including humans [1]. The first group A RV (RVA) was isolated from calves in 1967 during an investigation of calf diarrheal outbreaks, referred to as "scours" [2]. A few years later in 1976, RVA was discovered via electron microscopy in the feces of piglets, shedding virus [3]. In 1973, in humans, Melbourne scientists Ruth Bishop, Geoffrey Davison, Ian Holmes, and Brian Ruck identified RVA particles in the epithelial cells of patients with severe dehydrating diarrhea as RVA [1].

To date, RVAs have been isolated from multiple species including humans, other mammals, and various avian species [4-6]. Rotaviruses remain a significant cause of severe diarrheal illness and dehydration in multiple species [7]. This includes over 210,000 deaths in children under 5 years of age, a significant impact causing one-third of all childhood diarrheal-associated deaths globally [7]. Nearly every child experiences RVA-associated gastroenteritis infection by the age of 5 [8]. In the United States alone, RVA infection costs approximately $12 million in healthcare expenses, and $32 million from societal impacts [9, 10]. Additionally, RVAs have a substantial impact on the production swine industry. In commercial swine farms,

the morbidity rate varies below 20% but higher rates have been also reported. Interestingly, less severe diarrhea has been shown in studies of piglets >5 days of age [11]. Recently there have been reports of almost 90% of porcine commercial pig operations being infected with RVA, and 78% with rotavirus C (RVC), leading to major economic losses [12-14].

1.2 Nomenclature

RVs are nonenveloped viruses and members of the *Reoviridae* family, with the genome consisting of 11 segments of double-stranded RNA [15]. These 11 segments encode 6 structural proteins, VP1-4, VP6, VP7, and 6 nonstructural proteins NSP1-5/6 [15]. This enables frequent reassortment of dsRNA segments between different strains and plays an essential role in the genomic diversity of RVs [16, 17]. RVs are classified based on VP6 inner capsid protein reactivity, forming nine genetically distinct groups from RV A-D, F-J [4, 18, 19]. Of these groups, RVA was historically considered to be the most prevalent and pathogenic [13, 15, 18]. However, recently, new evidence has emerged demonstrating the increased prevalence and significance of RVs of groups B, C and H (RVB, RVB and RVH) in swine, foals and even adult humans [18, 20-26]. Less common groups like RVI and RVJ have also been detected in dogs and bats within the last seven years [27, 28].

Within each genogroup, RVs are further classified into distinct genotypes using a binary system based characteristics on the protease-sensitive spike protein VP4 and the outer surface glycoprotein VP7 [15]. This system is universal and follows a simplified GxP[x] format to denote individual RV strains based on nucleotide sequence identity. This system fully expands to

Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx which designates the VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6 genotypes [16, 29, 30].

1.3 Rotavirus Prevalence

RVAs prevalence rates fluctuate greatly based on geographical location [20, 31-33]. This group of RVs has historically been associated with diarrheal disease in mammals including pigs, since the initial detection in 1976 [31, 34-36]. Recently the genetic diversity and zoonotic potential of RVAs was proven after llamas, sheep, alpacas, and the children working with these animals all tested positive for one or more RVA strains in common in Cusco, Peru suggesting intraspecies transmission [37]. The RVA has also been shown to have significant environmental resilience via monitoring RVAs have been detected in water supplies, demonstrating a serious risk for both human and animal health [38]. RVA G9 genotype and mixed genotypes prevalence has been growing among infections of children as well, supporting potential risk of interspecies transmission [39]. The wide prevalence of RVA has been demonstrated recently by a study in Shanghai in 2012-2018, revealing a seasonal pattern of RVA infection reaching the highest rates in children (87%) every winter, with G9P[8] being the dominant variant [40].

RVAs also have a significant impact on production animals, including swine. RVAs have been consistently recognized in pigs and there is a pattern of re-emergence of common RVA genotypes, specifically of G1 and G9 with prevalence rates 61%–74% among the farms [18, 41-46]. Multiple G and P genotypes have been associated with disease development in swine; however G3, G4, G5, G9 and G11 in combination with genotypes P[5], P[6], P[7], P[13] and

P[28] are historically dominant [18, 30]. In the US G5 has been found to be the most prevalent G type of porcine RVA (71.4%), while the G9 prevalence was (2.31%) [18]. P-type prevalence has also been characterized revealing the abundance of P[7] genotype (77.2%) in US. Further, G5P[7] was found to be the most prevalent combination. However, our data obtained from 2004 to 2012 demonstrated a remarkable switch from G5P[7] to G9P[13] detected in 60.9% of positive samples while G5 strains were not detected [18, 31, 32].

The impact RVA the swine industry is multifaceted, as the infection leads to decreased growth performance, higher incidence of secondary bacterial infections and up to 15% mortality rates in conventional piglets [47]. Furthermore RVA-infected pigs may serve as a source of heterologous RVA infections of humans, cattle, and other farm animals [48-50]. In swine especially, there is a critical need to address prophylactic measures to decrease the enteric infection in swine, reduce financial losses to the pork production industry, and minimize the risk of RVA zoonotic transmission into other hosts.

1.4 Rotavirus A interactions with host cell surface glycans and other receptors

RVAs target the differentiated intestinal epithelial cells (IECs) on the small intestine, specifically the ileum. Although heavily studied, the RVA entry mechanism remains incompletely understood. In general, RVAs attach to various host cell receptors including sialic acids (SAs), histo-blood group antigens (HGBAs), heat shock cognate protein 70 (hsc 70), integrins, junctional adhesion molecule A, occludin, and tight junction protein ZO-I to name a few [51-55]. Of these, SAs, known to be abundant in the intestine, have been found to play a critical role in RVA attachment/entry [56].

Earlier works have demonstrated that removal of SAs from cells by neuraminidase (NA) treatment led to significant decrease of replication of some RVA strains [51, 57]. Interestingly, this effect had been demonstrated for some (not all) animal but not human RVAs. Due to this phenomenon, findings have led to classification of RVAs as SA-dependent (NA-sensitive) and SA-independent (NA-insensitive), respectively [58]. However, further studies have demonstrated that even in the absence of terminal SA moieties, SA-dependent RVAs still interact with internal SA moieties in gangliosides that are resistant to NA treatment [59]. Moreover, interactions with internal SAs have been demonstrated even for SA-independent strains [59]. Due to these findings, it was proposed to classify RVAs as ganglioside (internal SAs)-dependent and ganglioside-independent [59, 60]. Moreover, our recent studies have shown that NA treatment may lead to increased replication of even some RVA and RVC strains [61, 62]. For example, replication of RVA G9P[13] but not RVA OSU G5P[7] was increased after terminal SAs removal. After comparative sequence analysis, we have found that this could be associated with mutation D385N located near the VP5* hydrophobic loop that was found in several RVA strains including porcine G9P[13] compared with PRVA OSU G5P[7] [62, 63]. The key role of this mutation has been demonstrated by a study where a similar mutation in this region of VP5* was shown to significantly affect virus entry. Thus, these novel data suggested that the wide prevalence and cross-species transmission of this emergent genotype of RVA may be supported by the unique properties of its VP4 involving interactions with internal SAs [64-68]. Furthermore, there may be some other essential attachment factors that can be masked by terminal SAs [62]. Altogether, these data have led to recognition of other RVA attachment/entry factors.

Histo-blood group antigens (HBGAs) are carbohydrates found on red blood cells and mucosa in reproductive, urinary, respiratory, gastrointestinal tracts, and biological fluids such as saliva and milk [61, 69-71]. The HBGA biosynthesis pathway is initiated by the generation of disaccharide type 1 precursor (Galβ1-3GlcNAcβ-R), which is converted into subsequent antigen types [ABH(O), Lewis, and secretor/non-secretor] by addition of saccharide residues through a series of enzyme catalyzed glycosyltransferases [70, 72, 73]. This pathway is also developmentally regulated and could contribute to why children and young animals are at higher risk of severe RVA infections [74]. Further, similar polymorphic HBGAs are observed in both humans and animals, resulting in shared HBGAs that could be responsible for the cross-species transmission [74, 75]. Specifically, swine and humans share A and H type 1 HBGAs [62]. HBGAs are recognized as co-receptors and attachment factors for other viruses, such as coronaviruses and noroviruses [69, 76-78]. Clinical studies have shown a link between the presence of certain HBGAs and RVA genotype [51]. Despite this initial discovery, the RVA-HBGA interactions are not fully understood, and results have often been contradicting. For example, one study using binding assays concluded P[6] interacted with H type 1 exclusively, while a nuclear magnetic resonance (NMR) study stated that P[6] recognized A type HBGA [79, 80]. In turn, the severity of infection had a direct association with secretion status, strengthening the support of HBGAs serving as initial attachment factors for RVs [24, 81-83]. The critical role of HBGA in RV infection was further supported by our *in vitro* studies where replicative preferences of RVAs and RVCs were shown after infection of porcine intestinal organoids expressing A or H [61]. This has also been supported by multiple studies showing that nearly all human RVAs bind to various HGBAs in secretions [84]. For strains that are known to be SA-

independent (ex. human RVAs), HBGAs are thought to serve as a main attachment factor similarly to SA-sensitive strains interactions with terminal SAs [62]. Further, similarly to ganglioside-dependent RVAs, blocking of HBGA-expression resulted in decreased replication of RVAs [61].

Integrins are also identified as co-receptors for RVs [85-89]. These are heterodimeric transmembrane receptors, utilized by multiple viruses for attachment and infection. Several integrins including α2β1, α4β1, αxβ2, and αvβ3 have been described as potential receptors for RVA cell entry [85-89]. Several studies have also been performed, showing that HSPs, specifically heat shock conjugate 70 (hsc70), can be targeted by both enveloped and nonenveloped viruses by interacting with the early cell attachment stages [90-92]. The interactions between the enterocyte basement membrane and IECs are integrin-mediated and control overall differentiation [90, 91]. Integrins are primarily recognized on a variety of cells and recognize matrix and cell-surface ligands. However, their specific location varies. The positioning can be apically in crypts, inside villous enterocytes, or basolaterally [93, 94]. One study has found that the varied positioning of integrins may not only be essential for an initial binding step, but also a post-attachment step capable of facilitating RVA infection in suboptimal conditions [86]. Supporting this theory, Hewish et al also used poorly permissive cell lines to express integrins and showed similar results of increased permissibility and infection [93]. Interactions of at least some of integrins with RVA have been shown to be facilitated by VP4 and VP7 proteins [81]. As with other RV receptors, interactions with integrins have not been demonstrated for all RVAs. Moreover, there is no correlation between RVA dependence on SA

and integrin presence [95]. However, the mechanisms and understanding of these interactions are unclear.

Heat shock proteins (HSPs) are molecular chaperones that have historically been investigated as therapeutic targets [90, 96, 97]. These molecules play a critical role in protection of cells against various physical, chemical stresses and pathogen invasion by regulating cellular innate and adaptive immunity [98]. One of these proteins, heat-shock cognate protein hsc70 has been shown to be involved in RVA infection as a receptor or co-receptor [99]. Interactions of hsc70 with VP5* domain of VP4 spike protein and VP6 of RVA have been demonstrated during the early attachment phase of cell entry [91]. Additionally, Gualtero and colleagues reported an interaction between RVA double layered particles (DLPs) with hsc70 and revealed an indirect role of VP6 in RV cell entry [100]. In summary, studies have shown that RVAs interacts in multiple ways with hsc70, involving VP4 and VP6. Additionally, HSPs are highly conserved in all organisms, suggesting their potential role in RVA interspecies transmission [99]. Presence of HSPs in established cell lines such as MA104, BHK and caco-2 allows researchers to evaluate the role of these molecules in RVA infection *in vitro* [92, 96, 97, 99]. Therefore, an in-depth understanding of the mechanisms of hsc70-RV interactions is essential to define the hsc70 role in RVA pathogenesis and the feasibility of its use as a drug development target.

The remaining hypothesized co-receptors relate to specialized membrane domains that engage in the passage regulation. Components such as tight junctions can be hijacked by viruses after initial attachment to complete their infectious cycle [101, 102]. Specifically, reoviruses have been shown to utilize Junction Adhesion Molecule-A (JAM-A) for cell entry [103]. Other viruses have been shown to perform the opposite and regulate the JAM area to improve the exit

process and disseminate into other host cells more efficiently [104, 105]. Specific to RVAs, the VP8 protein affects tight junctions (ZO-1, claudin-3, and occludin), greatly diminishing these in general to the point that TJs have little functionality and forces these to be open [104]. These tight junctions have a crucial role in passage through the paracellular pathway, where ZO-1 is known for scaffolding characteristics, and is supported by claudin-3 and occludin which are integral proteins that act adhesively to complementary molecules, such as ZO-1 [106]. Although this seems to have negative impacts for the ongoing infection, RVA VP8 could be used for administration of macromolecules and gene therapies by keeping TJs open [104].

1.5 RV virion structure and replication

The RVA virion structure resembles a wheel, with short spikes and smooth outer rim when viewed using an electron microscope [107]. The size of the virion is ~100-nm and consists of the icosahedral capsid surrounding the 11 segments of dsRNA. Each of these segments represents one gene apart from 11, which in some RVAs strains encodes for 2 proteins, NSP5 and NSP6 [107]. The role of NSP6 is unclear with its encoding of an alternative reading frame of gene segment 11 and is suggested to only regulate the role of NSP5 [15, 107]. The components of the triple-layered capsid itself is composed of the inner capsid layer (VP2), the middle layer (VP6), and the outer capsid layer of VP7, with the spike protein denoted as VP4 [108, 109]. Inclusion of all three layers forms the triple layer particle (TLP) which is the complete infectious virion that can bind to target cells. This outer layer consisting of VP4 and VP7 can be removed via chelating reagents and reveals the subunits of the inner capsid known as the double-layer particle (DLP) [110, 111]. The DLP is a noninfectious particle but is transcriptionally active and

can produce virus in target cells [110, 111]. RVA structure can be simplified further by removal of the two outer layers to reveal a single-layered particle (SLP) [110]. The SLP is also referred to as the core and is rarely seen in normal conditions and is usually only seen in structural analysis studies [110, 111].

The three types of particles relate to the various stages of RV replication. The outermost RVA layer is associated with cell attachment, membrane penetration, and subsequent host cell entry [110]. However, this is highly variable in the composition of the proteins, and have alternative mechanisms of entry, assuming to be related to their wide host range [110]. Regardless, proteolytic cleavage of VP4 results in formation of subunits VP8 and VP5 and then the initiation of replication begins with the attachment of the TLP to host attachment receptors via the VP8* subunit [15]. The role of VP7 in the initial events of infection is less clear structurally, but it is understood that VP7 works with VP5 to interact with one of several RVA co-receptors at lipid rafts to mediate viral entry [107]. Depending on the strain and whether it is a clathrin-dependent or caveolin-independent endocytosis pathway, the VP4 and VP7 outer layer is lost via endocytosis, revealing the subviral DLP and subsequently released into the cell cytosol, due to low calcium concentrations in endosomes. Clathrin-dependent strains are internalized via endocytosis, primarily dependent on β1 integrins after initial attachment to junction adhesion molecules [112]. Strains that are independent of both clathrin and caveolin rely on the presence of dynamin 2, a regulator of membrane trafficking on host cell surfaces [112]. As stated before, in both types of entry pathways, this DLP is transcriptionally active and releases capped, non-polyadenylated, positive sense ssRNA from the genomic RNA segments after the loss of the outer-layer proteins [113].

The uncoating-to-RVA transcription process is very quick and has been reported to occur within 15 minutes after viral introduction [113, 114]. From this point, the ssRNA is used for translation or serving as templates for negative sense RNA for replication [113]. NSP2 and NSP5 interact with each other to form viroplasms (simple cytoplasmic inclusions). These viroplasms contain the components required for viral genome assortment and replication, further termed as "viral factories" [110, 113, 114]. From this point, VP1 and VP2 form interactions to trigger dsRNA synthesis [113, 115]. VP1 specifically has shown that the presence with the core shell protein of VP2 allows catalyzation of dsRNA synthesis, allowing for template recycling and start of transcription [115, 116]. Immediately after, RNA capping enzyme (VP3) together with VP1 are bound near the fivefold axes formed by the VP2 decamers [117]. From here, VP6 assembles into DLPs and NSP4 acts as an intracellular receptor recruiting the DLPs formed in viroplasms to the endoplasmic reticulum [118]. The endoplasmic reticulum is an essential part of RVA replication, since this is where RVA is assembled, matured, and retained. From this point, these particles (including VP4 and VP7) are converted to a triple-layer particle via a budding process [15, 113, 119]. As of 2021, the key formation steps are still undergoing investigation. The complete understanding of the specific steps, timing, and protein spatial-temporal control of the virion is limited.

1.6 Pathogenesis

The pathology of RVA is multifactorial, beginning with the interactions in the small intestine [51, 120]. The small intestinal tract consists of four layers: the mucosa, submucosa,

muscle layer (muscularis), and serosa (adventitia), in the order from superficial to deep [121]. The mucosa consists of epithelium and lamina propria. The epithelial layer (=epithelium) includes various epithelial cell types including absorptive (columnar) enterocytes, secretory (goblet), undifferentiated, tuft, M, cup-like, enteroendocrine, transient amplifying and stem cells [121]. The enterocytes are mature cells covering the villi of the small intestine that maintain digestive and absorptive functions [122]. RVAs have been reported to enter IECs via two routes; via passive diffusion or active transport. Water transport is a key factor in the intestine and passively absorbed through osmotic gradients such as the sodium-glucose cotransporter 1(SGLT1) [123]. Crypt cells in comparison are not well-defined and have no absorptive functions. Within these crypts reside proliferating stem cells that intermingle with Paneth cells and mucous secreting goblet cells [124]. These cells secrete Cl- ions, and result in a bidirectional transport of electrolytes and water in the epithelium [122, 125]. Within these crypts are stems cells, which are capable of extensive replication and self-maintenance. If the intestine is damaged, these cells are capable of using their properties of self-maintenance to reestablish and regenerate tissues [126]. The regeneration process often leads to colonies of these cells, which allows them to also be referred to as *clonogenic cells* or *clonogens* [127]. All of these components work in unison for both absorption and secretion maintenance.

The majority of the understanding of RVA pathophysiology is derived from animal models [122]. RVs preferentially infect the tips of the small intestinal villi and replicate in the mature enterocytes that are no longer dividing. This supports the idea that differentiated enterocytes have essential factors to support RVA replication [61, 128]. Although severity,

location, and titers vary between species and study methodology, the pathogenesis is almost exclusive to the proximal part of the villi in the distal small intestine [129-131].

Causing villous atrophy, RVA alters the environment of the intestinal epithelium during infection, resulting in the quite common symptom association of diarrhea. This symptom is caused by the secondary reaction to enterocyte destruction and resulting in decreased absorption of sodium, water, and mucosal disaccharides [132-135]. Despite this impact, RVA has been shown to not alter the cyclic adenosine monophosphate (AMP), which is involved in Na+/H+ exchange in crypt cells [122, 132]. The malabsorption associated with RVA infection causes undigested components such as carbohydrates, proteins, and fats to be pushed into the colon. However, the colon is not able to absorb water at this stage of RV infection, resulting in osmotic diarrhea. There have been additional attempts to define the mechanism of diarrhea. One group suggested that the malabsorptive diarrhea was directly correlated with the repair mechanisms initiated from epithelial damage and villus ischemia, while another hypothesized that infection caused elevated levels of prostaglandin E2 (PGE2) [136, 137].

Another potential mechanism related to RVA diarrhea is due to the NSP4-mediated activation of the enteric nervous system (ENS) [138]. The specific portion of NSP4 causing these activities is unknown, although it is associated with stimulation of vagal afferent neurons [138]. It has been suggested that it is one or the combination of all these components that mediate the diarrheagenic changes: the nonstructural protein itself, a secreted fragment, or individual peptides that have been found to have toxin properties in mouse studies [132, 138-140]. In more recent years, the investigation of several drugs showed the capability to decrease RVA symptoms by directly blocking the ENS responses. This can be due to the fact that enterotoxins from RV

NSP4 protein influence the intestinal motility response of the ENS [138]. There have been two studies to determine ENS involvement, one of which tested transit time of charcoal in patients with RVA infections, and the other investigated myoelectrical activity of the jejunum in neonatal piglets [139, 141]. Both studies showed that there was less transit time during infections, and the normal motility pattern was altered. The extent of the ENS involvement remains unknown, but various data support that intestinal transit time is shortened and this may explain how the few cells on the villous tips can greatly influence the crypt responses during RV infection [139, 141].

Regardless of the host immune system alterations, NSP4 is considered to be an important factor of RVA-associated diarrhea development although knowledge of the mechanisms behind the enterocyte damage is limited [119, 138, 142]. NSP4 characteristically increases calcium levels in the cytoplasm, resulting in hyperactivity in calcium-dependent chloride channels, and leading to excess chloride in the lumen to force additional water transport, giving the common symptom of diarrhea [142, 143]. This theory was supported when Seo and colleagues established mutated NSP4 proteins, and the mutants failed to signal after binding to integrins α1 and α2, resulting in reduced symptoms in neonatal mice [142]. The study additionally supports that NSP4 is released from infected cells and continues to interact with the integrins on neighboring healthy cells leading to damage and resulting in diarrhea. Another group has suggested that NSP4 has the capability to induce the production of 5-hydroxytryptamine (5-HT), a component released by enterochromaffin cells (ECs) serving as the release mediators to activate the ENS [144]. This is significant because the EC cells are the only cells to synthesize serotonin in the digestive tract, which plays a key role in essential homeostasis regulation of gut motility [144-148]. Serotonin regulates a multiple systems, including intestinal secretion, blood flow, and

cytokine levels [146, 148, 149]. Specifically, the serotonin receptor 5HTR influences cytokine transport and cell activation, while also influencing T cell and B cell proliferation [150-154]. These have been previously shown to be affected during viral infections, inducing gastroenteritis and enterotoxin-induced vomiting [144-149]. The significant consequence of serotonin and 5HT release is that enteric nerves undergo activation, and intestinal motility is increased, leading to decreased transport times, spreading of RVA virions (including NSP4), resulting in significant water transport, viral induced diarrhea, and subsequent dehydration. At this point it is important to highlight impact of age of the host for RVA infections, as the rate of epithelial cell replacement, mucin production, and absorption rates are less adaptive at younger ages [155, 156]. In contrast, RVA infection requires the protease-mediated cleavage of VP4 into VP5 and VP8, and newborns have low levels of protease [157]. In combination these are the possible causes of the increased severity of RVA infection between ages 3 months to 5 years for children [158].

As previously stated, enterotoxin induced vomiting from the NSP4-dependent 5HT release is another common symptom. Therefore, anti-emetic 5HTR antagonists are commonly used in combination with supportive therapies to treat RV infections [159]. The severe dehydration caused by RVA infection is also the reason for the characteristic signs of RVA including dry mouth, crying with no tear production, and decreased urination [160].

Besides other factors affecting RVA replication, cellular cholesterol has been shown to play a prominent role in RVA assembly. Lipids rafts contain sphingolipids, cholesterol, protein kinases, and glycosylphosphatidylinositol (GPI)-anchored proteins [161-165]. Of these, cholesterol is a key component of lipid rafts - specialized membrane microdomains responsible

for membrane trafficking, signaling, cell polarization and signal transduction. For viruses, the cholesterol domains are an essential first step for viral binding and cell entry [161, 164, 165]. The presence of lipid rafts and their cholesterol domains is a necessary factor for replication of RVAs of different origin. Cell entry of most RVAs such as bovine RVA, human strain Wa, and porcine strain TFR-41 is clathrin-mediated, meaning they depend on endocytosis to be performed in the presence of cholesterol [166]. Furthermore, cholesterol depletion has been shown to decrease RVA and RVC replication [61]. Cholesterol depletion can be achieved using a pharmacological depletion agent Methyl-β-cyclodextrin (MβCD) which can often reveal viral mechanisms and their dependence on cholesterol. This method has been used to test many strains including bovine RVA to evaluate the role of cholesterol in virus cell entry [164]. We have recently shown that RVC Cowden is dependent on cholesterol, as replication was inhibited with MβCD, but partially reversed with cholesterol restoration [61]. Another way to explore the role of cholesterol in RVA infection is using bile extracts. Interestingly, bile extracts have been shown to either decrease or increase replication of enteric viruses *in vivo* and *in vitro*. Primary BAs such as cholic acid and chenodeoxycholic acid have been shown to have an inhibitory effect on RVA infection, leading to significant investigations on the role of BA on enteric virus replication [167]. The replication of other viruses such as porcine deltacoronaviruses have also been examined with the addition of BAs, showing similar antiviral and inhibitory effects post-entry into cells [168]. We have further explored this for RVCs; however, no effect on RVC replication in intestinal enteroids after treatment with a bile extract has been demonstrated [61]. This is likely due to the fact that bile extracts contain various bile acids that may have opposing effects on viral replication.

Furthermore, polycation DEAE-dextran has been shown to facilitate virus adsorption to cells suggesting that cell culture additives could be used to provide optimal cell-growth conditions [169]. This evidence may be especially important for viruses which poorly replicate *in vitro*. In our lab, we have found that DEAE significantly increased RVC attachment, but it did not influence replication [61].

1.7 Immunity

Innate Immunity

Like majority of pathogens, RVA is initially recognized by pattern recognition receptors (PRR) that are present in immune cells and enterocytes [170]. The innate immune response is rapidly triggered after infection with RVs. This initial reaction induces the production of cytokines, including interferons (IFN, type 1 and 3) to reduce viral replication [170]. Type 1 IFNs such as IFN-α isoforms and IFN-β (IFN-α/β) often play vital roles in defense against infections, and typically have antiviral properties [170]. Type 3 IFNs have also began to be extensively investigated, particularly IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) that have been shown to have similar response mechanisms in cells [170-173]. Recently, interferon-λ has proven to be a more potent antiviral compared to type 1 IFNs, α and β for RVs [172][170-173]. Recently, interferon-λ has proven to be a more potent antiviral compared to type 1 IFNs, α and β for RVs [172].

Differential IFN signaling occurs as type III signaling through heterodimeric surface receptors IFNLR1 (lambda receptor, specific only for IFN-λ) and IL10RB (shared by IL-10

cytokines) [170-173]. Type 1 uses IFNAR, with subunits IFNAR1 and IFNAR2 (alpha receptors) [174]. After binding for both type 1 and III, they both trigger the Jak-STAT signal transduction pathway, which is an essential pathway in immune response as it mediates cell response to cytokines, interleukins, growth factors, and other homeostatic processes by upregulation of the genes called IFN-stimulated genes (ISGs) [170, 175-177]. ISGs such as interferon regulatory factors (IRFs) and PRRs mediate viral infection and modulate immune responses [178]. ISGs perform a range of functions and control many viral infections by inhibiting pathways required for pathogen life cycle [178, 179]. Type 1 uses IFNAR, with subunits IFNAR1 and IFNAR2 (alpha receptors) [174]. After binding for both type 1 and III, they both trigger the Jak-STAT signal transduction pathway, which is an essential pathway in immune response as it mediates cell response to cytokines, interleukins, growth factors, and other homeostatic processes by upregulation of the genes called IFN-stimulated genes (ISGs) [170, 175-177]. ISGs such as interferon regulatory factors (IRFs) and PRRs mediate viral infection and modulate immune responses [178]. ISGs perform a range of functions and control many viral infections by inhibiting pathways required for pathogen life cycle [178, 179].

Lin and colleagues have found that both type I and III are associated with RV response, inducing retinoic acid-inducible gene (RIG-I) or melanoma differentiation-associated gene 5 (MDA5) [119]. Additionally, MDA5 and RIG-1 have been proven previously to participate in the recognition of RVs on human IECs, and in bone marrow macrophages [180-182]. The cytoplasmic RNA sensors of RIG-I and MDA5 can also be found on toll-like receptors (TLRs) 3, 7, and 8 [183]. TLR3 induces production of IFN-β, while TLR7 and 8 signal myeloid differentiation response 88 (MyD88), activating IFN expression and IFN response factors (IRF)

[183]. However, there was no consensus regarding the role of TLR3 during RVA infection, possibly due to replication capacity of the models used [158]. The main inconsistences stem from a report by Broquet showing that TLR3 induced Trif was required for the induction of IFNs in adult mice, but not in young mice [184]. This seemed to imply that TLR3 expression is increased with age in mice [183]. However, there was no consensus regarding the role of TLR3 during RVA infection, possibly due to replication capacity of the models used [158]. The main inconsistences stem from a report by Broquet showing that TLR3 induced Trif was required for the induction of IFNs in adult mice, but not in young mice [184]. This seemed to imply that TLR3 expression is increased with age in mice [184]. However, another study demonstrated an upregulation of TLR3 in infant mice during RVA infection, but also that defense at this age is Trif independent [185]. Comparatively, this study concluded that adults rely heavily on TLR3/Trif immune responses for RVA defense.

Nonetheless, TLRs are essential for pathway regulation receptors that control innate immune responses to a majority of viral pathogens, as they are the primary transmembrane proteins of immune cells. Several studies have concluded that T helper (Th) cytokines Th1 and Th2 are released into serum in response to RV infections [186-188]. These results have also showed that the Th1 response is the primary result of RV infection, as CD4 cells secreted more IFN-γ than IL-13.

Adaptive Immunity

Acquired virus-specific cellular and humoral immune responses are also elicited by RVA infection and vaccination [189, 190]. A previous study followed children that were infected with

RVA and investigated the associated immune responses, demonstrating their protective role against subsequent RVA infection [191]. While the first infection provided 87% protection against moderate or severe disease, a second infection led to 100% protection. It was also found that both symptomatic and asymptomatic infections provided a similar degree of protection, indicating the significance of RVA exposure regardless of symptoms [191, 192]. A study from the UK that aimed to evaluate natural RVA infection found that previous infections reduce risk of disease development, but do not protect against reinfection [192]. Further, Chiba and colleagues were the first to present evidence of serotype specific protection against RVA by evaluating the amount of neutralizing (Nt) antibodies using stool samples and ELISA techniques. [193]. In their conclusions, a Nt antibody level of >1/128 seemed to be protective against RV infection. However, this study also demonstrated that immunity was short lasting and possibly not through classic neutralization. .[189, 190]. A previous study followed children that were infected with RVA and investigated the associated immune responses, demonstrating their protective role against subsequent RVA infection [191]. While the first infection provided 87% protection against moderate or severe disease, a second infection led to 100% protection. It was also found that both symptomatic and asymptomatic infections provided a similar degree of protection, indicating the significance of RVA exposure regardless of symptoms [191, 192]. A study from the UK that aimed to evaluate natural RVA infection found that previous infections reduce risk of disease development, but do not protect against reinfection [192]. Further, Chiba and colleagues were the first to present evidence of serotype specific protection against RVA by evaluating the amount of neutralizing (Nt) antibodies using stool samples and ELISA techniques. [193]. In their conclusions, a Nt antibody level of >1/128 seemed to be protective against RV

infection. However, this study also demonstrated that immunity was short lasting and possibly not through classic neutralization.

The reason behind the reduced disease severity in recurrent RVA infections is due to the primary roles of T and B cells. Mice were initially studied to investigate the role of T cells, specifically cytotoxic CD8 cells known to induce cell lysis/apoptosis. Their roles were explored with the use of B cell deficient mice and CD8 depletion, proving that both B cells and CD8 production are essential for RVA disease burden resolution [194]. Other studies have been performed using various models, including the use of gnotobiotic (Gn) piglets to examine RVA specific antibody and IFN-γ producing CD4+, CD8+ and CD4+CD8+ T cell responses [195, 196]. Data generated in both our lab and studies by others emphasized the role of intestinal IFN-γ producing CD4+ T cells and their significant association with protective immunity and infer that this role is possibly more important than CD8+ T cells [196-198]. Compared to studies of mice, these studies generated additional conclusions on the importance of CD4+ T cells that they were the key lymphocytes required for RV clearance [196-198]. This was an important conclusion, as there have been few studies regarding the inclusion of double positive T cells and their role in RV immunity, as a majority of studies focus on individual T cells [196-199]. A study in rhesus macaques demonstrated similar findings, where intestinal double positive T cells had increased capacity to produce protective cytokines [200]. The key protective role of CD8+ cytotoxic T cells has been demonstrated for RVA clearance, however, they did not provide long term protection, leading to a risk of reinfections [201, 202]. These studies performed by Franco and colleagues concluded that CD8+ T cells were able to produce protection for up to 5 months from viral reinfection.

Our lab has investigated the role of immunological B-cell memory, development, and protection [196, 203-205]. We have demonstrated that significant amounts of IgA and IgG memory B-cells offered complete protection against virulent Wa HRV (mimicking natural infections) strain shedding and symptoms [205]. Paired with this, we observed a high number of ASCs in the ileum, reporting that IgA ASCs in the intestinal propria correlated with protection against subsequent infections when challenged with additional HRVs. In contrast, IgG did not have the same correlation. It is important to note that memory B-cells must be activated by recall antigens, which can subsequently differentiate into ASC, and secrete high number of antibodies, paired with high affinity, to protect against reinfection [205, 206]. This is contributed that memory B cells need lower activation requirements, increased capacity to present antigens, and are capable to colonize specific sights to present antigens where T cells would be needed (such as in mucosal epithelium) [205, 206]. In summary, IgA ASC in serum could correlate to protective active immunity against RV disease [204].

1.8 Host evasion and evolution mechanisms

Despite the induction of host immune system involving sophisticated mechanisms in response to RVA infections, RVs still have a significant worldwide impact today [207]. RVAs continuously evade the immune system and evolve several strategies to continue effective transmission and infection [51, 138]. This goes hand in hand with the previous discussion of multiple co-receptors being recognized by various RVAs, as they have adapted mechanisms such as degradation of IFN regulation factors, inhibition of signal transducer and activator of transcription 1 (STAT1) and STAT2 to inhibit the JAK-STAT pathway, degrade IRF3/7, block

the NF-kB pathway used to alternatively activate IFNs, cloak viral clusters behind cell membrane-derived vesicles to go undetected, and create calcium waves just to name a few [121, 158, 208-210].

Nonstructural protein NSP1 often serves as the primary antagonist for the host immune system, as its main purpose is to enable infection [211]. Both studies in murine and bovine species have shown that NSP1 is capable of degrading IRF3/7 and thus the IFN induction pathway is shut down, decreasing cellular defense mechanisms against RVA infection [212-214]. An *in vitro* study demonstrated that NSP1 is capable of translocating IRF3 to the nucleus by inducing proteasome-dependent degradation, preventing its ability to signal the IFN pathway [212]. A similar role of NSP1 in proteasome-mediated degradation of inhibitor κB (IκBα) further emphasized the importance of this non-structural protein in RVA infection [212]. NSP1 has further been proven to induce degradation of IRF5 and IRF7 in a similar manner, all concluding in the ability to cease the IFN pathway [208-214]. Regarding STAT1 and STAT2, these functions are also inhibited by RVA by an unknown mechanism [51, 210]. However, the ability of RRV and human RVA, Wa G1P[8] to prevent nuclear accumulation of the STAT1 and STAT2 proteins has been documented. [158]. Interestingly, the role of NSP1 has been found to vary among RVA strains, as several studies have shown that NSP1 of porcine RVA G5P[7] OSU failed to translocate IRF3, but prevented the NF-kB induction pathway [184, 210]. Regarding STAT1 and STAT2, these functions are also inhibited by RVA by an unknown mechanism [51, 210]. However, the ability of RRV and human RVA, Wa G1P[8] to prevent nuclear accumulation of the STAT1 and STAT2 proteins has been documented. [158]. Interestingly, the role of NSP1 has been found to vary among RVA strains, as several studies have shown that

NSP1 of porcine RVA G5P[7] OSU failed to translocate IRF3, but prevented the NF-kB induction pathway [184, 210].

In conclusion, while the IFN-signaling pathway is complex, RVAs have the ability to subvert it, protecting themselves from the IFN-signaling cascade. It has been inferred that RVA NSPs constantly work to impede this host response, and the details of this are still unknown. However, it is known that the role of NSP1 and NSP2 is vital to viral replication, and these proteins work to impede apoptosis and immune responses to allow for RVA infection.

1.9 Rotavirus A control and prevention

Although RVA is a major cause of acute diarrhea, the mechanisms of RVA attachment/entry remain poorly understood. Together with the emergence of new strains, monitoring of RVA infections are essential to combat the high RVA prevalence and RVA-associated disease by revisiting both currently available vaccines and other preventative strategies. As was mentioned before, the most vulnerable group for RVA infection is young mammals including children from 3 to 12 months of age; thus, updating vaccination protocols to enhance lactogenic or mucosal protection is a key component to control RVA infection. For example, supplementation of pregnant sows with vitamin A has been shown to enhance passive immunity against another enteric pathogen - porcine epidemic diarrhea virus [40, 48].

Treatment of RVA-associated gastroenteritis relies heavily on supportive care, such as oral rehydration therapy (ORT) or treatment with intravenous fluids to counter dehydration [215]. Due to the high RVA transmissibility and impact of RVA infection, the prevention with

vaccines was essential [215, 216]. Development began in the mid-1970's, but there was little success in inducing protection against multiple heterologous RVA strains [217]. Careful evaluation of these data prompted the process of multivalent vaccine development that had a primary focus on several dominant VP7 genotypes [122, 215, 217]. The first of these was a multivalent, live, oral vaccine named RotaShield, a rhesus tetravalent vaccine [215, 216]. Development began in the mid-1970's, but there was little success in inducing protection against multiple heterologous RVA strains [217]. Careful evaluation of these data prompted the process of multivalent vaccine development that had a primary focus on several dominant VP7 genotypes [216]. This was a very successful vaccine that demonstrated high efficacy in RVA infection prevention, while reducing disease symptoms [218, 219]. However, this vaccine was associated with an increased risk of ileocolic intussusception, a condition where segments of the intestine would slip inside another and cause obstruction .[122, 215, 217]. The first of these was a multivalent, live, oral vaccine named RotaShield, a rhesus tetravalent vaccine [220-222]. This was a very successful vaccine that demonstrated high efficacy in RVA infection prevention, while reducing disease symptoms [218, 219]. However, this vaccine was associated with an increased risk of ileocolic intussusception, a condition where segments of the intestine would slip inside another and cause obstruction [220-222]. This resulted in subsequent removal of this vaccine from commercial use. However, it was concluded that the risk was associated with age of the recipient and possibly some other environmental/host factors, and not the vaccine itself [220-222].

To date, there are two oral, live attenuated vaccines that have been used since 2006. They include the three-dose RotaTeq (Merck & Co) and the two dose ROTARIX (GlaxoSmithKline).

ROTARIX consists of a single strain of human RVA G1P[8], while RotaTeq is comprised of five strains of human-bovine reassortant RVAs. Both show 80-90% protection against severe diarrhea and mortality in developed countries [223, 224]. However, in developing countries efficacy is less than half of this [223, 225, 226]. However, in developing countries efficacy is less than half of this [223, 225, 226]. This can be partially attributed to factors such as malnutrition (more specifically: vitamin A deficiency, protein deficiency), intestinal dysbiosis as well as poor hygiene and sanitation resulting in high exposure to RVAs and other pathogens [227, 228]. Additionally, the current emergence of genetically divergent strains such as G9 and G12, may negatively impact human RVA vaccine efficacy rates [229]. Furthermore, RVA Wa G1P[8] has been shown to elicit only partial protection against emergent PRVA G9P[13] suggesting that along with the factors mentioned above, efficacy of RVA G1P[8] based vaccines may be limited by the unique properties of G9 strains [230]. This is crucial, as emerging G9 RVs in humans such as G9P[4], G9P[6], and G9P[2] to name a few are phylogenetically similar to PRV rather than historical G9 strains. This illustrates the potential for zoonotic transmission of animal RVs, and the potential source for heterologous infections in humans.

RVA vaccines have also been introduced for calves and swine, although their efficacy is limited [231]. Induction of maternal immunity is a key to protect newborn piglets against RVA infection [120]. The current system includes the vaccination of sows and gilts with ProSystem Rota vaccine based on G4 and G5 genotypes of RVA that stimulates production of maternal antibodies that are transferred to suckling piglets [232-234]. There are ongoing efforts to expand this and develop a live attenuated trivalent RVA vaccine consisting of predominant in other countries such as G8, G9, and G5 [235]. Of importance, there is speculation regarding a previous

commercial vaccine based on the A2 strain (that bears serotype G9 specificities), which could have contributed to the diversity and spread of G9 in swine in the United States [229, 230]. The prolonged use of this vaccine potentially could have introduced G9 into swine populations that had no herd immunity, as A2 VP7 was shown to belong to lineage 3 (strains that have emerged in the mid-1990s) of G9. The current RVA vaccine for swine named ProSystem Rota (Intervet Inc./Merck Animal Health) is less used in sow or piglet vaccination routine as it does not guarantee prevention of RVA infection and the efficacy is limited [18, 231, 236]. Moreover, the American Veterinary Medical Association, the board of the American Association of Swine Veterinarians, and the Pork Quality Assurance® Plus (PQA Plus®) programs do not recommend using of vaccination against RVA as there is variable success, often suboptimal cost-benefit ratio, and the speculation of risk from live vaccine contribution to genetic diversity via reassortment among vaccine and wild-type stains. Thus, most farms omit the use of a RVA vaccine until diarrhea becomes a concern. Among other factors, including specific features of RVA G9P[13], wide prevalence of this genotype may be due to the absence of commercially available vaccines based on this genotype. However, more data needed to evaluate efficacy of RVA vaccines available in field experiments.

There are two commercially available vaccines for calves, based on two genotypes of RVAs (G6 and G10) that have been developed by Merck (Bovilis Guardian) and Zoetis (Scourguard 4KC). These are administered to pregnant cows to pass antibodies to calves through colostrum to reduce calf mortality and the incidence of diarrhea. RVAs have been found to be the most predominant species found in cattle, with one study finding 85% of samples testing positive in Germany [237, 238]. RVC was also detected, at a much lower percentage (31%), and

RVB was only found in 3% of samples. However, RVB and RVC RT-PCR detection assays were relatively new and are potentially understudied due to the comparative high prevalence of RVA [238].

Despite their recognized significance, there are no current vaccines available for protection against RVA infection caused other than group A RVs. Thus, while some successful tools have been developed to control RVA infection, protection against RVB and RVC as well as against new emergent RVA strains is poorly investigated.

1.10 Animal models

Rotaviruses have been studied in multiple animal models including mice, rabbits, rats, lambs, calves, goats, swine, and even neonatal monkeys [239-242]. Historically, mice have been the primary animal model for RVA infection due to many factors such as price, abundance, availability of various gene knock-out models, immunologic reagents, and ease of housekeeping. However, this model has been limited due to a variety of factors: only 15 days of susceptibility to RVA infection, the inbred status, the lack of clinical disease associated with heterologous RVA infections, etc [242]. Despite these factors mice are still used today to investigate RV pathogenesis, host immune response and the role of other factors such as the role of the gut microbiota [243]. Models such as lambs, goats, calves, and camelids are typically not suitable for RVA studies unless colostrum deprived and manipulated in high security isolation, as most of them have been infected previously with RVAs [244]. This does not mean there is no current disease burden in these species, as lambs alone have been found to have remarkable diarrhea-related mortality rates of up to 46% [245-247]. RVA has been shown to be an important

pathogen for these animals reaching as high as 60% prevalence worldwide and being associated with 15% mortality [245, 248, 249]. The lack of exploration in sheep can also be attributed to limited information on ovine specific RVA strains. Caprine RVAs have approximately the same prevalence rate as ovine RVA but have a wider range of infectious strains where RVA, RVB, and RVC have been found [248][239-242]. Historically, mice have been the primary animal model for RVA infection due to many factors such as price, abundance, availability of various gene knock-out models, immunologic reagents, and ease of housekeeping. However, this model has been limited due to a variety of factors: only 15 days of susceptibility to RVA infection, the inbred status, the lack of clinical disease associated with heterologous RVA infections, etc [242]. Despite these factors mice are still used today to investigate RV pathogenesis, host immune response and the role of other factors such as the role of the gut microbiota [243]. Models such as lambs, goats, calves, and camelids are typically not suitable for RVA studies unless colostrum deprived and manipulated in high security isolation, as most of them have been infected previously with RVAs [244]. This does not mean there is no current disease burden in these species, as lambs alone have been found to have remarkable diarrhea-related mortality rates of up to 46% [245-247]. RVA has been shown to be an important pathogen for these animals reaching as high as 60% prevalence worldwide and being associated with 15% mortality [245, 248, 249]. The lack of exploration in sheep can also be attributed to limited information on ovine specific RVA strains. [245, 248, 249]. The lack of exploration in sheep can also be attributed to limited information on ovine specific RVA strains. Caprine RVAs have approximately the same prevalence rate as ovine RVA but have a wider range of infectious strains where RVA, RVB, and RVC have been found [248].

Animal model choices to study humans should be based on their ability to reproduce a multitude of aspects accurately and efficiently of the disease in children. In response, swine are widely accepted as a primary RVA model for several reasons including anatomy, genetics, physiology, metabolism, omnivorous eating habits, overall immunity, availability, and lifespan [250-253]. Pigs are less costly and ethically are accepted over primates, which would be most likely the ideal model for human medical comparisons [254]. These factors indicate that of all possible animal models, swine are likely to have predictive characteristics that can be compared to humans [255].

Additionally, Gn piglets provide a physiologically relevant and robust model, serving as an ideal representation from the lack of confounding commensal microbes that are typically found in nature [195, 204, 241, 255]. The models do have a unique housing requirement, as these piglets must be delivered by caesarian section and placed in sterile isolators to assure germfree status. Gn piglets can also be supplemented with various nutrients and colonized with microorganisms of individual choice to re-create complete or simplified human gut microbiota signifying that piglets provide useful models for human RV infections allowing for highly controlled in-depth pathogenesis, immunological, and vaccine studies [241]. These piglets are immunocompetent, but immunologically immature [227, 250, 255].

The Gn pig model has previously been shown to be an exceptional model to evaluate immune response by reducing environmental impacts. RVA immune responses have been investigated using this model due to the fact both porcine and human RVAs cause clinical symptoms in Gn piglets and the advantage of resemblance of human gastrointestinal physiology and mucosal development [18]. We have inoculated neonatal Gn piglets with human Wa RVA,

and determine Nt activity against RVAs [18, 241]. For optimal experimental design, neonatal piglets can be inoculated orally with RVA strains to mimic natural RVA infection. Furthermore, these piglets can be inoculated with attenuated RVAs to mimic the use of live oral vaccines [241]. In our lab, we have examined IgM, IgG, and IgA antibody secreting cells (ASCs) in both intestinal and systemic tissues [204]. In addition, we used enzyme-linked immunosorbent assays (ELISA) to detect antibodies in serum, and importantly in intestinal contents. In summary, IgA showed a significant positive correlation with the overall protection against RVA [195, 204]. Moreover, we have shown extensively the ability of this model for variety of research questions, including the role of malnutrition, vitamin A and gut microbiota on RVA infection and vaccines [61, 255-257]. In conclusion, Gn piglets are an extraordinary model to study RVA pathogenesis and immunity to infection and vaccines.

1.11 Organoids and enteroids

RVA interactions with cellular receptors are usually genotype specific suggesting the need to develop an *in vitro* model for the evaluation of RVA-host interactions. Along with a cost of animal use this also raises a concern of ethical treatments of animals, and there are few *in vitro* models that recapitulate the intestinal epithelium to an extent of allowing viral replication and analysis. However, for some RVs such as RVB an established cell culture system has not been developed. In recent years, enteroid cultures have been established that have proven closely mimic the events occurring in enteric virus infections [61, 62, 258].

Intestinal enteroids are *in vitro*, three-dimensional culture systems that mimic the anatomy, morphology and functions of the small intestine [61, 62, 258-260]. In our lab, we have

successfully established porcine small intestinal (ileal) enteroids (PIEs) by isolation of intestinal epithelial stem cells in crypts of the small intestine from gnotobiotic piglets and their culture in Matrigel to support the growth and 3D structure [61, 62]. Of significance, these crypt cells still maintain their *in vivo* morphology, physiology and functions giving rise to stem cells, creation of amplifying cells, and the maturation of enterocytes, goblet cells, and Paneth cells. Our lab was successful in using PIEs to acquire a physiologically functional model that express various HGBAs including A+, H+, and A+/H+. From this establishment, we were able to investigate RVA infection, and conclude the HGBA preference of both RVA and RVC strains, and their individual ability to replicate in established porcine intestinal enteroids (PIEs) [61]. For example, RVA Wa G1P[8] has been shown to reach higher titers after infection of PIEs expressing A antigen, compared to other RVAs: G9P[13] and G5P[7] OSU which demonstrated higher replication in H-antigen expressing PIEs. Additionally, this model was helpful to elucidate the RVA and RVC interactions with terminal SAs. These studies not only confirmed the previous findings regarding the critical role of SAs in replication of some RVA and RVC but also demonstrated that removal of terminal SAs led to increased replication of porcine RVA G9P[13] and porcine RVC G3P[18]. While the role of structural and non-structural proteins of RVA has been studied for a long time, little is known about host cellular response to RVA infection. Thus, enteroids/organoids are an appropriate tool for evaluation of cellular host response to RV infection. For example, a detailed transcriptome analysis usually requires use of an animal model or may be done during clinical studies compared to enteroids use that allows to control experimental conditions at a lower cost [261, 262].

Enteroids could also have the potential to create individual medical care, as they can be established using small amounts of intestinal tissue (biopsy size) obtained from a patient and will retain the unique identity of the tissue [263]. This model recently has been advanced, allowing for apical facing or "inside-out" enteroids to be established. The polarity of this system allows for a comparatively uncomplicated imitation of natural infection processes [264]. This alteration of the enteroids proves to show an even closer physiological model, while maintaining the inexpensive and uncomplicated establishment of enteroids to research host-pathogen interactions [264]. In conclusion, enteroids recapitulate cell lineage and allow for a complex and physiologically authentic model for study, with the downside of no immune cells.

1.12 Reverse genetics systems

Reverse genetics has been shown to be a powerful tool used to study aspects of viral biology, including virus-host interactions. This approach has been vital in the development of vaccines, viral vectors, and therapeutic interventions for many RNA viruses due to the ability to generate recombinant viruses [265-267]. Specifically, the reverse genetics system (RGS) generates replication-competent viruses formed from genetically manipulated and artificially synthesized RNA or DNA, and only recently has RGS become available for viruses such as RVAs. There were previous challenges such as genome stability due to the segmented nature of the RVA genome, and the addition of requiring the use of multiple plasmids [29]. With this overcome, RVAs now have the ability to be divided into two groups: helper-virus-dependent to use helper viruses, mainly influenza A, to serve as a gene replacement backbone and plasmid-

based, using 11 plasmids encoding cDNAs for the individual segments with each RV cDNA flanked by a T7 RNA polymerase promoter [29, 267, 268].

RGS have been used to rapidly engineer viruses with mutations to determine the impact it can have on viral pathogenesis [267, 268]. This is due to an ability to introduce mutations directly into the target DNA sequences in any gene [29]. The flexibility of the RGS is seemingly endless, allowing the design of viruses with reporter genes, fluorescent proteins, insertion of additional segments, and overall complete manipulation with the viral genome [266, 269]. Once established, RGS seemingly offer endless manipulation for study designs. These include the potential to use the previously stated flexible additions, paired with studies of host-virus interactions, protein function, and virus replication steps [29]. Over the last few years, RGS efficiency has greatly improved, and barriers are consistently being lifted as reports are being published discussing novel molecular breakthroughs.

This technology has been utilized for several viruses in the *Reoviridae* family, including RVA, and recently advanced for use for a 12-segmented dsRNA virus [266]. Previously, the process to fully study the genotypes of RVs involved the inclusion of mutations into an expression system, which was often severely time consuming and labor intensive, while also giving inconclusive results despite all efforts. Advances have also been made to this system already with establishment of simplified systems based on co-transfection of T7 transcription vectors and capping enzymes [270]. Together, these factors allow for generation of recombinant RVAs that retain completely functional viral proteins without deletion or alterations made to open reading frames, which has previously been unachievable .[266]. Previously, the process to fully study the genotypes of RVs involved the inclusion of mutations into an expression system,

which was often severely time consuming and labor intensive, while also giving inconclusive results despite all efforts. Advances have also been made to this system already with establishment of simplified systems based on co-transfection of T7 transcription vectors and capping enzymes [270]. Together, these factors allow for generation of recombinant RVAs that retain completely functional viral proteins without deletion or alterations made to open reading frames, which has previously been unachievable [270].

Prior to RV RGS development, there was a heavy reliance on traditional cell culture reassortment techniques. Currently, one of the few issues with RGS is the addition of large segments, which could be inefficient but seem to be overcome in plasmid-base systems [29]. Utilization of a plasmid-based system allows for interrogation of gene functions. This system is an incredible tool that is just recently being investigated [29]. The combination of the decades of RVA research and RGS has the potential to address the remaining knowledge gaps regarding RVA biology, including virus replication, pathogenesis, segment assortment, and the key steps of viral cell attachment/entry. The recent and growing success of RGSs continues to aim for next-generation vaccine development, while combining ideal characteristics of models such as little cost paired with high efficacy.

1.13 Summary

This literature review provides a comprehensive up-to-date summary of research performed on RVAs, which continue to be a leading cause of severe diarrheal illness worldwide.

Recent outbreaks such as RVA G9P[13] and growing reports of RVC infections show that despite the extensive research done on RVs, challenges remain for full comprehension. Moreover, interactions of RVA even with the first recognized RVA receptor – SA remain incompletely understood. Relevant studies may help to understand the wide and population-specific prevalence of new RVA strains including RVA G9P[13] and RVCs. Ongoing research often leads to identification of novel areas of exploration, including studies exploring HBGA and other receptors that have shown a strong association with replication of different RVA and RVC strains, but lack on how therapies and vaccine efficacy is impacted understanding. Furthermore, RVA replication is also affected by host cellular responses including immune response, cholesterol metabolism, and expression of RVA receptors on cellular surface and in the external environment such as the mucous layer. Thus, understanding of mechanisms involved in RVA interactions with cells, and the particular role of RVA proteins in replication is necessary to develop new tools for protection against RVA infection. To dissect genotype-specific interactions with cellular receptors, evaluate the host cellular response to RVA infection and study the role of cholesterol metabolism, we have chosen a complex of *in vitro* assays. First, PIEs which mimic the anatomy, morphology and functions of the small intestine allow us to assess host cell response to RVA infection. Second, the use of RGS is a well-established approach in order to evaluate the role of unique mutations in virus replication. Finally, *in vitro* studies have been used in the past to investigate the role of cholesterol and different additives on RVA attachment/replication. The growing prevalence and emergence of reassorted RVA strains additionally leads to the question if currently available vaccines remain sufficient to protect both commercial livestock, and to minimize infections in children. As we discussed, there is evidence

suggesting limited protection against RVA G9P[13] provided by vaccines. This emphasizes the need of continuous molecular evaluation as the introduction of novel and zoonotic RVA reassortments arise through complex evolutionary dynamics that compromise our current vaccine protection, and potentially have the capability to increase disease burden rates in both humans and animals.

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Chapter 2. Host cell response to rotavirus infection with the emphasis on virus-glycan interactions, cholesterol metabolism and innate immunity.

2.1 Summary

Although, rotavirus A (RVA) is the primary cause of acute viral gastroenteritis in the young of humans and animals, mechanisms of its replication and pathogenesis remain poorly understood. Recently we demonstrated that neuraminidase-mediated removal of terminal sialic acids (SAs) significantly enhanced RVA G9P[13] replication, while inhibiting RVA G5P[7] replication. This coincided with the presence of strain-specific mutations in the VP4 protein hydrophobic loop.

To identify the molecular mechanisms associated with this dichotomic pattern of RVA-SA interactions, we compared transcriptome responses of porcine ileal enteroids to G5P[7] vs. G9P[13] infection. The analysis demonstrated that G9P[13] infection significantly downregulated expression of multiple sialyltransferase genes. Further, G5P[7] affected several signaling pathways related to immune response, while the G9P[13] top affected signaling pathways were related to cell cycle regulation and receptor expression pathways. Both strains altered signaling pathways controlling immune cell proliferation, chemotaxis, and inflammation. Top canonical impacted cellular pathways were those associated with cancers, organismal injury/abnormalities, inflammation, endocrine system disorders, dermatological disease, and fertility.

Because cholesterol is a principal component of cell membrane, we compared the effects of cholesterol-related additives on G5P[7] and G9P[13] replication in MA104 cells. While

replication of both strains was significantly enhanced following addition of cholesterol, bile acids (BA), and diethylaminoethyl-dextran, transcriptome analysis demonstrated that the two strains (G9P[13] to a greater extent) differentially affected lipid metabolism and BA production. Our results highlight the mechanisms regulating cellular response to RVA infection relevant to RVA-receptor interactions, metabolic, and immune signaling pathways critical for design of effective control strategies.

2.2 Introduction

Rotaviruses (RVs) are a global leading cause of acute viral gastroenteritis in both children and production animals [1]. The global burden of this infection is estimated to be 258 million cases of diarrhea in children under the age of five, with 215,000 deaths attributable to RV infection [1-3]. This remains the third-rated pathogen associated with childhood mortality and 24% of overall deaths in piglets [3, 4].

As a member of the *Sedoreoviridae* family, *Rotavirus* genus, this nonenveloped virus possesses 11 segments of double-stranded RNA (dsRNA) in a triple-layer viral capsid. These 11 segments encode dsRNA for structural: VP1-VP4, VP6, VP7, and non-structural proteins (NSP): and NSP1-5/6 [5]. Major antigenic properties are determined by the viral capsid proteins (VPs), while NSPs are essential for viral replication and pathogenesis [6]. Two of the structural protein genes, VP7 (glycoprotein, G-genotype) and VP4 (protease-sensitive, P-genotype), encode the outer capsid proteins and are the primary focus for epidemiological and immunological studies as they are essential for initial attachment and penetration into cells, and they independently

elicit virus neutralizing antibodies. RVs are classified into nine genetically distinct groups as RV A-D, F-J which are further subdivided into distinct genotypes following the binary classification system [7]. The RV genotyping binary classification system is based on the two capsid proteins and follows GxP[x] based on the sequence diversity [8]. Group A rotaviruses (RVA) are endemic globally as the most prevalent and pathogenic among the nine RV groups, accounting for >90% of RV gastroenteritis cases [7, 9-12].

RVAs have been consistently recognized as a cause of diarrhea in young piglets, and there is a pattern of re-emergence of common RVA genotypes (specifically, G1 and G9) that reach the prevalence of 61%–74% across different pork production systems [13, 14]. Historic studies identified porcine RVA G5P[7] as the dominant G-P combination, while our recent research demonstrated its reduced prevalence, while G9P[13] has emerged as the most prevalent genotype combination [14, 15]. Interestingly, these two viruses, G5P[7] and G9P[13] have been shown to differentially interact with one of the major receptors for RV entry – sialic acid (SA) [16].

Besides presence of receptors for RVA entry/attachment, some components of the plasma membrane such as cholesterol have been demonstrated to play a critical role in RVA replication [17-19]. Moreover, there is strong evidence that cellular glycans can directly bind cholesterol and alter lipid membrane dynamics and organization [20, 21]. This suggests that differential interactions with host glycans are likely to be associated with variable effects of RVAs on cholesterol/lipid metabolism. Further, similar to many viruses, cholesterol was shown to be an essential factor for RVA infection while also being an important component in intestinal physiology and antiviral responses [22-24]. These processes are attributed to the initial

interaction between RVA VP4 and co-receptors found on lipid rafts and cholesterol rich domains promoting the cleavage of VP4 into VP5* and VP8*, endocytosis or direct membrane penetration into the host cell, followed by the assembly of RVA [23]. Although there has been evidence supporting the essential role of cholesterol in RVA replication, the relationship between RVA replication and the biosynthesis of cholesterol is unknown [25]. Finally, it is not understood how the immune signaling pathways are modulated in response to complex and variable interactions among RVAs, host glycans and cholesterol.

Thus, the main goal of this study was to dissect the host response profiles induced by infection of porcine ileal enteroids (PIEs) with two distinct RVA strains with the emphasis on virus-glycan interactions, cholesterol metabolism and innate immunity. Additionally, we evaluated the effect of multiple cholesterol-related additives (cholesterol, DEAE-dextran, BAs, and MβCD) on RVAs replication.

2.3 Materials and Methods

2.3.1 MA-104 cells and PIEs

MA-104 cells were cultured in complete medium consisting of advanced MEM (aMEM, Gibco) and supplemented with 1% Antibiotic-Antimycotic (Gibco), and 10% Fetal Bovine Serum (FBS, Gibco) in a humidified incubator at 37°C, 5% CO_2. Cells were split every 3-5 days. After 2-3 days of growth, medium was removed, and cells were trypsinized. Cells were counted Cellometer Auto T4 (Nexcelom Bioscience) and adjusted to a final concentration of 64000 cells/mL and loaded onto a 96-well plate with 100 µL in each well, and incubated at 37°C, 5%

CO_2 for 3-4 days until monolayer was 90-95% complete. PIEs were maintained as described by Guo et. al, 2021.

2.3.2 Rotavirus A strains

Gnotobiotic pig small intestinal contents containing RVA G9P[13] (G9P[13] strain) and RVA G5P[7] were used in the study. Intestinal contents were diluted at a 1:10 ratio in sterile Minimal Essential Media (MEM Gibco; Life Technologies, Grand Island, NY, United States). Contents were then centrifuged at 2,095 x g for 10 minutes at 4ºC and filtered through a 0.2 mm filter. Both, RVA strains were preactivated with 10 ug/mL trypsin for 30 minutes at 37°C. Virus titers were adjusted prior to infection.

2.3.3 Neuraminidase treatment and rotavirus infection of PIEs

PIE NA treatment and RVA infection were performed similarly as described previously with a few modifications [31]. Differentiated PIEs were treated with 10mU of NA from *Arthrobacter ureafaciens* (Sigma, USA) diluted in TNC buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3:). TNC buffer was used as a negative control for NA treatment. Following NA treatment, the PIEs were infected with RVAs and harvested as described below. RVA inoculums were activated prior to infection using 10ug/ml trypsin for 30 minutes at 37°C and diluted in complete medium without growth factors (CMGF-) to achieve the desired MOI (1.0). PIEs were incubated with RVAs for 1 hour at 37°C and then washed twice with CMGF- and placed in 96-well plates in triplicates and harvested at 0 and 24 hours post infection. Plates

were kept at -80°C until RNA was extracted from homogenized cells using MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific).

2.3.4 Cell treatment with cholesterol-related additives

For all treatments a complete MA-104 monolayer was washed with wash medium [MEM containing 1% Antibiotic-Antimycotic, and 1% Non-Essential Amino Acids Solution (Gibco)] and then incubated with wash medium at 37°C for an hour. Afterwards, medium was removed, and additives were added at desired concentrations to MA-104 cells and incubated at 37 °C for 1 h. For cholesterol-depletion assay we used MβCD (Sigma, USA) (or dimethyl sulfoxide, (DMSO) as a control) at 20 mM concentration [26]. BA final concentration was adjusted to 20 ug/mL and ethanol was used as a control. Cholesterol was prepared with MilliQ water, prepared at 1 mg/mL stock, and adjusted prior to treatment to 10ug. DEAE-dextran stock solution was filtered using a 0.2um filter and adjusted to a working concentration of 50 ug/ml solution with CMGF-. Following MA-104 treatment, cells were washed and infected with RVAs and harvested as described below.

2.3.5 MA-104 rotavirus infection

Additives-containing medium was discarded from cells, and viruses were added (in triplicates) onto cells. Plates were centrifuged at 1573 xg, at 22°C for 30 minutes. Plates were incubated at 37°C, 5% CO_2 at 37°C for an hour. To remove the non-attached virus, cells were washed four times with PBS containing 1% penicillin-streptomycin (Gibco). Afterwards, a wash medium containing 0.5 µg/ml trypsin was added to cells and plates were incubated for 24 hours

at 37°C with 5% CO_2. Afterwards, cells were fixed with 80% acetone and dried at room temperature for two hours followed by cell culture immunofluorescent (CCIF) assay for RVA quantification as described previously [16].

2.3.6 Transcriptome analysis

RNA extracted from RVA-infected PIEs with and without NA treatment (N=6 per treatment) were sent to Psomagen to preform RNA-seq and differential expressed gene (DEG) and gene ontology analyses. Quantification of RNA was completed by Ribogreen (Life technologies) method using Victor X2 fluorometry. Integrity of RNA was completed by Agilent RNA screentape. All samples had concentrations of >36.00 ng/ul and RIN values above 7. Ingenuity pathway analysis (IPA) of DEGs was performed using Qiagen Digital Insights and Ingenuity Systems (Qiagen, USA).

2.3.7 Statistical analysis

GraphPad Prism v 5.0 (GraphPad Software, San Diego, CA, USA) was used for data analysis. Analysis was performed by one-way analysis of variance (ANOVA) followed by using Šidák multiple comparisons test using GraphPad Prism software. The p-value ≤0.05 was considered statistically significant. Comparison of the viral RNA titers between different treatments was done using Šidák multiple comparisons test, unless specified. Differences were considered statistically significant when $p \leq 0.05$.

2.4 Results

2.4.1 NA treatment decreases replication of G5P[7] and increases that of G9P[13].

Consistent with our previous results [16], we observed that NA treatment of PIEs led to inhibited replication of G5P[7] strain compared to control **(Figure 2.1B**). In contrast, removal of terminal SAs resulted in significantly increased replication of G9P[13] **(Figure 2.1A)**. In order to analyze the host cellular responses to RVA infection the infected/NA treated PIEs were used for total RNA extraction to conduct transcriptome analysis.

2.4.2 Cholesterol-related additives enhanced RVA replication

In order to further dissect the role of cholesterol in RVA infection we used several cholesterol-related additives: (BA, cholesterol, MβCD, and DEAE) on attachment (0 hpi) and replication (24 hpi) of G5P[7] and G9P[13] was evaluated in MA104 cells **(Figures 2.2 A-D)**. Our data demonstrated that cholesterol depletion (MβCD treatment) did not decrease replication of G9P[13] and G5P[7]. In contrast, treatment of MA-104 cells with BA resulted in significant increase in replication of both RVAs. Further, while cholesterol treatment enhanced replication of both G5P[7] and G9P[13] numerically, using DEAE-dextran as a cell culture additive led to significant enhancement of replication of both viruses and even relatively higher attachment of G9P[13] compared to G5P[7].

2.4.3 PIE transcriptome response to G9P[13] vs G5P[7] infection with or without NA pre-treatment

2.4.3.1 G9P[13] infection was associated with more profound alterations of PIE gene expression.

To dissect the differential effects of G9P[13] vs G5P[7] replication on the host (PIE) gene expression profile we used transcriptome analysis. We demonstrated that G9P[13] replication in H+ PIEs resulted in significant modulation of expression of 3,539 genes **(Figure 2.3)** with 1,616 upregulated and 1,923 downregulated. In contrast, infection with G5P[7] led to modulation of significantly fewer genes – 277 with 110 – upregulated and 167 – downregulated (**Figure 2.3**). Additionally, our data demonstrated that terminal SA removal had a differential effect on the host transcriptome response associated with G5P[7] and G9P[13] strains. While G5P[7] infection of the NA-treated PIEs resulted in significant modulation of 504 host (310 upregulated and 194 downregulated) compared with G5P[7]-infected non-treated PIEs, expression of only 329 genes (179 – downregulated and 150 - upregulated) was differentially affected following G9P[13] infection of the NA-treated vs. non-treated PIEs **(Figure 2.3, supplementary tables 2.1-2.4).** Gene-enrichment and annotation transcriptome analysis **(Figures 2.4 & 2.5)** demonstrated that NA treatment prior RVA infection perturbed several critical cellular and molecular processes including cell surface and plasma membrane modulation, cell migration, development and differentiation, transmembrane transport, ion transport, dsRNA binding, protease inhibition and immune response.

2.4.3.2 G9P[13] significantly modulated expression of genes encoding enzymes regulating glycan and sialic acid synthesis.

Sialyltransferase related genes

In order to understand the host response following the variable RVA-SA interactions we conducted targeted analysis of the expression of genes encoding sialyltransferases - enzymes responsible for SA transfer to nascent carbohydrates. (**Table 2.1**). Our data demonstrated that while infection of PIEs with G5[7] strain did not significantly modulate expression of these genes, inoculation of PIEs with G9P[13] led to prominent downregulation of expression of genes of ST3, ST6Gal, and ST8Sia families while upregulated expression of genes belonging to ST6GalNAc family, which have been shown to be major synthases of mucin-associated glycans [27].

Consistent with the above, comparison of RVA infection with and without NA treatment revealed that terminal SA removal induced genotype-specific effects (**Table 2.1**). For both RVA strains used in this study, NA treatment increased numbers of sialyltransferase genes (6 out of 12) whose expression was upregulated. Noteworthy, NA treatment had a more profound effect on the sialyltransferase gene expression following G5P[7] infection, where it reversed the respective RVA infection effects on the expression of 8 out of 12 sialyltransferase genes. This is in sharp contrast to G9P[13] infection, where the reversal effect following NA treatment was observed only for 3 out 12 genes.

Fucosyltransferases related genes

Similarly, to genes encoding sialyltransferases, only infection with G9P[13] but not G5P[7] resulted in significant modulation of fucosyltransferase-encoding gene – ABO glycosyltransferase expression (enzyme involved in HBGA synthesis) (**Table 2.2**). Moreover, terminal SA removal led to further upregulation of expression of this gene after infection with G9P[13] while infection with G5P[7] strain had a reverse effect. In contrast, no considerable modulatory effect on the expression the gene encoding *FUT1* [enzyme involved in H antigen (precursor) synthesis was observed. Further, NA treatment prior RVA infection had a reverse effect for both RVA strains.

2.4.3.3 G5P[7] and G9P[13] infection affected multiple DEGs associated with immune responses

The innate immune system is critical for the host defense against viruses. Our data demonstrated that infection caused by both RVA strains significantly altered innate immune signaling pathways (**Supplementary Tables 2.1-2.4**). IPA identified the top significantly affected molecules, and G9P[13] infection had a staggering >200 fold-change increase of ANPEP and TMIGD1, suggestive of increased granulocyte and cell recovery response **(Supplementary Table 2.1)**. Of interest, another significantly upregulated gene that was associated with the G9P[13] infection was ACE2 that encodes for an important cellular receptor playing an essential role in reducing disease burden due to its role in anti-inflammatory signaling. For comparison, the highest increased fold change for RVA G5P[7] infection was only 17-fold for THAP8 which is involved in cell apoptosis regulation. Overall, infection with G5P[7] led to fewer number of

innate immunity-related DEG. However, we detected decreased expression of CD274 (also known as PDL1), NCF1, and HDAC9. These genes are involved in the inflammatory response regulating programmed cell death, formation of NADPH oxidase used by neutrophils to engulf pathogenic microorganisms, and endothelial cell apoptosis/inflammatory factor expression. G5P[7] infection significantly decreased all these functions.

NA treatment further altered expression of some genes associated with innate immune response **(Supplementary Table 2.2 & 2.3)**. Of significance, following G9P[13] infection + NA pre-treatment there was a decrease of CORO1A expression, shown to decrease T-cell numbers leaving hosts susceptible to infection and immune dysregulation. Other findings included decreased CCN1, CD38, IL6R, PTGS2, and TNFRSF11B. Following G5P[7] strain infection with the NA pre-treatment, majority of innate immunity related genes seemed to exclusively be associated with decreased host cell movement and migration. The most significantly downregulated gene was ZNF23, a key molecule that is involved in cell migration. This gene has an opposite effect where its expression leads to inhibition of cell migration suggesting that G5P[7] infection following NA treatment slows down cell movement. Some of these genes included CXCL8 (previously known as IL-8) which attracts neutrophils, T-cells, and basophiles, ICAM-1 that recruits leukocytes to sites of inflammation, and decreased IL-7/16/20/23A. The majority of these can promote cell apoptosis or proliferation. However, there was no significant up- or down-regulation of the genes associated with immune evasion.

2.4.3.3 G9P[13] affects cholesterol metabolism related genes

RVA G9P[13] infection was found to downregulate the expression of ACSL4 (acyl-CoA synthetase long chain family member 4) - gene involved in inflammation, cell death, female fertility, and cancer regulation and FASN (fatty acid synthase regulating virus entry, host IFN response) (data not shown). The RVA impact on fertility observed in our study supports a previous study performed by Ciarlet et al. where Chinese hamster ovary cells were found to be susceptible to RV infection [28]. Importantly, G9P[13] also upregulated the expression of ACSL5 (long chain acyl-CoA synthetase) and DGAT1 (The ER-localized enzymes diacylglycerol acyltransferase) both involved in lipid droplet formation [29-32]. Based on the discussed above and other DEG, the IPA analysis reconstructed the lipid metabolism pathway affected by the RVA G9P[13] infection based on prediction activation/inhibition **(Figure 2.6).** In contrast to RVA G9P[13], infection with G5P[7] did not lead to a robust modulation of genes involved in lipid metabolism **(Figure 2.7)**. However, IPA analysis indicated that two lipid metabolism pathways were affected by G5P[7] infection. Lipid metabolism, molecular transport, and small molecule biochemistry networks were the most significantly affected by infection with this strain.

NA treatment of PIEs significantly affected several cell signaling pathways (**Figure 2.8**). For G9P[13], SA removal changed predicted networks of lipid metabolism and molecular transport pathways to be the most significantly affected **(Figure 2.8)**. Of interest, ABCB11 was one of the most significantly impacted genes. This is a membrane associated protein known to support cholestasis and provide instructions for bile salt transport pump, and in turn increasing production of bile acids. Furthermore, our data also demonstrated a significant upregulation of

expression of gene-encoding production of the 3-oxo-5-beta(β)-steroid 4-dehydrogenase enzyme (AKR1D1), responsible for producing of bile acids. For the G5P[7] RVA infection, NA treatment also impacted lipid metabolism but to a lesser extent. There were fewer significantly affected genes found but they were related to increased host regulatory mechanisms. In the presence of NA, Akt seems to be one of the key genes which is involved in cellular processes including metabolism, apoptosis, and proliferation. With IPA analysis, we found that the presence of NA with G5P[7] infection all leads to either increased or predicted activation of the Akt protein **(Figure 2.9)**.

2.4.3.4 Differentially modulated canonical pathways

Further, to identify the functional role of DEG obtained by IPA, we compared the major canonical pathways that were significantly affected by the RVA infection. Results of this analysis demonstrated that infection with G9P[13] modulated canonical pathways to a greater extent compared to G5P[7] **(Figure 2.10)**. Besides, surprisingly, among most affected canonical pathways (top 20 pathways) there were no common ones between the two RVA strains **(Figure 2.10)**. Direct comparison of most affected canonical pathways between G9P[13] and G5P[7] further demonstrated the unique cellular response to infection caused by the two RVAs used in this study **(Figure 2.10)**. Similar to the effect on the overall gene expression, NA treatment resulted in more prominent response after PIE infection with G5P[7] rather than G9P[13]. Furthermore, this treatment also resulted in modulation of six common canonical pathways between the two viruses reflecting similarities between host response to infection with different

genotypes of RVA. Taken together, our analyses highlighted both the common and unique major cellular pathways modulated in PIEs following the infection with G9P[13] and G5P[7] with or without NA treatment.

As shown in **Figures 2.10-2.12**, there are seemingly some surprising pathways affected by RVA infection. For example, one of the top pathways affected by G9P[13] infection is related to molecular mechanisms of cancer, mitosis, and DNA damage response. Further, IPA analysis indicated diseases and disorders that could be affected by G9P[13] and G5P[7] infection: gastrointestinal disease, cancers, organismal injury/abnormalities, and dermatological disease. Likewise, for G9P[13] infection IPA analysis demonstrated a close relation with endocrine system disorders, while for infection with G5P[7] strain it was inflammatory/autoimmune disease (data not shown).

Apart from potential link to T1 diabetes, RVAs have not been demonstrated to have long term health effects to the best of our knowledge [33]. Based on the DEG analysis and IPA, we have discovered an interesting finding of key genes showing significance that relate to fertility and spermatogenesis. G5P[7] infection showed a similar pattern with decreased cell movement, showing decreased expression of cation channel sperm association 4 **(Supplemental Table 2.4)**, decreased sperm maturation **(Supplementary Table 2.1)**. Furthermore, one of the top 5 canonical pathways being affected by this RVA strain is sperm motility **(Figure 2.10)**. Similarly, infection with G9P[13] also affected a sperm motility **(Figure 2.10)**.

2.5 Discussion

Globally we face the prevalence of emerging RVA strains with seemingly increased interspecies transmission potential, such as G9P[13] [34-38]. In this study, we have further explored the molecular mechanisms associated with the differential interactions of RVAs with the host cell SAs during cell entry. The differential effects of NA treatment on infection with G9P[13] vs G5P[7] RVAs agrees with the fact that these viruses may differentially modulate expression of genes associated with cell attachment/entry. Currently, there are several receptors associated with RVA attachment/entry and the key role of SAs and HBGAs has been demonstrated by several studies [25, 39]. A variety of SAs are produced by reactions involving sialyltransferases encoded by CAZy GT29 genes family. Our analysis has shown that infection of PIEs with G9P[13] led to significantly more prominent modulation of host cell responses. Specifically, in contrast to G5P[7] strain, it downregulated the expression of the most sialyltransferase genes (8 out of 12). Consistent with our previous results, these data suggest that this might be the molecular mechanisms evolved by G9P[13] allowing it to suppress terminal SA expression, thus allowing it to access gangliosides (GM1) and possibly other cellular receptors, enhancing its replication.

Synthesis of another group of receptors for RVA attachment/entry - HBGAs - is provided by a family of glycosyltransferases encoded by the *ABO, FUT1, FUT2*, and *FUT3* genes [40, 41]. Our analysis showed that only expression of the ABO gene was significantly or numerically upregulated after the PIE infection with G9P[13] or G5P[7], respectively. While FUT1 expression was only down-regulated numerically, no noticeable effect was observed for other genes associated with antigens of ABO and Lewis families. Of interest, NA treatment has

reversed the effects the PIE infection with G9P[13] or G5P[7] on ABO/FUT2 expression, suggestive of the fact that SA and ABO HBGAs can influence one another's presentation and expression subsequently altering their interactions with RVAs as was hypothesized for influenza A virus previously [18].

Membrane rafts also called lipid rafts are extremely important components of plasma and intracellular membranes known to regulate activity of membrane proteins [42]. Further, entry of several non-enveloped viruses has been demonstrated to be membrane lipid-raft associated [43]; while cholesterol depletion usually results in decreased replication of raft-dependent viruses [44-46]. A critical role of cholesterol in earlier steps of RVA replication and association of RVA particles and lipid rafts has been shown *in vitro* and *in vivo* [44, 47]. More specifically, VP4 protein of RVA was demonstrated to directly interact with lipid rafts, thus contributing to the assembly of RVA particles [43, 47-49]. Further, the association with lipid rafts was shown for RVA-related cellular receptors including SA, HGBAs, integrins, and heat shock cognate protein 70 [16, 25, 50-52]. This suggests that SA removal may affect RVA interactions with lipid rafts or even lead to alterations in the lipid by-layer structure and dynamics [20, 21].

Our current data demonstrated that while cholesterol and cholesterol-related additives affected attachment/replication of G9P[13] and G5P[7] in MA104 cells in a similar way, the associated cellular signaling differed drastically. Specifically, our data demonstrated that the presence of DEAE, bile acids, and cholesterol can increase or facilitate the replication of both RVA strains. Our transcriptome analysis results suggests that the entry mechanisms utilized by the two strains of RVAs could modify the way in which they depend on cholesterol, shown by

the modulation of lipid metabolism pathways. Exogenous cholesterol has been shown to recover infectivity of RVA after treatment of cells with cholesterol-disrupting agents [53]. However, the lack of the effect of MβCD on RVA replication is consistent with the findings of Cui and colleagues who also observed that cholesterol depletion did not affect replication of bovine RVA [53]. Thus, our findings with cholesterol related treatments and the lipid metabolism pathway analysis have expanded our knowledge regarding the role of cholesterol in RVA infection.

The dual role of BAs in replication of enteric viruses (including RVs) has been shown *in vivo* and *in vitro* [54, 55]. For example, treatment of cell culture with BAs (chenodeoxycholic acid and deoxycholic acid) led to significantly reduced replication of RVA Wa and SA11 strains [55]. In contrast with these findings, our data demonstrated significantly increased replication of both G5P[7] and G9P[13] RVAs after MA-104 treatment with BA. We hypothesize that this contrasting finding was due to the fact that we used a BA extract containing a mix of hyodeoxycholic acid (a secondary BA) and other BA salts. In our analysis results, we found that G9P[13] upregulates gene expression of proteins related to production of bile acids such as ABCB11 and AKR1D1, which could lead us to hypothesize that bile acids benefit replication of RVAs. Future *in vivo* studies on the current topic with RVAs used in this study are therefore recommended.

DEAE dextran, a branched glucan polysaccharide is known to promote virus entry and transduction efficacy [56, 57]. Further, this reagent has been shown recently to increase virus attachment of RVC strains [26]. Therefore, our current data support previous findings. Treatment of cells with DEAE-dextran increased attachment of G9P[13] while this effect on G5P[7] was less prominent. Further, we demonstrated that replication of both RVAs was increased after this

treatment suggesting that DEAE may be used as an additive to promote virus replication for viruses not adapted to cell culture.

Our current data suggest that increased cholesterol levels support RVA infection and infer that low cholesterol could create an unfavorable environment for replication. This inference is especially important for children as they are primarily at risk for RVA infection. It is understood that in the fetus cholesterol is obtained from both de novo (endogenous) synthesis and maternal dietary cholesterol [58], while neonates are more dependent on dietary cholesterol consumption via milk [58]. At this point, sterol (exogenous) synthesis of cholesterol can be maintained. The impact of RVA infection on infants seems to be increased with the decline of maternal antibodies and influenced by environmental factors, especially diet [59]. Together, these conclusions and knowledge about cholesterol levels and metabolism in children highlights the significance of an optimized nutritional portfolio to reduce the risk of severe RVA infections. We conclude that there is a complex interaction between RVAs, host glycans and cholesterol, providing novel insights for research regarding antivirals to approach block with the focus of cholesterol homeostasis.

Our transcriptome analysis has also highlighted immunomodulatory functions and genes of two RVA strains G9P[13] and G5P[7], along with the comparative impact of NA (**Table 2.1**) and without NA treatment (**Table 2.2**). Essential functions of G9P[13] were unveiled, showing that regulation, transporter activity, and host cellular components were modified by terminal SA removal. G5P[7] showed similar significant components regarding cell periphery but was greatly impacted by protein binding and a high variety of localization processes while G9P[13] was significantly less affected.

Our findings provide further supporting evidence of the pivotal role of SAs in replication of RVA G5P[7] and G9P[13] strains and unveil contrasting cellular and molecular mechanisms affected by both viruses allowing them to regulate SA availability. Additionally, this study elaborates on the significance of cholesterol metabolism and provides novel insights into biological outcomes of differential interactions between RVAs and host cell glycans. Significantly, it presents novel data on the existing interactions between cholesterol/lipid metabolism and cellular receptor expression. Differential transcriptome response of the genes related to immune function further highlights substantial differences in the pathogenesis associated with both strains. Finally, the canonical pathway analysis identified several important health disorders (including endocrine diseases) that may be associated with RVA infection reinforcing previous observations on possible links between childhood RVA infection and T1 diabetes development. We plan to further investigate these results using additional innovative tools such as a reverse genetics system to design and conduct studies to fully analyze G9P[13]-specific interactions with SAs to increase its replication, and how to manipulate this mechanism to reduce its growing global prevalence.

2.6 Acknowledgements

Conceptualization, M.R. and A.N.V.; methodology, M.R.; formal analysis, M.R.; investigation, M.R.; resources, A.N.V.; writing—original draft preparation, M.R.; writing—review and editing, A.N.V., and L.J.S; visualization, M.R.; supervision, A.N.V.; project administration, A.N.V.; funding acquisition, A.N.V. All authors have read and agreed to the published version of the manuscript.

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Sialyltransferase families	**UniProt Annotation**	**G5P[7]**	**G9P[13]**	**G5P[7]/G5P[7]-NA**	**G9P[13]/G9P[13] -NA**
ST3Gal	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 1 (ST3Gal I)	↓	↓	↑	↓
	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 2 (ST3Gal II)	↓	↓	↑	↓
	CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase 3 (ST3Gal III)	↑	↓	↓	↑
	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 4 (ST3Gal IV)	↑	↑	↑	↑
	CMP-NeuAc:lactosylceramide alpha-2,3-sialyltransferase 5 (GM3 synthase)	↓	↓	↓	↑
	CMP-NeuAc:beta-galactoside alpha-2,3-sialyltransferase 6 (Type 2 lactosamine alpha-2,3-sialyltransferase)	↑	↓	↓	↑
ST6GalNAc	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1 (ST6GalNAc I)	↑	↑	↑	↓
	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2 (ST6GalNAc II)	↓	↓	↑	↓
	Alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3-N-acetyl-galactosaminide alpha-2,6-sialyltransferase (ST6GalNAc IV)	↓	↑	↓	↑
	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 6 ST6GalNAc VI)	↑	↑	↓	↑
ST6Gal	Beta-galactoside alpha-2,6-sialyltransferase 1 (ST6Gal I)	↓	↓	↑	↓
ST8Sia	CMP-N-acetylneuraminate-poly-alpha-2,8-sialyltransferase (ST8Sia V)	↓	↓	↑	↓

Table 2. 1 Modulation of expression of genes encoding sialyltransferases in PIEs (with or without NA treatment) infected with G9P[13] and G5P[7] OSU.

Bolded arrows indicate significantly impacted regulation (fold change greater than 2 for upregulation, lower than -2 for downregulation). Colors are indicated where red is significantly downregulated, and blue is significantly upregulated.

Fucosyltransferase	UniProt Annotation	G5P[7]	G9P[13]	G5P[7]/G5P[7] -NA	G9P[13]/G9P[13] -NA
FUT1	Galactoside alpha-(1,2)-fucosyltransferase 1	↓	↓	↑	↑
ABO	ABO glycosyltransferase	↑	↑	↓	↑

Table 2. 2 Modulation of expression of genes encoding fucosyltransferases in PIEs (with or without NA treatment) infected with G9P[13] and G5P[7] OSU.

Colors are indicated where red is significantly downregulated, and blue is significantly upregulated.

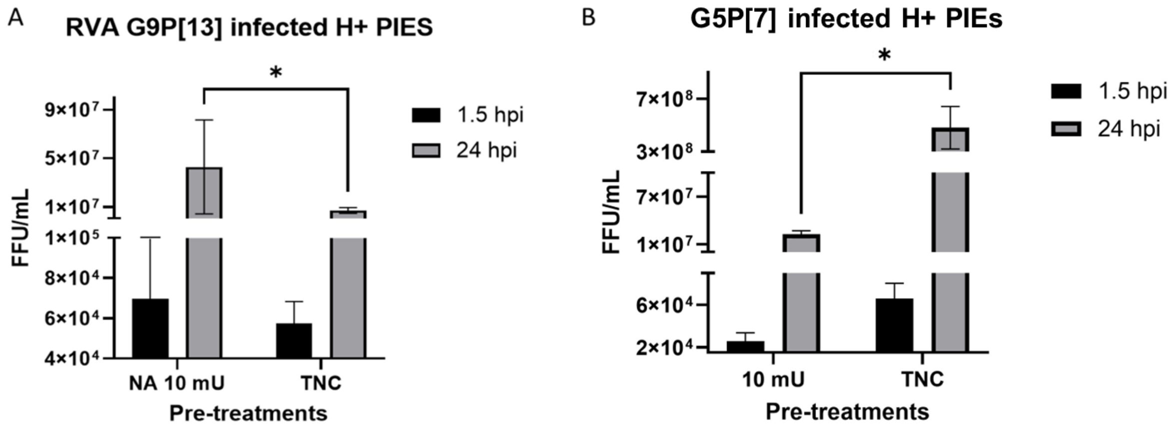

Figure 2. 1 Replication of G9P[13] (A) and G5P[7] G5P[7] (B) in PIEs with or without NA treatment, respectively.

Differentiated PIEs were pre-treated with 10 mU NA from *Arthrobacter ureafaciens* diluted in TNC buffer or TNC buffer for 1 h at 37 °C before RVA infection. Then, PIEs were inoculated (MOI 1.0) with RVAs and incubated at 37 °C. PIEs were harvested at 1.5 h and 24 h post infection, and the virus titers were measured by CCIF. The error bars represent the standard deviation from triplicate samples. Error bars are denoted as standard deviations obtained for 6 experimental replicates. Analysis was performed using two-way ANOVA followed by Šidák multiple comparisons test. Differences were considered statistically significant when $p \leq 0.05$.

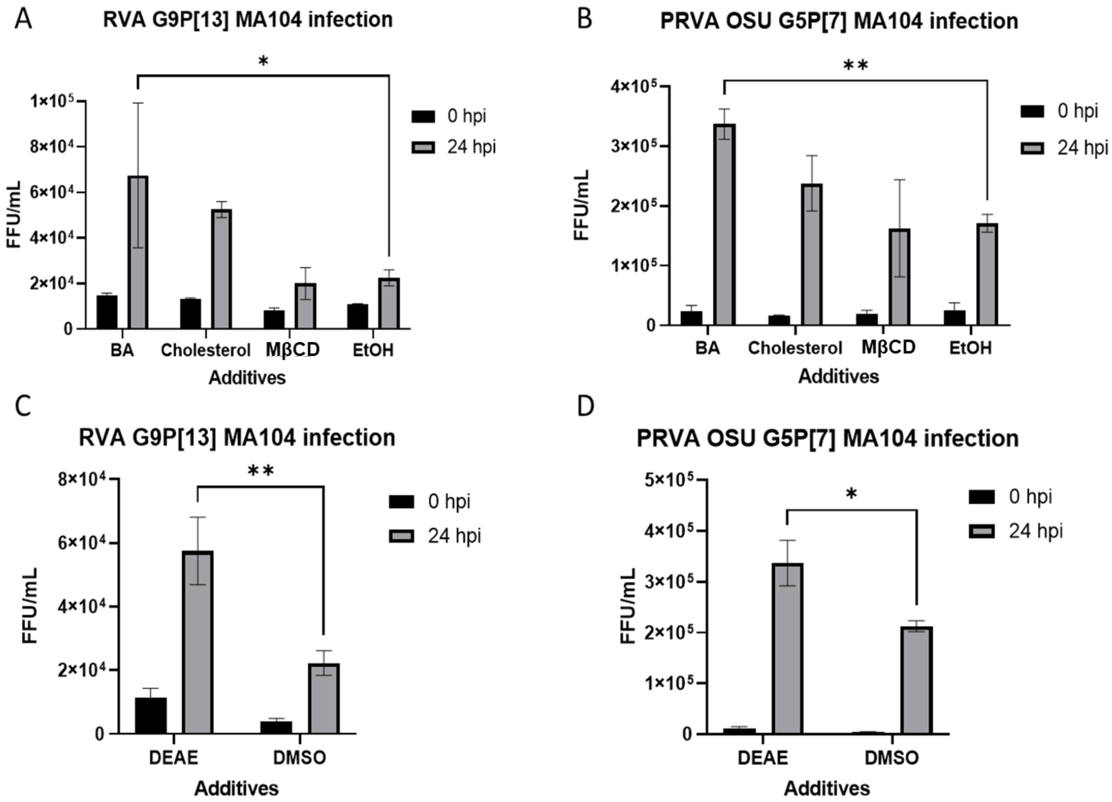

Figure 2. 2 RVA replication in MA104 cells with various additives.

RVA load was examined at 0 hpi and 24 hpi. Values were determined using CCIF. Error bars are denoted as standard deviation, and all experiments were repeated at least twice, performed in duplicates. Analysis was performed using two-way ANOVA followed by Šidák multiple comparisons test. Differences were considered statistically significant when $p \leq 0.05$.

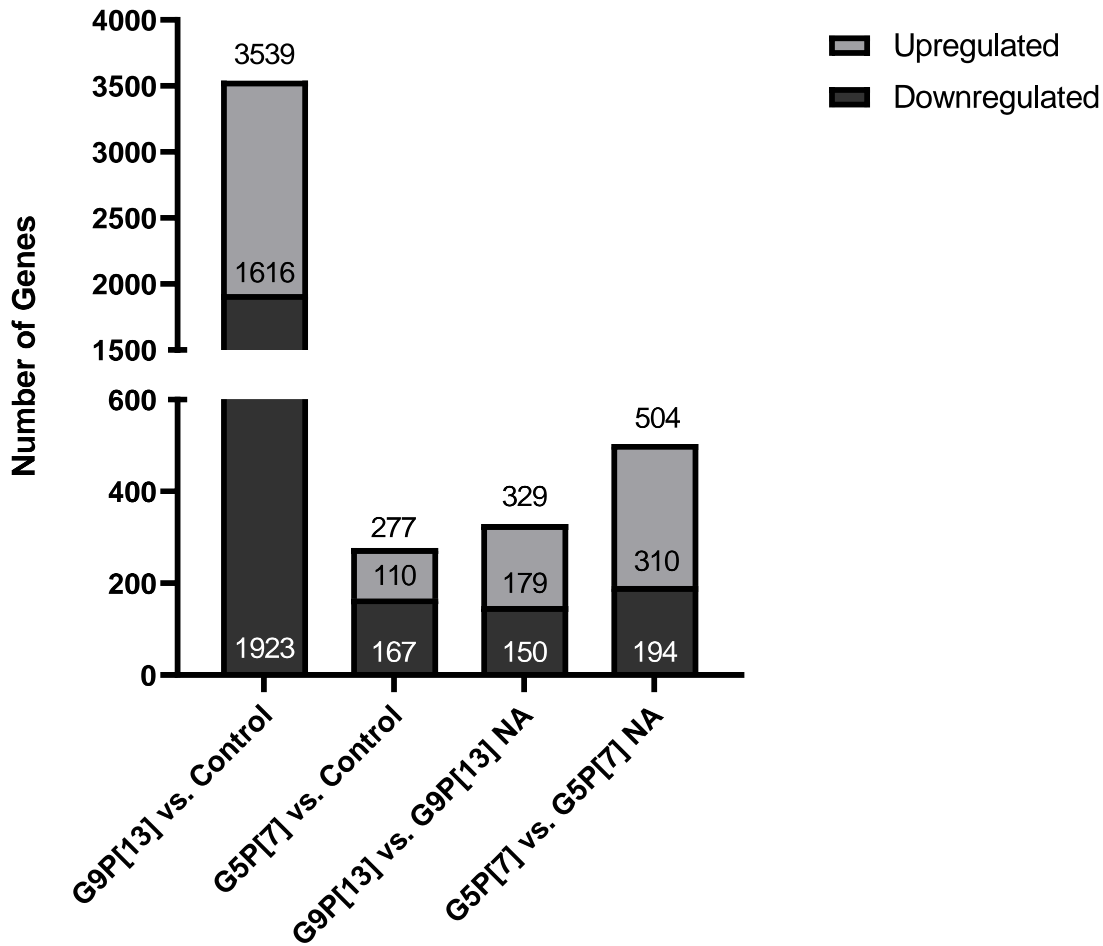

Figure 2. 3 Summary of the total gene numbers that were either upregulated or downregulated based on DEG comparisons.

Total number of genes is placed at the top of the individual bars and split accordingly to represent upregulated (light gray) and downregulated (dark gray) genes. Genes that account for these numbers can be seen in **Supplementary tables 1-4**.

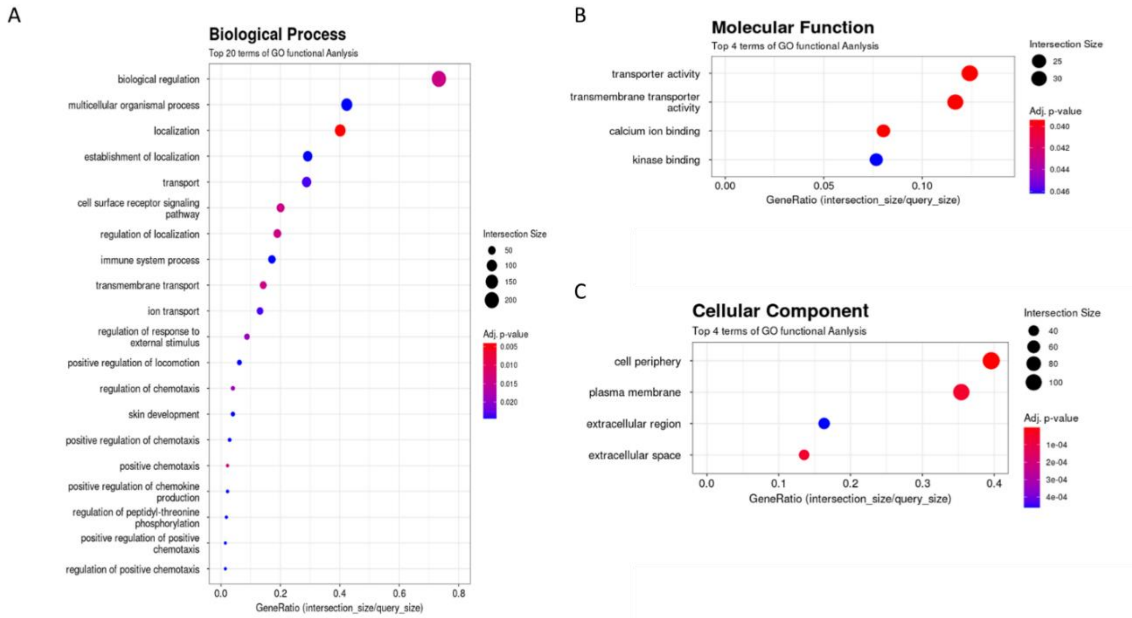

Figure 2. 4 Gene-enrichment and annotation transcriptome analysis summary for NA treated G9P[13]-infected PIEs.

This was performed using gProfiler, a tool to represent statistical enrichment analysis. gProfiler used significant gene expression for both upregulation and downregulation of genes found in (**Supplementary table 2.1**). A dot plot was used to show most significant Gene Ontology (GO) analysis, used to identify significant biological processes (A), function (B), and cellular components (C) that are most severely impacted in conditions that are represented by intersection size and p-value. In this figure, the dot plot represents the impact NA has on G9P[13] infection of PIEs, with the most significant genes being associated with GO term, size, and color of the dot. Size is shown to support high GO with dot size, depicting the p values. These are arranged by GeneRatio in descending order, provided by Psomagen's analysis.

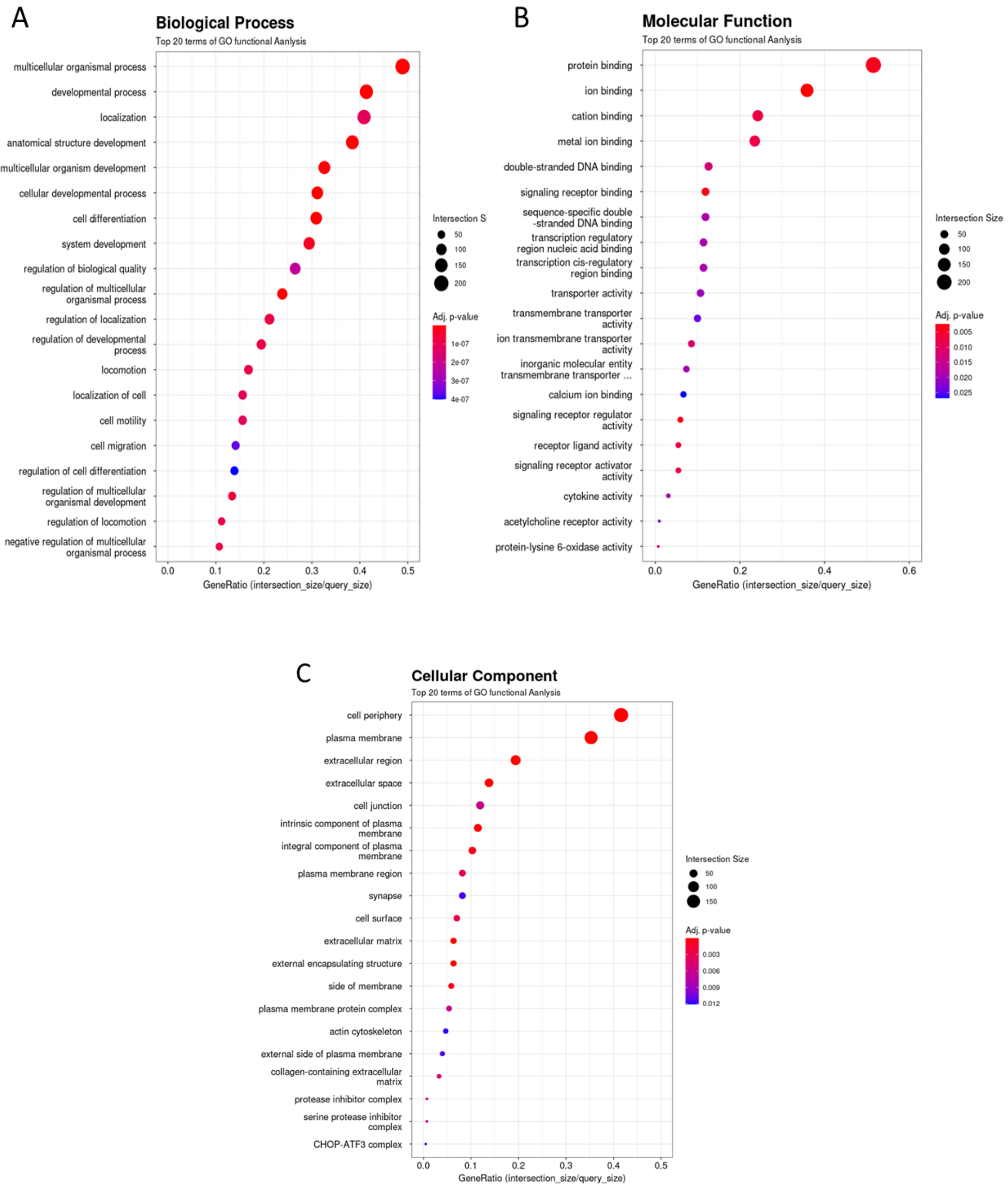

Figure 2. 5 Gene-enrichment and annotation transcriptome analysis summary for NA treatment of PIEs infected with RVA G5P[7].

This was performed using the same process and representation as (**Figure 2.4)**, to identify significant biological processes (A), function (B), and cellular components (C) that are most severely impacted in conditions that are represented by intersection size and p-value.

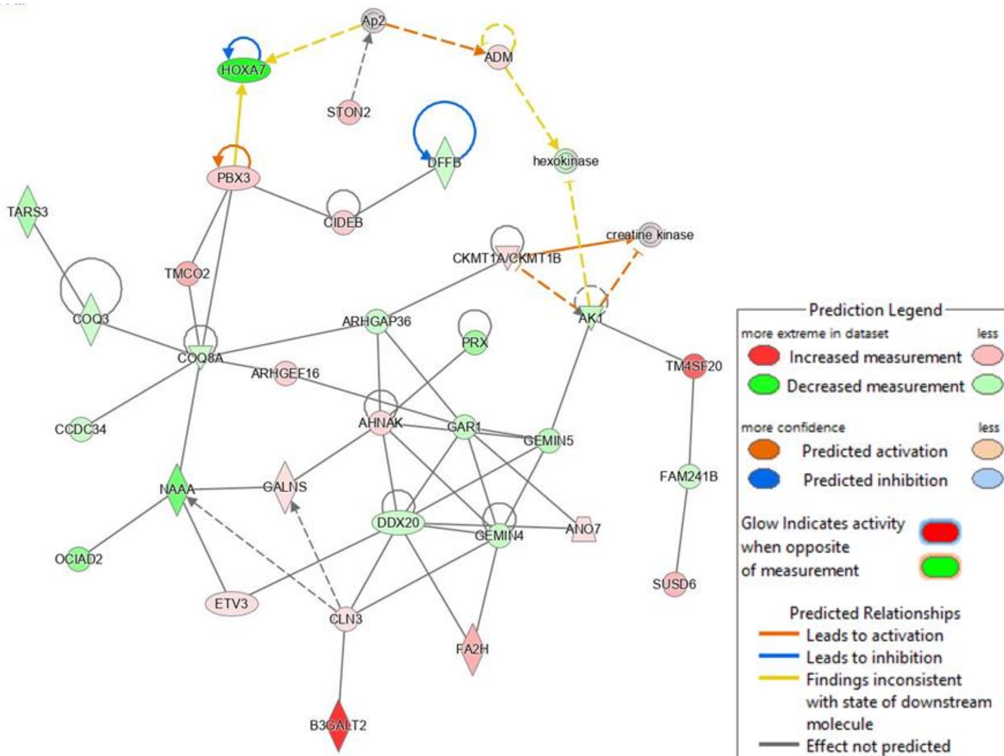

Figure 2. 6 Reconstructed lipid metabolism network based on G9P[13] infection of PIEs.

IPA generated pathways using the most significant entities from the analyzed transcriptome analysis data sets and connects to create a comprehensible synopsis of the analysis. The legend to the side signifies the colors and predicted altered pathway used by RVA G9P[13] infection.

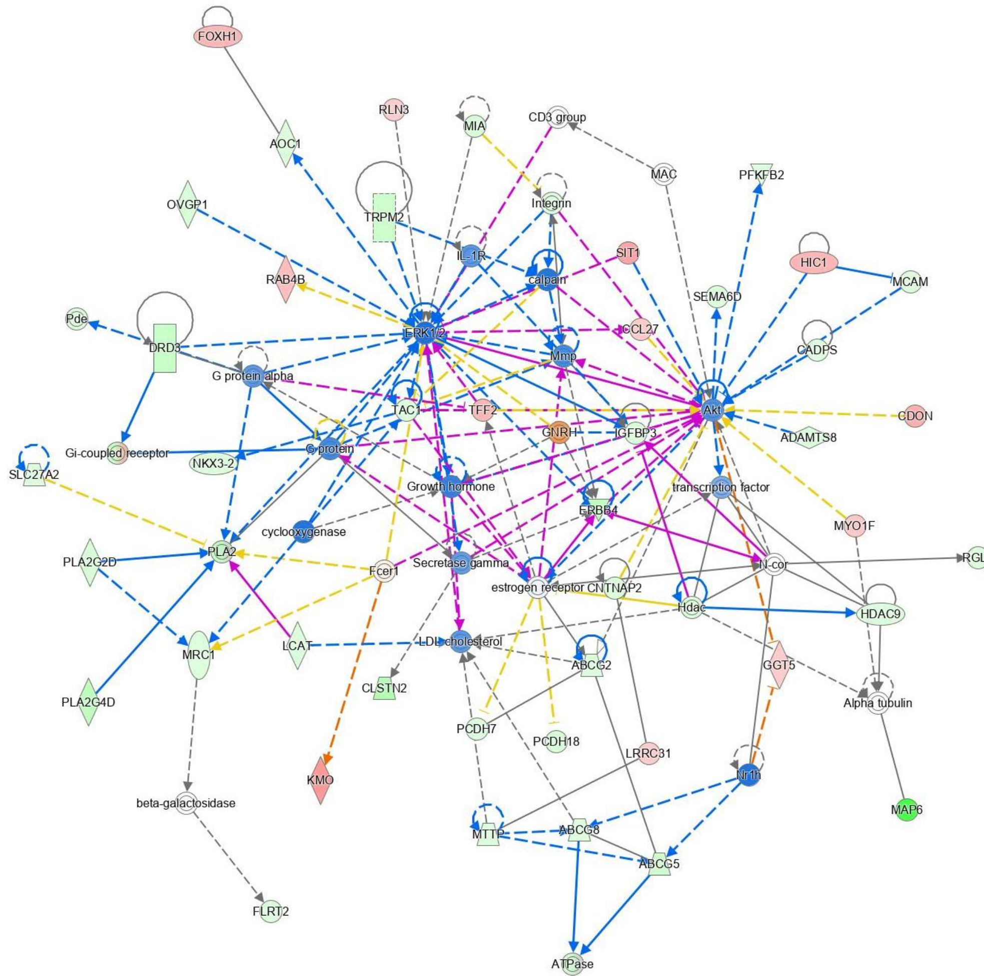

Figure 2. 7 Reconstructed lipid metabolism network based on RVA G5P[7]infection of PIEs.

IPA generated pathways using the most significant entities from the analyzed transcriptome analysis data sets and connects to create a comprehensible synopsis of the analysis. The legend from figure 6 applies to this as well, regarding PRVA G5P[7] infection.

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Figure 2. 8 Reconstructed lipid metabolism network for G9P[13] infection plus NA treatment of PIEs.

IPA generated pathways using the most significant entities from the analyzed transcriptome analysis data sets and connects to create a comprehensible synopsis of the analysis. The legend from figure 6 applies to this as well, regarding G9P[13] in the presence of NA infection.

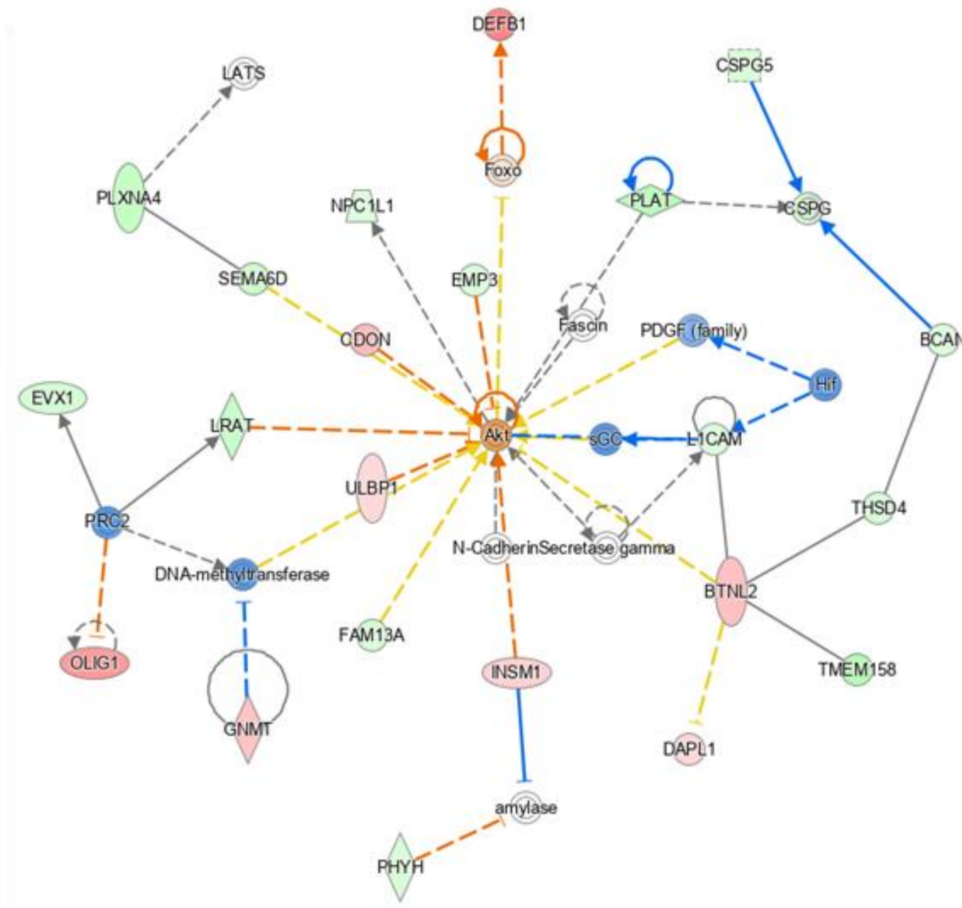

Figure 2. 9 Reconstructed lipid metabolism network based on the dataset for PIES infected with RVA G5P[7] following NA treatment of PIEs.

IPA generated pathways use the most significant entities from completed transcriptome analysis data set and connects to create a comprehensible synopsis of the analysis. All networks analyzing for the treatment with NA was associated with either confirmed increase of the Akt protein (serine-threonine protein kinase family that affects cellular metabolism), or predicted activation based on analysis results with high confidence.

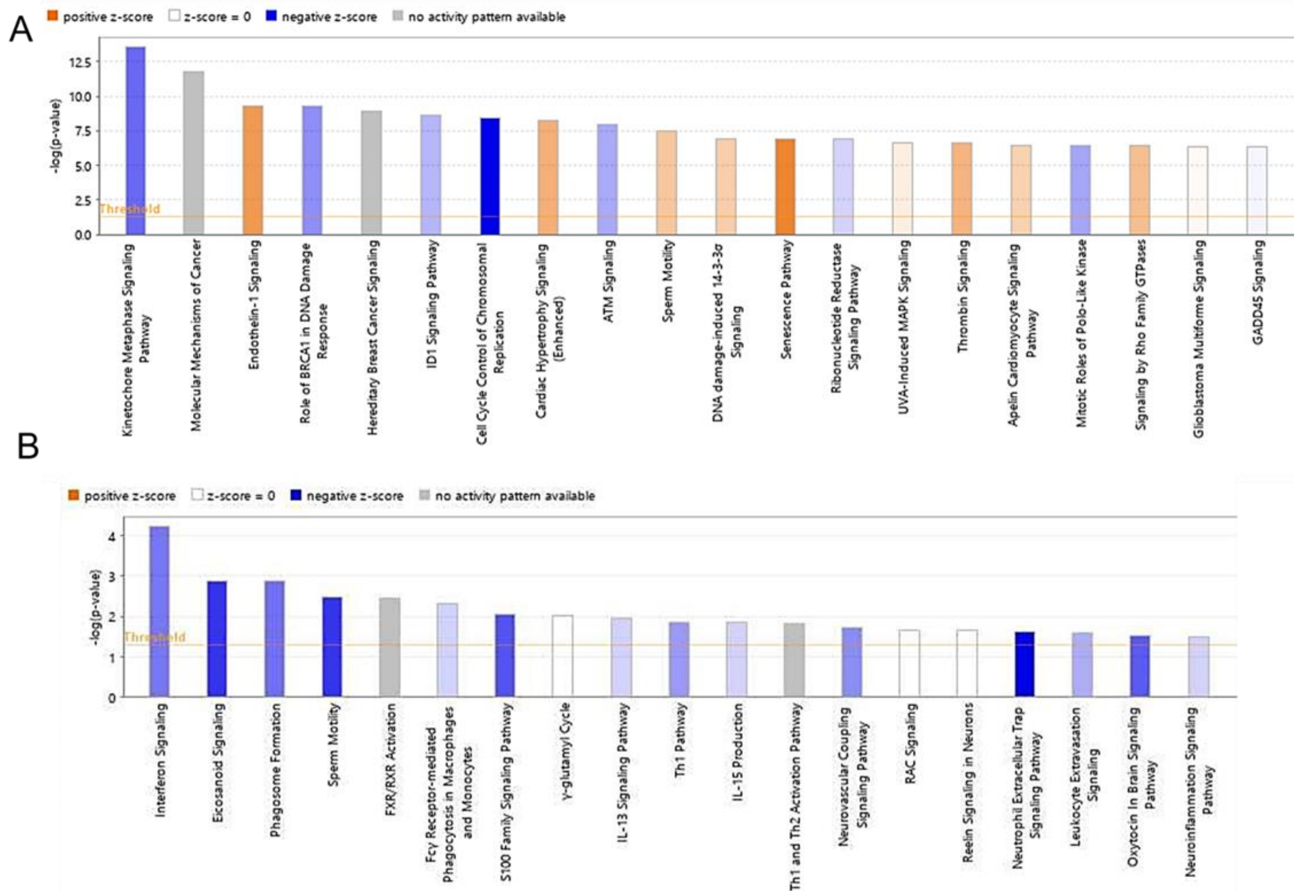

Figure 2. 10 Canonical pathway analysis of PIE response following G9P[13] infection vs control **(A)** and G5P[7] infection vs control **(B)** performed using Qiagen IPA.

Canonical pathway analysis was identified through the IPA library and shows the most significant contributions through the input data set. Parameters were set to show pathways based on total number of genes, and z-fold calculations to determine probability of association. Upregulated pathways are associated with positive z score shown in orange, and downregulated genes associated with negative z scores are shown in blue.

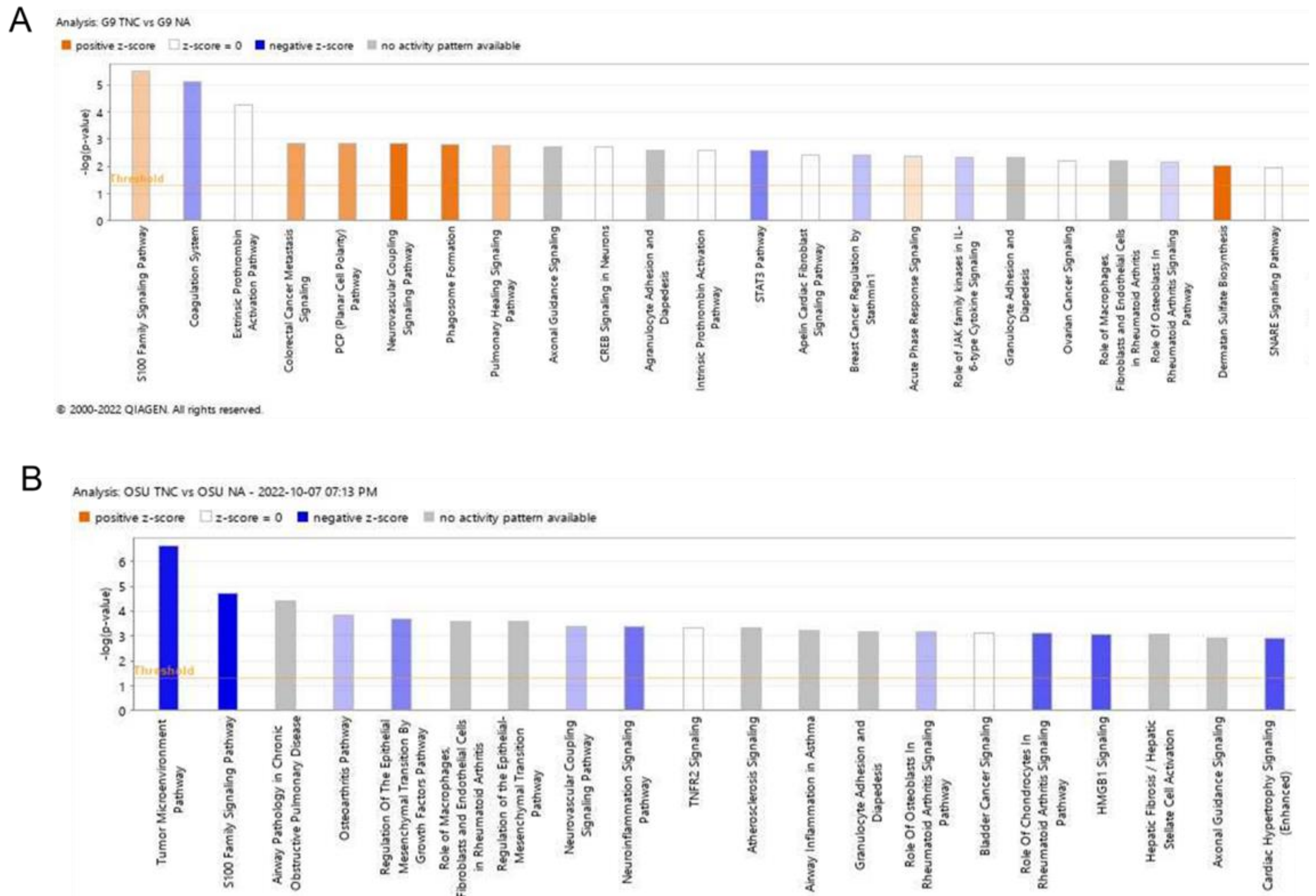

Figure 2. 11 Analysis of **c**anonical pathways differentially affected by G9P[13] vs G9P[13]+NA **(A)** and G5P[7] vs G5P[7]-NA **(B)**..

The parameters and coding are the same as used in Figure 2.10.

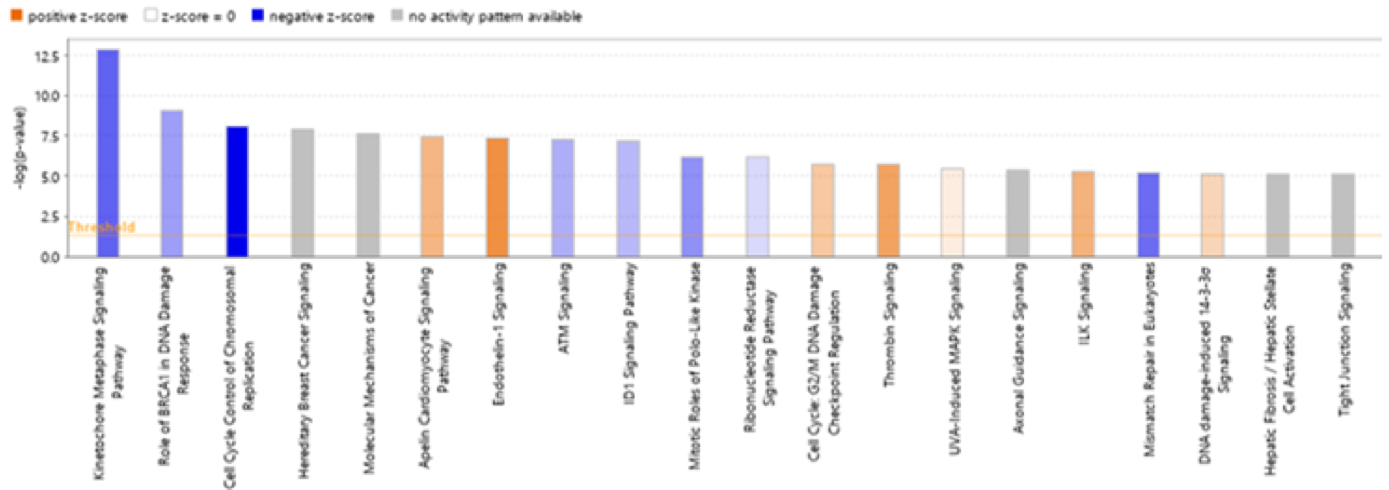

Figure 2. 12 Analysis of **c**anonical pathway differentially affected by G9P[13] vs G5P[7].

The parameters and coding are the same as Figure 2.10.

Supplemental data

Supplementary table 2. 1 Summary of DEGs common for both G9P[13] and G5P[7] strains in the presence of NA.

Genes are shown with '↓' and'↑' referring to down or upregulation. The upregulated/downregulated genes were determined using a cut off ± ≥ 2 or ≤ 2 when respectively compared with the control group (non-treated). All genes listed account for active protein coding.

Gene symbol	Description	G5P[7]	G9P[13]
ACKR4	atypical chemokine receptor 4	↓	↓
AGER	advanced glycosylation end-product specific receptor	↓	↑
AKR1D1	aldo-keto reductase family 1 member D1, transcript variant X3	↑	↑
ALOXE3	arachidonate lipoxygenase 3	↓	↓
BCL6B	B-cell CLL/lymphoma 6B	↓	↓
BPIFC	BPI fold containing family C	↑	↓
CD48	CD48 molecule	↓	↓
CDH6	cadherin 6	↑	↓
CHRNA4	cholinergic receptor nicotinic alpha 4 subunit	↓	↑
DNAH14	dynein axonemal heavy chain 14	↑	↑
DOK2	docking protein 2	↑	↓
EFEMP2	EGF containing fibulin like extracellular matrix protein 2	↓	↑
ENO3	enolase 3	↑	↓
ERRFI1	ERBB receptor feedback inhibitor 1	↓	↓
FAM25A	family with sequence similarity 25 member A	↓	↓
GAL3ST2	galactose-3-O-sulfotransferase 2	↓	↑
GIMAP4	GTPase, IMAP family member 4, transcript variant X1	↓	↑
GJD4	gap junction protein delta 4	↑	↑
GNG8	G protein subunit gamma 8, transcript variant X7	↓	↑
GOLGA7B	golgin A7 family member B, transcript variant X3	↓	↑
GP9	glycoprotein IX platelet	↓	↓
GPAT2	glycerol-3-phosphate acyltransferase 2, mitochondrial, transcript variant X3	↓	↑
IL17RE	interleukin 17 receptor E	↓	↑
KLHL4	kelch like family member 4, transcript variant X2	↑	↓
LOC100157711	sodium/potassium-transporting ATPase subunit alpha-4	↓	↓
LOC100623096	uncharacterized LOC100623096	↓	↓

LOC100624329	zinc finger X-chromosomal protein, transcript variant X13	↓	↓
LOC100628233	cationic amino acid transporter 3-like	↓	↓
LOC102165602	coiled-coil domain-containing protein 92	↑	↑
LOC102165847	**zinc finger protein 664-like, transcript variant X2**	↑	↓
LOC106504436	myeloid-associated differentiation marker-like	↓	↓
LOC110255332	acyl-coenzyme A thioesterase 4-like	↑	↓
LOXL3	lysyl oxidase like 3, transcript variant X2	↑	↓
LRMP	lymphoid restricted membrane protein, transcript variant X4	↓	↓
LYL1	LYL1, basic helix-loop-helix family member	↑	↓
MCIDAS	multiciliate differentiation and DNA synthesis associated cell cycle protein	↑	↑
MMP28	matrix metallopeptidase 28, transcript variant X2	↓	↑
MORC1	MORC family CW-type zinc finger 1	↑	↓
MTERF2	mitochondrial transcription termination factor 2	↓	↓
NID2	nidogen 2, transcript variant X1	↓	↑
P2RY6	pyrimidinergic receptor P2Y6	↓	↓
PARD6G	par-6 family cell polarity regulator gamma	↓	↑
PHYH	phytanoyl-CoA 2-hydroxylase	↓	↑
PLAT	plasminogen activator, tissue type	↓	↓
PLET1	placenta expressed transcript protein	↑	↑
PLS3	plastin 3	↓	↓
PPP1R32	protein phosphatase 1 regulatory subunit 32	↓	↑
PRCD	photoreceptor disc component, transcript variant X2	↑	↓
PYGO1	pygopus family PHD finger 1	↓	↑
SERPINB10	serpin family B member 10	↑	↓
SLC23A3	solute carrier family 23 member 3	↑	↑
SPAG11B	sperm associated antigen 11B	↑	↓
TMEM255A	transmembrane protein 255A, transcript variant X3	↓	↓
TRAF1	TNF receptor associated factor 1, transcript variant X1	↑	↓
TRIB2	tribbles pseudokinase 2, transcript variant X1	↑	↓
WNT3	Wnt family member 3, transcript variant X2	↓	↑
YOD1	YOD1 deubiquitinase, transcript variant X1	↓	↓

Supplementary table 2. 2**.** Summary of DEGs common for both G9P[13] and G5P[7] strains in the absence of NA.

Genes are shown with '↓' and'↑' referring to down or upregulation, respectively. These are also depicted with red arrows signifying upregulation, and blue representing down regulation. Differentially expressed genes are bolded. The upregulated/downregulated genes were determined using a cut off ± ≥ 2 or ≤ 2 when respectively compared with the control group (non-treated). All genes listed account for active protein coding.

Gene Symbol	Description	G5P[7]	G9P[13]
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	↓	↑
ABCG5	ATP binding cassette subfamily G member 5, transcript variant X1	↓	↑
ABCG8	ATP binding cassette subfamily G member 8, transcript variant X1	↓	↑
ANPEP	alanyl aminopeptidase, membrane	↓	↑
AOC1	amine oxidase, copper containing 1, transcript variant X5	↓	↑
BATF3	basic leucine zipper ATF-like transcription factor 3	↓	↓
C1H9orf16	chromosome 1 C9orf16 homolog	↑	↑
C6H19orf81	chromosome 6 C19orf81 homolog, transcript variant X1	↑	↑
C8H4orf48	chromosome 8 C4orf48 homolog	↑	↑
CATSPER4	cation channel sperm associated 4	↓	↑
CDK5R1	cyclin dependent kinase 5 regulatory subunit 1	↓	↓
CFAP61	cilia and flagella associated protein 61, transcript variant X3	↓	↓
CLIC5	chloride intracellular channel 5	↓	↑
CMC4	C-X9-C motif containing 4, transcript variant X1	↑	↑
CNTNAP2	contactin associated protein-like 2	↓	↑
CORO2B	coronin 2B, transcript variant X2	↓	↓
CPA6	carboxypeptidase A6, transcript variant X1	↓	↓
CRTAC1	cartilage acidic protein 1, transcript variant X2	↑	↑
CSDC2	cold shock domain containing C2	↓	↑
CYP26A1	cytochrome P450, family 26, subfamily A, polypeptide 1	↓	↓
CYS1	cystin 1	↑	↑
DCST1	DC-STAMP domain containing 1	↓	↑
DENND1C	DENN domain containing 1C	↓	↓
DENND2A	DENN domain containing 2A, transcript variant X5	↓	↓
DPCR1	diffuse panbronchiolitis critical region 1	↓	↑

EGR2	early growth response 2	↓	↓
ELMOD1	ELMO domain containing 1, transcript variant X4	↓	↓
ENKD1	enkurin domain containing 1, transcript variant X1	↑	↑
FAM129C	family with sequence similarity 129 member C	↑	↑
FAM25A	family with sequence similarity 25 member A	↑	↑
FAM71E1	family with sequence similarity 71 member E1, transcript variant X2	↑	↑
FAR2	fatty acyl-CoA reductase 2, transcript variant X1	↓	↑
FGF22	fibroblast growth factor 22	↑	↑
FOSB	FosB proto-oncogene, AP-1 transcription factor subunit	↑	↑
FOXH1	forkhead box H1, transcript variant X3	↑	↑
GAREM2	GRB2 associated regulator of MAPK1 subtype 2, transcript variant X2	↓	↓
GGN	gametogenetin	↑	↑
GGT5	gamma-glutamyltransferase 5, transcript variant X2	↑	↑
GPAT2	glycerol-3-phosphate acyltransferase 2, mitochondrial, transcript variant X3	↑	↑
GPR156	G protein-coupled receptor 156, transcript variant X6	↓	↓
HDAC9	histone deacetylase 9, transcript variant X22	↓	↓
HHLA2	HERV-H LTR-associating 2, transcript variant X22	↓	↑
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2	↓	↑
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	↓	↓
IGFBP3	insulin like growth factor binding protein 3	↓	↓
JPH1	junctophilin 1, transcript variant X1	↓	↓
KIF1A	kinesin family member 1A	↓	↑
LEAP2	liver enriched antimicrobial peptide 2	↑	↑
LEKR1	leucine, glutamate and lysine rich 1, transcript variant X1	↑	↑
LRRC23	leucine rich repeat containing 23, transcript variant X2	↑	↑
LRRC31	leucine rich repeat containing 31, transcript variant X2	↑	↑
LTB4R	leukotriene B4 receptor, transcript variant X1	↓	↑
LTF	lactotransferrin	↓	↓
MAF	MAF bZIP transcription factor, transcript variant X1	↓	↑
MCAM	melanoma cell adhesion molecule, transcript variant X3	↓	↓
MDFI	MyoD family inhibitor, transcript variant X4	↓	↓
MIA	melanoma inhibitory activity, transcript variant X1	↓	↓
MIR7138	ssc-miR-7138-3p	↓	↓
MOGAT2	monoacylglycerol O-acyltransferase 2	↓	↑
MRC1	mannose receptor C-type 1	↓	↑
MTERF2	mitochondrial transcription termination factor 2	↓	↓
MTTP	microsomal triglyceride transfer protein	↓	↑

MUC12	mucin 12, cell surface associated	↓	↑
MYH15	myosin heavy chain 15	↑	↓
NAGS	N-acetylglutamate synthase	↓	↑
NEURL3	neuralized E3 ubiquitin protein ligase 3	↑	↑
NKX3-2	NK3 homeobox 2	↓	↓
OASL	2'-5'-oligoadenylate synthetase like	↑	↑
ONECUT2	one cut homeobox 2	↑	↑
OVGP1	oviductal glycoprotein 1	↓	↓
PCDH7	protocadherin 7	↓	↓
PDGFRL	platelet derived growth factor receptor like, transcript variant X1	↓	↓
PECAM1	platelet and endothelial cell adhesion molecule 1	↓	↓
PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma	↓	↓
PIRT	phosphoinositide interacting regulator of transient receptor potential channels, transcript variant X2	↑	↑
PLBD1	phospholipase B domain containing 1	↓	↓
PLPPR2	phospholipid phosphatase related 2, transcript variant X1	↑	↑
PPP1R36	protein phosphatase 1 regulatory subunit 36, transcript variant X1	↓	↓
RAB39A	RAB39A, member RAS oncogene family, transcript variant X1	↓	↓
RAB4B	RAB4B, member RAS oncogene family, transcript variant X1	↑	↑
RET	ret proto-oncogene	↓	↓
RGL1	ral guanine nucleotide dissociation stimulator like 1, transcript variant X2	↓	↓
RGS16	regulator of G protein signaling 16	↑	↑
RLN3	relaxin 3	↑	↑
ROM1	retinal outer segment membrane protein 1	↑	↑
RSPH1	radial spoke head 1 homolog, transcript variant X3	↓	↓
RSPH9	radial spoke head 9 homolog, transcript variant X2	↑	↑
RTN4RL1	reticulon 4 receptor like 1, transcript variant X1	↓	↓
SCNN1A	sodium channel epithelial 1 alpha subunit	↑	↑
SECTM1	secreted and transmembrane 1	↑	↑
SLA-5	MHC class I antigen 5	↑	↑
SLC10A4	solute carrier family 10 member 4	↓	↓
SLC26A7	solute carrier family 26 member 7, transcript variant X5	↑	↑
SLC30A10	solute carrier family 30 member 10	↑	↑
SLC35G2	solute carrier family 35 member G2, transcript variant X1	↓	↓

SLC51B	solute carrier family 51 beta subunit, transcript variant X1	↓	↑
SLC6A4	solute carrier family 6 member 4, transcript variant X1	↓	↑
SNAI1	snail family transcriptional repressor 1	↑	↑
SNX22	sorting nexin 22, transcript variant X2	↓	↓
SPDEF	SAM pointed domain containing ets transcription factor	↑	↑
ST18	ST18, C2H2C-type zinc finger, transcript variant X18	↓	↓
STAG3	stromal antigen 3, transcript variant X2	↑	↑
STARD7	StAR related lipid transfer domain containing 7	↓	↓
SYBU	syntabulin	↑	↑
SYT12	synaptotagmin 12, transcript variant X4	↑	↑
TEX14	testis expressed 14, intercellular bridge forming factor	↓	↓
TFF2	trefoil factor 2	↑	↑
TGM3	transglutaminase 3	↑	↑
TMCO2	transmembrane and coiled-coil domains 2	↑	↑
TMEM255B	transmembrane protein 255B, transcript variant X2	↑	↑
TMEM88B	transmembrane protein 88B	↑	↑
TREX2	three prime repair exonuclease 2, transcript variant X2	↓	↑
TRIM31	tripartite motif containing 31	↓	↑
TUBAL3	tubulin alpha like 3	↓	↑
WFIKKN1	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 1	↑	↑

Supplementary table 2. 3**.** Summary of DEGs specific to G9P[13] with and without NA treatment, versus a control group (noninfected).

Bolded genes indicate genes that give contrasting regulation in the presence of NA. Genes are shown with '↓' and'↑' referring to down or upregulation, respectively, without the presence of NA. The upregulated/downregulated genes were determined using a cut off ± ≥ 2 or ≤ 2 when respectively compared with the control group (non-treated). All genes listed account for active protein coding, except "LOC" genes, indicating non-coding.

Gene symbol	Description	G9 vs. NA	G9 vs non-infected
ADGRF3	adhesion G protein-coupled receptor F3	↑	↑
AKAP2	A kinase (PRKA) anchor protein 2	↓	↓
APLN	**apelin**	↑	↓
ARL9	ADP ribosylation factor like GTPase 9, transcript variant X3	↓	↓
ATP4B	ATPase H+/K+ transporting beta subunit	↑	↑
C13H3orf70	**chromosome 13 C3orf70 homolog**	↑	↓
C15H2orf72	**chromosome 15 C2orf72 homolog**	↑	↓
CACNA2D2	calcium voltage-gated channel auxiliary subunit alpha2delta 2, transcript variant X1	↓	↓
CD177	CD177 molecule	↑	↑
CDH13	cadherin 13, H-cadherin (heart)	↓	↓
CEMIP	**cell migration inducing hyaluronan binding protein, transcript variant X2**	↓	↑
CENPL	**centromere protein L**	↑	↓
CHI3L2	**chitinase 3 like 2**	↑	↓
CHIA	**chitinase, acidic**	↑	↓
CHN1	chimerin 1, transcript variant X3	↓	↓
CHRNA4	cholinergic receptor nicotinic alpha 4 subunit	↑	↑
CLCA4	**calcium-activated chloride channel regulator 4**	↓	↑
COQ10A	**coenzyme Q10A, transcript variant X1**	↑	↓
CYP26A1	cytochrome P450, family 26, subfamily A, polypeptide 1	↓	↓
CYP27B1	25-hydroxyvitamin D3 1alpha-hydroxylase	↓	↓
CYR61	**cysteine rich angiogenic inducer 61**	↓	↑
CYS1	cystin 1	↑	↑
DACT2	dishevelled binding antagonist of beta catenin 2	↓	↓
DCST1	DC-STAMP domain containing 1	↑	↑
DLG2	discs large MAGUK scaffold protein 2, transcript variant X21	↓	↓

DOCK11	dedicator of cytokinesis 11	↓	↓
DOK2	docking protein 2	↓	↓
EFEMP2	EGF containing fibulin like extracellular matrix protein 2	↑	↑
EFR3B	**EFR3 homolog B, transcript variant X1**	↑	↓
ELMOD1	ELMO domain containing 1, transcript variant X4	↓	↓
ERRFI1	**ERBB receptor feedback inhibitor 1**	↓	↑
F12	coagulation factor XII	↓	↓
F7	**coagulation factor VII (serum prothrombin conversion accelerator)**	↑	↓
FAM25A	**family with sequence similarity 25 member A**	↓	↑
FBXO15	**F-box protein 15, transcript variant X1**	↑	↓
FFAR2	free fatty acid receptor 2	↑	↑
FOXQ1	forkhead box Q1	↑	↑
FRMPD4	FERM and PDZ domain containing 4, transcript variant X1	↓	↓
FSD1	fibronectin type III and SPRY domain containing 1, transcript variant X1	↑	↑
GAL3ST2	galactose-3-O-sulfotransferase 2	↑	↑
GFI1B	growth factor independent 1B transcriptional repressor, transcript variant X2	↓	↓
GIF	**gastric intrinsic factor**	↑	↓
GJD2	gap junction protein delta 2, transcript variant X1	↓	↓
GOLGA7B	golgin A7 family member B, transcript variant X3	↑	↑
GPAT2	glycerol-3-phosphate acyltransferase 2, mitochondrial, transcript variant X3	↑	↑
GRAMD1A	GRAM domain containing 1A	↓	↓
HOXA7	homeobox A7	↓	↓
HS3ST6	heparan sulfate-glucosamine 3-sulfotransferase 6, transcript variant X2	↑	↑
IFFO2	**intermediate filament family orphan 2, transcript variant X1**	↓	↑
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	↓	↓
IGSF9B	immunoglobulin superfamily member 9B, transcript variant X1	↓	↓
IKZF4	**IKAROS family zinc finger 4, transcript variant X9**	↑	↓
IL10RA	**interleukin 10 receptor subunit alpha, transcript variant X1**	↑	↓
IRAK1BP1	**interleukin 1 receptor associated kinase 1 binding protein 1, transcript variant X1**	↑	↓
KANSL1L	KAT8 regulatory NSL complex subunit 1 like	↓	↓

KCNF1	**potassium voltage-gated channel modifier subfamily F member 1**	↑	↓
KLF15	Kruppel like factor 15	↓	↓
KLHL4	kelch like family member 4, transcript variant X2	↓	↓
LBP	**lipopolysaccharide binding protein**	↑	↓
LHFPL4	**lipoma HMGIC fusion partner-like 4**	↑	↓
LOC100512420	histone H2B type 1-K	↑	↑
LOC100512780	**calcium-activated chloride channel regulator 4-like**	↓	↑
LOC100515533	oocyte-specific histone RNA stem-loop-binding protein 2	↓	↓
LOC100516628	UDP-glucuronosyltransferase 2B18-like, transcript variant X1	↑	↑
LOC100517025	uncharacterized LOC100517025, transcript variant X9	↓	↓
LOC100517451	olfactory receptor 1J4-like	↑	↑
LOC100519871	ATP synthase F(0) complex subunit C1, mitochondrial-like	↑	↑
LOC100522669	cytochrome P450 2W1	↑	↑
LOC100525798	nucleosome assembly protein 1-like 2, transcript variant X2	↓	↓
LOC100739463	**uncharacterized LOC100739463, transcript variant X2**	↑	↓
LOC100739694	.	↓	↓
LOC102157806	uncharacterized LOC102157806	↓	↓
LOC102158108	uncharacterized LOC102158108, transcript variant X8	↓	↓
LOC102159022	.	↓	↓
LOC102159167	uncharacterized LOC102159167	↓	↓
LOC102161056	uncharacterized LOC102161056, transcript variant X2	↑	↑
LOC102161999	.	↓	↓
LOC102162937	uncharacterized LOC102162937	↓	↓
LOC102164129	**uncharacterized LOC102164129, transcript variant X1**	↑	↓
LOC102164525	uncharacterized LOC102164525, transcript variant X1	↑	↑
LOC102165115	uncharacterized LOC102165115	↑	↓
LOC102165734	putative methyltransferase-like protein 21E pseudogene, transcript variant X2	↓	↓
LOC102165847	zinc finger protein 664-like, transcript variant X2	↓	↓
LOC102166590	putative methyltransferase NSUN6, transcript variant X1	↓	↓
LOC102168193	uncharacterized LOC102168193	↓	↓
LOC106504436	myeloid-associated differentiation marker-like	↓	↓
LOC106504476	.	↓	↓
LOC106504755	**uncharacterized LOC106504755**	↑	↓

LOC106505085	uncharacterized LOC106505085	↑	↑
LOC106505118	**uncharacterized LOC106505118**	↑	↓
LOC106505711	uncharacterized LOC106505711, transcript variant X3	↑	↑
LOC106505716	uncharacterized LOC106505716	↓	↑
LOC106506062	uncharacterized LOC106506062	↓	↓
LOC106507237	uncharacterized LOC106507237, transcript variant X2	↓	↓
LOC106509619	uncharacterized LOC106509619	↓	↓
LOC106509632	uncharacterized LOC106509632	↑	↑
LOC106509926	uncharacterized LOC106509926	↑	↑
LOC106510230	uncharacterized LOC106510230, transcript variant X2	↓	↓
LOC106510246	uncharacterized LOC106510246, transcript variant X1	↑	↑
LOC110255205	ABI gene family member 3-like	↑	↑
LOC110255332	acyl-coenzyme A thioesterase 4-like	↓	↓
LOC110255435	uncharacterized LOC110255435	↓	↓
LOC110255437	N/A	↓	↓
LOC110255528	uncharacterized LOC110255528	↓	↓
LOC110255709	uncharacterized LOC110255709	↑	↑
LOC110255719	uncharacterized LOC110255719	↓	↓
LOC110255896	uncharacterized LOC110255896	↓	↓
LOC110256062	uncharacterized LOC110256062	↓	↓
LOC110256264	translation initiation factor IF-2-like	↑	↑
LOC110256309	uncharacterized LOC110256309	↑	↑
LOC110256840	uncharacterized LOC110256840	↑	↑
LOC110257519	uncharacterized LOC110257519	↓	↓
LOC110257542	zinc finger and SCAN domain-containing protein 2-like, transcript variant X4	↓	↓
LOC110257799	uncharacterized LOC110257799, transcript variant X1	↓	↓
LOC110259339	uncharacterized LOC110259339	↓	↓
LOC110259834	proline-rich protein 36-like	↓	↓
LOC110259908	uncharacterized LOC110259908	↑	↑
LOC110260018	uncharacterized LOC110260018, transcript variant X6	↑	↑
LOC110260030	N/A	↓	↓
LOC110260197	ly-6/neurotoxin-like protein 1	↑	↑
LOC110260457	uncharacterized LOC110260457	↑	↑
LOC110260616	uncharacterized LOC110260616	↓	↓
LOC110260775	uncharacterized LOC110260775, transcript variant X1	↓	↓
LOC110260826	uncharacterized LOC110260826	↓	↓
LOC110261065	uncharacterized LOC110261065, transcript variant X2	↑	↑
LOC110261189	uncharacterized LOC110261189	↑	↑

LOC110261219	uncharacterized LOC110261219, transcript variant X2	↓	↓
LOC110261232	N/A	↑	↑
LOC110261270	uncharacterized LOC110261270	↓	↓
LOC110261421	uncharacterized LOC110261421, transcript variant X2	↓	↓
LOC110261673	histone H4	↓	↓
LOC110261772	uncharacterized LOC110261772	↓	↓
LOC733579	**tripartite motif protein TRIM5**	↑	↓
LYL1	LYL1, basic helix-loop-helix family member	↓	↓
MAP3K12	mitogen-activated protein kinase kinase kinase 12, transcript variant X2	↓	↓
MAP9	microtubule associated protein 9, transcript variant X4	↑	↓
MCAM	melanoma cell adhesion molecule, transcript variant X3	↓	↓
MDFIC	MyoD family inhibitor domain containing, transcript variant X1	↓	↓
MMP28	matrix metallopeptidase 28, transcript variant X2	↑	↑
MTERF2	mitochondrial transcription termination factor 2	↓	↓
MYH11	myosin heavy chain 11	↑	↑
NAPSA	napsin A aspartic peptidase	↓	↓
NID2	nidogen 2, transcript variant X1	↑	↑
NKD1	naked cuticle homolog 1, transcript variant X2	↓	↓
NRXN1	neurexin 1, transcript variant X33	↓	↓
OLFM4	**olfactomedin 4**	↑	↓
ORAI2	**ORAI calcium release-activated calcium modulator 2, transcript variant X3**	↑	↓
P2RY6	pyrimidinergic receptor P2Y6	↓	↓
PARD6G	**par-6 family cell polarity regulator gamma**	↑	↓
PECAM1	platelet and endothelial cell adhesion molecule 1	↓	↓
PFN2	**profilin 2, transcript variant X2**	↑	↓
PIH1D3	**PIH1 domain containing 3, transcript variant X2**	↑	↓
PKHD1	PKHD1, fibrocystin/polyductin, transcript variant X10	↑	↑
PLA2G5	**phospholipase A2 group V**	↑	↓
PLAT	**plasminogen activator, tissue type**	↓	↑
PLCH2	phospholipase C eta 2, transcript variant X2	↓	↓
PLET1	**placenta expressed transcript protein**	↑	↑
PLPPR2	phospholipid phosphatase related 2, transcript variant X1	↑	↑
PLS3	plastin 3	↓	↓
POU2AF1	**POU class 2 associating factor 1**	↑	↓
PPFIA4	PTPRF interacting protein alpha 4, transcript variant X1	↓	↓
PRCD	photoreceptor disc component, transcript variant X2	↓	↓
PRDM8	PR/SET domain 8, transcript variant X1	↓	↓

PTGS2	**prostaglandin-endoperoxide synthase 2**	↓	↑
PYGO1	**pygopus family PHD finger 1**	↑	↓
RAB33A	**RAB33A, member RAS oncogene family**	↑	↓
RAD54L2	RAD54-like 2 (S. cerevisiae), transcript variant X2	↓	↓
RHBDL3	rhomboid like 3, transcript variant X1	↓	↓
RIMS4	regulating synaptic membrane exocytosis 4	↓	↓
RNF112	ring finger protein 112, transcript variant X2	↑	↑
ROBO3	roundabout guidance receptor 3	↓	↓
RSPH1	radial spoke head 1 homolog, transcript variant X3	↓	↓
SAMD15	sterile alpha motif domain containing 15	↓	↓
SCLT1	sodium channel and clathrin linker 1, transcript variant X6	↓	↓
SECTM1	secreted and transmembrane 1	↑	↑
SEMA6B	semaphorin 6B, transcript variant X2	↓	↓
SH2B3	**SH2B adaptor protein 3, transcript variant X3**	↑	↓
SLC22A13	solute carrier family 22 member 13	↑	↑
SLC25A21	**solute carrier family 25 member 21, transcript variant X1**	↑	↓
SLC4A3	solute carrier family 4 member 3	↓	↓
SLC7A6	solute carrier family 7 member 6, transcript variant X6	↓	↓
SOX8	**SRY-box 8**	↑	↓
SPATA2L	spermatogenesis associated 2 like	↑	↑
SPHK1	**sphingosine kinase 1, transcript variant X6**	↑	↓
SPRED3	sprouty related EVH1 domain containing 3, transcript variant X3	↓	↓
STAG3	stromal antigen 3, transcript variant X2	↑	↑
STS	steroid sulfatase, transcript variant X1	↓	↓
SYCE2	**synaptonemal complex central element protein 2, transcript variant X3**	↑	↓
SYCP2	synaptonemal complex protein 2, transcript variant X1	↑	↑
SYTL3	synaptotagmin like 3, transcript variant X4	↓	↓
TARSL2	threonyl-tRNA synthetase like 2	↓	↓
TEX14	testis expressed 14, intercellular bridge forming factor	↓	↓
TFF1	trefoil factor 1	↑	↑
TGFB2	transforming growth factor beta 2, transcript variant X3	↓	↓
TGIF2	**TGFB induced factor homeobox 2, transcript variant X1**	↑	↓
TMEM220	transmembrane protein 220, transcript variant X2	↑	↑
TMEM255B	transmembrane protein 255B, transcript variant X2	↑	↑
TMEM88B	transmembrane protein 88B	↑	↑

TMOD1	**tropomodulin 1, transcript variant X1**	↑	↓
TNFRSF11B	TNF receptor superfamily member 11b	↓	↓
TRAF1	TNF receptor associated factor 1, transcript variant X1	↓	↓
TRIB2	tribbles pseudokinase 2, transcript variant X1	↓	↓
TSHZ2	teashirt zinc finger homeobox 2	↓	↓
TTC36	**tetratricopeptide repeat domain 36, transcript variant X1**	↑	↓
TUBA8	tubulin alpha 8	↑	↑
WNT10A	Wnt family member 10A	↑	↑
YOD1	**YOD1 deubiquitinase, transcript variant X1**	↓	↑
ZNF362	zinc finger protein 362, transcript variant X3	↓	↓
ZNF385C	zinc finger protein 385C	↓	↓

Supplementary table 2. 4**.** Summary of DEGs specific to G5P[7] with and without NA treatment, versus a control group (noninfected).

Bolded genes indicate genes that give contrasting regulation in the presence of NA. Genes are shown with '↓' and'↑' referring to down or upregulation, respectively, without the presence of NA. The upregulated/downregulated genes were determined using a cut off ± ≥ 2 or ≤ 2 when respectively compared with the control group (non-treated). All genes listed account for active protein coding, except "LOC" genes, indicating non-coding.

Gene Symbol	Description	G5P[7] vs. NA	G5P[7] vs. non-infected
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	↓	↓
ACBD7	acyl-CoA binding domain containing 7	↑	↑
ACKR4	atypical chemokine receptor 4	↓	↓
C4H1orf146	chromosome 4 C1orf146 homolog	↑	↑
CATSPER4	cation channel sperm associated 4	↓	↓
CCDC96	coiled-coil domain containing 96	↑	↑
CD274	CD274 molecule	↓	↓
CDH6	cadherin 6	↑	↑
CDON	cell adhesion associated, oncogene regulated, transcript variant X5	↑	↑
CHRM4	cholinergic receptor muscarinic 4	↑	↑
CLIC5	chloride intracellular channel 5	↓	↓
COL14A1	collagen type XIV alpha 1 chain, transcript variant X2	↑	↑
CPA6	carboxypeptidase A6, transcript variant X1	↓	↓
DEPDC4	DEP domain containing 4	↑	↑
DPCR1	diffuse panbronchiolitis critical region 1	↓	↓
DYRK4	dual specificity tyrosine phosphorylation regulated kinase 4	↑	↑
EFEMP1	EGF containing fibulin like extracellular matrix protein 1	↓	↓
ELOVL3	ELOVL fatty acid elongase 3	↑	↑
FAM129C	family with sequence similarity 129 member C	↑	↑
FAM133A	family with sequence similarity 133 member A, transcript variant X1	↑	↑
FAM25A	**family with sequence similarity 25 member A**	↓	↑
FBN2	fibrillin 2	↓	↓
FGF22	fibroblast growth factor 22	↑	↑
FOXP3	forkhead box P3	↑	↑

GP9	glycoprotein IX platelet	↓	↓
GPAT2	**glycerol-3-phosphate acyltransferase 2, mitochondrial, transcript variant X3**	↓	↑
GPR15	G protein-coupled receptor 15	↑	↑
HRH4	histamine receptor H4	↓	↓
IL9R	interleukin 9 receptor	↓	↓
KCNA7	potassium voltage-gated channel subfamily A member 7	↓	↓
KIAA1549L	KIAA1549 like, transcript variant X1	↑	↑
KIF1A	kinesin family member 1A	↓	↓
LOC100157711	sodium/potassium-transporting ATPase subunit alpha-4	↓	↓
LOC100513233	uncharacterized serine/threonine-protein kinase SgK494	↑	↑
LOC100514465	beta-1,4-galactosyltransferase 3-like, transcript variant X2	↑	↑
LOC100515156	.	↓	↓
LOC100521853	**.**	↑	↓
LOC100522141	tetratricopeptide repeat protein 9B, transcript variant X2	↑	↑
LOC100524499	cationic amino acid transporter 3-like	↑	↑
LOC100623534	uncharacterized LOC100623534	↑	↑
LOC102157909	.	↓	↓
LOC102158266	uncharacterized LOC102158266	↑	↑
LOC102158284	uncharacterized LOC102158284	↑	↑
LOC102159045	uncharacterized LOC102159045, transcript variant X1	↓	↓
LOC102163680	uncharacterized LOC102163680	↑	↑
LOC102163684	uncharacterized LOC102163684	↓	↓
LOC102163764	**uncharacterized LOC102163764, transcript variant X2**	↓	↑
LOC102163801	uncharacterized LOC102163801, transcript variant X1	↓	↓
LOC102165100	.	↓	↓
LOC102166523	RWD domain-containing protein 1-like	↓	↓
LOC102167177	uncharacterized LOC102167177, transcript variant X1	↑	↑
LOC106504286	.	↓	↓
LOC106506130	**uncharacterized LOC106506130**	↑	↓
LOC106507761	**uncharacterized LOC106507761**	↓	↑

LOC106508273	uncharacterized LOC106508273, transcript variant X2	↑	↑
LOC106509005	uncharacterized LOC106509005	↓	↓
LOC106509052	uncharacterized LOC106509052, transcript variant X1	↓	↓
LOC106509551	uncharacterized LOC106509551, transcript variant X2	↑	↑
LOC106510047	uncharacterized LOC106510047	↑	↑
LOC110255210	ATP-binding cassette sub-family G member 2-like	↓	↓
LOC110255442	.	↓	↓
LOC110255920	uncharacterized LOC110255920	↑	↑
LOC110256326	uncharacterized LOC110256326, transcript variant X2	↑	↑
LOC110257148	uncharacterized LOC110257148, transcript variant X2	↑	↑
LOC110257248	collagen alpha-2(I) chain-like	↓	↓
LOC110257628	uncharacterized LOC110257628	↑	↑
LOC110257707	uncharacterized LOC110257707, transcript variant X1	↓	↓
LOC110259227	uncharacterized LOC110259227, transcript variant X1	↓	↓
LOC110260061	uncharacterized LOC110260061, transcript variant X2	↑	↑
LOC110260079	uncharacterized LOC110260079	↑	↑
LOC110260387	uncharacterized LOC110260387	↓	↓
LOC110260443	uncharacterized LOC110260443	↓	↓
LOC110260746	uncharacterized LOC110260746	↑	↑
LOC110261075	uncharacterized LOC110261075	↓	↓
LOC110261160	uncharacterized LOC110261160	↓	↓
LOC110261421	uncharacterized LOC110261421, transcript variant X2	↓	↓
LOC110261594	uncharacterized LOC110261594	↓	↓
LOC110261683	uncharacterized LOC110261683, transcript variant X2	↓	↓
LOC110261743	uncharacterized LOC110261743	↓	↓
LOC110262049	uncharacterized LOC110262049	↓	↓
LOC110262129	uncharacterized LOC110262129	↓	↓
LOXL4	lysyl oxidase like 4, transcript variant X3	↓	↓
LRRC43	leucine rich repeat containing 43, transcript variant X3	↑	↑
LTB4R	leukotriene B4 receptor, transcript variant X1	↓	↓

MCIDAS	multiciliate differentiation and DNA synthesis associated cell cycle protein	↑	↑
MDFI	MyoD family inhibitor, transcript variant X4	↓	↓
MTERF2	mitochondrial transcription termination factor 2	↓	↓
MYH15	myosin heavy chain 15	↑	↑
NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2, transcript variant X1	↓	↓
NLRP6	NLR family pyrin domain containing 6, transcript variant X2	↓	↓
OLFM1	olfactomedin 1	↓	↓
PIRT	phosphoinositide interacting regulator of transient receptor potential channels, transcript variant X2	↑	↑
PLA2G4D	phospholipase A2 group IVD	↓	↓
PLPPR5	phospholipid phosphatase related 5	↑	↑
PPBP	pro-platelet basic protein	↑	↑
REP15	RAB15 effector protein	↑	↑
SEMA6D	semaphorin 6D, transcript variant X1	↓	↓
SLC16A4	solute carrier family 16 member 4, transcript variant X3	↓	↓
SLC1A2	solute carrier family 1 member 2, transcript variant X2	↓	↓
SLC23A3	solute carrier family 23 member 3	↑	↑
SLC30A10	**solute carrier family 30 member 10**	↓	↑
SNAI1	**snail family transcriptional repressor 1**	↓	↑
SNX22	**sorting nexin 22, transcript variant X2**	↑	↓
STARD7	StAR related lipid transfer domain containing 7	↓	↓
TKTL2	transketolase like 2	↑	↑
TMCC2	transmembrane and coiled-coil domain family 2, transcript variant X1	↑	↑
TMPPE	transmembrane protein with metallophosphoesterase domain	↓	↓
VSIG10L	V-set and immunoglobulin domain containing 10 like	↑	↑
WDR78	WD repeat domain 78	↑	↑
ZNF19	zinc finger protein 19	↑	↑
ZNF23	zinc finger protein 23	↓	↓
ZNF500	zinc finger protein 500	↓	↓

Chapter 3 Reverse Genetics Confirms the Key Role of Porcine Rotavirus A VP4 in Cell Attachment and Interactions with Sialoglycans

3.1 Summary

Rotaviruses (RVs) are a large genetically diverse and rapidly evolving population of double-stranded RNA viruses, causing severe gastroenteritis in a wide variety of species including humans. Re-emergence and high prevalence of G9 RVA genotype led to intensified studies on their pathogenesis, whole genome sequence analysis and immunogenicity. Studies on G9P[13] replication *in vitro* has revealed unique features in terms of interactions with cellular host receptors, especially sialic acids (SAs). Removal of external SAs from the target cell surface resulted in increased replication of G9P[13] in porcine intestinal enteroids (PIEs) while for several other RVAs this treatment was shown to either reduce or have no effect on replication. Furthermore, we have identified two unique mutations within the VP4 fusion region of prevents G9P[13] and an historically dominant porcine RVA G5P[7] OSU that might differentially regulate cell entry.

In our initial experiments, we used pT7 plasmids carrying the model simian RVA - G3P[2] SA-11 genes whereby its original VP4-coding gene was substituted by those from G9P[13] or G5P[7] OSU and replication of the chimeric viruses was compared with parental virus. Our study demonstrated that we were able to successfully rescue the chimeric G3P[2] SA-11 viruses containing VP4 proteins of G9P[13] or G5P[7] OSU; and the chimeric viruses possessed different growth kinetics. Next, to functionally characterize the role of the identified distinct VP4 mutations in virus-host interactions, we have established a completely plasmid

based reverse genetics system (RGS) for OSU RVA. In the future, this system will allow us to confirm that the unique features of interactions of G9P[13] with SAs are VP4-dependent and to evaluate the role of the OSU and G9P[13] strain-specific unique mutations.

3.2 Introduction

Rotaviruses (RV) are members of the *Reoviridae* family known to be a prevalent cause of severe acute gastroenteritis in infants and animals [1]. RVA genome consists of an 11 gene segmented, double-stranded RNA (dsRNA) genome that it is contained in a nonenveloped icosahedral virion formed by three concentric protein layers [2, 3]. RVs are further divided in groups based on distinct antigens, classifying them in groups A-D, F-J [4]. These groups are arranged by a binary classification system based on the two surface proteins of RVs, VP7 (glycoprotein, G protein) and VP4, (protease sensitive spike protein, P protein) each of which independently induces virus neutralizing antibodies [5, 6].Of these, group A RV (RVA) is associated with high prevalence and severity of enteric diseases (including the acute gastroenteritis) in children and young animals [1, 5, 6]. Despite the introduction of RVA vaccines, these infections still account for >200,000 deaths in children <5 years in low-income countries where efficacy is variable [1]. RVAs have been consistently recognized in pigs, including with the emergence of G9 and G1 strains. Abundance of G9P[13] demonstrated by Amimo and colleagues, have attracted attention of scientists in order to evaluate unique properties of this genotype contributing to its wide prevalence [7]. After detection and isolation on cell culture, our lab has extensively been working to dissect a wide spectrum of host-virus interactions of porcine G9P[13] [7-9]. We have demonstrated that the currently available

vaccines against RVA may not provide optimal protection against heterologous G9P[13] [9]. In order to evaluate the exact mechanisms on why this could be possible, we were able to demonstrate preference of G9P[13] in attachment to histo-blood group antigens (HBGAs), specifically H+. Sialic acids (SAs) are well known attachment/entry factors for RVAs [10, 11]. Removal of external SAs from cells by sialidase treatment led to significantly decreased replication of some RVA strains with no effect on replication of others. During evaluation of the role of sialidase treatment on replication of G9P[13] we have shown a unique characteristic of this virus – its replication was significantly increased after sialidase treatment of MA-104 and porcine intestinal enteroids (PIEs). Further, these unique features have been shown to reflect a mutation within the gene encoding spike protein of G9P[13] (VP4). More specifically, comparative sequence analysis of the genomes of G5P[7] OSU with G9P[13] revealed two amino acid (aa) substitutions (S385N and D393N) within the VP4 hydrophobic loop og G9P[13]. This G9P[13] genomic feature reveal heterogeneity among RVA strains in attachment/entry/replication mechanisms, proving this is an important target for investigation.[8-10]. VP4 is considered as a major protein contributing to RVA attachment/entry, VP7 outer capsid protein has also been shown to play a critical role in RVA entry [12]. Thus, whether mutations in VP4 are solely responsible for the unique host-virus interactions of G9P[13] remains unknown.

Reverse genetics systems (RGS) have been used recently to rapidly engineer viruses, with the ability to introduce desired mutations [12-14]. The first RGS for an RNA virus was established in 1978 using a simian strain RVA G3P[2] (SA11), and over the recent decades there have been a multitude of systems established for a variety of viruses including Bluetongue virus,

Coltivirus, *Vesivirus*, and has been used among SARS-CoV-2 for a coronavirus investigation tool [15-21]. Of interest, a RGS protocol for rotaviruses have been optimized previously by the Patton lab using the RVA SA11 strain.

Thus, in order to evaluate the role of VP4 in unique interactions of G9P[13] with cellular receptors, we have used recombinant simian RVA G3P[2] SA11 containing of G9P[13] or G5P[7] OSU VP4 spike proteins and developed a complete OSU-based reverse genetics system.

3.3 Materials and Methods

3.3.1 Cells and Porcine Intestinal Enteroids

African green monkey pure cell line (MA-104) cells were used. Cells were cultured in complete medium consisting of advanced MEM (aMEM, Gibco) and supplemented with 1% Antibiotic-Antimycotic (Gibco), and 10% Fetal Bovine Serum (FBS, Gibco) in a humidified incubator at 37°C, 5% CO_2. Cells were split every 3-5 days. After 2-3 days of growth, medium was removed, and cells were trypsinized.

Cells were counted with a Cellometer Auto T4 (Nexcelom Bioscience) and adjusted to a final concentration of 64000 cells/mL and loaded onto a 96-well plate with 100 μL in each well, and incubated at 37°C, 5% CO_2 for 3-4 days until the monolayers were 90-95% complete.

Porcine intestinal enteroids (PIE) were established and maintained as described by Guo et. al, 2021 [31].

3.3.2 Rotavirus A strains

Gnotobiotic pig small intestinal contents containing RVA G9P[13] and RVA OSU G5P[7] were used in the study. Intestinal contents were diluted at a 1:10 ratio in sterile Minimal Essential Media (MEM Gibco; Life Technologies, Grand Island, NY, United States). Contents were then centrifuged at 3000 rpm for 10 minutes at 4°C and the supernatants filtered through a 0.2 mm filter.

3.3.3 RNA extraction for genes amplification

RNA was extracted using RNAeasy kit (Qiagen). RT-qPCR was performed using One-step RT-PCR Kit (Qiagen, Germantown, MD, USA) using the primers and probe indicated (Table 3.1). Graph Pad Prism (GraphPad Software, San Diego, CA, USA) was used for data representation.

3.3.4 Structural Modeling

The 3D structure of the OSU VP4 protein carrying the original and G9P[13]-like amino acids in the positions 385 and 393 were modeled with SWISS-MODEL (https://swissmodel.expasy.org) using OSU VP4 protein sequence as the template. The structural analysis was carried out with UCSF Chimera (http://www.rbvi.ucsf.edu/chimera).

2.3.5 Cloning of VP1-4, VP6, VP7 and NSP1-5 OSU genes and G9P[13] VP4

We designed specific primers for VP4 of both G9P[13] and G5P[7] OSU to construct the recombinant pT7 vectors that include specific restriction enzyme recognition sites [XmaI for all forward primers, BseRI (VP1-4,6,7) and RsrII (NSP1-5) for reverse primers] **(Table 3.1)**. The RNA obtained from intestinal contents was converted into cDNA using SuperScript™ IV Reverse Transcriptase (Thermo Fisher Scientific). To obtain gene amplicons cDNA synthesis was performed using the following PCR conditions and using designed primers: VP1: 2 min amplification at 60°, and VP2-NSP5 at 55°, with NSP fragments amplified at 55° for 30s. PureLink™ Quick Gel Extraction Kit (Invitrogen) was used to extract the fragments from the gel. Results were analyzed using 1% agarose gel. Each fragment was then preserved via blunt-end cloning using CloneJET PCR Cloning Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Ligation mixtures were transformed into *E. coli* DH10B Competent Cells (Thermo Fisher Scientific). Recombinant clone selection was done using LB agar with carbenicillin (Thermo Fisher Scientific). Obtained cDNA fragments were cloned into a pT7 vector (Addgene). Plasmid restriction enzyme digestion was performed using XmaI, RsrII, and BseRI (NEB) enzymes and rCutSmart Buffer (NEB) following manufacturer's recommendations. Restriction enzyme digestion was also performed on pT7-VP1SA11 plasmid [a gift from Takeshi Kobayashi (Addgene plasmid # 89162; http://n2t.net/addgene:89162 ; RRID:Addgene_89162)] to obtain linearized pT7 vector [16]. Each insert was individually ligated into the pT7 plasmid vector using T4 DNA Ligase (Thermo Fisher Scientific), following the manufacturer's protocol. PureLink™ Quick Gel Extraction Kit (Invitrogen) was used for plasmid DNA extraction. Presence of the fragments of expected sizes in plasmids was confirmed using PCR with T7 promoter/terminator primers and Q5 high fidelity PCR (NEB) and by

restriction enzyme digestion with XmaI/BseRI or XmaI/RsrII, respectively. The recombinant plasmids were also analyzed by Sanger sequencing (using gene specific and T7 promoter/terminator primers) to confirm that no mutations were introduced during cloning. The pT7 plasmids were preserved at -80°C until used.

3.3.6 Generation of Recombinant Virus

We used commercially available plasmids from Addgene (from Takeshi Kobayashi's lab): pT7-VP1SA11, pT7-VP2SA11, pT7-VP3SA11, pT7-VP4SA11, pT7-VP6SA11, pT7-VP7SA11, pT7-NSP1SA11, pT7-NSP2SA11, pT7-NSP3SA11, pT7-NSP4SA11, and pT7-NSP5SA11, containing the full-length cDNA of the corresponding gene segment from of G3P[2] SA-11.

Generation of the recombinant viruses was conducted following the RGS protocols developed in the Patton lab at Indiana University [22]. Briefly, BHK-SR19T7 (BHK-T7) cells were kindly provided by Dr. Mark E. Peeples, Nationwide Children's Hospital, Columbus, Ohio. The cells were maintained in growth media containing MEM, supplemented with 10% FBS, 4 μg/mL Puromycin, and 1% Antibiotic-Antimycotic solution (Gibco), and passed at confluency. On day one, a monolayer of BHK-T7 was rinsed twice with PBS, trypsinized using a trypsin-EDTA (0.25%) solution and resuspended in growth media. Cells were counted by Cellometer Auto T4 (Nexcelom Bioscience) and concentration was adjusted to 2 x 10^5/mL and plated on a 12-well cell culture plate. Cells were incubated at 37°C, 5% CO_2 for 3-4 days until 90% confluency. Then BHK-T7 cells were transfected with SA-11 plasmids, or with SA-11 plasmids

where VP4 of SA-11 was substituted with those of G5P[7] OSU or G9P[13]. In a 0.5 mL microcentrifuge tube, 1 mg/mL of each plasmid was added as previously described: 0.8 µL of viral protein (VP)1, VP2, VP3, VP4, VP6, VP7, non-structural protein (NSP)1, NSP3, NSP4 and 2.4 µL of NSP2 and NSP5. Additionally, 0.8 µL of pCMV/NP868R was added to the mixture, which was gifted from Dr. John Patton, Department of Biology, University of Indiana. These concentrations remained constant with different strains. All plasmids were kept on ice until use. Once plasmids were combined, 110 µL of prewarmed Opti-MEM reduced serum medium (Gibco) was added and gently mixed. Afterwards, 32 µL of TransIT-LTI Transfection reagent (Mirus) was added to the plasmid/media mixture. The mixture was vortexed briefly and incubated at room temperature for 20 minutes. During the incubation, BHK-T7 monolayers were rinsed with 2 mL of media consisting of 500 mL of Dulbecco's modified Eagle's MEM, 4.5 g/L glucose, 1% glutamine, and 1% of 100x penecillyn-streptomicyn solution (incomplete media). After the 20-minute incubation period, the transfection mixture was added dropwise onto BHK-T7 cells and incubated at 37°C, 5% CO_2 for 48 hours. Monolayers of MA104 cells were trypsinized using trypsin and adjusted to a concentration of $8x10^5$ cells/mL in incomplete medium. 0.25 mL ($2x10^5$) MA-104 cells were added onto the transfected BHK-T7 cells. Three days later, the BHK-T7/MA-104 cells were subjected to three cycles of freezing and thawing. The lysates were transferred to a 1.5 mL tube, and centrifuged for 10 minutes at 500 x *g*, at 4°C. Supernatants were removed from the pelleted cells, collected for further amplification, and stored at -20°C for longer term. Monolayers of MA-104 cells was washed twice with PBS and 2 mL of incomplete media containing 0.5µg/mL of trypsin. Following this, 300 µL of BHK-T7/MA-104 cell lysate was added to MA-104 monolayer and incubated at 37°C, 5% CO_2 for 7 days, or until

cytopathic effect was observed. MA-104 cells were lysed by undergoing three freeze-thaw cycles and placed in 1.5 mL tubes, centrifuged, and the clarified supernatants were stored at -80°C.

3.3.7 Cell Culture Immunofluorescence (CCIF)

We used CCIF to determine infectious (replicating) RVA titers. MA-104 cells were maintained in T75 flasks and passed every 3-5 days. Cells were kept in complete medium consisting of advanced MEM (aMEM, Gibco) and supplemented with 1% Antibiotic-Antimycotic (Gibco), and 10% Fetal Bovine Serum (FBS, Gibco). After 2-3 days of growth, medium was removed, and MA-104 cells were trypsinized. Then 20 mL of complete medium was added to neutralize trypsin. Cells were counted using Cellometer Auto T4 (Nexcelom Bioscience) and adjusted to a final concentration of 64000 cells/mL. Cells were loaded onto a 96-well plate with 100 µL in each well, and incubated at 37°C, 5% CO2 for 3-4 days until monolayer was 90-95% complete. Once MA-104 monolayer were ready, cells were washed with medium containing MEM, 1% Antibiotic-Antimycotic, and 1% Non-Essential Amino Acids Solution (Gibco) (wash medium). 100uL wash medium was added to cells and incubated at 37°C for an hour. Afterwards wash medium was removed and cells were inoculated with cell lysates starting from diluted samples (1:10), then a 4-fold dilution (1:100), followed by two 10-fold dilutions (1:1000 and 1:10000). Plates were centrifuged at 2,600 rpm, at 22°C for 30 minutes. Then plates were incubated at 37°C, 5% CO_2 for 24 hours. Afterwards, cells were fixed with 80% acetone and dried at room temperature for two hours followed by use of acell culture immunofluorescent (CCIF) assay for RVA quantification as described previously [23]. Virus

titers (FFU/mL) were calculated under the formula of average number of fluorescent foci in duplicate wells multiplied by dilution/inoculum volume.

3.3.8 RNA extraction and PCR

RNA was extracted using MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific). RVA PCR was performed as described previously [8]. Ct values from RT-PCR were converted to FFU/mL based on a standard curve previously generated in our lab. Graph Pad Prism (GraphPad Software, San Diego, CA, USA) was used for data representation.

3.3.9 RVA growth kinetics

Complete MA-104 cells monolayers were infected with parent and recombinant viruses as described previously [24]. Briefly, all viruses were preactivated with trypsin as described above. MA-104 cells were washed twice, wash medium was removed, and cells were inoculated with viruses with at MOI 0.1 and incubated at 37°C, 5% CO_2 for 72 hours. Infected cells were harvested at various time points. Harvested cells were kept at -80°C until use. Cells were homogenized prior to RNA extraction/CCIF.

3.4 Results

3.4.1 G9P[13] and OSU unique VP4 mutation identification and VP4 Structural Modeling

We have previously identified the key mutations associated with RVA cell culture adaptation and attenuation in the VP4 aa positions 385 and 393 [25]. We have now additionally compared, the VP4 proteins of the virulent RVA OSU and G9P[13] strains and demonstrated that they possessed unique aa substitutions in these positions **(Fig. 3.1)**.

We hypothesized that S385N and D393N substitutions in the OSU VP4 will alter its 3D structure which subsequently may result in G9P[13]-like interactions with SAs of the OSU-based chimeric progeny virus. We have conducted structural analysis using Swiss-Model and demonstrated that the 3D structure was altered very slightly where mutations were substituted **(Fig. 3.2)**. These slight conformational changes could alter the structure of the fusion domain that was shown to play a significant role in the attachment of SA-independent RVAs but this remains to be investigated [26-28].

3.4.2 Generation and rescue of SA11 chimeric viruses carrying OSU or G9P[14] VP4

To compare the effects of the G5P[7] OSU and G9P[13] VP4 on virus replication/assembly we used the commercially available RVA SA-11 RGS and the pT7-OSU-VP4 and pT7-G9P[13]-VP4 we generated. In this experiment, the plasmid backbone of SA11 was used with a) the original SA11 VP4 as a control, b) OSU VP4 and c) G9P[13] VP4.

The RGS protocol shown here **(Figure 3.3)** describes the steps of RVA cloning, co-transfection of BHK-T7 cells with pT7 vectors, substitution of gene segments, and overseeding with MA104 cells. During the virus amplification in MA104 cells (following plasmid

transfection and BHK-T7/MA104 overlay stage), rescued SA-11 virus containing the VP4 of G9P[13] resulted in more prominent CPE effects within 24 **(Figure 3.4E)** and 48 hours **(Figure 3.4F)** compared to G5P[7] OSU **(Figures 3.4B and 3.4C, respectively)**. Surprisingly, G9P[13]-VP4-associated CPE was more pronounced even compared to the effect of infection with the parental G3P[2] SA-11 strain **(Figure 3.4 G-I)**. To confirm viral presence, qRT-PCR was performed **(Figure 3.5)**. Of interest, G3P[2] SA-11 rescued after transfection had median titers around 10^4 FFU/mL while the subsequent infection of MA-104 cells resulted in increased viral titer (~$3x10^6$ FFU/mL) by 48 hrs post-infection. In contrast, amplification of rescued G5P[7] OSU resulted in lowest final titer at $1.7x10^3$ FFU/mL, while G9P[13] had almost double final titer at $3.6x10^3$ FFU/mL. Results from this RT-PCR was confirmed by run on 3% agarose gel depicting positive bands to further support virus presence.

3.4.3 Generation of OSU G5P[7] RGS

Next, we proceeded with cloning of the remaining G5P[7] OSU VPs and all NSP genes. We have successfully cloned all OSU genes and validated the recombinant plasmids using PCR, restriction enzyme digestion and sequencing as described in Materials and Methods. We are now conducting experiments to ***generate and rescue OSU-G9P[13] VP4 chimeric viruses containing one (S385N or D393N) or two (S385N and D393N) mutations, to compare the growth kinetics of the parental and chimeric viruses and to test sialidase (neuraminidase, NA) sensitivity of the parental and chimeric viruses.***

3.5 Discussion

The genome manipulation using reverse genetics systems provides a critical tool essential for advanced viral replication and pathogenesis studies. With the validation of this process, we seek to further explore the molecular mechanisms of RVA interactions with the host cell glycans. Our 3D structural modeling analysis allowed us to predict the potential structural alterations in the OSU VP4 after introduction of the G9P[13]-like substitutions (S385N and D393N). This analysis suggested that these structural alterations could be responsible for the contrasting modes of G5P[7] OSU vs G9P[13] interactions with SAs.

Next, our initial data using the SA11-based RGS demonstrated that while we were able to rescue all the chimeric RVA viruses, introduction of heterologous VP4 altered RVA growth kinetics. This is consistent with the previous findings that demonstrated that the rescue of viable RVA reassortants bearing heterologous VP4s is strain-dependent [29]. We have generated and validated an entirely plasmid-based RVA RGS for G5P[7] OSU strain, which is characterized by robust *in vivo* and *in vitro* replication and is uniquely NA-sensitive compared to other porcine RVA we tested in our studies. We next plan to use this system to generate a series of chimeric virulent OSU RVAs carrying the full-length G9P[13] VP4, fusion domain of the G9P[13] VP4 or the G9P[13]-like substitutions S385N and D393N to establish if these alterations are responsible for G9P[13]-like interactions with host cell glycans.

Collectively, our data suggest that the overall VP4 structure affects the efficacy of the chimeric RVA rescue and alters their growth kinetics and likely due to the modulation of the mechanisms of the VP4 interaction with cell receptors including SAs. In conclusion we have established an RGS platform that allows for mechanistic studies of RVA-host glycan

interactions. Such knowledge is useful for basic and applied science, including the targeted identification and validation of potential therapeutics and vaccine developments.

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Contributions: Conceptualization, M.R. and A.N.V.; methodology, M.R.; formal analysis, M.R.; investigation, M.R.; resources, A.N.V.; writing—original draft preparation, M.R.; writing—review and editing, A.N.V., and L.J.S; visualization, M.R..; supervision, A.N.V.; project administration, A.N.V.; funding acquisition, A.N.V. All authors have read and agreed to the published version of the manuscript.

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Gene	Sequence
VP1-F	TATCGATCCCGGGTTAATACGACTCACTATAGGCTATTAAAGCTGTACAATGGGGAAGTACAAT
VP1-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCCGGTCACATCTAAGCGTTCTAATCTTGAAAGAAATTA
VP2-F	TATCGATCCCGGGTTAATACGACTCACTATAGG GGCTATTAAAGGCTCAATGGCGTACAGGAAG
VP2-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCC AACATATGCATTGACGCT CTAACAAATCGGAATAA
VP3-F	TATCGATCCCGGGTTAATACGACTCACTATAGG CTCTGATGGTGTAAAC ATGAAAGTATTAGCTTT
VP3-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCC GTAATTTAGTAGTCT TCACTCAGACATATC
VP4-F	TATCGATCCCGGGTTAATACGACTCACTATAGG GGCTATAAA ATGGCTTCGCTCATT
VP4-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCCGGTCACATCCTCTAGAAATTACAACTTACATTG
VP4G9-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCCGGTCACATCCTCTAGAAATCACAACTTACATTG
VP6-F	TATCGATCCCGGGTTAATACGACTCACTATAGGGGCTTTTAAACGAAGTCTTCAACATGGAGGTTCTGTAC
VP6-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCC CATCTGAGTGATTACT TCACTTAATCAACATGCTTC
VP7-F	TATCGATCCCGGGTTAATACGACTCACTATAGG GGTTAGCTCCTTTTA ATGTATGGTATTGAATATACCA
VP7-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCCTCTAACCTAAGTTATAC CTAGACTCGGTAATA
NSP1-F	TATCGATCCCGGGTTAATACGACTCACTATAGG TTTTTTGAAAAGT ATGCTTGCTATTATT
NSP1-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCCATAGTGACATAATTTC CTAGGCGCTACTCTAGTG
NSP2-F	TATCGATCCCGGGTTAATACGACTCACTATAGG AGCCTTGCGGTGTAGCC ATGGCTGAGCTAGCTT
NSP2-R	AGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCGGTCACATCTAAGCGTT ACTTCGTCCATTTTT TTAAATTCCAACATGTGA
NSP3-F	TATCGATCCCGGGTTAATACGACTCACTATAGG GCTTTTCAGTGGTTG ATGCTCAAGATGGAGTCTACTCAG
NSP3-R	AGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCGGTCACATCTAAGCGTT TGTTAGCTTTTAA CTATTCATATGTACATTC
NSP4-F	TATCGATCCCGGGTTAATACGACTCACTATAGG GCGTGCGGAAAG ATGGATAAGCTTGCC
NSP4-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCCCACTTTCCCATTCTT TCACATAGACGCAGT
NSP5-F	TATCGATCCCGGGTTAATACGACTCACTATAGG TAAAGCGCTACAGTG ATGTCTCTCAGCATTG
NSP5-R	CCGCGAGGAGGTGGAGATGCCATGCCGACCCTGGGGAGCTCC TTACAAATCTTCGAT
PT7-Promoter	CTGTGGATAACCGTATTACCG
PT7-Terminator	GCTAGTTATTGCTCAGCGG

Table 3. 1 Cloning primers.

List of designed gene specific primers used to confirm fragments and the presence of pT7 plasmid.

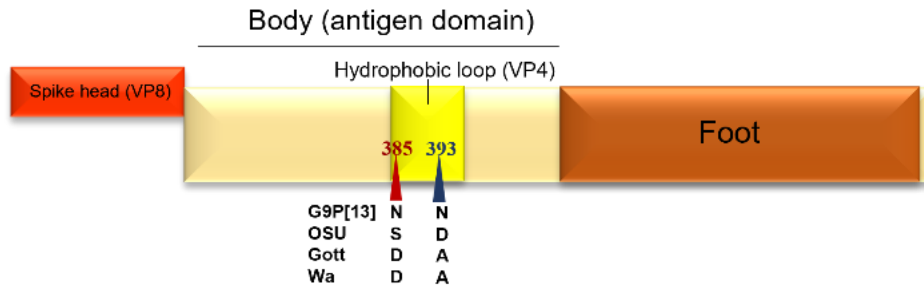

Figure 3. 1 Virulent RVA strain-specific mutations within the VP4 hydrophobic loop.

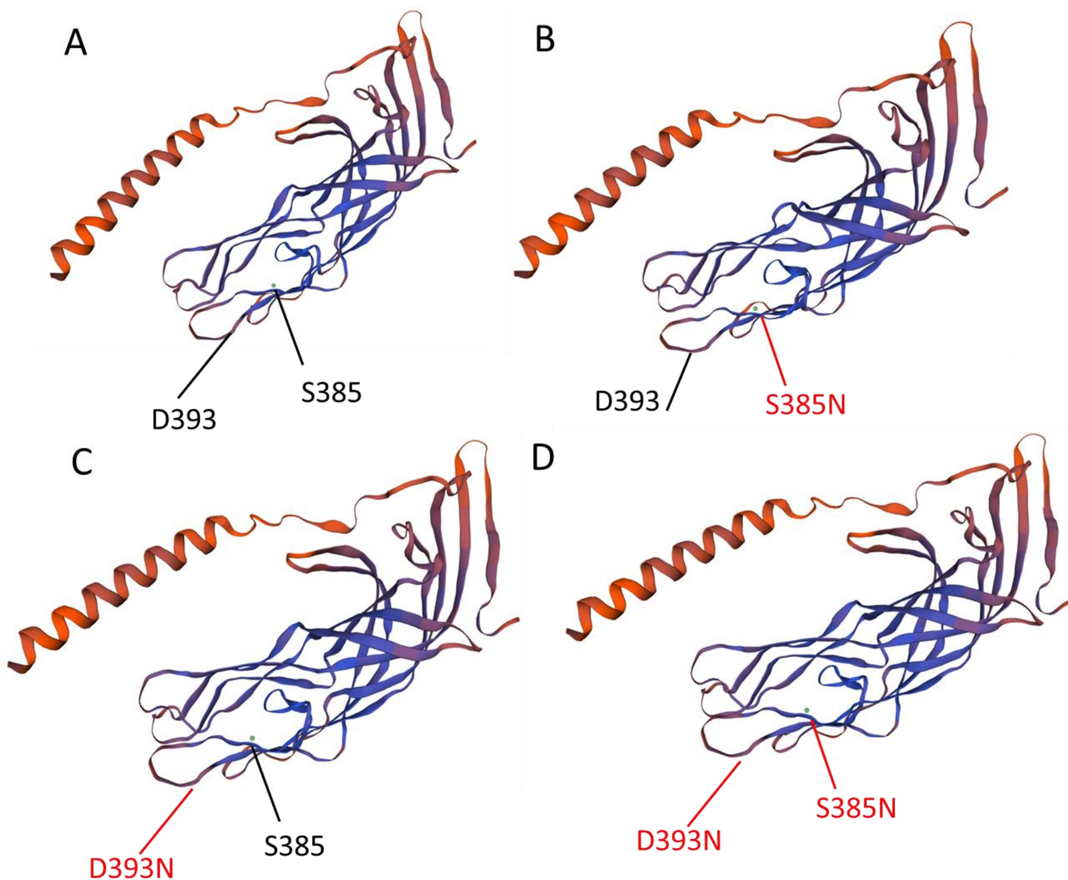

Figure 3. 2 SWISS-Model Expasy generated 3D models.

Structures of RVA OSU VP4 carrying the original S385 and D393 (A), G9P[13]-like N385(B), N393 (C), and both G9P[13]-like mutations (D). Red lines and mutations indicate G9P[13]-like mutations on OSU backbone.

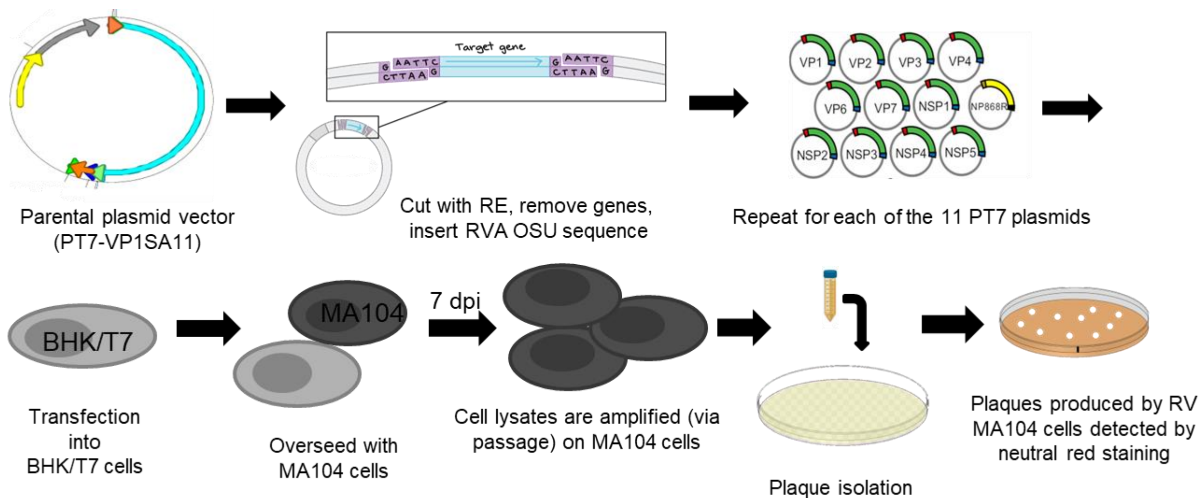

Figure 3. 3 Workflow of PRVA reverse genetics system establishment.

Parental plasmid vector PT7-VP1SA11 was used and cut with restriction enzyme digestion to insert each individual OSU VP and NSP sequences, and G9 VP4. NP868R was maintained as an addition to each of the recombinant viruses made. The presence of the specific inserts were confirmed using PCR, and the plasmids were transfected into BHK-T7 cells. Three days later, MA104 cells were overseeded onto plates. Seven days post infection, or until cytopathic effects were observed, cell lysates were amplified via passage on to MA104 cells. Afterwards, viral replication was confirmed using RT-PCR and then visually by using plaque isolation.

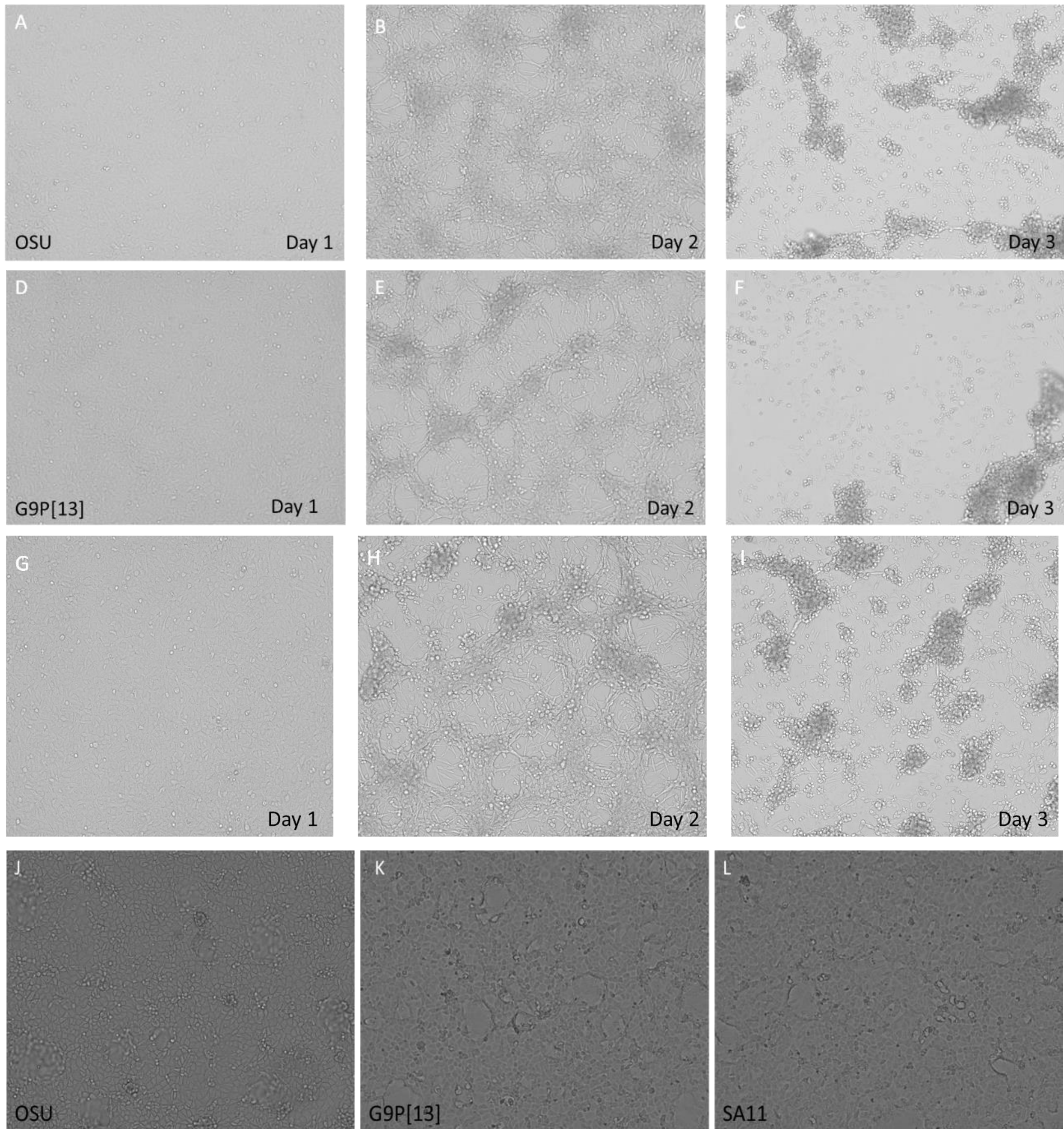

Figure 3. 4 The effect of replication of parental (G3P[2] SA-11) and two chimeric (G5P[7] OSU VP4) and G9P[13] VP4) viruses in MA-104 cells.

Microscopy photos following RGS protocol, with substituted VP4 of parental with or replication of G3P[2] SA-11 with G5P[7] OSU VP4 substitution after 24 (A), 48 (B) and 72 (C), **B** hours after transfection. **D-F:** replication of G3P[2] SA-11 with G9P[13] VP4 substitution after 24, 48 and 72 hours, respectively. Replication of parental virus G3P[2] SA-11 after 24 **(G)**, 48 **(H)** and

72 **(I). K-L:** Amplification completed on Day 6. Visually, G9P[13] showed CPE on Day 5 and had increased areas of CPE compared to G3[2] SA11 and OSU. CPE for G3[P2] SA11 began on Day 6. OSU showed very little CPE, following the previous trend where CPE was less compared to G9P[13] and G3P[2] SA11.

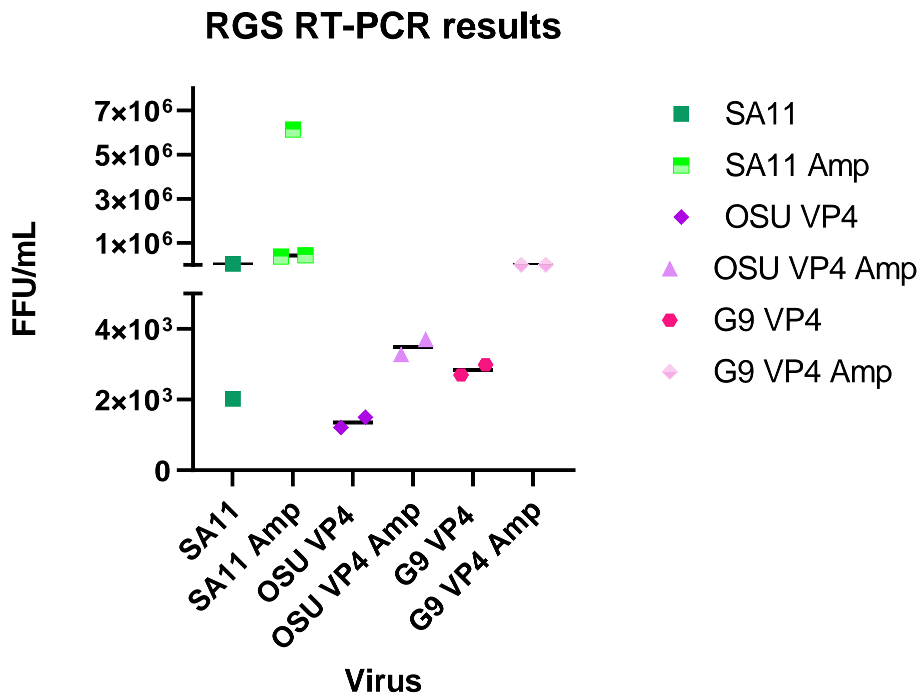

Figure 3. 5 Replication of parental (G3P[2] SA-11) and two chimeric (G5P[7] OSU VP4) and G9P[13] VP4) viruses.

Quantitative RT-PCR was used to detect RVA RNA and obtained Ct values were converted to FFU/mL based on a standard curve previously generated in our lab. . Denoted are the results from SA11 initial lysates, SA11 amplification (shown as "amp"), OSU VP4 substitution, and G9 VP4 substitution.

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