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Functional Comparison of IFN-α Subtypes Reveals Potent HBV Suppression by a Concerted Action of IFN-α and -γ Signaling

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Abbreviations:

HBV, hepatitis B virus; HBeAg, HBV e antigen; HBsAg, HBV surface antigen; pgRNA, pre-genomic RNA; cccDNA, covalently closed circular DNA; PHH, primary human hepatocyte; IFN, interferon; ISGs, IFN-stimulated genes; ISGF3, ISG factor 3; ISRE, IFN-stimulated response element; GAS, IFN-γ-activated sequence; IFNAR1/2, IFN-α/β Receptor Subunit 1/2; GBP, guanylate binding protein.

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Y. Li, F. Lai and J. Chen performed the experiments with contributions from Y. Wang, J. Ye, W. Zai, M. Liu, F. Shen, M. Wu, K. Hu and B. Li. K. Sutter and U. Dittmer contributed to preparation of the recombinant IFN-α subtypes and analysis of the data. M. Lu, X. Zhang, J. Zhang and J. Li

provided reagents and advice. J. Chen and Z. Yuan designed the study, and wrote the manuscript with help from the other authors. J. Chen, Q. Chen and Z. Yuan supported and supervised the study.

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ABSTRACT

Interferon-α (IFN-α), composed of numerous subtypes, plays a crucial role in immune defense. As the most-studied subtype, IFN-α2 has been used for treating chronic hepatitis B virus (HBV) infection with advantages of finite treatment duration and sustained virological response, but its efficacy remains relatively low. Here, we aimed to screen for IFN-α subtype with the highest anti-HBV potency and to characterize new mechanism of IFN-α-mediated HBV restriction. Using cell culture-based HBV infection systems and a human liver chimeric mouse model, IFN-α subtypes-mediated antiviral response and signaling activation were comprehensively analyzed. IFN-α14 was identified as the most effective subtype in suppression of HBV cccDNA transcription and HBeAg/HBsAg production, with IC_{50} values approximately 100-fold lower than the conventional IFN-α2. IFN-α14 alone elicited IFN-α and -γ signaling crosstalk similarly to the combined use of IFN-α2 and IFN-γ, inducing multiple potent antiviral effectors, which synergistically restricted HBV replication. GBP5, one of the most differentially-expressed genes between IFN-α14- and -α2-treated liver cells, was identified as a new HBV restriction factor. A strong IFN-α-IFNAR1 receptor subunit interaction determines the anti-HBV activity of IFN-α. The *in vivo* anti-HBV activity of IFN-α14 and treatment-related transcriptional patterns were further confirmed, and little adverse effect was observed. **Conclusion:** A concerted IFN-α and -γ response in liver, which could be efficiently elicited by IFN-α subtype 14, is associated with potent HBV suppression. These data deepen our understanding of the divergent activities of IFN-α subtypes and the mechanism underlying the synergism between IFN-α and -γ signaling, with implications for improved IFN therapy and HBV cure strategies.

Hepatitis B virus (HBV) infection causes acute and chronic liver diseases and increases the risk of hepatocellular carcinoma (HCC). Although a safe and effective HBV vaccine is available, HBV infection remains a major health burden worldwide with more than 250 million individuals chronically infected. Currently, nucleot(s)ide analogues (NAs)-based antiviral treatment can efficiently control viral replication. However, because NAs do not directly affect the covalently closed circular DNA (cccDNA) which serves as a viral persistence reservoir, a long-term or even life-long therapy is needed to maintain infection under control. Development of strategies to achieve HBV cure has thus become a central goal of academic and industrial research. As complete elimination of HBV in patients with chronic hepatitis B (CHB) is difficult to attain, a consensus has been reached aiming at "functional cure", which is characterized by sustained silencing of cccDNA and loss of HBsAg with finite treatment duration (1).

Interferon (IFN), a group of cytokines firstly described in 1957, is a crucial part of our built-in immune defense system, and has been used for treatment of viral infections and cancers because of its antiviral and antitumor activity. To date, more than twenty distinct IFN genes have been identified in humans. They are typically classified into three types: type I (α, β, etc.), type II (γ) and type III (λ) IFN, according to the types of IFN receptors through which they signal. IFN-α constitutes the largest IFN type and can be further divided into thirteen IFN-α subtypes in humans, all of which are clustered on chromosome 9 and signal through a shared type I IFN heterodimeric receptor complex comprising IFN-α receptor 1 (IFNAR1) and IFNAR2 subunits. Binding of IFN-α to IFNAR1/2 triggers the signaling transduction of JAK-STAT pathway, resulting in expression of interferon stimulating genes (ISGs), a heterogeneous group of proteins that serve as effectors to control viral infection and regulate the immune responses (2).

As an early-discovered and most-studied subtype, IFN-α2 (2a/2b) has been approved for treatment of chronic hepatitis B since 1990s. Compared to the NAs that target HBV reverse transcription to inhibit viral replication, IFN-α has been shown to restrict HBV by affecting multiple stages of the viral life cycle (3, 4), thus has unique advantages towards HBV cure, including a finite course of treatment, a relatively higher rate of HBeAg and HBsAg seroconversion and lower HCC incidence. However, the response rate to the current IFN therapy

in patients remains low (rate of HBeAg seroconversion after 48 weeks of PEG-IFN treatment is 20%-40%), hampering its clinical application. A longstanding question in IFN research that has both puzzled and inspired researchers is why there are so many IFN-α subtypes that appear to differ in biological functions, although all of which signal through shared IFNAR1/2 (2, 5). Our recent findings on the distinct antiviral effect of IFN-α subtypes against retrovirus have advanced the understanding of this issue (5, 6), however, the antiviral potencies of IFN-α subtypes against HBV and the mechanism that led to efficient IFN-α response remains unclear.

Here, we aimed to screen for IFN-α subtype with the highest anti-HBV potency and characterize the underlying mechanisms, thereby providing new insights for both research and practice. We identified IFN-α subtype 14 as the most effective IFN-α subtype in suppression of HBV cccDNA transcription and HBeAg/HBsAg production, with IC_{50} values approximately 100-fold lower than the conventional IFN-α2. Mechanistically, a concerted action of IFN-α and -γ signaling was elicited by IFN-α14, inducing multiple potent antiviral effectors for an efficient control of HBV infection, and this is determined by a strong and specific IFN-α-IFNAR1 receptor subunit interaction. The *in vivo* anti-HBV activity of IFN-α14 and transcriptional signature associated with potent HBV suppression were further confirmed in a human liver chimeric mouse model, and little adverse effect was observed.

Materials and Methods

Cell culture and HBV infection

The HepG2-NTCP cell line (provided by Prof. Stephan Urban), HepaRG cells (Biopredic International, France), and primary human hepatocytes (PHHs, purchased from RIDL Shanghai, China) were cultured and infected with HBV virions as we previously described (7). The HepG2-NTCP-STAT2 KD cells were constructed by transfected with the pX330-mCherry vectors (provided by Dr. Jinsong Li) followed by FACS screening and single colonies selection.

Recombinant IFN-α proteins

The recombinant proteins of 13 human IFN-α subtypes were prepared as described (8). As the amino acid sequences of IFN-α1 and IFN-α13 are exactly the same, they were noted as IFN-α1/α13. We normalized the working concentration of IFN-α subtypes according to their protein concentration. The plasmids encoding for IFN-α2-EIFK, IFN-α2-PF, IFN-α14-DTYR and IFN-α14-LL were synthesized by GeneScript and were transformed into competent BL21 *E.coli* cells for protein expression. All the recombinant IFNs were confirmed to be endotoxin-free (<0.1 endotoxin units/μg, Shanghai Labway Clinical Laboraory). The human IFN-α2 (Catalog No. 11101-1) and IFN-α14 (Catalog No. 11145-1) were purchased from PBL Assay Science. The recombinant human IFN-γ was purchased from Peprotech (Catalog No. 300-02).

Plasmids, antibodies, primers and reagents

The plasmids, antibodies and primers used in this study are listed in Table S1, S2 and S3, respectively. All plasmids were prepared using Endo-Free Plasmid Kits (Omega). Inhibitors including Ruxolitinib (S2789), Tofacitinib (S1378) and AS1517499 (S8685) were purchased from Selleck. The levels of HBeAg and HBsAg in the cell culture supernatant were determined by ELISA (Kits from Kehua, China). Cell viability was determined using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan). Dual-luciferase reporter assay was conducted as described (9). Quantitative PCR was performed using Thunderbird SYBR qPCR Mix (Toyobo) in a StepOne Real-Time PCR System.

Western blot, Northern blot and Southern blot

They were conducted as described (7, 10). The Native PAGE for detection of homodimers and heterodimers were conducted as we previously described (9).

Microarray and RNA sequencing

PHHs and HepG2-NTCP cells infected with HBV were mock-treated or treated with IFN-α2 or IFN-α14 for 6 h. The RNA samples were prepared with TRIzol Reagent. The RNA isolation, microarray analysis and data processing were performed by the Shanghai Biotechnology Corporation using the Affymetrix GeneChip Human Transcriptome Array 2.0 (HTA2.0). The RNA sequencing and analysis were done by GENEWIZ (Suzhou, China) using the Illumina

system. The data are accessible through the GEO series accession number GSE138566, GSE138567 and GSE138569. Functional annotation was performed using Gene Ontology (GO).

Surface plasmon resonance (SPR)

The affinities of the IFN-α subtypes for IFNAR were determined by Biacore T200. IFN-I receptor subunit 1 or 2 were immobilized on a Biacore Series S Sensor Chip CM5 via carboxyl groups on the dextran. The recombinant IFNs, tested for binding at concentrations from 0.1 nM to 2 μM, were flowed at a rate of 30 μl/min for 60 s for association, followed by 90 s for dissociation over immobilized recombinant IFNAR2 extracellular domain IFNAR2-EC (10359-H08H, Sino Biological Inc.) in HBS-EP running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.5% (v/v) Surfactant P20, pH 7.4). For IFNAR1-EC (13222-H08H, Sino Biological Inc.), the association and dissociation time were 30 s.

Humanized liver chimeric mouse model

Fah/Rag2/Il2rγ triple knockout (FRG KO) mice purchased from Yecuris Corporation were utilized in the establishment of human liver chimeric mice (11). Briefly, 1.0x10E6 PHH (Lonza) were injected intrasplenically into 4-6 weeks old mice followed by a number of NTBC cycling withdrawal conditions to facilitate mouse hepatocytes damage which in turn allow human hepatocytes to repopulate. hALB production was measured by ELISA (Bethyl Laboratories Inc.) whereby ~5mg/ml represents ~70% humanization of mouse liver at 2-3 months post-transplantation, and 1.0x10E7 IU/ml of HBV (Genotype D, produced by HepAD38 cell lines) was injected by tail vein. Serum HBV DNA (Qiagen) was monitored (Table S6). HBV-infected mice were randomized into different treatment groups. PBS control and IFNα treatments were administered intravenously daily for 14 days from Day 45 post-infection onwards. Monolisa HBsAg ULTRA ELISA (#72348, BioRad) were used to measure HBsAg production. All mouse liver tissues (209.8±10.64μg) were homogenised in PBS followed by Hirt DNA extraction for HBV cccDNA detection. All mice were sacrificed at 10 weeks post HBV-infection. Staining of FAH (ab140167, abcam) and HBsAg (MD-05-0186, Raybiotech) on liver sections was performed. All images were captured by the ZEISS Axio Scan.Z1 and processed using the Zen lite software.

All mice were bred and kept under specific pathogen-free with 12 light/12 dark cycle conditions in Biological Resource Centre, Agency for Science, Technology and Research, Singapore in accordance with the guidelines of the Agri-Food and Veterinary Authority and the National Advisory Committee for Laboratory Animal Research of Singapore.

Statistical analysis

Data were analyzed using Prism 7.0 (GraphPad). For comparison of two datasets, data were analyzed using Student's *t* test and presented as the mean ± standard deviation. Correlations between two datasets were determined by linear regression and evaluated by Pearson's r analysis. A *P*-value < 0.05 was considered to be statistically significant. Data are shown as mean ± SD.

Results

IFN-α subtype 14 exerts the most potent antiviral activity against HBV among all thirteen IFN-α subtypes.

Currently, only one IFN-α subtype (IFN-α2) is used therapeutically, the effect of other IFN-α subtypes on HBV control remains unknown. In an HBV-transfection-based cell culture model, IFN-α subtypes showed different efficacies in suppression of HBV core particle (CP)-associated-DNA production (Fig. 1A and S1A) and viral antigens expression (Fig. S1B), among which IFN-α14 was the most effective. This was further verified in HBV-infected primary human hepatocytes (PHHs) (Fig. 1B and S1C): IFN-α14 was very potent in inhibiting HBeAg, HBsAg, HBV DNA and pre-genomic RNA (pgRNA) (% inhibition: 85%, 81%, 88% and 93%, respectively) (Fig. 1C), with no cytotoxicity (Fig. S1D). Representative IC_{50} values of IFN-α14 for the reduction of HBeAg and HBsAg levels in PHHs were approximately 10-fold lower than those of IFN-α6 and 100-fold lower than those of IFN-α2 (Fig. 1D). Since the HBV genotype was reported to affect the therapy response to IFN-α, we examined this issue as previously described (7) and revealed that IFN-α14 has a pan-genotypic anti-HBV effect (Fig. S1E). Consistent with previous reports and our own results suggesting that IFN-α inhibits HBV mainly at the cccDNA

transcription and post-transcription levels (7, 12, 13) rather than directly degrades cccDNA, IFN-α14 did not significantly affect the cccDNA pool either (Fig. 1E). Studies using the HBV-infection-based dHepaRG and HepG2-NTCP models showed consistent results with those obtained in PHHs (Fig. 1F-G and S1F-G). IFN-α proteins from a different source (purchased from PBL) were applied to confirm the anti-HBV effect of IFN-α14. (Fig. S1H).

IFN-α14 elicits a crosstalk between IFN-α and IFN-γ signaling

IFNs exert antiviral effect mainly through JAK-STAT signaling (Fig. 2A). We showed that higher anti-HBV potency of an IFN-α subtype was generally associated with higher activation of STAT1 and ISRE, with IFN-α14 inducing the highest level of phosphorylation of STAT1, STAT2 and ISRE activation (Fig. 2B-D and S2A). We thus focused on IFN-α2 and -α14 for studying mechanisms of the differential biological activities of IFN-α subtypes. In addition to potently activating the canonical IFN-α signaling pathway, IFN-α14 also induced STAT6 phosphorylation in PHH (Fig. 2E), and exhibited IFN-γ-like properties that elicited the activation of GAS, which is known as IFN-γ-activated element (Fig. 2F and S2B), as well as led to STAT1 homo-dimerization (Fig. 2G and S2C) in addition to the classical STAT1-STAT2 heterodimer formation.

A concerted IFN-α and -γ signaling response is associated with potent HBV control.

To examine the role of the canonical and non-canonical signaling in IFN-α14-mediated antiviral responses, we used a neutralizing antibody against IFNAR2, the JAK1/2 inhibitor Ruxolitinib and the STAT6 inhibitor AS1517499 to globally or selectively block the signaling (Fig. 3A). As expected, the antiviral effect of both IFN-α2 and -α14 was diminished when the IFN signaling was blocked at the receptor or the JAK level, while it was not much affected when STAT6 phosphorylation was inhibited (Fig. 3B). We further knocked down *STAT2* in HepG2-NTCP to specifically block the canonical IFN-α signaling pathway (Fig. 3C). Subsequently, only STAT1 homo-dimerization was observed in STAT2-deficient cells (Fig. 3D),

and in turn, the inhibitory effect of IFN-α14 on HBV was reduced (Fig. 3E), suggesting a critical role of the canonical IFN-α signaling in eliciting anti-HBV response.

Intrahepatic IFN-γ-transcriptional signature has been reported to be associated with the response to IFN-α treatment in chronic hepatitis B (14) and a combination of IFN-α with IFN-γ could enhance IFN responses (15, 16). We found that combined use of IFN-α2 and IFN-γ elicited equivalent levels of phosphorylated and homo-/hetero-dimerized STAT1 as IFN-α14 alone (Fig. 3F and S2D), and this led to similar potent inhibition of HBs/HBeAg (Fig. 3G), demonstrating a synergistic effect between IFN-α and IFN-γ signaling for HBV control and suggesting a fascinating role of IFN-α14 in eliciting such actions. HBV has been shown to counter-regulate the IFN system (9, 17), particularly at a high viral load, which may contribute to the impaired IFN response. We further examined and revealed that although attenuated IFN signaling was observed in HBV-replicating cells treated with both IFNs, IFN-α14 outperformed IFN-α2 in conquering HBV-mediated antagonism of IFN signaling (Fig. S3).

IFN-α14 induces potent and multiple antiviral molecules, including a group of genes known as IFN-γ-regulated, among which GBP5 suppresses HBeAg and HBsAg expression.

Since the IFN-mediated antiviral action depends on the induction of multiple IFN-stimulated genes (ISGs) and the magnitude of ISG expression is closely associated with the anti-HBV efficacy of IFN-α (7, 18), we next analyzed the transcription profile of IFN-induced genes: 319 genes were induced by both IFN-α2 and IFN-α14 in HBV-infected PHHs and most of them were known as IFN-α-related genes that are associated with the innate and antiviral responses. A subset of genes, including GBPs, WARS and TNFSF10, were significantly more induced by IFN-α14 than by IFN-α2 (Fig. 4A and Table S4). In the HepG2-NTCP model, IFN-α14 was also shown to potently induce ISGs mRNA expression, including multiple genes previously known as IFN-γ-regulated (Fig. 4B-C and Table S5), supporting the observation that IFN-α14 stimulated a crosstalk between the canonical IFN-α and IFN-γ signaling. The induction of a number of differentially expressed genes were further confirmed by qPCR (Fig. S4A).

Three guanylate binding proteins (GBPs), including GBP2, 4 and 5, were found to be potently induced by IFN-α14 (Fig. 4D). The induction of GBP5 could also be observed in PHHs treated with IFN-α2 combined with IFN-γ (Fig. 4E). Since GBPs have been known as a subfamily of GTPases with well-established activity against intracellular bacteria and parasites, we further investigated the effects of GBPs on HBV by ectopic expression of GBPs in HBV-infected and -transfected cells. While GBPs did not really much affect the HBV RNAs production, GBP4 and GBP5 significantly reduced the HBeAg and HBsAg levels (Fig. 4F and S4B). Moreover, overexpression of GBP5 enhanced the antiviral effect of IFN-α2 and further reduced HBeAg and HBsAg levels in IFN-α14-treated cells (Fig. 4G and S4C). These findings suggest a novel role of GBPs in antiviral response.

Strong and specific IFN-α-IFNAR1 interaction determines the superior antiviral effect of IFN-α14.

The functional differences of various IFN subtypes have been suggested to lie in their binding affinity toward the receptor subunits (19, 20). Here, we observed a positive correlation of the inhibitory effect of specific IFN-α subtypes on HBV RNA and HBeAg expression levels with their binding affinity to IFNAR1, but not to IFNAR2 (Fig. 5A-B). As IFNAR1 has been shown to play a role in IFN-α/-γ signaling crosstalk (21), we reasoned that minor changes in the regions of IFN-α proteins that are responsible for the binding to IFNAR1 might critically affect their anti-HBV activity. To verify this, we constructed a series of mutated IFN-α2 and IFN-α14 proteins with mutual substitution of amino acids located in the regions that have been identified to be important for the binding of IFN-α to IFNAR1 or IFNAR2 (Fig. 5C and S5A) including IFN-α2-EIFK/-PF (IFN-α2 with α14-derived IFNAR1-/IFNAR2-binding sequence) and IFN-α14-DTYR/-LL (IFN-α14 with α2-derived IFNAR1-/IFNAR2-binding sequence). The binding affinities of the mutated IFNs to IFNARs were quantified by Biacore analysis (Fig. 5D and S5B). As expected, IFN-α2-EIFK showed a significantly increased binding affinity to IFNAR1 while that of IFN-α14-DTYR decreased, and the signaling activation patterns including the phosphorylation of

STAT1, STAT1 homo-dimerization (Fig. 5E) and ISGs induction were accordingly changed upon the substitution of the IFNAR1-binding region (Fig. S5C). Functional analysis showed that IFN-α2-EIFK exhibited potent activity in reduction of HBs/HBeAg similar to IFN-α14, while IFN-α14-DTYR showed only weak anti-HBV activity (Fig. 5F and S5D). In contrast, IFN-α2-PF and IFN-α14-LL exhibited similar signaling activation patterns and anti-HBV activity as IFN-α2 and IFN-α14, respectively. These result suggest that the superior anti-HBV effect of IFN-α14 is determined by strong and specific IFN-α14-IFNAR1 interaction.

Potent suppression of established HBV infection by IFN-α14 with little adverse effect in human liver chimeric mice.

We further used a human liver chimeric mouse model (FRG-KO mice) to validate the anti-HBV potency of IFN-α2 and -α14 and the treatment-related transcriptional signatures *in vivo*. With a total of 31 mice, we performed a pilot study (n=11) followed by study with larger scale (n=20). In the pilot study, each mouse with established HBV infection was injected intravenously with 250 ng of IFNs (based on 25g/mouse) or saline every two days for 10 days. The differences in transcriptional induction of GBP5 and TNFSF10 between IFN-α2- and α14-treated mice were clearly observed (data not shown). However, the antiviral effect was not statistically significant in both groups which may be due to the short half-life of the recombinant IFN proteins *in vivo*. Therefore, in the following study, we treated the mice for a longer time period with a higher frequency (treated daily for 2 weeks). We did a series of serological and intrahepatic measurements with samples collected at the indicated time points (Fig. 6A). The IFN treatment were overall well tolerated by the mice, even though the treatment protocol involved frequent handling and blood draws and daily injection of IFNs or saline. Both IFN-α2 and -α14 treatment interfered with a HBV DNA and HBsAg increase and significantly reduced the HBV RNAs production. Compared to IFN-α2, mice treated with equivalent doses of IFN-α14, even with lower doses of IFN-α14 (100 ng), showed approximately 1 log lower relative concentrations of serum HBV DNA (Fig. 6B and S6A). Analysis of the levels of intrahepatic HBV RNAs at one week after

the cessation of IFN treatment revealed that, compared to IFN-α2, IFN-α14 treatment resulted in an additional 0.7 Log and 0.6 Log reduction of HBV preC/pgRNA and total RNAs, respectively, even at the low dose (Fig. 6C). By mapping the sequencing reads to the HBV genome, we further showed that the expression of HBV pgRNA and subgenomic RNAs was globally inhibited by IFN-α14 (Fig. 6D and S6B-E). Compared with the control group, IFN-treated mice showed an inhibition of HBsAg production (Fig. 6E, $P<0.05$) and a trend toward cccDNA reduction (Fig. 6F and S6E). Although no statistical significance was found, the overall HBsAg and cccDNA levels in mice with IFN-α14 treatment were lower than those treated with IFN-α2. The transcriptional activity of cccDNA, which was calculated as the ratio of total HBV RNAs to cccDNA, was clearly reduced in IFN-α14-treated group (Fig. 6G).

The intrahepatic gene expression was analyzed by mRNA sequencing and the results were highly consistent with our *in vitro* data, showing that IFN-α14 treatment induced multiple antiviral genes including a subset of genes that has been recognized as usually IFN- γ -induced (Fig. 7A-B and S7). While the induction levels of the classical IFN-α-induced gene IFITM1 were comparable between the IFN-α2 and -α14 group, the genes GBP2, GBP4, GBP5, CXCL10 and WARS were more potently induced by IFN-α14 (Fig. 7C), strongly supporting the data obtained *in vitro*.

Since the side-effect of IFN-α treatment has always been a big concern in clinic, we performed some safety analysis in the control versus IFN-treated mice, also comparing IFN-α2-treated and IFN-α14-treated group. Although the majority of IFN-treated mice displayed a slight reduction in body weight between 6wpi and 9wpi (Table S6 and Fig. S8A), the mean serum hALB levels (Fig. 7D) were similar across all the groups. In addition, morphology and histological analysis of the livers, spleens and kidneys of mice from each group showed no significant damage occurred upon IFN treatment (Fig. 7E, S8B and S9). Moreover, blood chemistry analysis was performed to measure a series of parameters (Fig. 7F and S10). The serum ALT levels were increased in IFN-treated mice as expected, however, they were similar between the IFN-α2 and IFN-α14 group. The A/G ratio slightly increased in IFN-α2-treated mice while it remained unchanged in the IFN-α14-trated mice. A modest reduction of the serum levels of urea nitrogen

(BUN) and glucose (Glu) was observed in IFN-treated mice, while the serum levels of alkaline phosphatase (ALP), amylase (AMY), total bilirubin (TBIL), phosphorus, Ca^{2+}, Na^{+}, K^{+} and total proteins (TP) were similar across all the four groups. These data suggest that IFN-α14 exhibits much higher antiviral potency than IFN-α2 while the adverse effects induced by the two IFN-α subtypes were very similar at an overall tolerable level.

Discussion

Among thirteen human IFN-α subtypes, IFN-α2 is the most-studied and has been approved for treatment of chronic hepatitis B for decades. Low response in large proportion of patients and the potential side-effects have hampered its application in clinic. Here, we identified IFN-α14 as a highly effective IFN-α subtype against HBV *in vitro* and *in vivo*. Moreover, a concerted action of IFN-α and IFN-γ signaling, which could be efficiently elicited by IFN-α14, was found to be associated with potent induction of antiviral effectors and suppression of HBV transcription and antigens production. These results have deepened our understanding of the divergent antiviral properties of IFN-α subtypes and uncovered an association between the induction of a concerted IFN-α and -γ response in liver and potent HBV control, with implications for improved IFN therapy and HBV cure strategies.

At present, long-term therapy with NAs in CHB patients can efficiently control viral replication and mitigates inflammatory liver disease but does not result in a cure of HBV. How to achieve HBV cure remains a great challenge in basic and clinical research. IFN-α, with combined antiviral and immuno-regulatory activities, has unique advantages in disruption of HBV reproductive cycles and sustained control of HBV transcription and replication after the cessation of treatment (22). However, the efficacy of the currently-used IFN-α2 is not satisfactory. Both viral and host factors affecting the responsiveness to IFN-α therapy have been extensively studied with the aim of more effective therapy. Factors including female sex, high baseline alanine aminotransferase level, low baseline HBV-DNA level and HBV genotype A and B have been shown to be prognostic parameters for a better response following IFN therapy. However,

therapeutic regimens based on these parameters have not shown greatly improving IFN response. This study, from the perspective of IFN-α subtypes, showed that IFN-α14 is far more potent than the conventional IFN-α2 in HBV control. Particularly, as IFN-α14 elicits much more potent suppression of HBV antigen production and cccDNA transcription, the findings may help to develop new strategies towards HBsAg loss and sustained cccDNA silencing, which have been widely recognized as two essential steps in attaining the goal of HBV functional cure (1).

The inhibitory effect of IFN-α on HBV replication has been demonstrated in series of studies using *in vitro* and *in vivo* system (3, 4, 7, 23), and a number of ISGs like MxA, APOBEC3G, MyD88, ISG20 and TRIM22 have been reported as effectors that actively inhibit transcriptional and post-transcriptional HBV gene expression (24). Here, a group of guanylate binding proteins (GBPs) were found to be potently induced by IFN-α14. GBP5, which has recently been shown to interfere with HIV-Env processing (25) by suppressing the activity of the virus-dependency factor furin (26), was identified as a new anti-HBV effector that can potently restrict HBeAg and HBsAg production. Since the common SNP in the promoter of furin gene has been shown to affect HBeAg maturation and CHB progression (27), GBP5 might also target furin for HBV restriction, which needs further investigation. Given that GBPs have been known to be important for restriction of intracellular bacteria and parasites (28), these findings have revealed a novel role of GBPs in host defense against viral infection, and advanced the understanding of IFN-α-mediated cellular antiviral mechanisms. Besides, a consensus is emerging that it is the magnitude of cellular IFN response and a complex concerted action of multiple effectors that determine the overall antiviral effect of IFN-α (18, 29, 30). In this respect, activating efficient JAK-STAT signaling transduction and inducing potent and multiple ISGs are crucial for an optimal control of HBV infection. Moreover, it has long been obscured whether multiple IFNs are equally relevant to host survival, or some IFN subtypes are more essential for immune defense whereas others display immunological redundancy. A recent evolutionary analysis suggests that IFN-α subtype-6, -8, -1/13 and -14 and IFN-γ may have evolved under strong purifying selection, attesting to their non-redundant and essential function in host defense (31). Accordingly, IFN-α14 has been shown to be very often the most effective subtype in the treatment of various kinds of virus infection (5)

including HIV, EV71, HSV, HEV and HCV (Fig. S11), suggesting a non-redundant special role of IFN-α14 in antiviral immunity. These evidences also provide a possibility of utilizing a new IFN-α subtype to improve the efficacy of IFN-based therapy.

As all type I IFNs bind to and signal through a shared heterodimeric receptor complex IFNAR1/2, how these subtypes exert distinct biological functions represents an area of intrigue. Accumulating evidence now shows that the binding affinity and dynamics of each IFN-I for the two receptor subunit varies and, as a result, the stability of the IFN-IFNAR ternary complex for the various IFNs spans a range and may thus set a certain IFN subtype apart from one another (2, 19). Among type I IFNs, IFN-β has the highest binding affinities to both IFNAR1 and IFNAR2. However, IFN-β is more toxic in patients likely because of its high anti-proliferative activity (19), and thus might have a lower risk/benefit ratio than IFN-α14. Aiming for enhanced antiviral and antitumor activity, a series of genetically engineered IFNs have been developed (20, 32), but with potential problems such as immunogenicity and pharmacokinetics. Our data suggests that the functional properties of IFN-α14 is determined by a strong and specific IFN-α14-IFNAR1 receptor subunit interaction. Moreover, IFN-α2-EIFK, an IFN-α2 mutation with IFN-α14-specific IFNAR1-binding sequence, exhibited potent anti-HBV activity similar to IFN-α14. Compared to those genetically engineered IFNs, IFN-α14 (and the IFNα2-EIFK mutant) represent fascinating naturally engineered variants with more promising efficacy and benefits for the treatment of viral infections.

Since the IFN-IFNAR interactions are relatively species specific, we used a humanized mouse model (FRG-KO mice) to further characterize the action of IFN-α14 *in vivo*. The results generally confirmed the anti-HBV effect of IFN-α14 and suggested that the treatment was overall well tolerated. Further studies are required to evaluate the antiviral activity as well as the adverse effects of IFN-α14 in the presence of an adequate immune system, however, this is technically challenging and is almost unanswerable without human studies. It should be noted that there was a trend that the serum glucose levels were reduced in IFN-α14-treated mice compared to that of the HBV-infected mice with placebo treatment. In fact, some studies have already reported that HBV hijacks the intra-hepatic metabolism, including the glucose metabolism, for facilitating its

persistence and resulting pathogenesis (33). Considering the above transcriptome data showed that IFN-α14 could decrease the expression of several key enzymes in glucose homeostasis, such as G6PC, IFN-α14 may to some extent reverse the HBV-mediated changes to normal hepatocyte gene expression. More investigation of the potential links and underlying mechanisms would be interesting.

In addition, we showed that IFN-α14 exerted an IFN-γ-like activity that induce the expression of a group of genes previously known as IFN-γ-regulated. This actually recalls previous studies on the intrahepatic gene expression patterns using the woodchuck model (34, 35), liver biopsies from CHB patients (14, 36) and our own study which was mainly focused on the intrahepatic genes associated with the low viral replication in the inactive carrier phase (37). We can see that a IFN-γ transcriptional signature is closely associated with an effective response to IFN-α treatment and HBV clearance, and many of those differentially expressed genes, such as GBP5, WARS and TNFSF13, were overlapped with the genes identified here that were potently induced by IFN-α14 (Table S7). Notably, a high dose of IFN-a2 also elicited an IFN-γ-like response, but is more likely to lead to cytotoxic effects in parallel (data not shown). IFN-γ has been shown to be critical in non-cytolytic control of HBV in cell and animal models (38, 39), but its production in CHB patients is somehow impaired (40, 41). Some early reports have suggested that IFN-γ alone or combination with IFN-α efficiently inhibit virus replication (16), however, their application in clinic was unsuccessful and toxicity was frequently observed (42). As IFN-α14 naturally elicits a synergistic IFN-α2 and -γ signaling through IFNARs, more efficient and less toxic effect with IFN-α14 treatment are expected. Moreover, we showed that IFN-α14 outperformed IFN-α2 in conquering the inhibition of IFN signaling by HBV, implying a potency of IFN-α14 as an alternative tool for enhancing or restoring the impaired IFN response in non-responsive CHB patients treated with IFN-α2. These data may also facilitate the identification of novel markers of the host responsiveness to IFN-α in patients. Moreover, it would be interesting to examine whether other immune-based therapeutic interventions could also elicit a concerted IFN-α and -γ response in liver and how it links to HBV control and clearance. Altogether, these data broaden our

understanding of the mechanism of IFN-α-mediated HBV control and shed light on the development of improved IFN-based therapy towards HBV functional cure.

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Figure legends

Figure 1. Effect of IFN-α subtypes on HBV replication and gene expression *in vitro*. (A) Huh7 cells transfected with pHBV1.3 were treated with IFN-α subtypes (10 ng/ml) for 72 h, then the HBV CP-DNA was extracted and detected by Southern blot. The pCMV-HBV and pCMV-HBV-YMDD (replication-defective) were used as controls to ensure that there is no background from input plasmid (Fig. S1A). (B). Scheme outlining the HBV infection experiments. (C) The levels of extracellular HBeAg, HBsAg, CP-DNA and intracellular pgRNA of HBV-infected PHH cells treated with IFN-α subtypes (0.5 ng/ml) were examined. (D) The levels of HBeAg and HBsAg in the indicated groups were examined, based on which IC_{50} values were calculated. (E) The levels of cccDNA were examined in the indicated groups by qPCR (normalized to mitochondrial DNA). (F) The levels of HBeAg, HBsAg and HBV DNA in the culture medium of the HBV-infected dHepaRG cells treated with IFN-α subtypes (2 ng/ml) for 12 days were examined. (G) HBV-infected HepG2-NTCP cells were treated with 10 ng/ml of IFN-α. The CP-DNA and the intracellular HBV RNAs were determined by Southern blot and Northern blot, respectively. RC: relaxed circular; SS: single-stranded.

Figure 2. IFN-α14 elicits a crosstalk between IFN-α and IFN-γ signaling. (A) Schematic model of IFN-I-activated signaling pathways. (B and C) Huh7 cells treated with IFN-α subtypes (10 ng/ml) for 0.5 h and 8 h were extracted for analysis of pSTAT1(Y701), pSTAT2(Y690) and ISRE activation, respectively. (D) Graphic comparison of the antiviral effect of IFN-α subtypes with the activation levels of STAT1, STAT2 and ISRE. (E) PHHs treated with the indicated IFN-α for 0.5 h were extracted for immunoblotting. (F) HepG2-NTCP cells co-transfected with pRL-TK and pISRE-luc or pGAS-luc were treated with 10 ng/ml of IFN-α or 2 ng/ml of IFN-γ for 8 h and then harvested for the luciferase assay. (G) HepG2-NTCP cells were treated with 10 ng/ml of IFN-α or 2 ng/ml of IFN-γ for 0.5 h. Then the STAT1 hetero- or homo-dimer were examined by the native PAGE followed by immunoblotting in indicated cells.

Figure 3. IFN-α14-elicited IFN-α and -γ signaling synergistically induces potent HBV suppression. (A) PHHs were pre-treated with IFNAR2 neutralizing antibody (5 μg/ml), JAK inhibitor Ruxolitinib (1 μM) or the STAT6 inhibitor AS1517499 (1 μM) for 1 h, and then were treated with IFN-α2 (1 ng/ml) or IFN-α14 (0.2 ng/ml) for 0.5 h. The phosphorylation levels of STAT1 and STAT6 in the cells were examined by immunoblotting. (B, E and G) The extracellular levels of HBeAg and HBsAg were examined in the indicated groups. (C and D) The IFN-induced STAT1 and STAT2 phosphorylation and STAT1 hetero- or homo-dimer formation were examined in wild-type and in STAT2-deficient HepG2-NTCP cells treated with IFN for 0.5 h. (F) PHHs treated with IFN-α2 (0.4 ng/ml), IFN-α14 (0.4 ng/ml) or/and IFN-γ (0.2 ng/ml) for 0.5 h were lysed for examination of the phosphorylation of STAT1 and STAT2.

Figure 4. IFN-α14 induces multiple potent antiviral effectors. (A and B) HBV-infected PHHs (A) and HepG2-NTCP cells (B) were mock-treated or treated with IFN-α2 or IFN-α14 at 2 ng/ml and 5 ng/ml at day 3 post infection, respectively. Total RNA of the cells were extracted 6 h later for microarray and RNA-seq analysis, respectively. The heat map showed differentially expressed genes between IFN-α14- and IFN-α2-treated groups. (C) GO analysis of the differentially expressed genes (Table. S5). Top 15 significantly enriched biological processes were presented. (D) The mRNA levels of GBPs in PHHs at indicated time points post IFN-α14 (2ng/ml) treatment were determined by RT-qPCR; The expression of GBP5 at indicated time point with different doses of IFN-α treatment was analyzed by immunoblotting. (E) The GBP5 induction in PHHs treated with the indicated IFNs for 6 hours were determined by RT-qPCR. (F) HBV-infected HepG2-NTCP cells were transfected with plasmids encoding GBP2, GBP4 or GBP5 and the levels of intracellular HBV RNAs and extracellular HBeAg and HBsAg were analyzed by Northern blot and ELISA, respectively. (G) HBV-infected HepG2-NTCP cells were transfected with plasmids encoding GBP5 and treated with IFN-α2 (5 ng/ml) or IFN-α14 (1 ng/ml) and the levels of extracellular HBeAg and HBsAg were analyzed by ELISA.

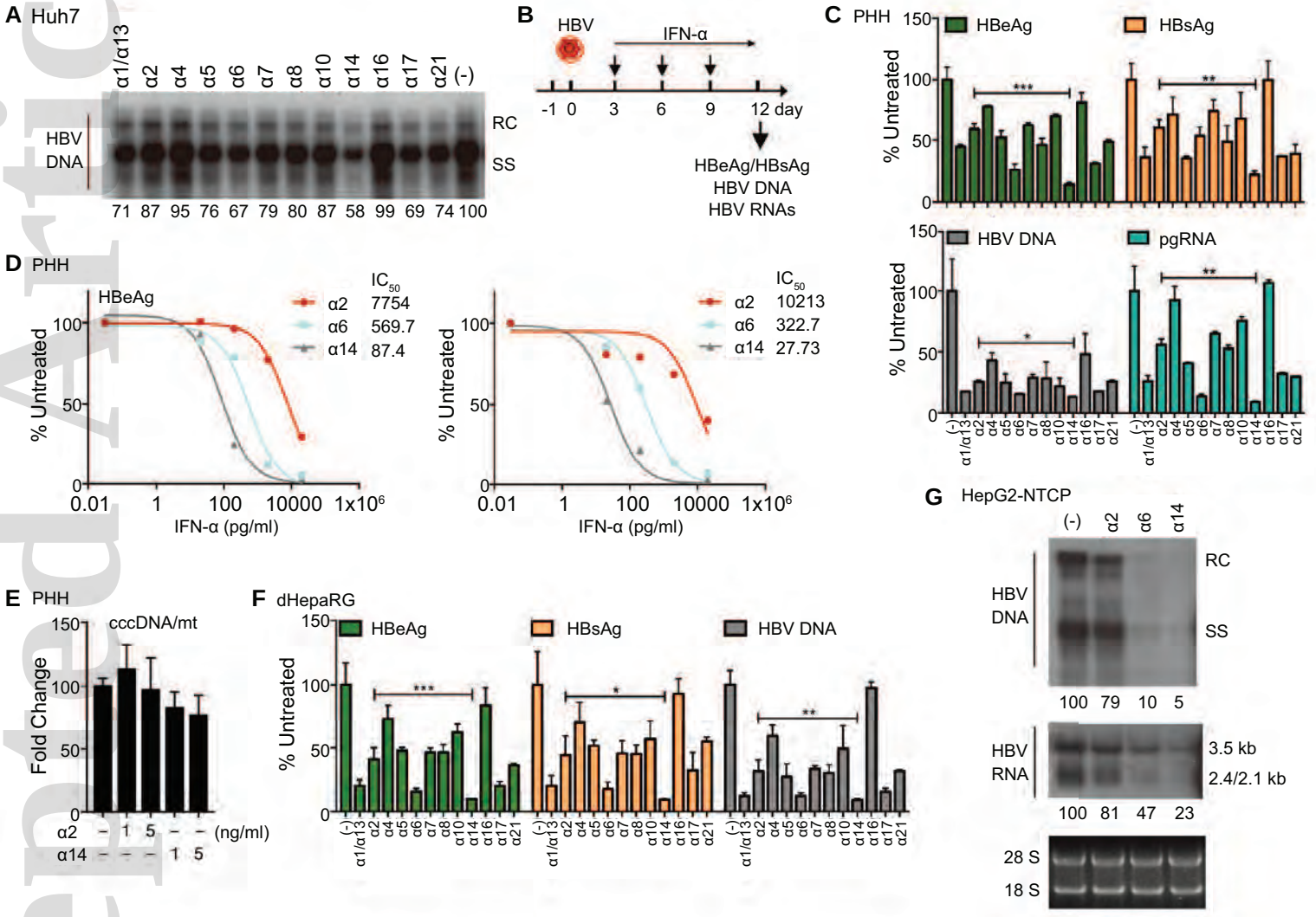
Figure 5. The IFN-α-IFNAR1 interaction determines the anti-HBV effect of IFN-α. (A and B)

Correlation between the non-log-transformed binding affinity ($1/K_D$) to IFNAR-1 and IFNAR-2 based on published data and the potency of IFN-α for reduction of HBeAg(A) or pgRNA(B). (C) Previously reported residues responsible for the binding of IFN-α to IFNAR1(colored in orange) and IFNAR2 (blue). The different sites between the protein sequences of IFN-α 2 and IFN-α14 for mutation were marked. (D) Biacore analysis of the binding of the indicated IFN-α proteins to the extracellular domain of IFNAR1 (IFNAR1-EC). (E) HepG2-NTCP cells were treated with the indicated IFN-α (0.4 ng/ml) for 30 min, and then extracted for the native- or SDS-PAGE followed by immunoblotting. (F) Extracellular HBeAg and HBsAg levels of the HBV-infected PHHs cells were treated with the indicated IFNs at 0.2 ng/ml from 3 dpi to 12 dpi. The extracellular levels of HBeAg and HBsAg were then evaluated by ELISA.

Figure 6. Potent suppression of established HBV infection by IFN-α14 *in vivo*. (A) Schematic model of study design. (B) The levels of serum HBV DNA before and after IFN/placebo treatment were examined. The data was shown as the ratio of post- to pre-treatment. (C) The levels of intrahepatic HBV preC/pgRNA and viral total RNAs at the endpoint were quantitated with specific primers. The data was shown as relative amounts. Sample X21 (Ctrl) was made relative to 1 for fold change analyses. hALB was used as housekeeping gene. (D) HBV transcript-level quantification by nucleotide mapping in the indicated mice were analyzed by RNA sequencing. (E) The levels of serum HBsAg before and after IFN/placebo treatment were examined. The data was shown as the ratio of post- to pre-treatment. (F) The levels of intrahepatic HBV cccDNA at the endpoint were quantitated with specific primers. The data was shown as copies per μg liver tissue. (G) The ratio of total HBV RNAs to cccDNA were calculated. Differences between groups were shown as *P*-values.

Figure 7. IFN-α14 elicits IFN-α/-γ signaling crosstalk *in vivo* with little adverse effect. (A and B) Total RNA of the liver samples at the endpoint were extracted for RNA-seq analysis. The heat map showed the top 50 differentially expressed genes between IFN-α2 (high) and IFN-α14 (high) groups. (C) The intrahepatic mRNA levels of indicated genes were quantitated using

specific primers. The data was shown as fold change compared with the control group. (D and F) The serum levels of ALB and ALT at the endpoint were quantitated. Differences between groups are shown as *P*-values. (E) Histological analysis of the tissue samples of indicated mice (cropped from Fig. S9A).



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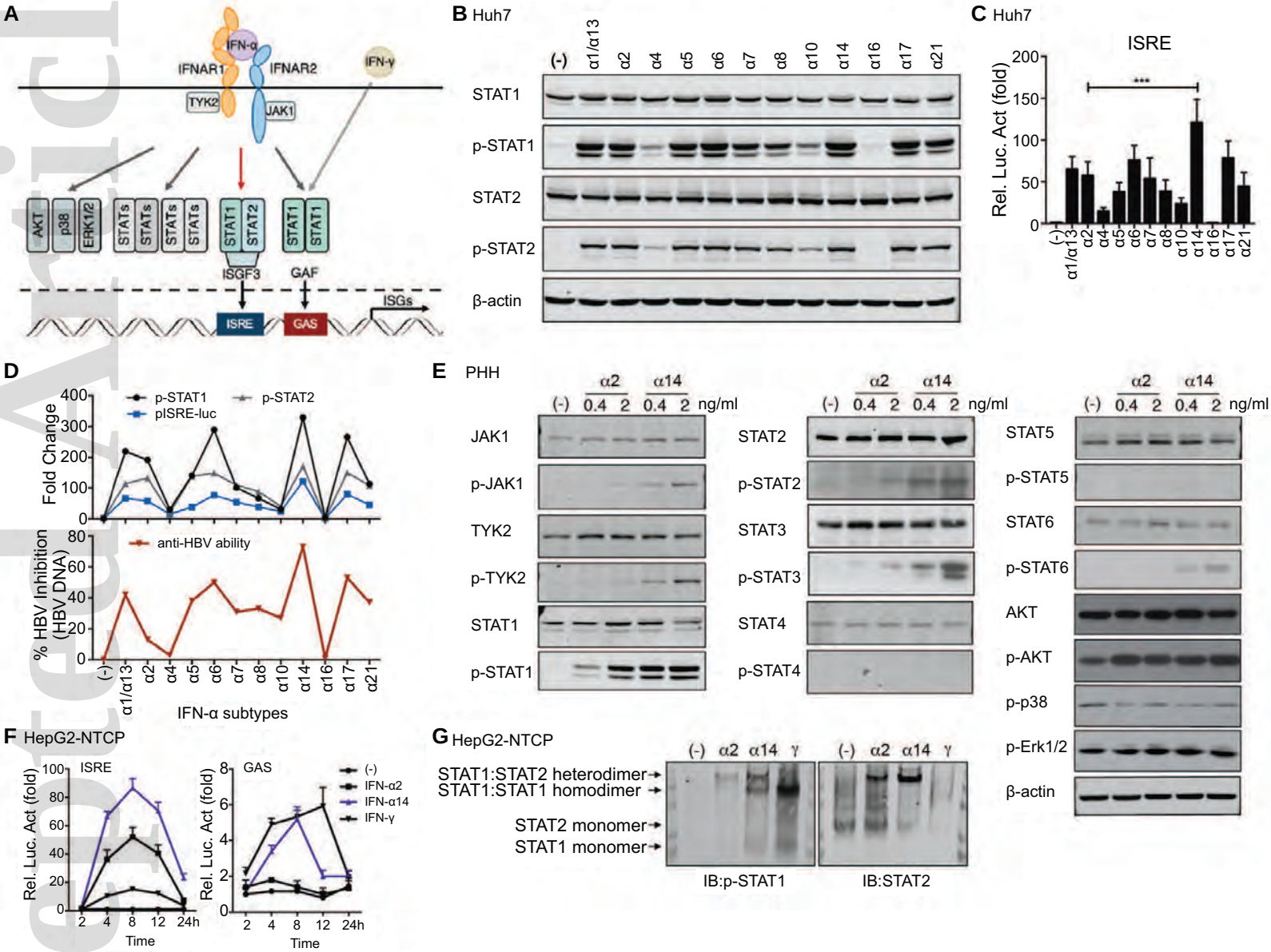

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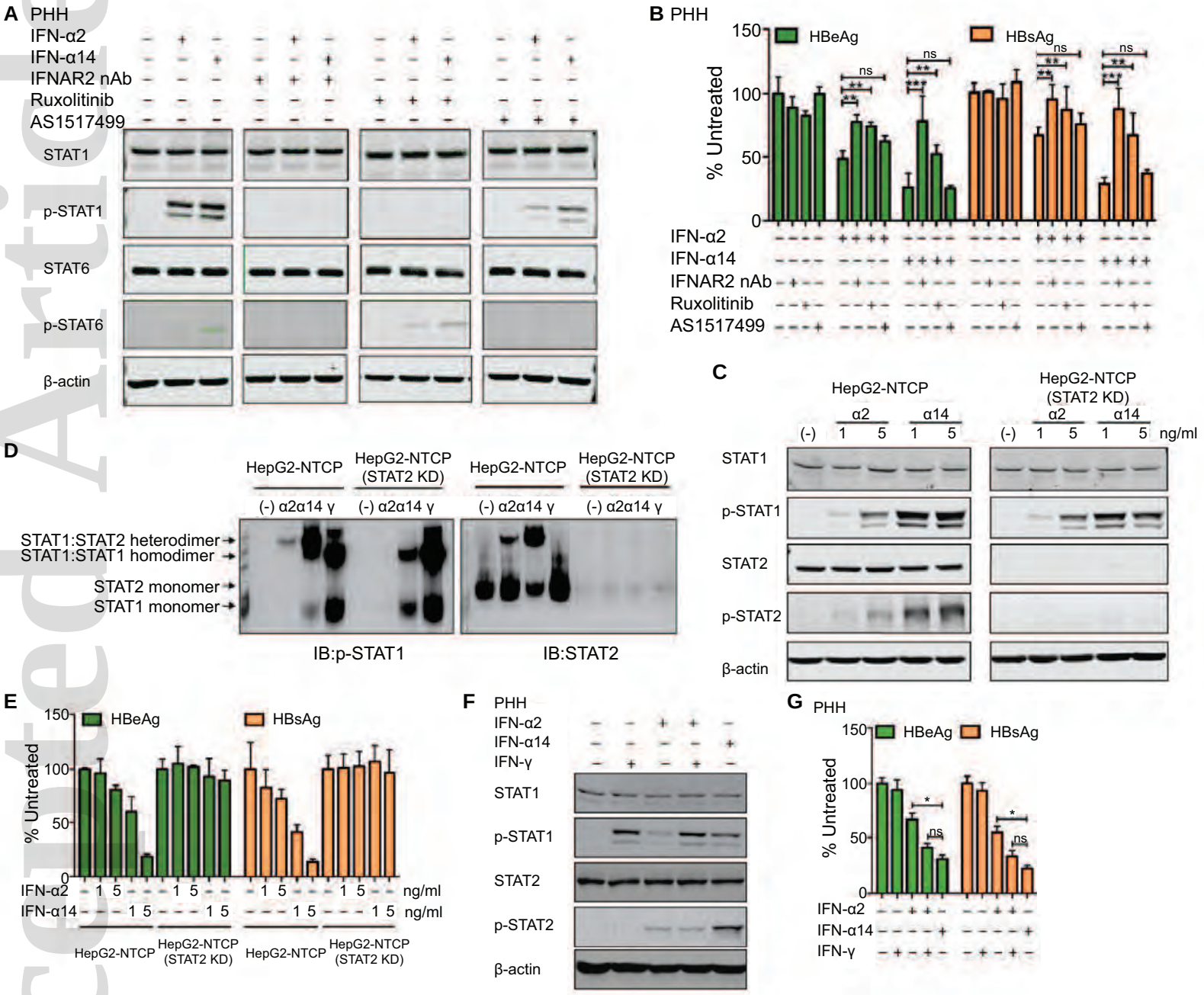

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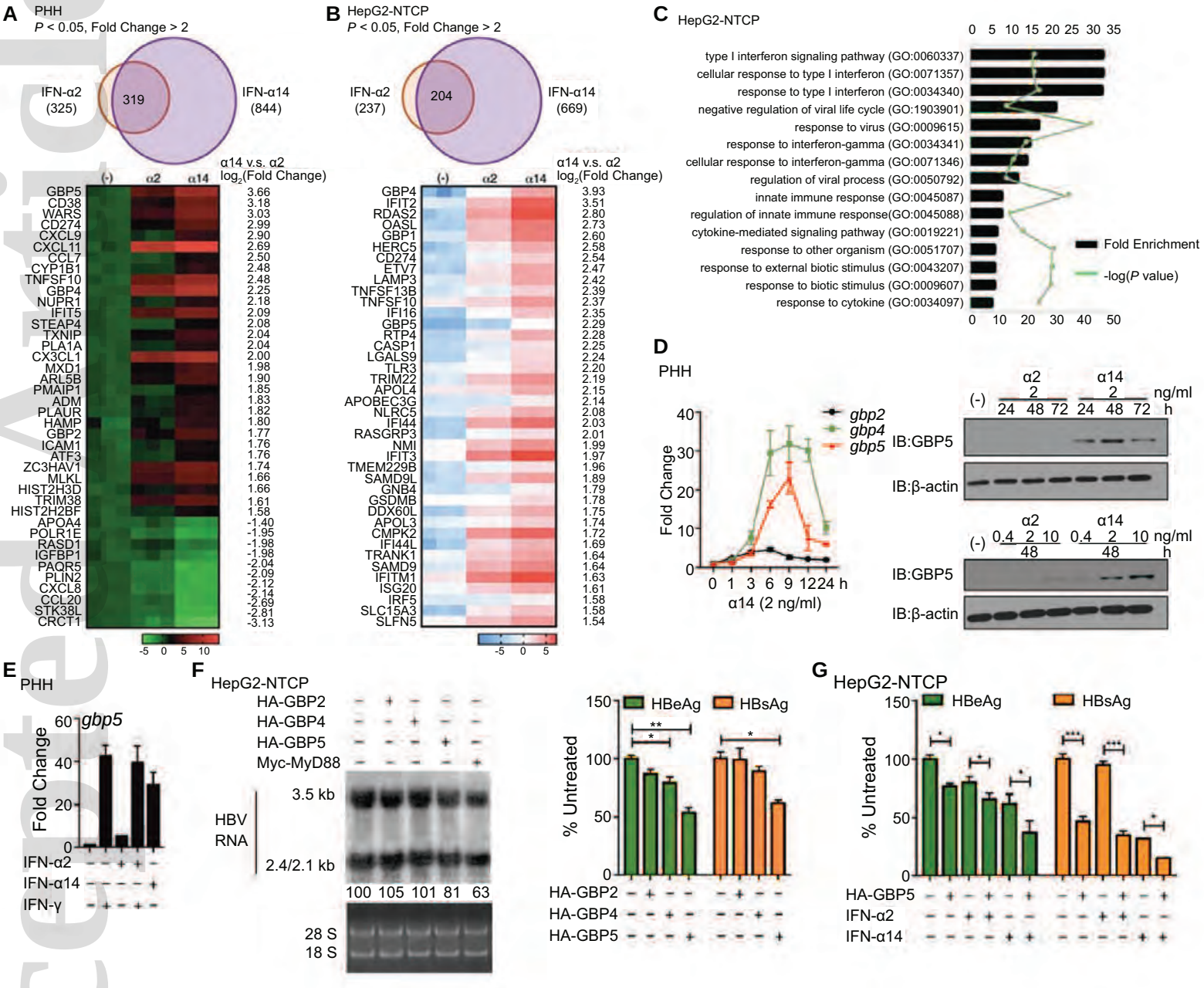

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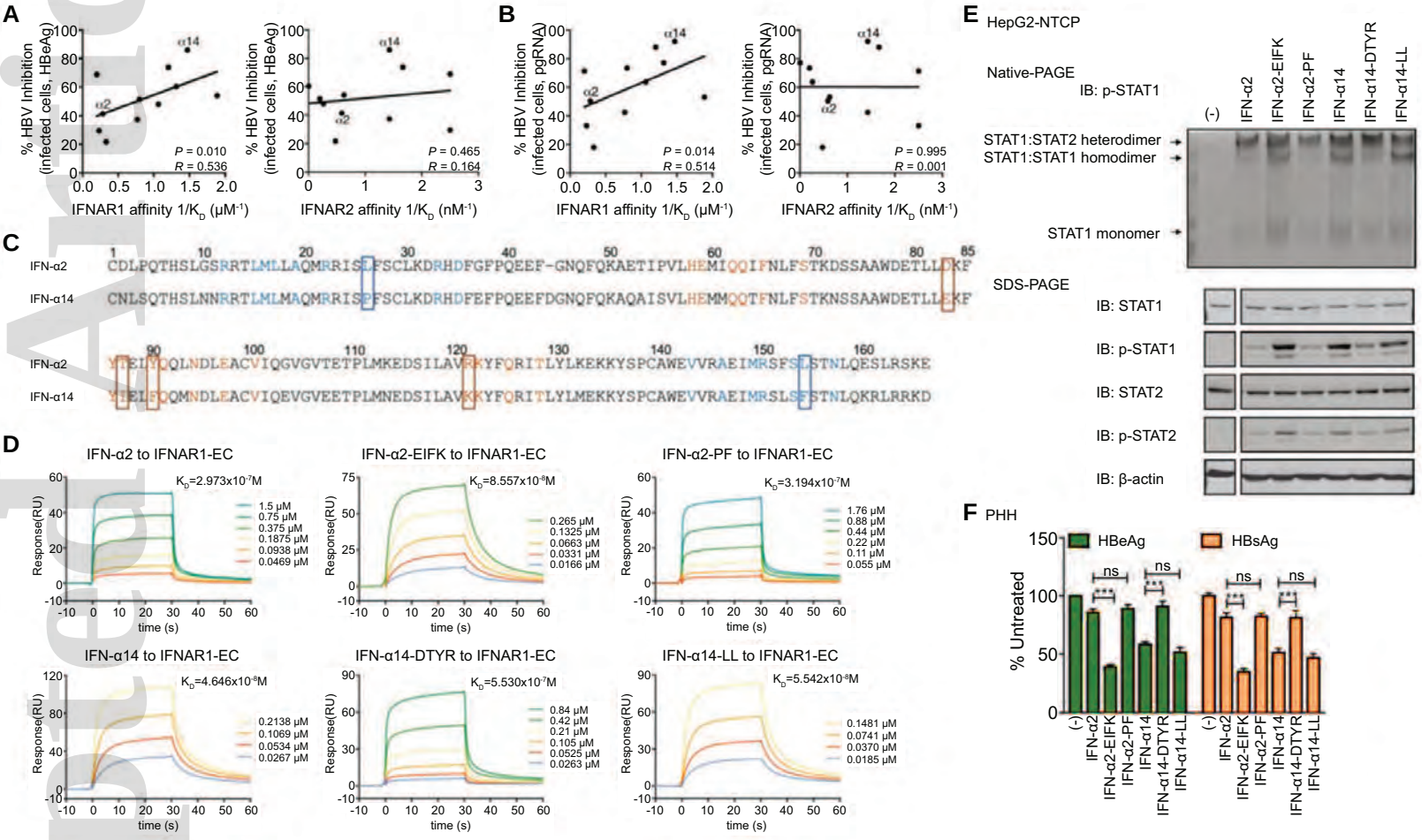

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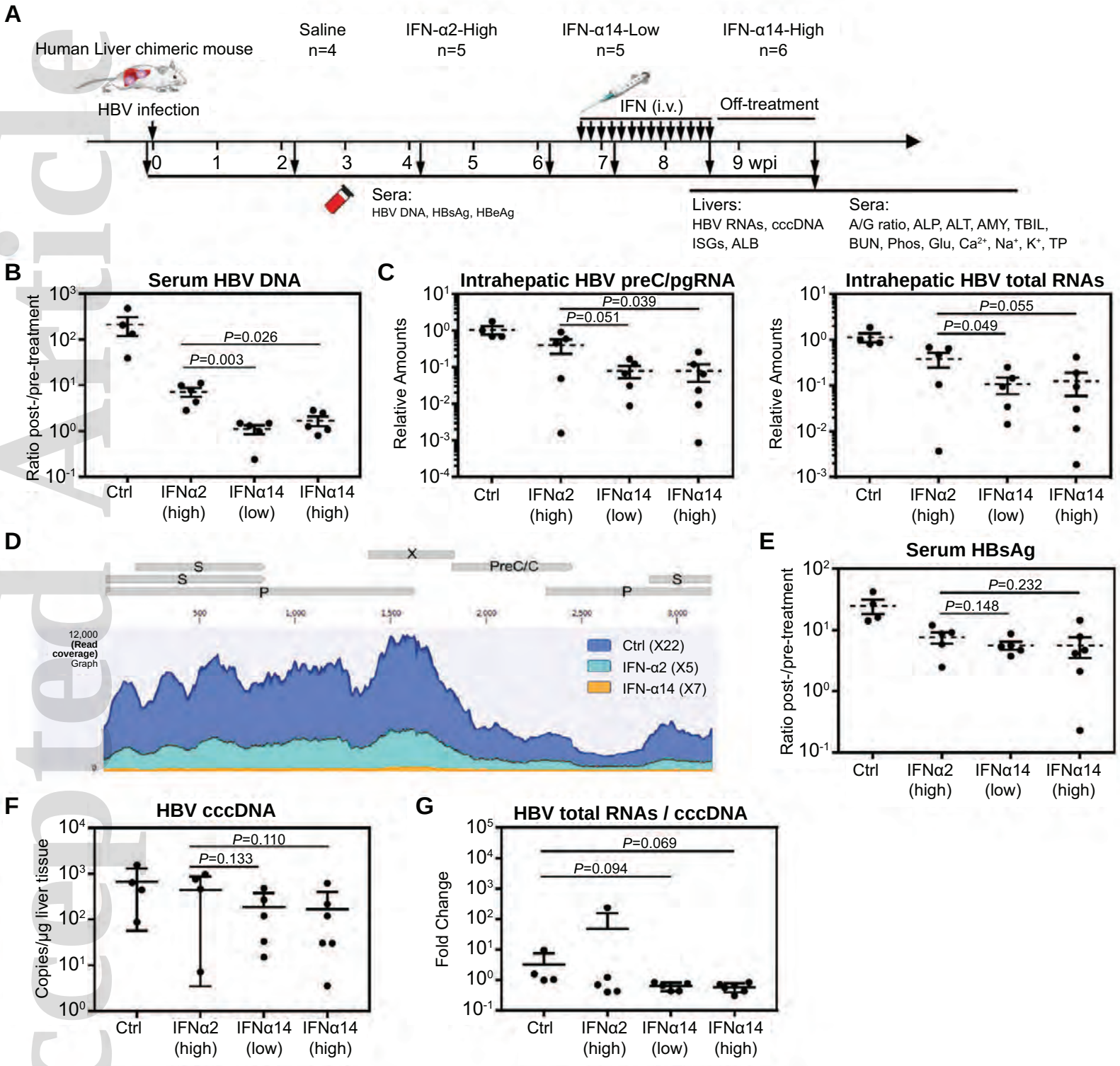

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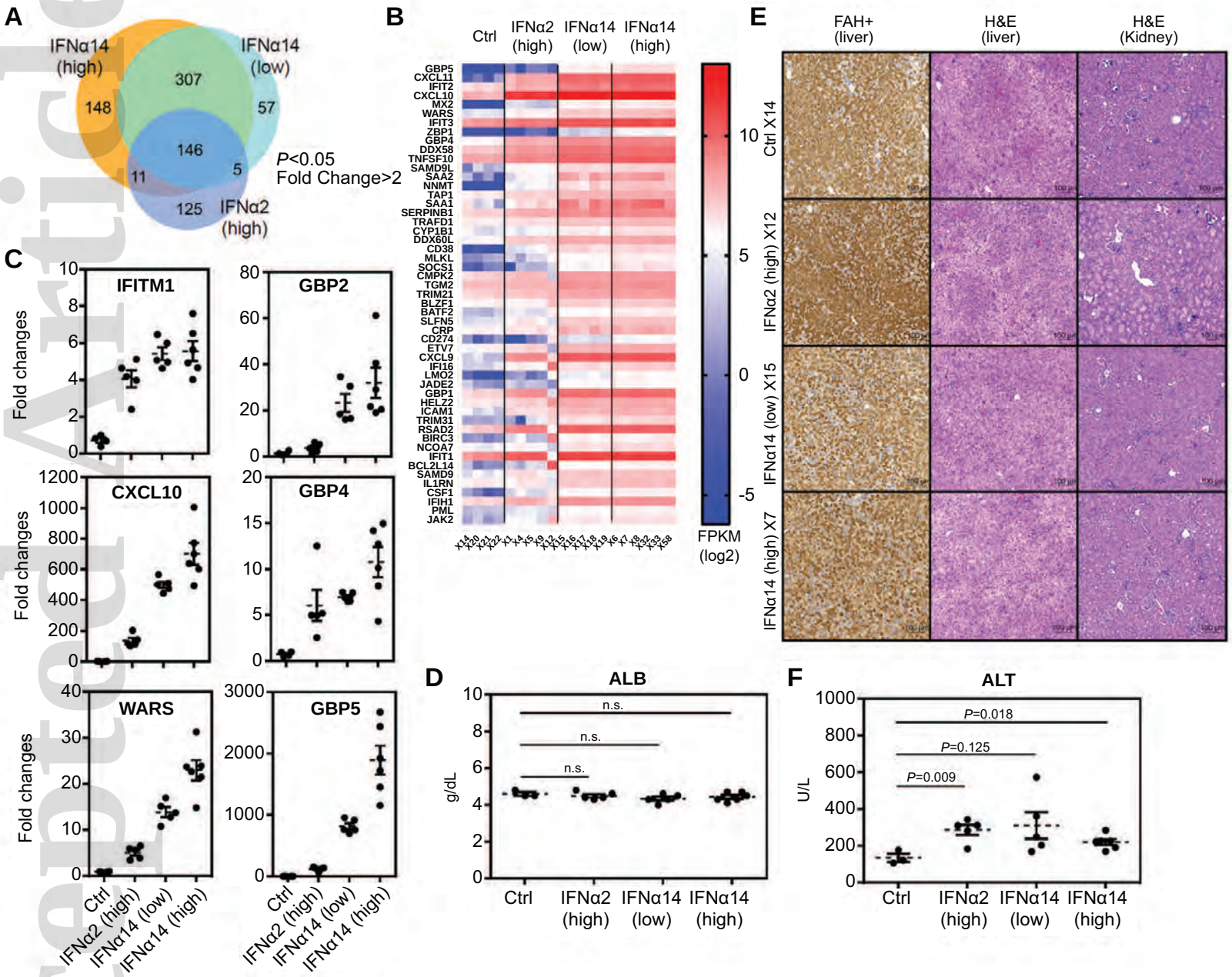

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