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MAJOR ARTICLE

H and B Blood Antigens Are Essential for In Vitro Replication of GII.2 Human Norovirus

Shigeyuki Tamiya,[1,2] Naomi Matsumoto,[2] Shiho Kurokawa,[3] Yutaka Nakamura,[1] Yu Takahashi,[4] Naomi Sakon,[5] Mikihiro Inoue,[6,7] Yuhki Koike,[6] Keiichi Uchida,[8] Yoshikazu Yuki,[3] Hiroshi Ushijima,[9] Hiroshi Kiyono,[3,10,11] and Shintaro Sato[1,2]

[1]Department of Microbiology and Immunology, School of Pharmaceutical Sciences, Wakayama Medical University, Wakayama , Japan, [2]Department of Virology, Research Center for Infectious Disease Control, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, [3]Department of Human Mucosal Vaccinology, Chiba University Hospital, Chiba, Japan, [4]Food Biochemistry Laboratory, Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan, [5]Department of Microbiology, Osaka Institute of Public Health, Osaka, Japan, [6]Department of Gastrointestinal and Pediatric Surgery, Mie University Graduate School of Medicine, Mie, Japan, [7]Department of Pediatric Surgery, Fujita Health University, Aichi, Japan, [8]Department of Pediatric Surgery, Mie Prefectural General Medical Center, Mie, Japan, [9]Division of Microbiology, Department of Pathology and Microbiology, Nihon University School of Medicine, Tokyo, Japan, [10]Future Medicine Education and Research Organization, Chiba University, Chiba, Japan, and [11]CU-UCSD Center for Mucosal Immunology, Allergy, and Vaccines (cMAV), Departments of Medicine and Pathology, University of California, San Diego, California, USA

Background. Human norovirus (HuNoV) is a major cause of enteric infectious gastroenteritis and is classified into several genotypes based on its capsid protein amino acid sequence and nucleotide sequence of the polymerase gene. Among these, GII.4 is the major genotype worldwide. Epidemiological studies have highlighted the prevalence of GII.2. Although recent advances using human tissue– and induced pluripotent stem cell (iPSC)–derived intestinal epithelial cells (IECs) have enabled in vitro replication of multiple HuNoV genotypes, GII.2 HuNoV could replicate only in tissue-derived IECs and not in iPSC-derived IECs.

Methods. We investigated the factors influencing GII.2 HuNoV replication in IECs, focusing on histo-blood group antigens. We also assessed the immunogenicity of GII.2 virus-like particles (VLPs) and their ability to induce neutralizing antibodies. Antibody cross-reactivity was tested to determine whether GII.2 VLPs could neutralize other HuNoV genotypes, including GII.4, GII.3, GII.6, and GII.17.

Results. Our findings indicated that GII.2 HuNoV replication in vitro requires the presence of both H and B antigens. Moreover, GII.2 VLPs generated neutralizing antibodies effective against both GII.2 and GII.4 but not against GII.3, GII.6, or GII.17. Comparatively, GII.2 and GII.17 VLPs induced broader neutralizing responses than GII.4 VLPs.

Conclusions. The findings of this study suggests that GII.2 and GII.17 VLPs may be advantageous as HuNoV vaccine candidates because they elicit neutralizing antibodies against the predominant GII.4 genotype, which could be particularly beneficial for infants without prior HuNoV exposure. These insights will contribute to the development of effective HuNoV vaccines.

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Correspondence: Shintaro Sato, PhD, Department of Microbiology and Immunology, School of Pharmaceutical Sciences, Wakayama Medical University, 25-1, Shichiban-cho, Wakayama 640-8156, Japan (shintas@wakayama-med.ac.jp); (shinta@biken.osaka-u.ac.jp).

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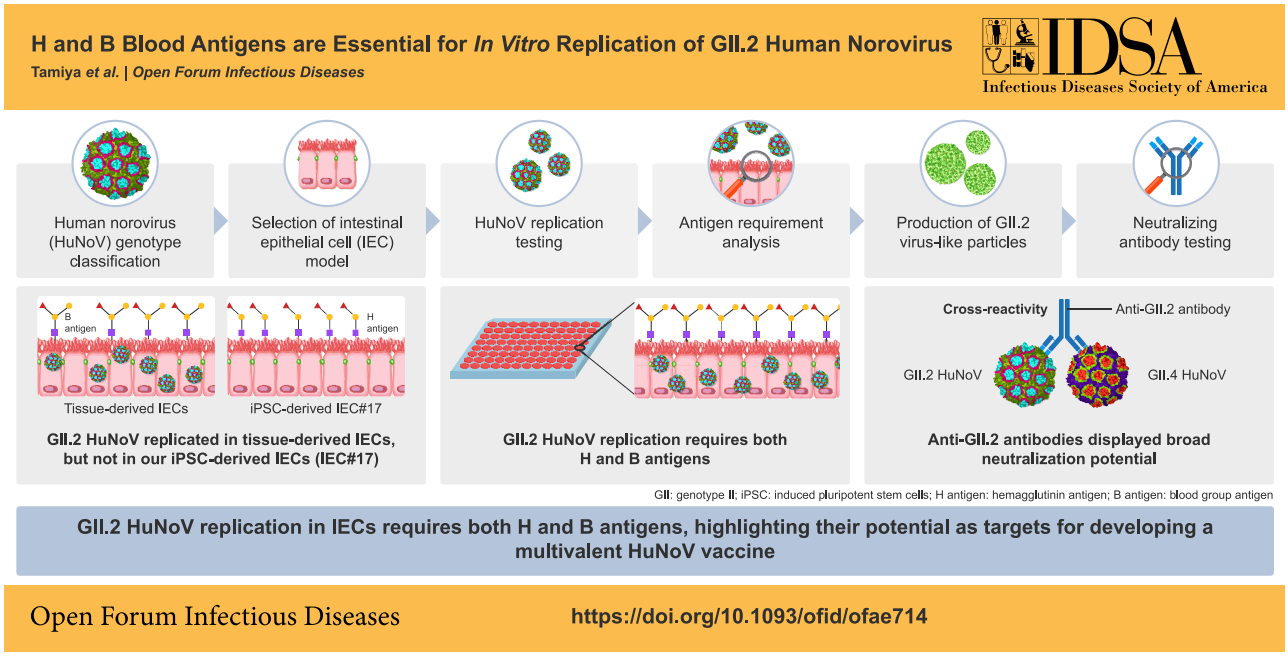
Graphical Abstract



Keywords. HBGA; human norovirus; in vitro replication; intestinal epithelial cells.

Human norovirus (HuNoV) is the leading cause of diarrheal infections in people of all ages worldwide [1]. HuNoV is highly contagious and causes symptoms like vomiting and diarrhea at an infectious dose as low as 10 000 viral genome copies [2–4]. It spreads predominantly through contaminated food and fecal-oral transmission, affecting individuals across all age groups. Notably, immunocompromised infants and elderly persons face heightened risks, which often culminate in severe and potentially fatal consequences, such as dehydration and aspiration pneumonia [5]. Therefore, HuNoV outbreaks in day-care facilities, schools, elderly care facilities, and hospitals necessitate prompt and meticulous decontamination and patient isolation, imposing a significant economic and social burden [6–9]. Nevertheless, the development of vaccines and specific therapeutic agents against HuNoV has been challenging, and no vaccines are currently available.

Norovirus is a non-enveloped virus with a single-stranded RNA genome containing 3 open reading frames (ORFs). ORF1 encodes a single polyprotein cleaved into ≥6 viral proteins (VPs), while ORFs 2 and 3 encode structural proteins VP1 and VP2, respectively. Currently, noroviruses are classified into 10 genogroups (GI–GX) based on their VP1 gene sequence, with GI, GII, GIV, GVIII, and GIX infecting humans [10]. Among these, the GII genogroup is the most common cause of HuNoV epidemics, with GII.4 the predominant genotype globally [11]; 7 HuNoV epidemics that occurred between 2002 and 2012 were attributed to mutations in amino acids within the highly antigenic region of VP1 of GII.4 [12]. However, other genotypes, such as GII.17, which was predominant in Asia during 2014–2015 and GII.2, the dominant HuNoV genotype causing infections during 2016–2017 in Europe and Asia, outpaced GII.4 [13–17]. Although the incidence rate of HuNoV infections has been shown to have declined temporarily in children during and after the coronavirus disease 2019 pandemic, GII.4 and GII.2 remain the most prevalent genotypes, particularly in Japan [18].

For years, the in vitro culture of HuNoV has remained unattainable. Consequently, virological studies on HuNoV relied on binding experiments with virus-like particles (VLPs) or, alternatively, on using mouse norovirus (genogroup GV) as a surrogate virus that can infect and propagate within the macrophage-based cell line RAW 264.7 [19]. However, although structurally similar to HuNoV, mouse norovirus does not infect humans or cause symptoms like diarrhea or vomiting in mice.

In 2016, Ettayebi et al [20] introduced an in vitro HuNoV replication method using intestinal epithelial cells (IECs) prepared from human small intestinal tissues. After terminal differentiation of monolayered IECs on Matrigel-coated culture plates, the addition of GII.4 HuNoV resulted in several-hundred-fold replication of the virus after only a few days of culture. Furthermore, GII.3, which could hardly multiply, showed a dozen-fold multiplication with the addition of bile to the cell culture medium. The addition of bile also increased the growth efficiency of GII.4 viruses. This advance in in vitro cultivation has

facilitated research on HuNoV to gain insights into the mechanisms of human cell invasion and symptom development, such as vomiting and diarrhea.

Studies using human intestinal tissue-derived IECs have reported that the HuNoV genotypes GI.1, GII.1, GII.2, GII.3, GII.4, GII.6, GII.8, GII.12, GII.13, GII.14, and GII.17 can replicate in vitro [20–22]. Our group's previous study also demonstrated replication of GI.7, GII.3, GII.4, GII.6, and GII.17 in IECs derived from human induced pluripotent stem cells (iPSCs) [23]. For non-enveloped viruses like HuNoV, infection often depends on interactions between viral capsid proteins and protein receptors on host mucosal epithelial cells. In mouse norovirus, CD300lf functions as a host protein receptor [24, 25]; however, the corresponding human protein receptor for HuNoV remains unknown. Nonetheless, the histo-blood group antigens (HBGAs), particularly the H antigen, plays an important role in HuNoV infection. GI.1, GII.4, and GII.17 HuNoV genotypes require H antigens for entry into IECs and subsequent replication in vitro [26]. The expression of H antigen in IECs and secretions, such as intestinal mucus and saliva, is regulated by fucosyltransferase 2 (FUT2). Individuals with normal FUT2 activity are termed "secretors" and those with defective or inactive mutations of FUT2 are called "non-secretors."

In the current study, we aimed to investigate the in vitro infection and replication requirements of HuNoV genotype GII.2, a recently emergent genotype. In addition, using the GII.2 HuNoV replication system with human iPSC–derived IECs, we assessed whether immunization with GII.2 VLPs could produce neutralizing antibodies that are cross-reactive with other HuNoV genotypes. The findings of this study may aid in vaccine development and enhance immunity, particularly in infants who lack prior exposure to HuNoV.

MATERIALS AND METHODS

Cell Culture

Each conditioned medium was prepared as described elsewhere [27, 28]. Human iPSC lines TkDN4-M [29], TkPP7 [30], TkC01, and TkD2 [31] were supplied by The University of Tokyo, and 1383D6 was provided by RIKEN BRC through the National BioResource Project of MEXT/AMED in Japan. TkC01-derived IECs (IEC#33) were prepared using a STEMdiff Intestinal Organoid Kit (STEMCELL Technology), according to the manufacturer's protocol. Differentiation of other iPSC lines into IECs was performed as described elsewhere [23, 28, 31, 32]. Human jejunal crypts were isolated as described elsewhere [28].

The experiments were approved by the human ethics committees of Wakayama Medical University, Osaka University, and Mie University. All tissue samples were obtained after obtaining informed consent. Intestinal organoids were cultured in Matrigel with organoid culture medium (Advanced Dulbecco's modified Eagle medium/F12 [Thermo Fisher Scientific] supplemented with 10 mmol/L HEPES [pH 7.3; Thermo Fisher Scientific]; 2 mmol/L GlutaMAX [Thermo Fisher Scientific]; 100 U/mL penicillin plus 100 μg/mL streptomycin; 25% mouse Wnt3a, human R-spondin 1, human noggin, and human hepatocyte growth factor [WRNH] conditioned medium; 1×B-27 [Thermo Fisher Scientific]; 50 ng/mL mouse epidermal growth factor [Peprotech]; 10 μmol/L SB202190 [Sigma-Aldrich]; and 500 nmol/L A83-01 [Tocris] plus 10 μmol/L Y-27632 [Wako]). For HuNoV inoculation, IECs dissociated using TrypLE Express (Thermo Fisher Scientific) were seeded on 2.5% Matrigel-coated 96-well plates at 2×10^4 cells per well in 100 μL of organoid culture medium.

After 2 days of culture, the medium was changed to differentiation medium (Advanced Dulbecco's modified Eagle medium/F12 supplemented with 10 mmol/L HEPES [pH 7.3]; 2 mmol/L GlutaMAX; 100 U/mL penicillin plus 100 μg/mL streptomycin; 1×B-27; 12.5% human R-spondin 1 and human noggin conditioned medium; 50 ng/mL mouse epidermal growth factor; and 500 nmol/L A83-01). After another 2 days, the medium was changed to a differentiation medium with or without 0.03% porcine bile (Sigma-Aldrich). The cells were incubated for another 2 days and used for subsequent experiments.

HuNoV Preparation and Infection

GII.4_2006b[P4]–containing stool samples (identification no. 13499) were collected from a child with acute gastroenteritis who visited a pediatric outpatient clinic in Osaka Prefecture, Japan. All other viral samples used in this study were HuNoV-positive stool specimens collected from Osaka Prefecture, Japan, during the 2016–2019 endemic seasons.

HuNoV preparation and infection were performed as described elsewhere [23, 32]. Briefly, HuNoV-positive stool samples were suspended in 10% (wt/vol) phosphate-buffered saline by means of vigorous vortexing. The suspensions were centrifuged at 12 000*g* for 30 minutes, and the supernatants were serially filtered with 0.45 μm and 0.22 μm filters. The filtered samples were aliquoted and stored at −80°C as an undiluted virus solution (see Table 1 for strain details). Immediately before use, each virus solution was diluted to 2×10^7 genome equivalents/mL with base medium (Advanced Dulbecco's modified Eagle medium/F12 supplemented with 10 mmol/L HEPES [pH 7.3], 2 mmol/L GlutaMAX, and 100 U/mL penicillin plus 100 μg/mL streptomycin).

The prepared IECs (3–6 wells per sample) were inoculated with 100 μL (2×10^6 genome equivalents) of diluted virus solution and then left for 1 hour in a 5% carbon dioxide incubator at 37°C. The inoculum was then removed, and the cells were washed twice with 150 μL of base medium. Differentiation medium (100 μL), with or without 0.03% porcine bile, was added to the cells, which were pipetted lightly twice and collected.

Table 1. Human Noroviruses Used in Current Study

Sample ID No.	Genotype	Titer, Genome Equivalents/μL	Collection Date	Replication of Virus in IECs
16-464	GII.2[P16]	7.85×10^6	March 2017	In current study
16-482	GII.2[P16]	1.34×10^8	March 2017	In current study
OSN1926	GII.2[P16]	2.68×10^6	June 2019	In current study
17B93	GII.4[P31]	1.60×10^6	October 2017	[23, 31]
17-53	GII.4[P31]	3.14×10^7	June 2017	[23, 31, 32]
17-231	GII.4[P31]	3.88×10^7	October 2017	[23, 32]
13499	GII.4[P4]	1.69×10^6	April 2015	[33, 34]
16-50	GII.3[P12]	6.64×10^6	April 2016	[23, 32]
18-78	GII.6[P7]	8.70×10^5	May 2018	[23, 32]
16-421	GII.17[P17]	9.48×10^7	February 2017	[23, 32, 34]

Abbreviations: ID, identification; IECs, intestinal epithelial cells.

This step was performed again, and the samples were collected at 1 hour after infection as reference samples (total, 200 μL). Another 100 μL of differentiation medium was added to each well, with or without 0.03% bile, and the mixtures were then cultured for 72 hours in a 5% carbon dioxide incubator at 37°C. The supernatants were collected with one wash in the same manner as the reference samples collected 1 hour after infection (total 200 μL).

For blocking experiments, the diluted virus solutions were incubated with 100 ng of anti-VLP polyclonal antibody (pAb) or normal rabbit immunoglobulin G (IgG) at 37°C for 90 minutes before inoculation into the prepared IECs (6 wells per sample).

Statistical Analysis

Each experiment was performed at least twice, with 3–6 technical replicates. All statistical analyses were performed using GraphPad Prism 8 and 9 software. Comparison between normal antibody and either anti-GII.2, anti-GII.3, anti-GII.4, or anti-GII.17 antibody at 72 hours after infection was performed using Student t test or 1-way analysis of variance with Dunnett multiple comparisons test. Significance was set at $P < 0.05$.

For details on the following methods not listed here, please refer to the Supplementary Materials and Methods, which includes genotyping of HBGA, quantification of virus genome equivalents, preparation of VLPs, generation of antibodies to GII.2 and GII.3 VLPs, HBGA-binding assay of HuNoV VLPs, lentiviral infection, and immunofluorescence staining.

RESULTS

Replication of GII.2 HuNoV in Tissue-Derived IECs but Not Human iPSC–Derived IEC#17

HuNoV genotypes, including GII.3, GII.4, GII.6, GII.17, and GI.7, can infect and replicate in IECs from the human iPSC TkDN4-M line (IEC#17) [23, 32]. In the current study, we showed that GII.2 genotype HuNoV could not replicate in IEC#17 (Figure 1*A*), even in the presence of bile, an essential factor for GII.3 HuNoV replication [35]. Although electron microscopy showed that our specimens contained virus particles of the appropriate diameter (Figure 1*B*), these particles may have lost their ability to infect or replicate in IECs.

Costantini et al [21] observed non-GII.4 HuNoV strains, including GII.2, replicating in human tissue-derived IECs. Therefore, we checked whether our GII.2 samples could replicate in such IECs. Human IECs prepared from 2 jejunal samples (jej#1 and jej#2) and inoculated with 3 GII.2 HuNoV samples showed replication in all tested GII.2 viruses in both IECs in a bile addition–dependent manner (Figure 1*C*).

Both H and B Antigens Required for GII.2 HuNoV Replication In Vitro

Gene expression analysis revealed that pluripotent stem cell–derived IECs are more similar to fetal IECs than adult IECs [36]. To confirm whether the in vitro replication of GII.2 HuNoV is specific to adult tissue–derived IECs, we prepared IECs from several other human iPSC lines: 1383D6 (to IEC#25), TkPP7 (to IEC#29), TkC01 (to IEC#33), and TkD2 (to IEC#34), and infected them with GII.2 HuNoV. Similar to IEC#17, GII.4 HuNoV replicated in IEC#29 and IEC#33 but not in IEC#25 and IEC#34 (Figure 2*A*). In IEC#33, GII.2 HuNoV, which could not replicate in IEC#17, replicated sufficiently, although at a lower rate than GII.4 HuNoV (Figure 2*A*). These results suggest that GII.2 HuNoV can replicate in human iPSC–derived IECs regardless of the derivation method (from intestinal tissues or from iPSCs).

Next, we evaluated H antigen expression in each IEC using UEA1 staining. UEA1-positive H antigens were detected in all cells except IEC#25 and IEC#34 (see Supplementary Figure 1), suggesting that these may have been derived from non-secretor individuals. To confirm this, we amplified exon 2 of *FUT2* by polymerase chain reaction and analyzed the nucleotide sequence of the resulting amplicon. These findings revealed that both IEC#25 and IEC#34 possess a homozygous A385T mutation, which is known to result in a non-functional FUT2 enzyme [37] (Table 2).

Subsequently, we analyzed the ABO(H) blood group types of the cell lines. Jej#1 and Jej#2 IECs, derived from individuals with blood types B and AB, respectively, were confirmed to express these antigens through immunostaining (Supplementary Figure 2). Similarly, immunostaining of iPSC-derived IECs revealed that none of the cells expressed A antigens, but B antigens were detected in IEC#25 and IEC#33 (Supplementary Figure 2). The ABO genotypes of each IEC were identified using the polymerase chain reaction–restriction fragment length polymorphism method [38]. IEC#17, IEC#29, and IEC#34 were of the OO type, whereas IEC#25 and IEC#33 were of the BO type (Table 2). These findings indicated that cells supporting

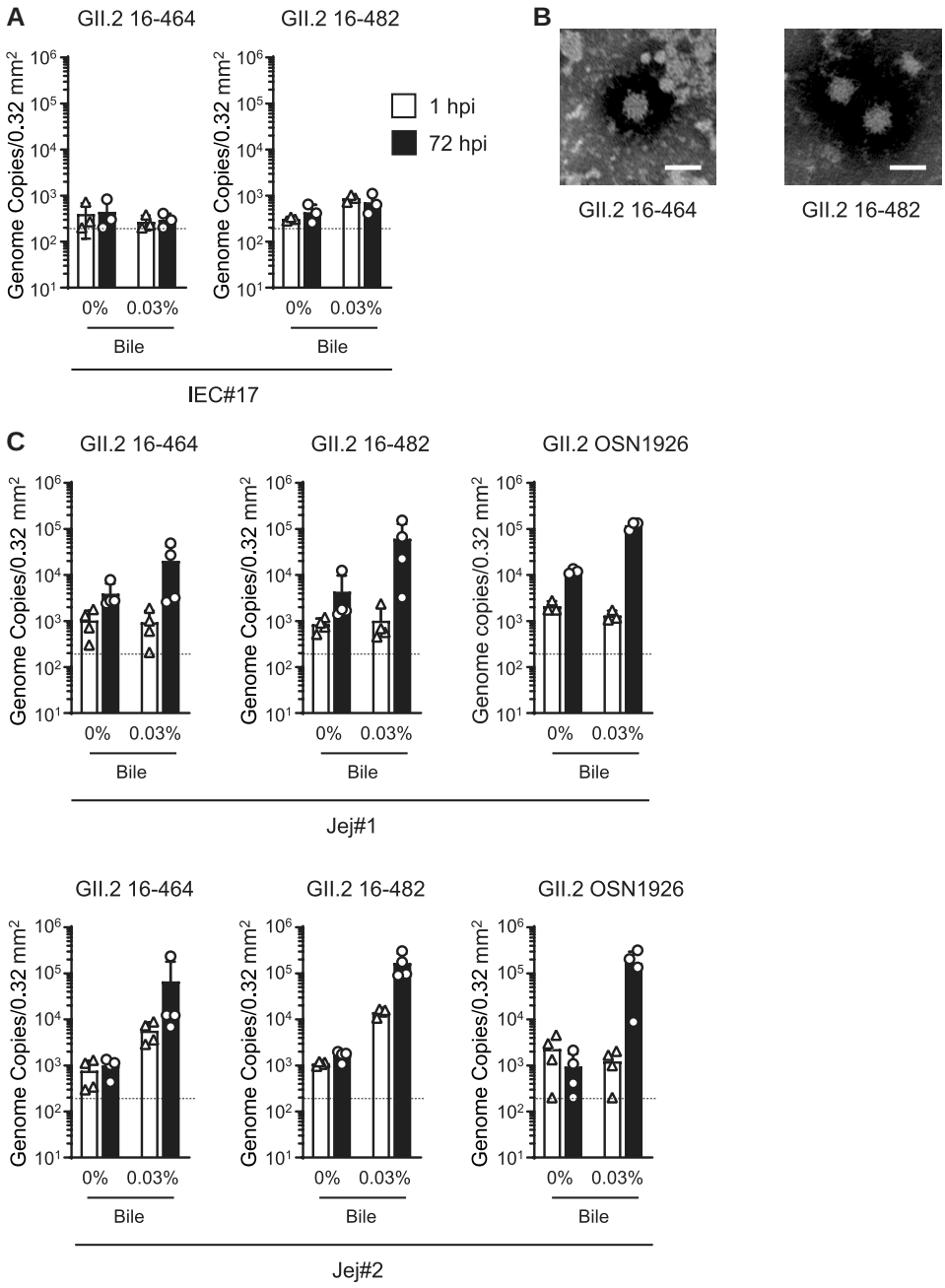


Figure 1. Replication of GII.2 genotype human noroviruses (HuNoVs) in intestinal epithelial cells (IECs) derived from human induced pluripotent stem cells (iPSCs) or human jejunum tissues. *A, C,* Monolayered human iPSC–derived IECs (IEC#17) (*A*) and human jejunum-derived IECs (Jej#1 and Jej#2) (*C*) were inoculated with 2×10^6 genome equivalents of GII.2 HuNoVs. Inoculation and sampling were performed as described in Materials and Methods. Viral genomic RNA was extracted from both supernatants (1 and 72 hours post infection [hpi]), and genome equivalents were quantified by means of reverse-transcription quantitative polymerase chain reaction. Samples collected 1 hour after infection were used as references. Each value is representative of ≥3 independent experiments and is shown as the mean (SD) of 4–6 wells of supernatants from each culture group. Dashed lines represent limits of detection. *B,* Electron microscopic images of GII.2 HuNoV in fecal suspensions (scale bars represent 50 nm).

GII.2 HuNoV replication (Jej#1, Jej#2, and IEC#33) express type B antigens.

Therefore, we hypothesized and tested whether the group B antigen is essential for GII.2 replication, similar to the H antigen in GII.4 HuNoV. Using lentiviruses, we previously established a highly efficient gene transfer method for IECs through monolayer formation [39]. Genes encoding α-1,3-N-acetylgalactosaminyltransferase and α-1,3-galactosyltransferase, which are responsible for adding group A and B antigens, respectively, were forcibly expressed in IEC#17, IEC#29, and IEC#34. The expression of each antigen was confirmed using immunocytochemistry (Supplementary Figure 3).

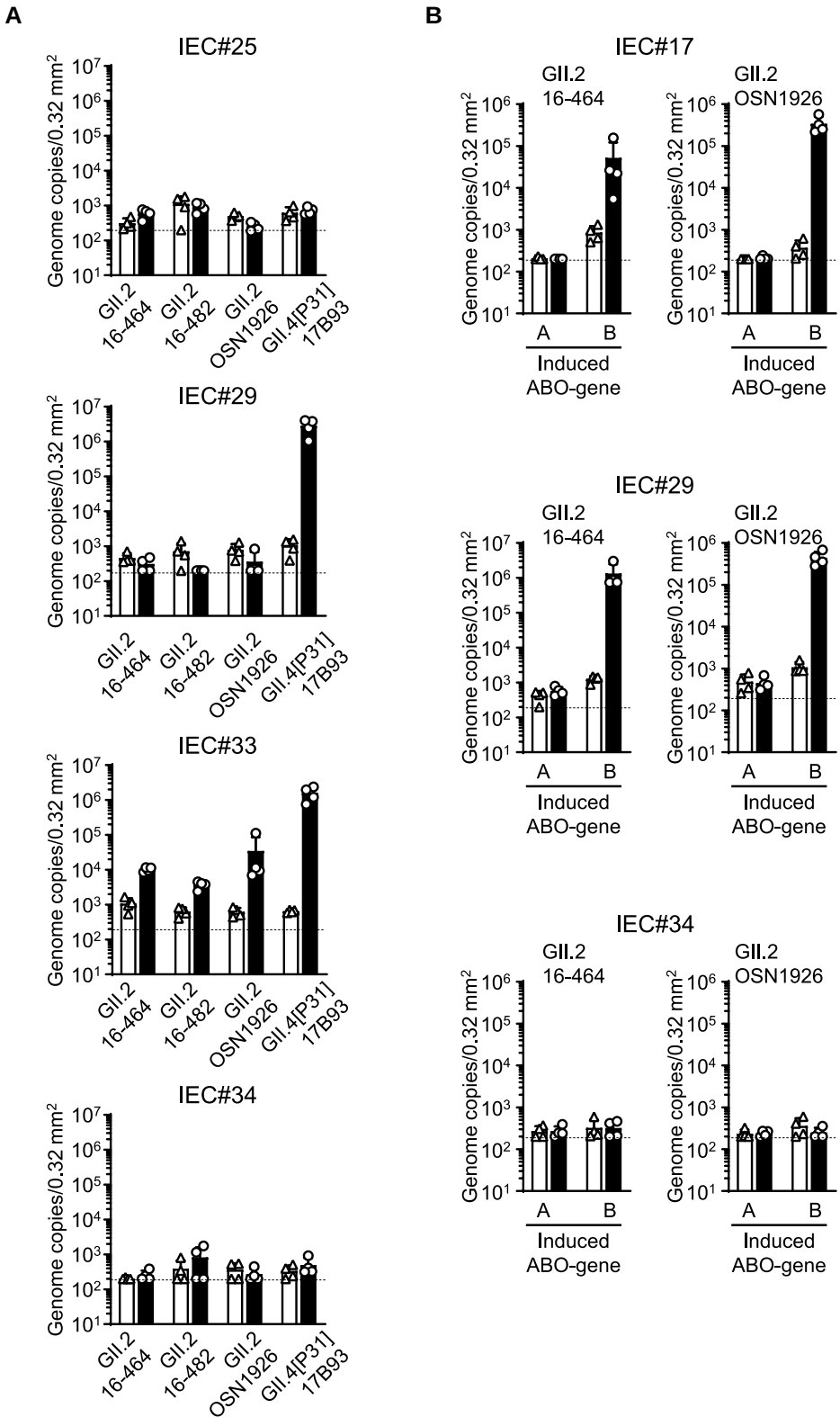


Figure 2. Replication of GII.2 and GII.4 genotype human noroviruses (HuNoVs) in intestinal epithelial cells (IECs) established from different human induced pluripotent stem cells (iPSCs). *A*, Monolayered IECs established from the indicated human iPSCs were inoculated with 2×10^6 genome equivalents of the indicated HuNoVs. Inoculation and sampling were performed as described in Materials and Methods. Viral genome equivalents present in culture supernatants at 1 (*white bars*) or 72 (*black bars*) hours after infection were quantified using reverse-transcription quantitative polymerase chain reaction. Each value is representative of ≥2 independent experiments and is shown as the mean (SD) of 4–6 wells of supernatants from each culture group. *B*, Monolayered IECs transfected with genes encoding α-1,3-N-acetylgalactosaminyltransferase (for A-type antigen) or α-1,3-galactosyltransferase (for B-type antigen) were inoculated with 2×10^6 genome equivalents of the indicated GII.2 HuNoVs. Sampling and quantification of genome equivalents were performed as shown in Figure 1. Dashed lines represent limits of detection.

Infection of each cell with GII.2 revealed no replication in group A antigen-transfected cells but strong replication in IEC#17 and IEC#29 transfected with the group B antigen (Figure 2*B*). Furthermore, confocal laser-scanning microscopy showed that GII.2 HuNoV adhered to and invaded only B-type antigen-positive cells (Supplementary Figure 4). These findings indicate that group B antigens on IECs are essential for GII.2 virus growth in vitro. We also speculated that H antigens are necessary for the replication of this genotype, similar to GII.4 HuNoV. This is because IEC#25 and IEC#34, categorized as non-secretory types, lack H antigen expression on the cell surface due to a homozygous loss-of-function mutation in *FUT2* [31] (Table 2 and Supplementary Figure 1).

To verify this hypothesis, cells expressing the H antigen in IEC#25 and the B or H antigen, or both, in IEC#34 were prepared and confirmed by means of immunocytochemistry (see Supplementary Figure 5). Forced expression of the H antigen restored the in vitro replication of GII.4 HuNoV in both IEC#25 and IEC#34, whereas expression of the B antigen in IEC#34 had no effect (Figure 3*A* and 3*C*). In contrast, GII.2 HuNoV replication was observed in H antigen–expressing IEC#25 (Figure 3*B*). GII.2 HuNoV replication was not observed when the H and B antigens were independently expressed in IEC#34 cells but only when they were simultaneously expressed (Figure 3*D*). These findings indicate that the in vitro replication of GII.2 HuNoV in IECs requires both H and B antigens and that these antigen complexes serve as viral receptors for the entry of GII.2 HuNoV into cells.

Table 2. Human Induced Pluripotent Stem Cell Line and Jejunum Intestinal Epithelial Cells Used in Current Study

iPSC Line	IEC	ABO Type	*FUT2*	Secretor
TkDN4-M	#17	OO	*Se*, *se*^{428}	Positive
1383D6	#25	BO	*se*^{385}, *se*^{385}	Negative
TkPP7	#29	OO	*Se*, *Se*	Positive
TkC01	#33	BO	*Se*, *Se*	Positive
TkD2	#34	OO	*se*^{385}, *se*^{385}	Negative
Not applicable	Jej#1	BO or BB	*Se*, *se*^{385}	Positive
Not applicable	Jej#2	AB	*Se*, *se*^{385}	Positive

Abbreviations: FUT2, fucosyltransferase 2; IEC, intestinal epithelial cell; iPSC, induced pluripotent stem cell; Se, means normal gene; se, means mutated gene.

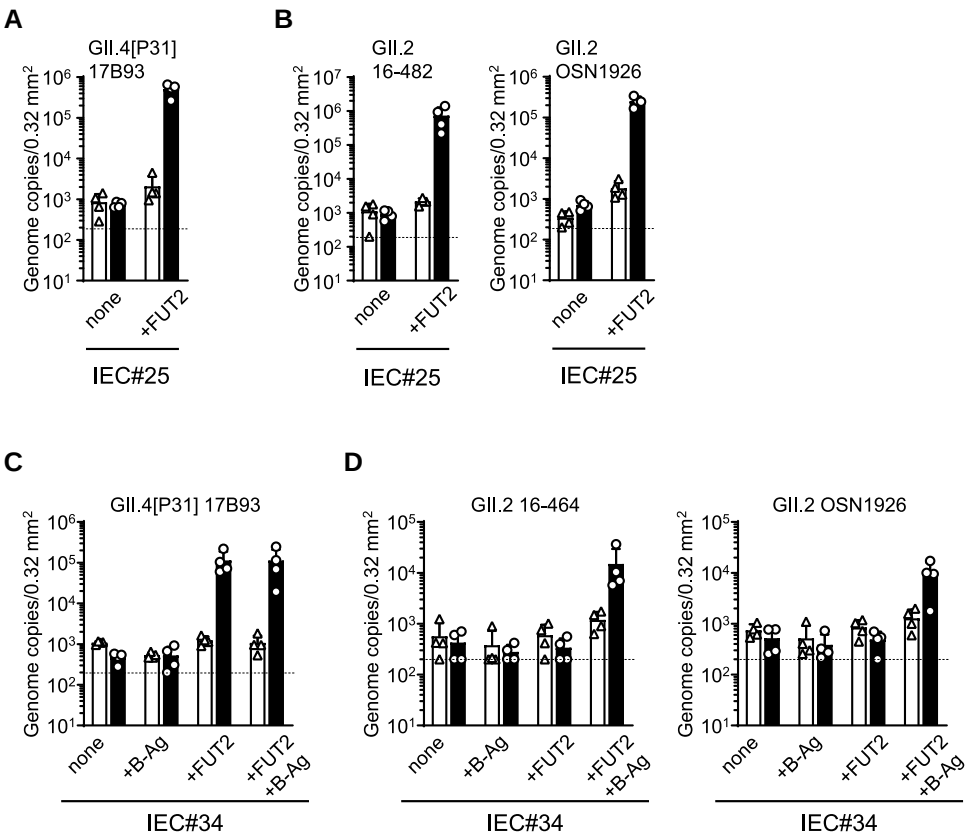


Figure 3. Replication of GII.2 and GII.4 human noroviruses (HuNoVs) in fucosyltransferase 2 (FUT2)–inactivated intestinal epithelial cells (IECs). *A, B,* Non-secretory type (FUT2-negative) IEC#25 cells were transfected with or without genes encoding FUT2. *C, D,* FUT2-negative IEC#34 cells were transfected with or without genes encoding α-1,3-galactosyltransferase (B antigen [B-Ag]), FUT2, or both. Monolayer cells were inoculated with 2×10^6 genomic equivalents of HuNoVs. Sampling and quantification of genome equivalents were performed as shown in Figure 1. Dashed lines represent limits of detection.

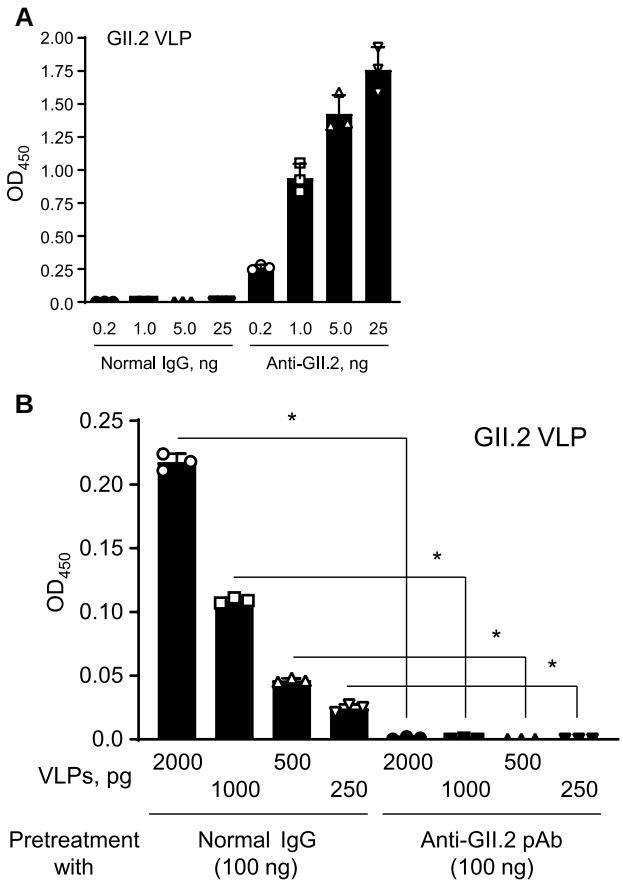


Figure 4. Inhibitory experiment of binding between GII.2 genotype human norovirus (HuNoV) virus-like particles (VLPs) and histo-blood group antigens (HBGAs) with anti-VLP polyclonal antibody (pAb). *A,* Titers of rabbit anti-GII.2 pAb were quantified using enzyme-linked immunosorbent assays. Data are expressed as the mean (SD) from 1 experiment representative of 2 independent experiments. *B,* Blocking activity of 100 ng of normal rabbit immunoglobulin G (IgG) or anti-GII.2 pAb against HBGA binding of the indicated amounts of GII.2 VLPs was measured by means of enzyme-linked immunosorbent assay using plates coated with 1 μg of porcine gastric mucin. Data are shown as the mean (SD) from 1 representative of 2 independent experiments. Comparison between normal antibody and anti-GII.2 pAb at each VLP amount was performed using Student *t* test. $^*P < 0.05$. Abbreviation: OD_{450}, optical density at 450 nm.

Cross-Reactivity of Anti-GII.2 HuNoV VLP Antibodies That Can Neutralize GII.4 HuNoV Infection

The GII.4 genotype has often been the most prevalent HuNoV genotype, making it a suitable candidate for vaccine antigens. Previous studies have reported that antibodies induced by injectable immunization with GII.4 or GII.17 VLPs neutralize GII.4 and GII.17 viruses, respectively [23]. Immunization with GII.4 VLPs showed no cross-neutralization against other genotypes, whereas immunization with GII.17 VLPs showed similar cross-reactivity against the GII.4 virus as GII.4 VLPs. Consequently, we explored the potential of GII.2 VLPs as vaccine antigens along with GII.4 and GII.17 VLPs [23]. Polyclonal antibodies from rabbits immunized with GII.2 or GII.3 VLPs, produced via a baculovirus expression system, bound to each VLP in a dose-dependent manner in enzyme-linked immunosorbent assays (Figure 4*A* and Supplementary Figure 6). Although HuNoV VLPs bind to porcine gastric mucin containing HBGA, this binding was inhibited by preincubation with anti-GII.2 antibodies dose dependently (Figure 4*B*).

We investigated the virus-neutralizing effect of anti-GII.2 antibody using an in vitro GII.2 HuNoV replication system. Preincubation with non-immunized rabbit IgG did not affect GII.2 HuNoV replication, but the anti-GII.2 antibody completely blocked it (Figure 5*A*). Anti-GII.3, anti-GII.4, and anti-GII.17 antibodies did not inhibit GII.2 HuNoV replication (Figure 5*B*). The anti-GII.2 antibody did not affect the replication of GII.3, GII.6, and GII.17 HuNoVs but exhibited cross-reactivity against GII.4_2012[P31], which was concurrent with GII.2, similar to the cross-reactivity of anti-GII.17 antibodies (Figure 5*C*). Both anti-GII.2 and anti-GII.17 antibodies also completely inhibited the in vitro growth of the older GII.4 epidemic strain (GII.4_Den Haag; GII.4_2006b[P4]) (Figure 5*D*). These findings, along with our previous report [23], suggest that GII.2 HuNoV VLPs are similar to GII.17 VLPs and have the potential as multivalent vaccine antigens for HuNoVs.

DISCUSSION

In vitro replication is essential to understand HuNoV biology and antibody development. The current study aimed to understand why the GII.2 HuNoV genotype, although increasingly prevalent, replicated in tissue-derived IECs but not in our human iPSC–derived IECs. We confirmed that GI.7, GII.3, GII.4_2006b[P4], GII.4_2012[P31], GII.6, and GII.17 [23, 32, 33], but not GII.2, could replicate in our system using IEC#17. As in vitro HuNoV replication currently requires virus-positive stool as the virus source, it is difficult to determine whether the system is inadequate or whether the stool specimens lack infectious particles when replication fails. We checked whether GII.2 HuNoVs could replicate using IECs from human jejunal tissue as per Costantini et al [21], using the jejunal strain (J2) developed by Ettayebi et al [20]. Viral replication was confirmed in ≥3 specimens. These results suggest that the failure of GII.2 HuNoV to replicate in human iPSC–derived IECs may be due to an issue with the cells (IEC#17). In contrast, other HuNoV genotypes replicated in IEC#17, indicating that GII.2 invades host cells via a mechanism different from that of other HuNoV genotypes.

Studies on VLPs of various HuNoV genotypes and synthetic oligosaccharides have shown that GII.2 VLPs (rBUDS) strongly bind to type A or B antigens [40, 41]. Although binding to synthetic oligosaccharides has not been confirmed, Ao et al [42] discovered that a segment of GII.2 VP1 (*P* domain) binds to the B phenotype saliva (HBGA). They also found that mutating the 256th valine of GII.2 VP1 to isoleucine allows a portion of

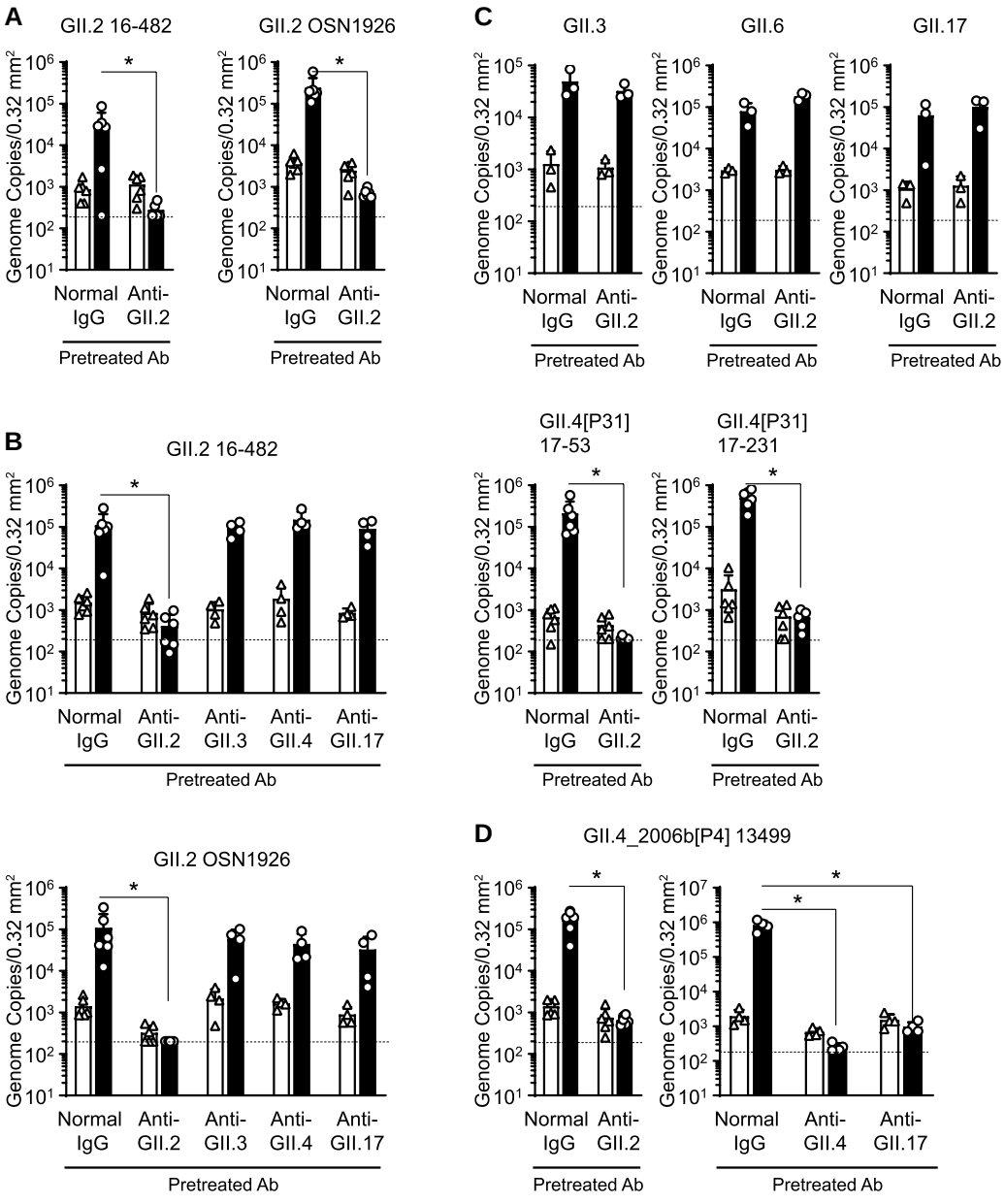


Figure 5. Neutralization activity of anti-GII.2 pAb against replication of GII.2 and GII.4 human noroviruses (HuNoVs). Before inoculation of monolayered intestinal epithelial cells, 2×10^6 genome equivalents of each HuNoV genotype (shown in the upper part of each graph) were incubated with 100 ng of the indicated anti-HuNoV virus-like particle (VLP) antibody (Ab) or with normal rabbit immunoglobulin G (IgG), for 1.5 hour. Inoculation, sampling, and quantification of genome equivalents were performed as described in Materials and Methods. Each value is representative of ≥3 independent experiments and is shown as the mean (SD) of 4–6 wells of supernatants from each culture group. $^*P < .05$.

VP1 to bind to both A and B phenotype saliva [42]. Our findings indicated that group B antigen expression is essential for in vitro GII.2 HuNoV replication in the fecal samples we tested, whereas group A antigen had no effect. In addition, the 256th amino acid of VP1 in all GII.2 strains used in this study was valine, matching the *P*-domain sequence reported to bind A phenotype saliva. These findings suggest that while the *P* domain of VP1 is highly antigenic and crucial for HBGA binding, the actual binding of complete viral particles to HBGA on IECs requires comprehensive verification. Experiments using partial proteins or synthetic oligosaccharides may offer limited insights into the interaction between HuNoV VP1 and HBGA.

We demonstrated that active FUT2 expression (secretors) was crucial for GII.2 HuNoV propagation in vitro. However, Lindesmith et al [43] found that non-secretors also exhibit vomiting and diarrhea with GII.2 HuNoV infection. They confirmed that GII.2 VLPs bind to saliva from secretors and noted that adding 1% bile enables binding to the saliva of

nonsecretors. Given the presence of various host factors and intestinal bacteria with sugar-metabolizing enzymes, host FUT2 activity may be substituted by environmental factors for GII.2 HuNoV infection and propagation in vivo. In addition, a human challenge study showed that non-secretors developed symptoms that were dependent on the viral dose [44].

Recent reports have shown that GII.2 HuNoV can replicate in vitro using J4^{FUT2} cells created by introducing the *FUT2* gene into J4 cells (*se*^{428}, *se*^{428}; OO blood genotype) [26, 45]. This indicates that the B antigen may not be essential for all GII.2 genotypes, necessitating further investigation.

Type A and B glycosyltransferases use H-antigens as substrates. Thus, the presence of type B antigens on the cell surface of IEC#25, which has an inactive *FUT2* mutation (A385T) and should not express H antigens, was surprising. The B antigen expression level did not change after the introduction of active *FUT2* into IEC#25 and IEC#34. However, both the H antigen produced by the active *FUT2* gene and the B antigen produced by B-type glycosyltransferase were necessary for the in vitro replication of GII.2 HuNoV in IEC#25 and IEC#34.

These findings, along with those of a previous study [46] suggesting the weak activity of the A385T *FUT2* mutation, led us to conclude that sufficient levels of both H and B antigens on the cell surface are required for GII.2 HuNoV in vitro replication. Regardless of FUT2 activity levels, B-type glycan expression was confirmed by immunostaining using an anti–B-type antigen monoclonal antibody (BG-3). This implies that α-1,3-galactosyltransferase may use the H antigen without α1,2-fucose (Bombay phenotype) as an acceptor substrate. To explore this, *FUT2* knockouts in IEC#33 and IEC#25 are required to determine whether these cells can also react with the BG-3 antibody. Future analyses using in vitro and in vivo systems will provide detailed insights into the GII.2 HuNoV life cycle and explain the mechanisms bridging in vitro findings and in vivo behavior, including the roles of the B antigen.

Historically, HuNoV epidemics were mainly caused by the GII.4 genotype, but the GII.2 genotype has recently become more prevalent in Europe and Asia [14–17, 47]. Vaccines developed against HuNoV have primarily targeted GII.4; however, it remains unclear whether the immune response to GII.4, especially neutralizing antibodies, extends to other HuNoV genotypes. We observed that rabbit polyclonal antibodies from GII.4 VLP immunization failed to inhibit GII.3, GII.6, and GII.17 replication in vitro, whereas antibodies from GII.17 VLP immunization exhibited cross-neutralizing activity, effectively suppressing both GII.4, and GII.17 HuNoV propagation [23]. Immunization with GII.2 VLPs in mice produces antigen-specific antibodies, although their neutralizing activity has not been confirmed [48].

In the current study, newly generated GII.2 VLPs were used, and immunization of rabbits produced antibodies with neutralizing activity against both GII.2 and GII.4. Although antibodies from GII.2 and GII.17 VLP immunizations did not cross-react with genotypes other than GII.4, they showed neutralizing activity against the GII.4_2006b[P4] genotype, which is now rare. These findings suggest that GII.2 and GII.17 VLPs may be more effective than GII.4 VLPs as vaccine antigens against HuNoV, particularly in infants not exposed to HuNoVs.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Author contributions. S. T. and N. M. performed most of the experimental infections. S. K., Y. Y., and H. K. produced viruslike particles and antibodies. Y. N. performed immunohistochemical staining. Y. T. prepared and provided L-WRNH cells as a source for producing WRNH CM. N. S. and H. U. prepared and provided human norovirus–positive stool specimens. M. I., Y. K., and K. U. prepared and provided jejunal tissue. S. S. designed and directed the overall research, performed the experiments, analyzed the data, and wrote the manuscript.

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References

1. Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. N Engl J Med **2009**; 361:1776–85.
2. Atmar RL, Opekun AR, Gilger MA, et al. Determination of the 50% human infectious dose for Norwalk virus. J Infect Dis **2014**; 209:1016–22.
3. Lindesmith L, Moe C, Marionneau S, et al. Human susceptibility and resistance to Norwalk virus infection. Nat Med **2003**; 9:548–53.
4. Teunis PF, Moe CL, Liu P, et al. Norwalk virus: how infectious is it? J Med Virol **2008**; 80:1468–76.
5. Hall AJ, Lopman BA, Payne DC, et al. Norovirus disease in the United States. Emerg Infect Dis **2013**; 19:1198–205.
6. Bartsch SM, Lopman BA, Ozawa S, Hall AJ, Lee BY. Global economic burden of norovirus gastroenteritis. PLoS One **2016**; 11:e0151219.
7. Cates SC, Kosa KM, Brophy JE, Hall AJ, Fraser A. Consumer education needed on norovirus prevention and control: findings from a nationally representative survey of U.S. adults. J Food Prot **2015**; 78:484–90.
8. Jump RLP, Crnich CJ, Mody L, Bradley SF, Nicolle LE, Yoshikawa TT. Infectious diseases in older adults of long-term care facilities: update on approach to diagnosis and management. J Am Geriatr Soc **2018**; 66:789–803.
9. Leone CM, Jaykus LA, Cates SM, Fraser AM. A review of state licensing regulations to determine alignment with best practices to prevent human norovirus infections in child-care centers. Public Health Rep **2016**; 131:449–60.
10. Chhabra P, de Graaf M, Parra GI, et al. Updated classification of norovirus genogroups and genotypes. J Gen Virol **2019**; 100:1393–406.
11. Lopman BA, Steele D, Kirkwood CD, Parashar UD. The vast and varied global burden of norovirus: prospects for prevention and control. PLoS Med **2016**; 13: e1001999.
12. Kroneman A, Vega E, Vennema H, et al. Proposal for a unified norovirus nomenclature and genotyping. Arch Virol **2013**; 158:2059–68.
13. Chan MCW, Hu Y, Chen H, et al. Global spread of norovirus GII.17 Kawasaki 308, 2014–2016. Emerg Infect Dis **2017**; 23:1359–4.

14. Niendorf S, Jacobsen S, Faber M, et al. Steep rise in norovirus cases and emergence of a new recombinant strain GII.P16-GII.2, Germany, winter 2016. Euro Surveill **2017**; 22:30447.
15. Ao Y, Wang J, Ling H, et al. Norovirus GII.P16/GII.2-associated gastroenteritis, China, 2016. Emerg Infect Dis **2017**; 23:1172–5.
16. Sakon N, Komano J, Tessmer HL, Omori R. High transmissibility of norovirus among infants and school children during the 2016/17 season in Osaka, Japan. Euro Surveill **2018**; 23:18-00029.
17. Thongprachum A, Okitsu S, Khamrin P, Maneekarn N, Hayakawa S, Ushijima H. Emergence of norovirus GII.2 and its novel recombination during the gastroenteritis outbreak in Japanese children in mid-2016. Infect Genet Evol **2017**; 51: 86–8.
18. Pham NTK, Khamrin P, Shimizu-Onda Y, et al. Genetic diversity and declining norovirus prevalence in infants and children during Japan's COVID-19 pandemic: a three-year molecular surveillance. Arch Virol **2023**; 168:231.
19. Wobus CE, Karst SM, Thackray LB, et al. Replication of norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol **2004**; 2:e432.
20. Ettayebi K, Crawford SE, Murakami K, et al. Replication of human noroviruses in stem cell-derived human enteroids. Science **2016**; 353:1387–93.
21. Costantini V, Morantz EK, Browne H, et al. Human norovirus replication in human intestinal enteroids as model to evaluate virus inactivation. Emerg Infect Dis **2018**; 24:1453–64.
22. Ettayebi K, Tenge VR, Cortes-Penfield NW, et al. New insights and enhanced human norovirus cultivation in human intestinal enteroids. mSphere **2021**; 6: e01136–20.
23. Sato S, Hisaie K, Kurokawa S, et al. Human norovirus propagation in human induced pluripotent stem cell-derived intestinal epithelial cells. Cell Mol Gastroenterol Hepatol **2019**; 7:686–8.e5.
24. Orchard RC, Wilen CB, Doench JG, et al. Discovery of a proteinaceous cellular receptor for a norovirus. Science **2016**; 353:933–6.
25. Haga K, Fujimoto A, Takai-Todaka R, et al. Functional receptor molecules CD300lf and CD300ld within the CD300 family enable murine noroviruses to infect cells. Proc Natl Acad Sci U S A **2016**; 113:E6248–E55.
26. Haga K, Ettayebi K, Tenge VR, et al. Genetic manipulation of human intestinal enteroids demonstrates the necessity of a functional fucosyltransferase 2 gene for secretor-dependent human norovirus infection. mBio **2020**; 11:e00251-20.
27. Takahashi Y, Inoue Y, Sato S, et al. Drug cytotoxicity screening using human intestinal organoids propagated with extensive cost-reduction strategies. Sci Rep **2023**; 13:5407.
28. Takahashi Y, Sato S, Kurashima Y, et al. A refined culture system for human induced pluripotent stem cell-derived intestinal epithelial organoids. Stem Cell Rep **2018**; 10:314–28.
29. Takayama N, Nishimura S, Nakamura S, et al. Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. J Exp Med **2010**; 207:2817–30.
30. Izumida E, Suzawa T, Miyamoto Y, et al. Functional analysis of PTH1R variants found in primary failure of eruption. J Dent Res **2020**; 99:429–36.
31. Matsumoto N, Kurokawa S, Tamiya S, et al. Replication of human sapovirus in human-induced pluripotent stem cell-derived intestinal epithelial cells. Viruses **2023**; 15:1929.
32. Sato S, Matsumoto N, Hisaie K, Uematsu S. Alcohol abrogates human norovirus infectivity in a pH-dependent manner. Sci Rep **2020**; 10:15878.
33. Masuda A, Man Lee J, Miyata T, et al. High yield production of norovirus GII.4 virus-like particles using silkworm pupae and evaluation of their protective immunogenicity. Vaccine **2023**; 41:766–77.
34. Sasou A, Yuki Y, Kurokawa S, et al. Development of antibody-fragment-producing rice for neutralization of human norovirus. Front Plant Sci **2021**; 12: 639953.
35. Murakami K, Tenge VR, Karandikar UC, et al. Bile acids and ceramide overcome the entry restriction for GII.3 human norovirus replication in human intestinal enteroids. Proc Natl Acad Sci U S A **2020**; 117:1700–10.
36. Fordham RP, Yui S, Hannan NR, et al. Transplantation of expanded fetal intestinal progenitors contributes to colon regeneration after injury. Cell Stem Cell **2013**; 13:734–44.
37. Maroni L, van de Graaf SF, Hohenester SD, Oude Elferink RP, Beuers U. Fucosyltransferase 2: a genetic risk factor for primary sclerosing cholangitis and Crohn's disease–a comprehensive review. Clin Rev Allergy Immunol **2015**; 48: 182–91.
38. Yamamoto F, Clausen H, White T, Marken J, Hakomori S. Molecular genetic basis of the histo-blood group ABO system. Nature **1990**; 345:229–33.
39. Nishi N. Aqueous molecular clusters isolated as liquid fragments by adiabatic expansion of liquid jets. Z Phys D Atoms Mol Clusters **1990**; 15:239–55.
40. Huang P, Farkas T, Zhong W, et al. Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns. J Virol **2005**; 79: 6714–22.
41. Harrington PR, Lindesmith L, Yount B, Moe CL, Baric RS. Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice. J Virol **2002**; 76:12335–43.
42. Ao Y, Cong X, Jin M, et al. Genetic analysis of reemerging GII.P16-GII.2 noroviruses in 2016–2017 in China. J Infect Dis **2018**; 218:133–43.
43. Lindesmith LC, Brewer-Jensen PD, Mallory ML, et al. Virus-Host interactions between nonsecretors and human norovirus. Cell Mol Gastroenterol Hepatol **2020**; 10:245–67.
44. Rouphael N, Beck A, Kirby AE, et al. Dose-response of a norovirus GII.2 controlled human challenge model inoculum. J Infect Dis **2022**; 226:1771–80.
45. Atmar RL, Ettayebi K, Ramani S, et al. A bivalent human norovirus vaccine induces homotypic and heterotypic neutralizing antibodies. J Infect Dis **2024**; 229:1402–7.
46. Henry S, Mollicone R, Fernandez P, Samuelsson B, Oriol R, Larson G. Molecular basis for erythrocyte Le(a+ b+) and salivary ABH partial-secretor phenotypes: expression of a FUT2 secretor allele with an A–>T mutation at nucleotide 385 correlates with reduced alpha(1,2) fucosyltransferase activity. Glycoconj J **1996**; 13:985–93.
47. Bidalot M, Théry L, Kaplon J, De Rougemont A, Ambert-Balay K. Emergence of new recombinant noroviruses GII.p16-GII.4 and GII.p16-GII.2, France, winter 2016 to 2017. Euro Surveill **2017**; 22:30508.
48. Han JC, Li QX, Fang JB, et al. GII.P16-GII.2 recombinant norovirus VLPs polarize macrophages into the M1 phenotype for Th1 immune responses. Front Immunol **2021**; 12:781718.