Original Article
Replication and transcriptionomic analysis of human noroviruses in human intestinal enteroids

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Abstract: Human noroviruses (HuNoVs) are a major cause of epidemic and sporadic cases of acute gastroenteritis worldwide. Recently, human intestinal enteroids (HIEs) have been shown to support the replication of HuNoVs, and be an excellent model to study HuNoV-host interactions. We implemented the HIE system in our laboratory and investigated the global molecular events associated with the mechanism of HuNoV-host interactions. Successful replication was observed for several norovirus GII genotypes, and totally 5,376 genes with different expression in HIEs were identified during infection. Bioinformatics analysis revealed that several important pathways, especially the "Signal transduction" and "Immune system" pathways, were involved in the HuNoV-host interaction. Quantitative PCR results validated that IFN-λ instead of IFN-β was elevated in HIEs after infection. Our study showed the holistic understanding of the transcriptome events in the HIE model infected by HuNoVs, and highlighted the important role of IFN-λ signaling in the HuNoV-host interactions.

Keywords: Human norovirus, human intestinal enteroid, RNA sequencing, interferon

Introduction

Human noroviruses (HuNoVs), a genus in *Caliciviridae*, are a major cause of epidemic and sporadic cases of acute gastroenteritis worldwide, which account for at least 95% of viral outbreaks and more than 50% of all outbreaks of gastroenteritis [1-3]. In the United States alone, HuNoVs are responsible for about 20 million cases of acute gastroenteritis annually, resulting in over 70,000 hospitalizations and nearly 800 deaths [4]. In developing countries, these infections lead an estimated 200,000 deaths annually in children under 5 years of age [5]. In addition to causing morbidity and mortality in patients, norovirus disease also causes significant economic burden as a result of health care costs and loss of productivity [6]. Up to now, effective vaccines and antiviral drugs are still lacking because of the limited knowledge of mechanisms of norovirus persistence and immune evasion in human [7].

HuNoVs cause a species-specific infection, and elucidation of the pathophysiology of HuNoV infections relies on the studies using healthy adult volunteers. Thus, the lack of a robust HuNoV cell culture system is one of the key challenges in assessing the efficacy of vaccines and antiviral drugs. Human intestinal enteroids (HIEs), or "mini-guts", are generated from stem cells in the intestinal crypts isolated from human intestinal tissue, which recapitulate the cell diversity and complexity of the human gastrointestinal tract [8-10]. Recently, HIEs have shown to support the replication of HuNoVs, and be an excellent model to study HuNoV-host interactions [11, 12]. In this study, we used HIEs to investigate the host cell response to norovirus infection by RNA sequencing.

Materials and methods

HIEs cultures

Wnt3a-producing cells (CRL-2647 cells) were purchased from ATCC company. R-spondin-producing cells (R-spondin1) were purchased from Trevigen company. Recombinant human

Table 1. Primer sequences used in this study

Genes	Primer sequence
IFN-β Forward	CGCCGCATTGACCATCTA
IFN-β Reverve	TTAGCCAGGAGGTTCTCAACAATAGTCTCA
IFN-λ Forward	GGACGCCTTGGAAGAGTCACT
IFN-λ Reverve	AGAAGCCTCAGGTCCCAATTC
ISG15 Forward	GGACCTGACGGTGAAGATGCT
ISG15 Reverve	ACGCCAATCTTCTGGGTGATCT
ISG56 Forward	TACAGCAACCATGAGTACAA
ISG56 Reverve	TCAGGTGTTTCACATAGGC
GAPDH Forward	GCCAAAAGGGTCATCATCTC
GAPDH Reverve	GGGGCCATCCACAGTCTTCT

Noggin were purchased from Peprotech company. Complete media with and without growth factors (CMGF⁺ and CMGF⁻, respectively), differentiation media, and Wnt3a- R-spondin- and Noggin-conditioned media were prepared as previously studies [11, 12].

Three-dimensional (3D) cultures of HIEs were generated and cultured as detailed by previous studies [8, 9]. Briefly, HIE cultures were derived from biopsies from surgical specimens. HIEs were grown and maintained as multilobular, 3D cultures in Matrigel (BD). For all infections, single cell suspensions were prepared from the 3D HIEs by trypsin digestion, and then seeded on a 96-well plate using CMGF⁺ media. After 24 hours of growth, the culture media was changed to differentiation medium to differentiate the cells for 5 days.

Stool filtrates

To prepare 10% stool suspensions, sterile PBS was added to HuNoV-positive stools, which were then homogenized by vortexing and sonicated three times for 1 min. The sonicated suspensions were centrifuged at 1,500 × g, 10 min, at 4°C. The supernatant was passed serially through 5 µm, 1.2 µm, 0.8 µm, 0.45 µm and 0.22 µm filters. The filtered samples were aliquoted and frozen at -80°C until used.

HuNoV infection of HIE monolayers

For inoculation, the HIE cell monolayers were washed once with CMGF⁻ media and inoculated with HuNoV diluted in CMGF⁻ media containing or not containing bile substitute glycyrene deoxycholic acid (GCDCA) (Sigma, Cat. G0759) for 1 hour at 37°C. The inoculum was removed

and monolayers were washed twice with CMGF⁻ media to remove unbound virus. Differentiation media was then added and the cultures were incubated at 37°C for the indicated time points.

RNA extraction and real-time PCR

We isolated total RNA from HIE monolayers using Trizol reagent (Invitrogen) according to the manufacturer's protocol. For each sample, 1 ug of RNA was reverse transcribed using the reverse transcription kit (Takara). We performed real-time PCR amplification using SYBR Green Master Mix kit (Takara) in an Applied Biosystems Q6 platform. GAPDH was used as the internal control for mRNA quantification, and the fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study was provided in **Table 1**.

RNA sequencing in BGISEQ-500 platform

18 total RNA was extracted using Trizol (Invitrogen), and the quantity and quality of mRNA were assayed with an Agilent 2100 Bioanalyzer and NanoDrop. Approximately 1 µg total RNA was initially used for BGISEQ-500 library construction, and samples were then sequenced on the BGISEQ-500 platform [13]. Raw sequencing reads were filtered to get clean reads by using SOAPnuke, and HISAT pipeline was applied to align reads against reference genome. The identification of differentially expressed genes (DEGs) was based on the negative binomial distribution of DEseq2 package, and the cut-off of DEGs was Fold Change ≥ 2 and adjusted *P* value ≤ 0.05.

Bioinformatics analysis

Gene ontology (GO) enrichment analysis was performed using the GOseq R package, in which gene length bias was corrected [14]. For DEGs, GO terms with corrected *P* < 0.05 were considered significantly enriched. Additionally, the statistical enrichments of DEGs in the Kyoto encyclopedia of genes and genomes (KEGG) related pathways were implemented by the KEGG orthology based annotation system [15].

Statistical analysis

Each experiment was performed two or more times, with three technical replicates of each culture, condition and time point in each experiment. All results are reported as means ± SEM.
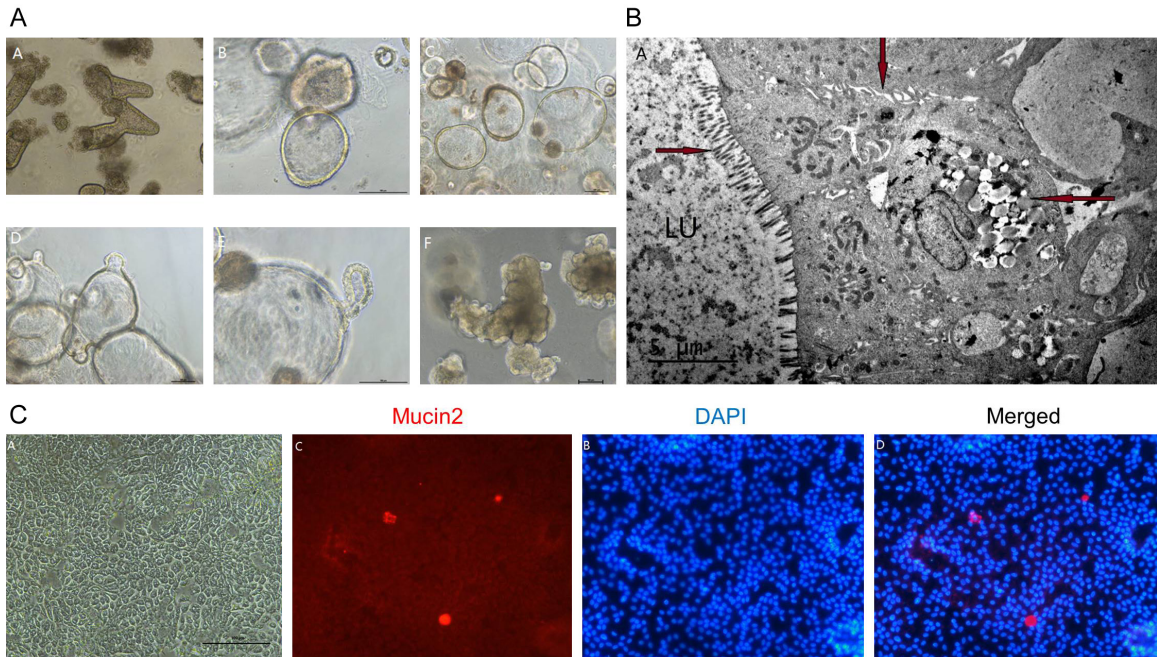
Figure 1. Growth of 3-dimensional human intestinal enteroids (HIEs). A. The representative figures of undifferentiated HIEs under microscopy. B. A representative figure of undifferentiated HIEs under transmission electron microscopy. C. Monolayer cells of HIEs after differentiation stained with Mucin2-PE.
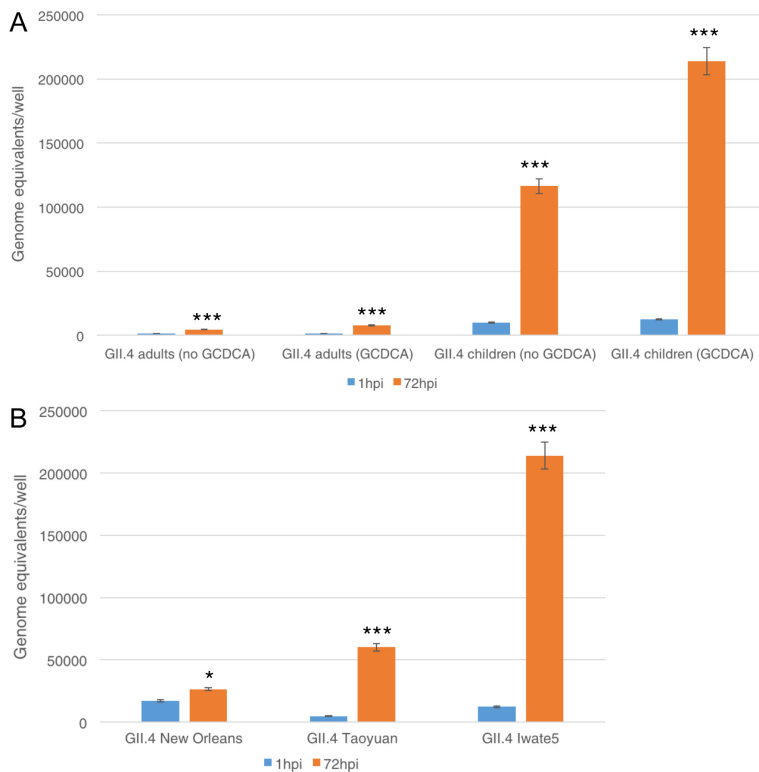

Figure 2. Evaluation of human norovirus (HuNoVs) replication in HIEs detected by qPCR. A. Replication of HuNoVs GII.4 from stool filtrates of children or adult patients in the monolayer cells of HIEs in the absent or presence of glycyrene deoxycholic acid (GCDCA). B. Replication of HuNoVs GII.4 New Orleans, GII.4 Taoyuan, and GII.4 Iwate5 in the monolayer cells of HIEs in the presence of GCDCA. *, $P < 0.05$; ***, $P < 0.001$; compared with the data in 1 hpi.

Statistical evaluation between groups was performed by two-tailed Student's t-test or one-way ANOVA and is presented in the Figures. *P*-values < 0.05 were considered statistically significant.

Results

Establishment of human intestinal enteroid model

Intestinal crypts were isolated from fresh adult jejunal tissue specimens, then seeded on Matrigel-coated plates in the presence of growth factors, including Wnt-3A, R-Spondin, Noggin, etc. Microscopic observation showed that the cells in HIE were similar to the cells in human intestinal tissue with polarized growth (**Figure 1A**). Transmission electron microscopic observation revealed that the villus faced the cavity surface, the complete tight connection was present between cells, the typical vesicle bodies of
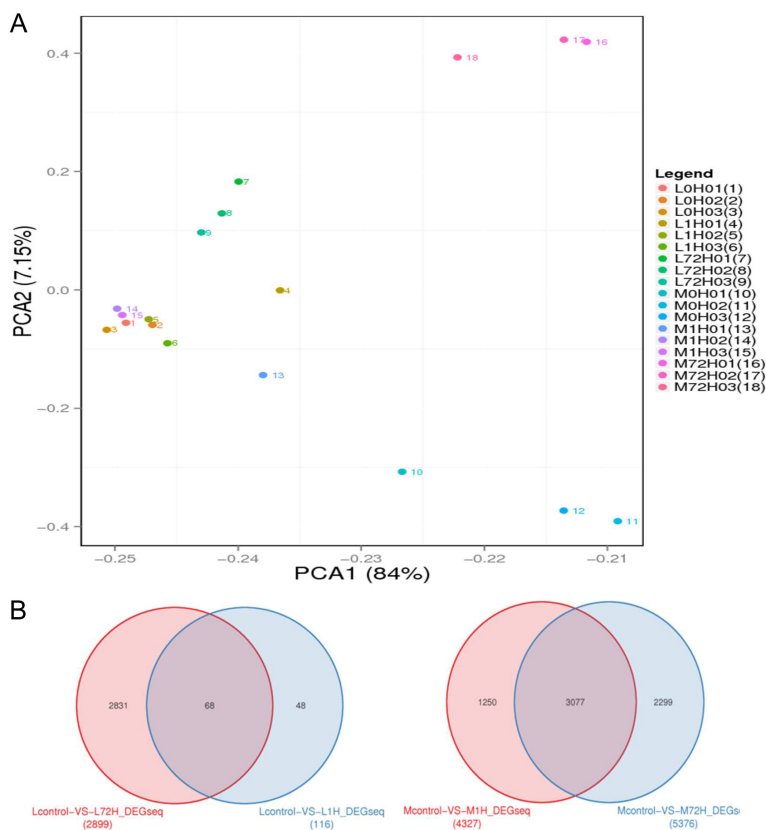
Figure 3. Transcriptional response to HuNoVs infection in HIEs assessed by RNA sequencing. A. Principal component analysis of the difference of samples. B. Venn diagrams depict the overlap among each individual's deregulated genes at 1 and 72 hpi.

the amount of genomic RNA detected at 1 hpi; while the virus genome of HuNoVs from children patients increased 17.23 folds at the same time point (**Figure 2A**). In addition, we observed the expansion rate of different GII.4 variants in HIE monolayers from the same source. Three strains of HuNoVs GII.4, including GII.4 New Orleans, GII.4 Taoyuan, and GII.4 Iwate5, were used to infected the HIE monolayers. In the presence of GC-DCA, at 72 hpi, the virus genome of these three HuNoV strains increased 1.55 folds, 12.60 folds, and 17.23 folds, respectively, compared with that detected at 1 hpi (**Figure 2B**).

RNA-sequencing analysis of gene expression profile in HIE infected by HuNoV

We systematically investigated the mRNA transcriptome in HIE during the infection of HuNoV using RNA sequencing. To this end, monolayers of HIEs from two different donors (L and M) were inoculated with GII.4 HuNoVs. At 0, 1, and 72 hpi, cells were harvested to detect the gene expression profiles. Principal component analysis showed that the intraculture transcriptional profiles for a specific infection status clustered together, but the basal human epithelial transcriptome differs significantly between HIEs from different individuals (**Figure 3A**). In total, after normalization, 116 and 2,899 genes were found differentially expressed in the HIE from donor L at 1 and 72 hpi, respectively, compared with the uninfected control; while 4,327 and 5,376 genes were deregulated in the HIE from donor M at 1 and 72 hpi, respectively, compared with the uninfected control (**Figure 3B**).

The aberrant genes were subjected to Gene Ontology (GO) analysis for functional classification (**Figure 4**). Differential genes associated with biological processes were mainly enriched in "cellular process", "biological regulation", "metabolic process", "regulation of biological

goblet cells accumulated above the nucleus, and hormone-rich vesicles in endocrine cells accumulated under the nucleus (**Figure 1B**). The result of immunofluorescence showed that the goblet cells were dispersed in the monolayer cells after differentiation (**Figure 1C**).

HuNoV replication in HIE

To evaluate whether the HIE support replication of the HuNoVs, monolayers of HIEs were inoculated with HuNoVs GII.4 from stool filtrates of children or adult patients. In the absence of GCDCA, at 72 hours post-infection (hpi), the genome equivalents of viral progeny of HuNoVs from adult patients increased 3.38 folds compared with the amount of genomic RNA detected at 1 hpi; while the virus genome of HuNoVs from children patients increased 11.88 folds at the same time point. By contrast, in the presence of GCDCA, at 72 hpi, the genome equivalents of viral progeny of HuNoVs from adult patients increased 6.10 folds compared with
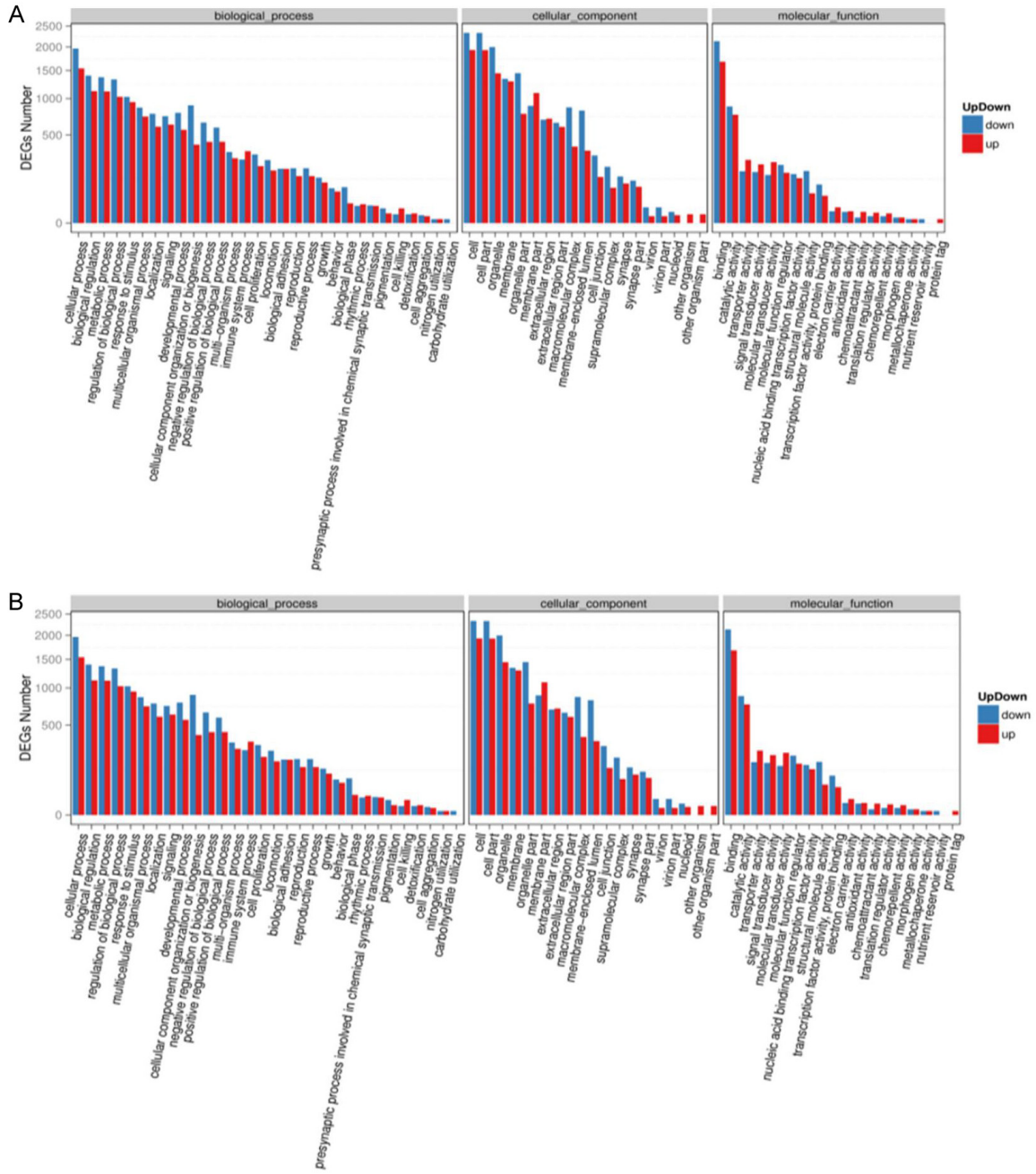
Figure 4. GO category analysis of differential genes in HIEs during HuNoV infection. A. HIEs from donor L. B. HIEs from donor M.

process", "response to stimulus", "multicellular organismal process", "localization", "signaling", "developmental process", "cellular component organization or biogenesis", "multi-organism process", "immune system process", which implied that HuNoV infection significantly activated the cellular responses, intestinal cell-related metabolic processes, and biological regulation and immune response of HIE cells. Deregulated genes related to cellular component were ma-

inly enriched in "cell", "cell part", "organelle", "membrane", "extracellular region", "extracellular region part", "macromolecular complex", "membrane-enclosed lumen", which suggested that multiple cellular components were involved in the infection and replication of HuNoV. Differential genes associated with molecular function were primarily enriched in "binding", "catalytic activity", "transporter activity", "signal transducer activity", "molecular function

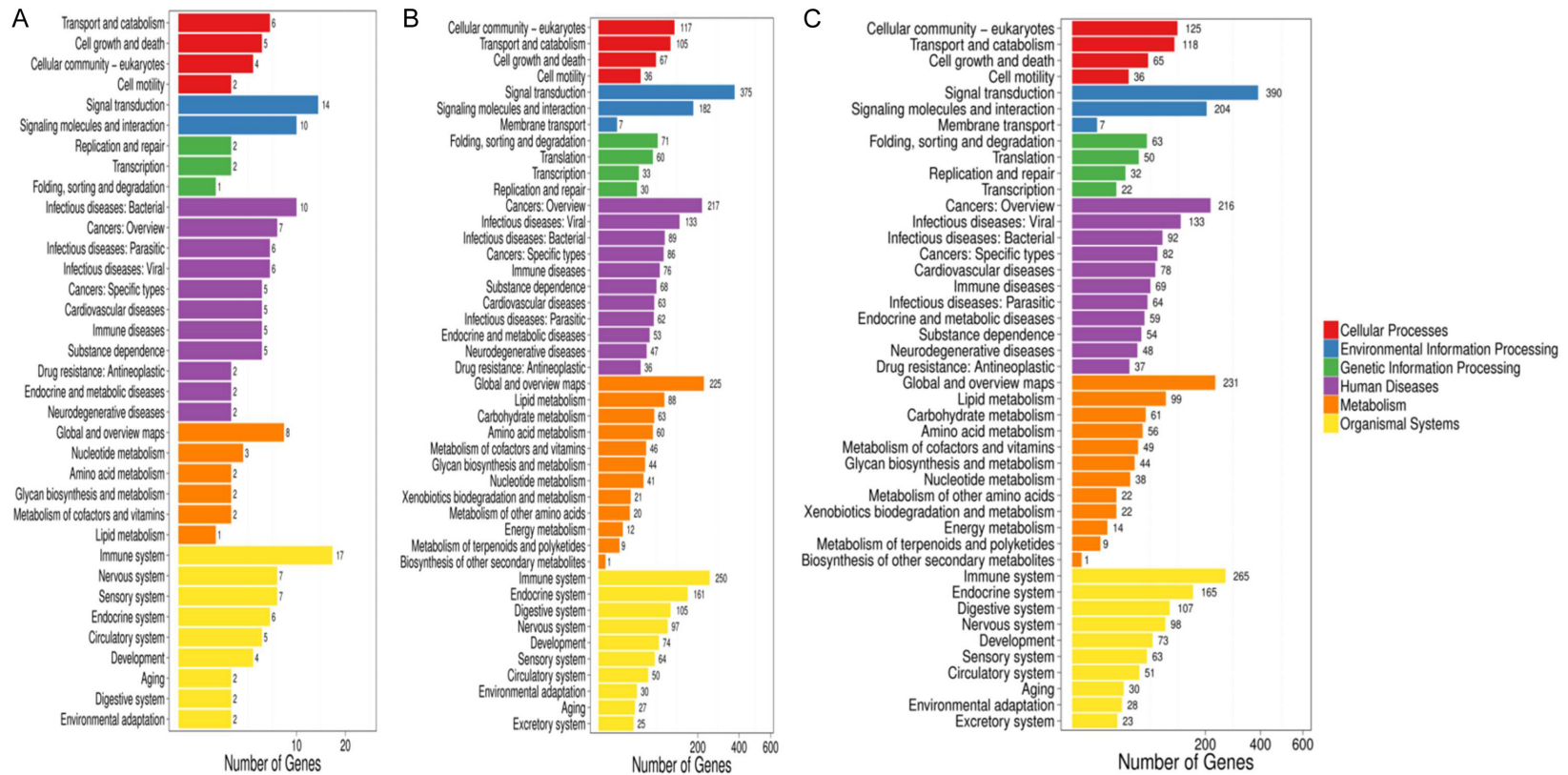**Figure 5.** Pathway analysis of differential genes in HIEs during HuNoV infection. A. 0 h vs 1 h. B. 1 h vs 72 h. C. 0 h vs 72 h.
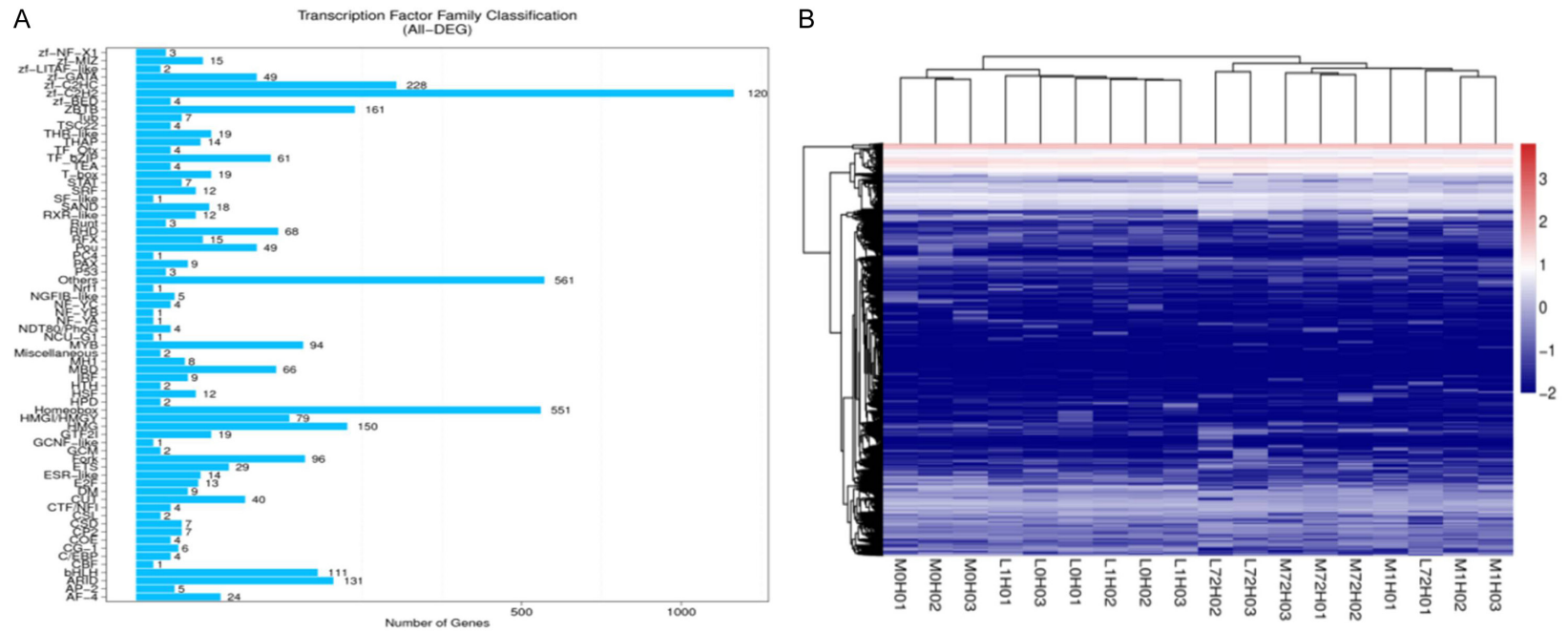
Figure 6. Analysis of differential transcription factors in HIEs during HuNoV infection. A. Classification map of the enriched transcription factor family. B. Gene expression heat map of transcription factors.
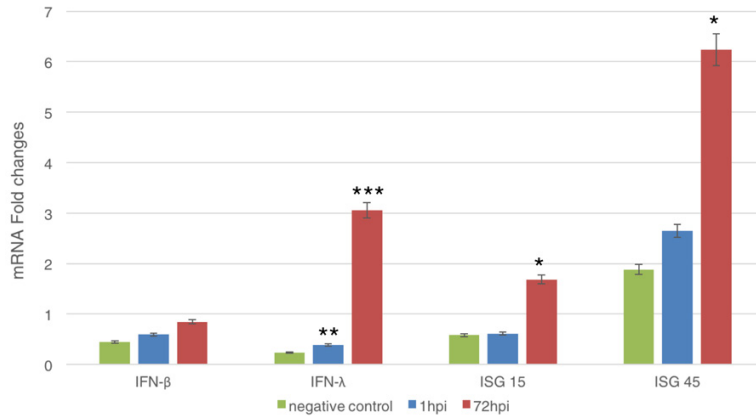
Figure 7. Expression of IFN-β, IFN-λ, ISG-15, and ISG-45 in HIEs during HuNoV infection detected by qPCR. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; compared with the data in control group.

regulator", "binding transcription factor activity", and "structural molecule activity", which indicated that HuNoV infection resulted in complicated interaction between pathogen and host cell.

The differential genes also were subjected to pathway analysis for functional classification (**Figure 5**). Six significantly enriched pathway were found, including "cellular Processes", "Environmental Information Processing", "Genetic Information Processing", "Human diseases", "Metabolism", and "Organismal systems". These differential genes could be further allocated to 45 pathways, in which "Signal transduction" and "Immune system" were the most markedly enriched pathways.

At last, we analyzed and classified differentially expressed genes with transcription factor activity. The results showed that differential transcription factors were mainly enriched in several families, such as zf-C2H2, zf-C2HC, Homeobox, HMG and ARID, suggesting that these transcription factor families played a crucial role in the regulation of gene expression after infection (**Figure 6A**). In addition, cluster analysis that the expression pattern of transcription factors in different samples were similar (**Figure 6B**).

The expression of IFNs and IFN-stimulated genes in HIE infected by HuNoV

GO analysis showed that differentially expressed genes in HIE cells were enriched in type III interferon (IFN) signaling pathway after infection (*P* < 0.001, **Figure 7**). To verify the sequenc-

ing results, we examined the expression of IFN-β, IFN-λ, and IFN-stimulated genes (ISGs) using qPCR. Consistent with the results of RNA sequencing, the qPCR result revealed that the expression of IFN-β in HIE cells was not changed after infection, but the expression of IFN-λ, ISG-15, and ISG-45 was significantly elevated after infection.

Discussion

HuNoVs have resisted great efforts to establish in vitro culture models for over 40 years. Most efforts were unsuccessful, or the results could not be reproduced by other groups. A primary breakthrough in the field of in vitro expansion of intestinal epithelium is the successful long-term culture of intestinal epithelial organoids. Recently, several studies have shown that HIEs support the replication of HuNoVs and other enteric viruses, which enable analysis of the host response against these viruses. In this study, we successfully constructed the HIEs as ex vivo, 3D cultures in growth factor enriched media, which contained a stem cell niche and the diversity of intestinal epithelial cell type. GII.4 remains the dominant genotype of HuNoVs worldwide [16]. Our data showed that the HuNoV GII.4 from children patients replicated quickly than the HuNoVs from adult patients in HIEs, and bile substitute GCDCA significantly promoted the replication rate of HuNoVs in HIEs. In addition, the expansion rates of different GII.4 variants were varied in HIEs. These results demonstrated that the HIE model is robust for HuNoV replication. However, our study also revealed that the basal human epithelial transcriptome differs significantly between HIEs from different individuals, which is consistent with previous study [17]. This inherent variability captured in the HIE model could provide a unique opportunity to investigate the similarities and differences in susceptibility of HuNoVs infection among individuals with different gene background.

Elucidation of the mechanism of norovirus persistence and immune evasion in human cell is critical for the prevention and therapy of Hu-

NoVs infection. RNA sequencing is a powerful tool to explore the mechanism of cell response to stimulus. In this study, we used HIEs to investigate the host cell response to norovirus infection by RNA sequencing. We found that the expression of thousands of genes in HIEs were changed after infection. Though bioinformatics analysis, several important pathways, especially the "Signal transduction" and "Immune system" pathways, were identified, which were involved in the interaction with the virus. These data reveal that host cell response to norovirus infection was a very complicated process, and mining the mechanism behind the transcriptome data will be beneficial for understanding the mechanism of norovirus persistence and immune evasion, which is the primary task in future study.

An early innate immune response mounted by epithelial cells is crucial in limiting viral replication [18]. IFNs are potent antiviral cytokines during innate immune. Three types of IFNs have been identified, including type I, type II, and type III IFN. Type II IFN (IFN-γ) is primarily produced by immune cells, while type I (IFN-α/β) and type III IFN (IFN-λ) are synthesized by immune and epithelial cells, which are believed to be involved in the host-pathogen interactions at epithelial cells [19, 20]. In addition, the receptor of type I IFN is located on most cell types, whereas the receptor of type III IFN is mainly restricted to epithelial cells [21]. The lack of a type I IFN response was found in murine and human intestinal epithelial cells following rotavirus infection [17, 22]. Instead, the innate type III IFN response was observed to be preserved in HIEs irrespective of rotavirus strain or replication competency [17]. Consistent with these studies, our study found that the expression of IFN-β in HIE cells was not changed after infection, but the expression of IFN-λ, ISG-15, and ISG-45 was significantly elevated after infection. Therefore, it is possible that human intestinal epithelial cells are programmed to respond to HuNoVs with type III instead of type I IFN.

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Disclosure of conflict of interest

None.

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