Human Cytomegalovirus-IE2 Affects Embryonic Liver Development and Survival in Transgenic Mouse

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SUMMARY

In this study, a transgenic mouse model that could specifically and stably express the immediate-early protein 2 (IE2) in the liver was constructed to simulate the pathology of the effect of IE2 expression on cells after human cytomegalovirus infection. Results showed that a consistent IE2 expression affects the orderly process of embryonic liver development by disrupting hepatic morphogenesis and hepatocyte maturation, resulting in lethality at embryonic day 17.5 to day 1.

BACKGROUND & AIMS: Congenital human cytomegalovirus (HCMV) infection is a common cause of liver injury. The major immediate-early protein 2 (IE2) of HCMV is critical for the progression of HCMV infection. As a result of species isolation, there are no animal models suitable for HCMV infection, which aimed to study the long-term effects of IE2 on embryonic liver development in vivo. Hence, this study aimed to investigate the role of IE2 in liver development using a transgenosis mouse model.

METHODS: Rosa26-Loxp-STOP-Loxp (LAS)-IE2+/−, cre mice that could specifically and stably express IE2 in the liver, were constructed. Phenotypic analysis, immunolocalization studies, messenger RNA analyses, transcriptome sequencing, and flow cytometry analysis were performed on Rosa26-LSL-IE2+/−, cre mice during hepatogenesis.

RESULTS: Rosa26-LSL-IE2+/−, cre mice could consistently express IE2 at different embryonic stages in vivo. With the development of Rosa26-LSL-IE2+/−, cre embryos from embryonic day 17.5 to postnatal day 1, progressive liver hypoplasia and embryonic deaths were observed. Furthermore, molecular evidence that IE2 expression inhibited hepatocyte proliferation, increased cell apoptosis, and impaired hepatocyte maturation was provided.

CONCLUSIONS: Rosa26-LSL-IE2+/−, cre mice could stably express IE2 in the liver. IE2 expression resulted in embryonic liver hypoplasia by disrupting hepatic morphogenesis and hepatocyte maturation, which may be responsible for embryonic deaths. This study is helpful in understanding the mechanism of liver injuries induced by HCMV infection. (Cell Mol Gastroenterol Hepatol 2022;14:494–511; https://doi.org/10.1016/j.jcmgh.2022.05.002)

Keywords: Human Cytomegalovirus; IE2; Liver Development; Mouse Model.
Human cytomegalovirus (HCMV), a member of the β-herpesviruses, is the most common virus that causes congenital infection, affecting 0.4%–1% of live births. HCMV infection is a major cause of birth defects including liver injury, hearing and visual loss, neurologic deficits, and intrauterine growth retardation, and may contribute to adverse consequences such as stillbirth and premature delivery. Because liver structure and function are critical for circulation, metabolism, and nutrition, liver injuries caused by HCMV infection should be taken seriously. As a result of the strict species-tropism of HCMV, an animal model of HCMV infection has not been established yet. Hence, the in vivo mechanism of liver injuries caused by HCMV remains unclear.

Immediate-early protein 2 (IE2), a 579-amino acid protein, is encoded by the immediate-early gene IE2. As a master transcriptional regulator, IE2 could induce the expression of other virus-related genes and independently regulate host cell promoters. The perturbation of cell growth control by IE2 may be one of the ways in which virus promotes the development of some diseases, such as mental retardation and microcephaly. However, the effects of HCMV-IE2 on liver development are unclear, and further research is needed to elucidate the specific effects and mechanisms involved.

In this study, a transgenesis mouse Rosa26−Loxp-STOP-Loxp (LSL)-IE2+/−, cre that could specifically and stably express IE2 in the liver was established. The long-term effects of IE2 on the liver and their underlying mechanisms were investigated in vivo. The results suggest that IE2 expression in the liver has a negative impact on hepatic morphogenesis and hepatocyte maturation during hepatogenesis. Moreover, IE2 accumulation resulted in late embryonic lethality. This study provides a new insight on the underlying mechanisms of molecular pathogenesis through which HCMV induces liver damage.

**Results**

### Liver Hypoplasia and Late Embryonic Lethality Were Observed in IE2-Expressing Mice

To explore the effects of IE2 on the liver, a transgenic mouse model (Rosa26−LSL-IE2+/−, cre) that could long-term stably express IE2 in the liver was constructed. Rosa26−LSL-IE2+/−, cre mice were genotyped by polymerase chain reaction (PCR) and further confirmed by Western blot (Figure 1A and B). Surprisingly, among 88 neonatal mice, no live Rosa26−LSL-IE2+/−, cre mice were obtained. Embryonic lethality occurred between embryonic day (E)17.5 and day (D)1 (Table 1).

Consistent with this lethality, in Rosa26−LSL-IE2+/−, cre mice, the whole liver became smaller, with lower liver weight and liver/body weight ratio at E17.5–D1 compared with those of control embryos (Rosa26−LSL-IE2+/−) (Figure 1C and D). The histologic staining of liver tissue sections showed that the red blood cells were nearly all anucleated at hepatic vessels, and hepatocytes were beginning to organize and form mature hepatic cords from E17.5 in control embryos. However, Rosa26−LSL-IE2+/−, cre embryos showed an abnormal architecture; at E17.5, the cellularity of the liver was reduced, and the empty space was increased; by D1, the liver structure was disordered, with large necrotic areas and irregularly arranged hepatocytes. Meanwhile, abundant nucleated red blood cells were observed in the liver and cardiac cavity (Figure 1E).

Then, IE2 expression was detected in Rosa26−LSL-IE2+/−, cre livers. As shown in Figure 1F, IE2 was expressed consistently at different embryonic stages E15.5, E17.5, and D1, while it was strongly expressed from E17.5. All of these data indicate that the late lethality of Rosa26−LSL-IE2+/−, cre embryos was probably linked to severe liver hypoplasia and phenotypic defects that may result from the strong activation of IE2 signaling at E17.5–D1, which is a critical period for liver morphogenetic and metabolic development.

### Loss of Hepatoblasts and Hepatocytes in IE2-Expressing Liver During Liver Development

The specific expression of albumin (ALB) in hepatoblasts and hepatocytes led to the activation of IE2 transcription and translation processes in both cells of our mouse model. To understand the causes of the abnormal liver architecture in Rosa26−LSL-IE2+/−, cre mice at the cellular level, the numbers of hepatoblasts and hepatocytes were measured by α-fetoprotein (AFP)/β-like non-canonical notch ligand 1 (DLK1) and ALB immunostaining, respectively (Figure 2A and B). AFP and DLK1 are established hepatoblast markers, whereas ALB is a hepatocyte marker. Overall, DLK1- and AFP-positive cells were decreased continuously, while ALB-positive cells were increased gradually in 2 groups with liver development, but the expression levels in the 2 groups were significantly different at the same stage. Specifically, at E15.5, there was no significant difference in DLK1-, AFP-, and ALB-positive cells between the 2 groups, whereas at E17.5, DLK1-, AFP-, and ALB-positive cells were significantly lower than those in the control group. By D1, DLK1- and AFP-positive cells almost disappeared in the control group, but still existed in the IE2-expressing group. ALB-positive cells were dramatically lower in the IE2-expressing group than in the control group.

Abbreviations used in this paper: AFP, α-fetoprotein; ALB, albumin; C/EBPa, CCAAT/enhancer binding protein alpha; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats; CrisprR-associated 9; D, day; DEG, differentially expressed gene; DLK1, β-like non-canonical notch ligand 1; E, embryonic; GO, Gene Ontology; GSEA, gene set enrichment analysis; HCMV, human cytomegalovirus; HNF4a, hepatic nuclear factor 4α; IE2, immediate-early protein 2; KEGG, Kyoto Encyclopedia of Genes and Genomes; LSL, Loxp-STOP-Loxp; mRNA, messenger RNA; PCR, polymerase chain reaction; PPI, protein–protein interaction; TNF, tumor necrosis factor.

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in the control group. Because IE2 expression was significantly higher at E17.5–D1 than at E15, a small amount of IE2 expression had no significant effect on the number of hepatoblasts and hepatocytes, but its long-term and strong expression might disrupt liver morphogenesis by affecting the number of hepatoblasts and hepatocytes.
A Dramatic Alteration in Gene Expression in IE2-Expressing Liver

To rule out the possibility that clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) technology exerted a negative influence on liver development and to further explore the molecular mechanism of liver injury caused by IE2 expression, total RNA was isolated from the livers of D1 IE2-LSL-IE2+/− mice, wild-type mice, and Rosa26-LSL-IE2+/−, cre mice for transcriptome sequencing. A total of 6 Gb of data per sample were obtained, and the average data quality 30 (Q30) was greater than 93%, which indicates that the results of the RNA-sequencing analyses were reliable (Table 2). Correlation and principal component analyses indicated that the gene expression patterns of Rosa26-LSL-IE2+/− mice were highly similar to those of wild-type mice, but were significantly different from those of Rosa26-LSL-IE2+/−, cre mice (Figure 3A and B). In addition, genomic region distribution analysis showed that the proportions of the 3′ untranslated region (3′ UTR) and coding sequence (CDS) expression were significantly increased and decreased, respectively, in Rosa26-LSL-IE2+/−, cre mice (Figure 3C). These data showed that the CRISPR/Cas9 technology used to construct the Rosa26-LSL-IE2+/− mouse model did not affect the liver, while the liver transcriptome alterations may be attributed to the specific expression of IE2.

Then, differentially expressed genes (DEGs) were analyzed by edgeR packages of R (https://www.r-project.org/. Supplementary Table 1). In total, 3723 genes were expressed differentially (1717 genes were up-regulated, and 2006 genes were down-regulated) in the Rosa26-LSL-IE2+/−, cre group compared with those in the Rosa26-LSL-IE2+/− group (Figure 3D and E). DEGs were identified based on an adjusted P value less than .05 and absolute value of log2 fold change greater than 1. The differences in the expression of top DEGs in the 2 groups were verified by quantitative real-time PCR (Figure 3F).

IE2-Specific Expression Affects Multiple Steps of Liver Development In Vivo

To further verify the effect of IE2 expression on the liver in vivo, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment were performed to analyze the biological functions of the DEGs. KEGG analysis showed that the up-regulated genes were enriched mainly in herpes simplex infection, cell cycle, and apoptosis (Figure 4A, Supplementary Table 2), while the down-regulated genes were enriched significantly in metabolic pathways, chemical carcinogenesis, complement and coagulation cascades, and fatty acid degradation (Figure 4C, Supplementary Table 3). GO analysis showed that the enriched categories of up-regulated genes were associated mainly with DNA replication, including nucleosome assembly, DNA packaging, nucleosome organization, and chromatin assembly (Figure 4B, Supplementary Table 4). However, the enriched categories of down-regulated genes were related mainly to hepatocyte functions, including metabolic and oxidation reduction processes (Figure 4D, Supplementary Table 5).

Based on the string protein interaction database (http://StringDB.org), a protein–protein interaction (PPI) network was used to illustrate the interaction among the DEGs of the Rosa26-LSL-IE2+/−, cre group. By ranking their degrees, the top 30 hub regulatory genes in the PPI network were determined (Table 3). Interestingly, these hub DEGs with higher degrees were associated mainly with cell cycle (Ccnb1, Ccn2, Jun, and Pena) and DNA damage (Rad21 and Bub1), suggesting that the overall changes of the liver transcriptome in Rosa26-LSL-IE2+/−, cre mice may be caused by abnormal cell proliferation and DNA damage response. Taken together, these results indicate that IE2 affects multiple processes of liver development, including cell proliferation, apoptosis, liver metabolism, and liver synthetic function.

Failure of Proliferation in IE2-Expressing Liver

To further determine how IE2 affects the cell-cycle progression, a network of cell-cycle–related genes was
evaluated using PPI (Figure 5A). The network showed that cyclin dependent kinase interacting protein/kinase inhibitory protein (CIP/Kip) family members, such as cyclin-dependent kinase inhibitor 1a (cdkn1a, also known as P21) and cyclin-dependent kinase inhibitor 1b (cdkn1b, also known as P27) have a high degree of connectivity. In addition, the cell cycle of D1 mice also was tested by flow cytometry. It was found that the number of S-phase cells was

Table 2. Quality Control of the RNA-Sequencing Analysis

| Group         | Sample         | Total reads | Total mapped | CleanGC | CleanQ20 | CleanQ30 |
|---------------|----------------|-------------|--------------|---------|----------|----------|
| Rosa26-LSL-IE2<sup>+/c</sup>,cre | B              | 26,578,833  | 92.51%       | 49.51; 49.38 | 97.57; 95.40 | 93.61; 90.77 |
|               | C              | 28,219,745  | 93.02%       | 48.81; 48.78 | 97.55; 95.53 | 93.46; 90.30 |
|               | E              | 23,773,646  | 93.17%       | 49.92; 49.60 | 97.71; 95.19 | 94.00; 90.52 |
| Rosa26-LSL-IE2<sup>+/c</sup>     | IE2            | 25,531,997  | 95.93%       | 50.49; 50.31 | 97.64; 96.87 | 93.85; 92.57 |
|               | IE3            | 23,681,012  | 95.71%       | 50.42; 50.18 | 97.74; 97.13 | 94.03; 93.07 |
|               | IE4            | 21,074,033  | 96.35%       | 50.20; 50.04 | 97.56; 96.50 | 93.64; 91.82 |
| Wild-type     | WT1            | 25,010,863  | 93.38%       | 50.27; 50.19 | 97.41; 93.92 | 93.02; 87.89 |
|               | WT2            | 25,536,067  | 94.87%       | 50.30; 49.75 | 97.82; 95.43 | 94.17; 90.64 |
|               | WT3            | 31,992,416  | 95.30%       | 50.38; 50.33 | 97.67; 95.52 | 93.76; 90.42 |

GC, Guanine and cytosine; Q20, Quality_20; Q30, Quality_30; WT, Wild-Type.
Figure 3. IE2-specific expression altered the expression level of genes in the liver. (A) Heatmap representing the correlation coefficient of samples between Rosa26-LSL-IE2+/− mice, wild-type mice, and Rosa26-LSL-IE2+/−, cre mice. The closer the correlation coefficient (R2) is to 1, the higher the similarity of expression patterns between samples. (B) Principal component analysis (PCA) was used to evaluate the differences between 3 groups and the repeatability of samples within groups. (C) Comparing the distribution of exon, intron, and intergenic regions of the genome to detect the source of sequence in the genome. (D) The gene number of up-regulation and down-regulation in the Rosa26-LSL-IE2+/−, cre group compared with the Rosa26-LSL-IE2+/− group. (E) Heatmap representing alteration of hepatic gene expression. Blue represents a down-regulation in expression and red represents up-regulation when compared with Rosa26-LSL-IE2+/−. (F) Validation of top DEGs by quantitative real-time PCR. Top DEGs were up-regulated (up) and down-regulated (down). Error bars represent SEM. The Student t test was used to analyze statistical differences. ***P < .001. n = 3 mice per group.
increased in Rosa26-LSL-IE2+/−, cre mice compared with that in Rosa26-LSL-IE2+/− mice (Figure 5B and C). Ki67 is a nuclear antigen expressed during the proliferative phase, except in cells in the G0 phase. The proportion of Ki67-positive cells was increased significantly in Rosa26-LSL-IE2+/−, cre mice compared with that in Rosa26-LSL-IE2+/−/C0 mice (Figure 5D). These results suggest that numerous cells have a stagnant cell cycle that does not function properly.

However, the molecular mechanisms leading to S-phase arrest remain unknown. To understand the molecular basis underlying this, the messenger RNA (mRNA) expression and protein levels of P21 and P27 were evaluated. It was found that P21 and P27 were highly expressed in Rosa26-LSL-IE2+/−, cre mice (Figure 5E and F). The arrest in cell-cycle progression possibly was owing to P21 and P27 inhibition. Furthermore, gamma histone variant H2AX (γH2AX), as a marker of DNA damage and repair,18 was used to measure DNA damage, which evidently was increased in Rosa26-LSL-IE2+/−, cre mice (Figure 5G). These data indicate that IE2 may block cell proliferation and induce DNA damage, thereby inhibiting the normal development of the liver.

Dysregulated Apoptosis in IE2-Expressing Livers

An increase in apoptosis may be a key factor in hepatic hypoplasia. To determine whether IE2 expression is involved in regulating this programmed death, flow cytometry was performed on the hepatocytes of Rosa26-LSL-IE2+/− and Rosa26-LSL-IE2+/−, cre mice. The proportion of apoptotic cells was increased significantly in Rosa26-LSL-IE2+/−, cre mice, which was consistent with the KEGG enrichment analysis (Figure 6A and B). The expression levels of the death receptor TRAIL-R (Tnfrsf10b), the pro-apoptotic gene PMAIP1, and the apoptotic gene caspase3 were up-regulated in Rosa26-LSL-IE2+/−, cre mice (Figure 6C).

Next, the protein level of caspase 3 in the liver was estimated. As shown in Figure 6D, there was no caspase 3 expressed at E15.5. However, its expression was increased significantly in Rosa26-LSL-IE2+/−, cre livers at D1, indicating
liver with more CD4+ CD45 and CD3+ CD8+ T cell infiltration (Figure 7F). These data suggest that IE2 expression may induce a certain cellular inflammatory immune reaction, but it is subject to evaluation in further studies.

**Impaired Hepatocyte Maturation in IE2-Expressing Livers**

Glycogen synthesis is a biochemical function of mature hepatocytes. Periodic acid–Schiff staining showed a marked decrease in glycogen synthesis at 17.5 days, and it was almost none at D1 in Rosa26-LSL-IE2+/−/− mice, compared with that in Rosa26-LSL-IE2+/−/− mice. However, glycogen synthesis increased continuously with liver development in Rosa26-LSL-IE2+/−/− mice (Figure 8A). Next, the expression level of zonula occludens-1, which is an indicator of tight junctions, was determined to assess hepatocyte maturity (Figure 8B). Several tight junctions evidently were increased in Rosa26-LSL-IE2+/−/− livers, whereas those in Rosa26-LSL-IE2+/−/−/−, cre livers showed a dramatic decrease in number. In addition, the whole liver’s metabolism at stage D1 was evaluated by gene set enrichment analysis (GSEA), and the data showed that hepatocyte maturation and metabolic function remained low overall (Figure 8C).

DEGs related to hepatic differentiation and maturation are shown in Figure 8D. CCAT/enhancer binding protein alpha (C/EBPα) and hepatic nuclear factor 4α (HNF4α), which are key regulators of hepatocyte differentiation and maturation, controlled the expression of multiple liver-specific transcriptional genes. The mRNA and protein expression levels of C/EBPα and HNF4α were decreased dramatically in Rosa26-LSL-IE2+/−/−/−, cre livers at stage D1 (Figure 8E and F). Moreover, several transcriptional genes regulated by C/EBPα and HNF4α were observed to be decreased significantly in Rosa26-LSL-IE2+/−/−/−, cre livers at stage D1, including apolipoprotein M, A-I, A-II, C-II, and C-IV and coagulation factors XI, XII, and XIII (Table 4). Thus, the significant decrease in C/EBPα and HNF4α in hepatocytes appears to be a major mechanism of hepatocytes’ inadequate maturation.

**Discussion**

IE2 (also named IE86 and UL122), is essential for HCMV replication. Previous studies have confirmed that HCMV-induced neurodevelopmental disorders can be explained by IE2 action alone. Although our previous research suggested that IE2 can promote hepatic steatosis in adult mice, its effect on embryonic liver development remains rarely reported. In this study, transgenic mice (Rosa26-LSL-IE2+/−/−/−, cre) were constructed to simulate the pathology of the effect of IE2 expression on cells after HCMV infection and explore the effect of IE2 on the embryonic liver. It was found that long-term IE2 expression disrupts the liver’s normal development by impairing the program of hepatocyte proliferation, apoptosis, and maturation, which may be responsible for the lethality at E17.5–D1.

The death of Rosa26-LSL-IE2+/−/−, cre mice at late embryonic stage was unexpected. Our research team also constructed an animal model that specifically expressed that cell death was serious at this stage. Furthermore, it was expressed mainly in IE2-positive cells, suggesting that it may be an IE2 target responsible for the decrease in the number of hepatoblasts and hepatocytes of Rosa26-LSL-IE2+/−/−, cre mice.
IE2 in the hippocampus; it showed that IE2 expression only causes hippocampus damage in mice, but it does not affect their survival. The development of fetal liver plays a key role in the whole life cycle. Based on these findings, we proposed here that late embryonic lethality could be owing to hepatic hypoplasia caused by long-term and stable IE2 expression in the liver. However, the precise causes leading to this occurrence must be investigated and debated further.

During embryogenesis, the timing of the hepatoblast-to-hepatocyte transition occurs between E13.5 and E15.5, after which hepatocytes increase until adulthood. In contrast, IE2-expressing mice showed a tremendously slower increase in the number of hepatocytes from E17.5 to D1 compared with that in the control group of this study. The hypoplasia observed in the livers of Rosa26-LSL-IE2+/−, cre mice was associated with the inhibition of hepatocellular proliferation. Most cells were halted in S-phase in Rosa26-LSL-IE2+/−, cre mice. Murphy et al. also confirmed that IE2 protein could induce more cells to enter the S-phase and to halt the cell cycle. Han et al. showed that IE2 expression, but not IE1, induces host cell-cycle arrest by cell-cycle-inhibitory and DNA binding activities, leading to the impairment of brain development. We also identified 2 potential target genes that might account for the growth arrest in hepatocytes: P21 and P27. A previous study showed that P21, overexpressing in hepatocytes, which dramatically impaired hepatocyte proliferation, resulting in aberrant tissue organization, impeded liver and body growth, and increased mortality. These data might suggest that the expression of IE2 hindered cell-cycle processes, which may be an important factor for developmental liver arrest.

On the other hand, we also observed that apoptosis of hepatocytes significantly increased. In Rosa26-LSL-IE2−/−, cre mice. Studies have confirmed that cell apoptosis is one mechanism by which hypocellularity can occur in the developing liver. Previous studies have reported that HCMV disrupts apoptosis through the external (extracellular ligands and death receptors) and internal apoptosis pathways (endoplasmic reticulum stress, lysosomal dysfunction, and mitochondrial dysfunction). Chien and Dix detected significant amounts of TNF-α, TNF receptors 1 and 2, active caspase 8, active caspase 3, TRAIL, TRAIL-R, FAS, and FASL mRNAs and/or proteins in murine cytomegalovirus-infected eyes. Chiou et al. reported that IE2, but not IE1, up-regulates FASL expression and promotes HCMV-infected human retinal pigment epithelium cell apoptosis. In our study, Tnfrsf10b, Pam1p1, and Caspase 3 were highly expressed in IE2-expressing mice. In addition, immunofluorescence results showed that the expression of caspase 3 was higher in D1 than in E17.5 mice, which was contrary to the trend of ALB expression during liver development. In addition, IE2-expressing liver showed higher expression of inflammatory factors, which may promote cell apoptosis. Hence, the continuing accumulation of IE2 damaged hepatocyte survival and resulted in serious hepatic hypoplasia.

In addition to the dramatic decrease of hepatocytes in IE2-expressing livers, we also found inadequate maturation of hepatocytes, characterized by diminished glycogen storage, decrease of drug metabolic activity (Figure 4C), and significantly lower expression of factors associated with hepatocyte function such as apolipoproteins, transthyretin, aldehyde dehydrogenase, and coagulation factors. Cell metabolic studies have evaluated the primary infection of HCMV involved in the dysfunction of cellular metabolism, leading to a variety of amino acid, fatty acid, and energy metabolism disorders. Furthermore, in our study, we also observed a lower expression in the regulators of hepatocyte maturation, such as C/EBPα and HNF4α. C/EBPα is a critical regulator of hepatocyte metabolic function by transcriptional control of multiple downstream factors, such as apolipoprotein M and A-I, several coagulation factors, and transthyretin. HNF4α is essential for morphologic and functional differentiation of hepatocytes, accumulation of hepatic glycogen stores, and generation of a hepatic epithelium. Further research is necessary to verify whether IE2 directly controls the expression of C/EBPα and HNF4α. Overall, impaired hepatocyte maturity was inadequate to sustain hepatic or fetal viability.

This study had several limitations. First, given that HCMV has a large and complicated genome, our results could not sufficiently explain the mechanism of liver diseases with congenital HCMV infection. Second, the expression level of IE2 after HCMV infection in human beings is unknown because of a lack of research. Thus, the results of this study only speculate the effect of IE2 on liver after HCMV infection. Further studies and clinical data are needed to support the specific situation of fetal liver with HCMV infection.

In summary, the Rosa26-LSL-IE2+/−, cre mice that stably expressed IE2 in the liver were constructed successfully. Based on this transgenesis mouse model, our results showed that IE2 may play a key role in HCMV-caused liver disorders. The long-term expression of IE2 inhibited hepatocyte proliferation, increased cell apoptosis, and impaired their maturation, resulting in liver hypoplasia. Moreover, our findings provide valuable resources to further elucidate the potential target genes that might account for the growth arrest in hepatocytes: P21 and P27. A previous study showed that P21, overexpressing in hepatocytes, which dramatically impaired hepatocyte proliferation, resulting in aberrant tissue organization, impeded liver and body growth, and increased mortality. These data might suggest that the expression of IE2 hindered cell-cycle processes, which may be an important factor for developmental liver arrest.

Figure 5. (See previous page). Effects of IE2 expression on cell cycle and DNA damage. (A) Network of PPI of cell-cycle-related DEGs with Rosa26-LSL-IE2+/− and Rosa26-LSL-IE2−/−, cre mice. Green represents a regulatory gene, red represents a target gene. (B) The cell cycles were detected by flow cytometry between D1 Rosa26-LSL-IE2+/− and Rosa26-LSL-IE2−/−, cre mice. (C) Proportional distribution of cells in GO, S, and G2/M stages of the cell cycle. (D) D1 liver sections stained with immunostained Ki67 (left). Quantification of Ki67-positive cells (right). (E) mRNA expression levels of P21 and P27 were measured by quantitative real-time PCR. (F) The protein expression levels of P21 and P27 were measured by Western blot (left). Quantification of P21 and P27 expression (right). (G) Comparison of DNA damage between D1 Rosa26-LSL-IE2+/− and Rosa26-LSL-IE2−/−, cre mice. γH2AX is a marker of DNA damage. Green arrows represent DNA damage. Scale bars: 20 μm. Ctrl represents Rosa26-LSL-IE2+/−, IE2 represents Rosa26-LSL-IE2−/−, cre. Error bars represent SEM. The Student t test was used to analyze statistical differences. *P < .05, ***P < .001. n = 3 mice per group. Ctrl, control; DAPI, 4', 6-diamidino-2-phenylindole.
the potential cellular and molecular mechanisms of HCMV infection and to develop novel strategies to overcome related pathologies during liver development.

Materials and Methods

Animals

CRISPR/cas9 technology was used to construct Rosa26-LSL-IE2+/− mice. Rosa26-LSL-IE2+/− and albumin-Cre mice were used to obtain Rosa26-LSL-IE2+/−, cre mice (Figure 9). All mice were housed and maintained under specific pathogen-free conditions on a 12-hour day/night cycle at 23°C ± 2°C and received an autoclaved standard diet and water ad libitum. Mice were mated overnight, and the vaginal plug was detected at the noon as E0.5. The Rosa26-LSL-IE2+/−, cre mice were identified by PCR. The Rosa26-LSL-IE2+/− mice were control mice. The sequences of primers are listed in Table 5. All animal experiments were performed according to the guidelines of the Animal Welfare and Research Ethics Committee of Qingdao University.

Sample Collection

Embryos were collected on E15.5, E17.5, and D1, and fixed in 4% paraformaldehyde for 24 hours. Embryos were embedded in paraffin and sectioned at 5–6 μm. Liver tissues were stored in liquid nitrogen.

Figure 6. IE2-specific expression promotes cell apoptosis. (A) The cell apoptosis was detected by flow cytometry between D1 Rosa26-LSL-IE2+/− and Rosa26-LSL-IE2+/−, cre mice. (B) Proportional distribution of fluorescein isothiocyanate (FITC)+ PI+ cells. (C) mRNA expression levels of the apoptosis-related genes were measured by quantitative real-time PCR. (D) Immunofluorescence for E15.5, E17.5, and D1 livers, with primary antibodies against IE2 (red), caspase 3 (green), and Alexa Fluor 488– and 555–conjugated secondary antibodies. Error bars represent SEM. The Student t test was used to analyze statistical differences. **P < .01, ***P < .001. n = 3–5 mice per group. DAPI, 4', 6-diamidino-2-phenylindole.
Total RNA Extraction and Transcriptomic Analysis

Total liver RNA was extracted by TRIzol reagent following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The quantity and purity of RNA were analyzed using Bioanalyzer 2100 and the RNA 6000 Nano LabChip Kit (Agilent, Folsom, CA). After purification, the poly (A) - or poly (A) + RNA fractions was fragmented into small pieces by using divalent cations under increased temperature. Then, the cleaved RNA fragments were reverse-transcribed to create the final complementary DNA library in accordance with the protocol for the mRNA-sequencing sample preparation kit (Illumina, San Diego, CA), the average insert size for the paired-end libraries was 300 bp.
Figure 8. Inadequate maturation of remnant hepatocytes in Rosa26-LSL-IE2+/−, cre livers. (A) Hepatocellular cytoplasmic glycogen stores stained with periodic acid–Schiff. Scale bars: 20 μm. (B) Immunofluorescence for zonula occludens-1 (ZO-1) showed tight junctions in the D1 livers. Scale bars: 20 μm. (C) GSEA showing target metabolism pathways. Heatmap representing alteration of gene expression enriched in hepatocyte differentiation and maturation. Green represents a down-regulation in expression and red represents up-regulation when compared with the Rosa26-LSL-IE2+/− group. (D) mRNA expression levels of the major cell markers and important transcription factors were measured by quantitative real-time PCR. (E) The protein expression levels of CEBPα and HNF4α were measured by Western blot (left). Quantification of CEBPα and HNF4α expression (right). Ctrl represents Rosa26-LSL-IE2−/−, IE2 represents Rosa26-LSL-IE2+/−, cre. Error bars represent SEM. The Student t test was used to analyze statistical differences. **P < .01, ***P < .001. n = 3–5 mice per group. Ctrl, control; DAPI, 4′, 6-diamidino-2-phenylindole.
Then, the paired-end sequencing on an Illumina Novaseq was performed at the Berry Genomics Corporation (Beijing, China) following the vendor’s recommended protocol.

**Bioinformatics Analysis**

First, the spliced, repetitive, low-sequencing-quality reads were filtered out by Cutadapt (https://cutadapt.readthedocs.io/en/stable/installation.htm). Then, the sequence quality was verified by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The reads were mapped to the reference genome of mouse (mm10) by hisat2 software (http://daehwankimlab.github.io/hisat2/) for similarity analysis.

Quantitative analysis of the gene expression level was performed by feature count software and the DEGs were analyzed by edgeR software. The fold change of mRNA greater than 1.5 and \( P < .05 \) were considered to be the DEGs. GO and KEGG pathways were analyzed by topGO (https://rpubs.com/aemoore62/TopGo_colMap_Func_Troubleshoot) and KOBAS software (http://kobas.cbi.pku.edu.cn/download.do). GSEA was performed by GSEA 3.0 software (http://software.broadinstitute.org/gsea/index.jsp) to determine whether a predefined set of genes was significantly different between the 2 groups. The PPI network was created by STRING 11.0 (https://string-db.org), and cytoscape 3.2 (https://cytoscape.org/download.html) was used to screen the most important gene (hub gene) among these DEGs. The hub genes were ranked by the degree in PPI network.

**Quantitative Real-Time PCR**

RNA was extracted by the RNEasy kit (TIANGEN, Beijing, China), and complementary DNA was synthesized by using the Superscript kit (Roche, Basel, Switzerland). The interested genes were quantified using quantitative real-time PCR, and performed on a Roche 480 Real Time System. The primers were synthesized by Sangon Biotech (Shanghai, China). The primer sequences are listed in Table 6. The \( 2^{-\Delta\Delta C_T} \) method was used to determine the relative expression of the target genes normalized to GAPDH.

**H&E staining**

Approximately 3-μm paraffin-embedded tissue sections were stained with H&E and examined by a light microscope (Olympus, Tokyo, Japan).

**Immunofluorescence**

Paraffin-embedded sections were deparaffinized, rehydrated, and placed in water for 5 minutes, and then antigen retrieval was performed using a sodium citrate buffer (pH 6.0) for 10 minutes in a microwave oven. After, the sections were blocked in 5% bovine serum albumin and 0.05% Triton X-100 (Solarbio, Beijing, China) for 30 minutes before application of primary antibody overnight at 4°C. The following day, sections were washed and incubated with a secondary antibody at 1:50 for 60 minutes. Sections were washed, incubated with 4′, 6-diamidino-2-phenylindole containing a fluorescent inhibitor for 5 minute, and then sealed by coverslip. Primary antibodies were as follows: DLK1 (1:200, cat. BF0433; Affinity, Colorado, USA), AFP (1:100, cat. A17898; ABclonal, Wuhan, China), ALB (1:50, cat. 66051-1-lg; Proteintech, Chicago, IL), CD3 (5 μg/mL, cat. NB600-1441; NOVUS, Centennial, CO), CD4 (1:200, cat. NBP1-19371; NOVUS, Centennial, CO), CD8 (1:100, cat. NB200-578; NOVUS, Centennial, CO), zonula occludens-1 (1:100, cat. A0659; ABclonal, Wuhan, China), caspase 3.

**Table 4. Transcriptome Sequencing Analysis of D1 Livers Show Differences in Hepatocyte Maturation**

| Gene name | Rosa26-LSL-IE2+/− | Rosa26-LSL-IE2+/−,cre | log₂ fold change |
|-----------|------------------|------------------------|-----------------|
| Apom      | 20,732.8         | 3823.803               | -2.4            |
| Apoa1     | 539,948          | 161,932.1              | -1.7            |
| F11       | 6166.915         | 580.1111               | -3.4            |
| Cebpa     | 8919.249         | 2988.301               | -1.6            |
| Tr         | 92,307.05        | 21,216.03              | -2.1            |
| Aldh8a1   | 7154.736         | 483.6135               | -3.9            |
| Hp        | 151,217.9        | 15,128.83              | -3.3            |
| F7        | 1882.091         | 436.3027               | -2.1            |
| F10       | 23,025.82        | 5290.734               | -2.1            |
| F13b      | 10,956.96        | 2364.134               | -2.2            |
| F5        | 10,192.95        | 3992.128               | -1.4            |
| Trf       | 1,344,075        | 313,306.7              | -2.1            |
| Pzp       | 181,941.8        | 20,681.85              | -3.1            |
| Hnf4a     | 18,749.25        | 3782.18                | -2.3            |
| Apob      | 105,442.6        | 43,189.55              | -1.3            |
| Apoa2     | 70,310.33        | 5747.637               | -3.6            |
| Apoc4     | 8514.516         | 779,1263               | -3.5            |
| Apoc2     | 540.3241         | 38,43124               | -3.8            |
Figure 9. Construction of Rosa26-LSL-IE2<sup>+/−</sup>, cre mice. First, Rosa26-LSL-IE2<sup>+/−</sup> model mice were constructed. Next, Rosa26-LSL-IE2<sup>+/−</sup>, cre mice were obtained by Rosa26-LSL-IE2<sup>+/−</sup> mice mating with albumin-Cre mice. IE2<sup>+/−</sup> represents Rosa26-LSL-IE2<sup>+/−</sup>; Cre<sup>+/−</sup> represents Rosa26-LSL-IE2<sup>+/−</sup>, cre. Amp, ampicillin resistance screening gene; CAG, CAG promoter; loxp, locus of X-overP1; SacII represents SacII Restriction Endonuclease; STOP_represents transcription termination element; WPRE, Woodchuck hepatitis virus post-transcriptional regulatory element; −/−, wild-type, IE2<sup>+/−</sup>.

Table 5. The Sequence of Real-Time PCR Primers of Rosa26-LSL-IE2<sup>+/−</sup>, cre Mice

| Primer name | Forward, 5′-3′ | Reverse, 5′-3′ | Product size |
|-------------|----------------|----------------|--------------|
| Alb         | TTAGGCCCCCTTACCATAACTG | GAGGCAGAGGCTTAGGAAGATGG | 390 bp |
| Wild type   | TCAGATCTTTTTATAGGGGACACAC | TAAAGGCCACTCAATGCTCACTAA | 994 bp |
| IE2         | TCAGATTTTTATAGGGGACACAC | ACGCTATGTGGGATACGCTGCT | 414 bp |
indicated in the figure legends and processed with ImageJ software (National Institutes of Health, Bethesda, MD).

**Immunohistochemistry Staining**
Liver tissues were fixed with 4% formalin and embedded in paraffin. Paraffin-embedded liver sections (5 μm) were deparaffinized, rehydrated, and soaked in boiling water diluted with sodium citrate buffer (pH 6.0) for 6 minutes to repair the antigen. Endogenous catalase was inactivated with 3% hydrogen peroxide. Then, sections were incubated with anti-mouse Ki67 (1:100, cat. A16919; ABclonal, Wuhan, China) and anti-mouse CMV-IE1 and IE2 (1:100, cat. ab53495; Abcam, Cambridge, UK) overnight at 4°C. The following morning, sections were incubated with the secondary antibodies in the incubator for 40 minutes. After washing with distilled water and phosphate-buffered saline, sections were exposed to 3,3′-diaminobenzidine tetrahydrochloride for 1 minute, hematoxylin for 1 minute, differentiate solution for 1 second, and observed after the neutral gum seal.

**Western Blot**
The liver tissues were isolated and pestled, and were lysed in radioimmunoprecipitation assay buffer. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed to separate equal amounts of cell lysates, and were transferred to a polyvinylidene difluoride membrane (Millipore, Burlington, MA). After incubation with the primary antibodies overnight at 4°C, and after washing with phosphate-buffered saline, secondary antibodies for 2 hours at room temperature. Finally, dropped electrochemiluminescence liquid for 1 minute, signals were detected using a chemiluminescence instrument (Imagequant LAS500, Cytiva, USA). Primary antibodies were as follows: CMV-IE1 and IE2 (1:500, cat. ab53495; Abcam, Cambridge, UK), P27 (1:1000, cat. A16722; ABclonal, Wuhan, China), P21 (1:1000, cat. bs-55160R; Bioss, Beijing, China), CEBPα (1:1000, cat. A0904; ABclonal, Wuhan China), HNF4α (1:1000, cat. A13998; ABclonal, Wuhan China), and β-actin (1:2000; Santa Cruz, CA). The secondary antibodies used were horseradish peroxidase–conjugated sheep anti-mouse IgG (1:500; Absin, Shanghai, China) and anti-rabbit (1:10,000; Absin, Shanghai, China).

**Analysis of Flow Cytometry and Cell Staining**
The livers were taken to prepare single-cell suspensions. Single-cell suspensions were added to an Eppendorf tube (Eppendorf, Hamburg, Germany, 1 × 10^5 per tube) in 100 μL phosphate-buffered saline. Annexin V-fluorescein isothiocyanate/Propidium Iodide (Biolegend) was used to detect cell apoptosis. PI dye (Abcam) was applied to the cell cycle. Specific antibodies were used for staining on the cell surface. The antibodies used were as follows: anti-CD3-Pacific Blue (cat. 100214; Biolegend), anti-CD4–Brilliant Violet 605 (cat. 100451; Biolegend), and anti-CD8–Allophycocyanin (cat. 100714; Biolegend); all antibodies were anti-mouse antibodies. Finally, the cells were analyzed using a fluorescence-activated cell sorter,

### Table 6. The Sequence of the Primers for Quantitative Real-Time PCR

| Gene name | Forward, 5′-3′ | Reverse, 5′-3′ | Product size |
|-----------|----------------|----------------|--------------|
| Gm8300    | AGAGTATGCCGGAGCTGACCAAAATG | CTCTTATGTGGCAGCAGCTCTTAC | 89 bp |
| Gm2022    | TCAGGTACCCAAATGCTGGGATG | CTCTTATGTGGCAGCAGCTCTTAC | 80 bp |
| Gm5662    | TCAGGTACCCAAATGCTGGGATG | CTCTTATGTGGCAGCAGCTCTTAC | 80 bp |
| Gm2016    | TCAGGTACCCAAATGCTGGGATG | CTCTTATGTGGCAGCAGCTCTTAC | 80 bp |
| Fam162b   | GTCTGTTGCTGTATGTTGTTCTG | CTTCAAGCCCTATCGCTCTTTGC | 95 bp |
| Cyp4a10   | GCTGTTGGCTCTGCTGGTAAAGGATG | CATCTGCGTGCTTTCCAGTGTTGAC | 85 bp |
| Apoa4     | GCTGTTGGCTCTGCTGGTAAAGGATG | CATCTGCGTGCTTTCCAGTGTTGAC | 85 bp |
| Smpc      | GTCACTCTCTGCTGCTGCTCTTCTC | ACTTCTCTGCTTTCCAGTGTTGAC | 133 bp |
| Cyp4a14   | GCAATCTTGGGTGCAGCTGCTCAATC | AACGAAATGGCAGCTCGCTTTCCAC | 150 bp |
| Sult2a1   | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 135 bp |
| Cdkn1b    | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 140 bp |
| Cdkn1a    | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 144 bp |
| HNF4a     | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 147 bp |
| HNF6      | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 149 bp |
| TBX3      | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 152 bp |
| ALB       | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 154 bp |
| AFP       | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 156 bp |
| PMAIP1    | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 158 bp |
| TNFRSF10b | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 160 bp |
| CASPASE3  | TGGACGGCTCAGGCTCAGCTCTTCTC | AGCCATCGTCTTTCCAGTGTTGAC | 162 bp |
and the data were analyzed using FlowJo-V10 (https://www.flowjo.com/solutions/flowjo).

Statistical Analysis
All statistical analyses were performed using GraphPad Prism V4.0 (GraphPad Software, Inc, La Jolla, CA). Data are represented as means ± SEM and were analyzed by a 2-tailed Wilcoxon rank-sum test. \( P < .05 \) was accepted as statistically significant.

All authors had access to the study data and reviewed and approved the final manuscript.

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Conflicts of interest
The authors disclose no conflicts.

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Data Availability Statement
The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.