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B 型肝炎病毒核心蛋白與壓力顆粒形成和先天性免疫

反應之關聯性

Hepatitis B virus core protein in stress granules formation

and innate immune responses

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逝者如斯，兩年的碩班時光如梭，兩年前剛踏入臺灣大學這所學術殿堂的懵懂學生也將要畢業了。在這兩年裡要說的感謝數不盡。其中最要感謝的就是陳培哲老師。每一次與陳老師的討論都是一次學習，老師總是利用專業科學家的思維提出令人耳目一新的觀點，另外老師常會反問我們與題目相關的問題，並要我們去整理相關文獻以提升我們對於專業知識的了解度。因此，在陳老師的指導下，我不僅提升了我在設計實驗上的邏輯思考能力以及在面對困難時的應對能力，也學習到如何判斷實驗的結果並從中發想出另一個問題的能力。此外，陳老師在日常生活中也非常照顧我們，老師常會拿許多好吃的水果以及餅乾到休息室與我們分享，也時常提醒我們要好好保持運動習慣。因此不論是在學術上或是日常中，在老師的指導及照顧下我真的學到了許多，很謝謝老師當時願意讓我加入您的實驗室，使我能夠在既充滿學術氣息又充滿溫情的實驗室中完成我的碩士學位。

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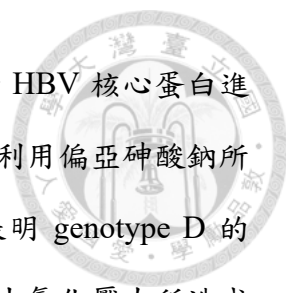
碩班期間我常用一句出自村上村樹所作小說《海邊的卡夫卡》中的一句話：「你將成為世界上最強悍的十五歲少年」來勉勵自己，相信在經歷了碩班的訓練後，我也能像故事中的主角一樣將這段經歷化作養分，並突破生活中的層層困難。



摘要

先天性免疫系統對於啟動適應性免疫反應以及病毒的清除至關重要。然而，B 型肝炎病毒（Hepatitis B virus; HBV）長久以來一直被認為不能夠誘導有效的先天性免疫反應，因此被認為是一種「隱形病毒」。在被 B 型肝炎病毒感染的成年患者之中，大部分患者體內都能夠檢測到適應性免疫反應，並能有效地在 6 個月內將病毒清除，這表示作為連接適應性免疫反應橋樑的先天性免疫反應仍應有被病毒感染所誘導。近年來，越來越多病毒被證明能夠在宿主體內誘發壓力顆粒（Stress granule）的形成，許多病毒蛋白或核酸也被發現會與壓力顆粒中的分子交互作用，而壓力顆粒的產生與某些病毒誘導的先天性免疫反應具有一定關係。雖然目前有一些間接證據顯示 B 型肝炎病毒可能具有誘發壓力顆粒產生的能力，但對於 B 型肝炎病毒與壓力顆粒之間相關性的研究仍較為缺乏，因此本研究的目的是在於探討 B 型肝炎病毒與壓力顆粒形成以及先天性免疫反應之間的關聯性，我們假設在 B 型肝炎病毒存在的情況下，宿主細胞會形成壓力顆粒並介導 B 型肝炎病毒誘發的先天性免疫反應，我們亦假設具有高度免疫原性的 HBV 核心蛋白（HBV core protein）會參與在 HBV 誘發壓力顆粒生成的機制當中。

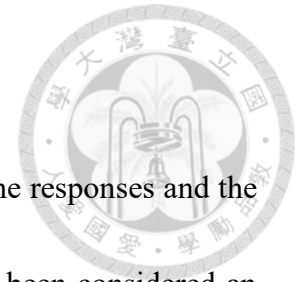
在本研究中，我們利用可穩定表達 HBV genotype D 的 HepAD38 細胞株以及轉染 HBV genotype A 質體的人類肝炎細胞株 Huh7 作為我們實驗的細胞株；同時也利用對細胞中壓力顆粒的標記蛋白 G3BP1 進行染色來確立壓力顆粒的生成。我們首先透過施加不同濃度以及時間的偏亞砷酸鈉（Sodium arsenite; NaAsO₂）給予細胞氧化壓力，以建立可於細胞中穩定形成壓力顆粒的 positive control，並篩選了適合對細胞中 HBV 抗原進行染色的抗體。接著我們觀察了在不同誘導天數的 HepAD38 中壓力顆粒的形成情形。我們觀察到在大多數細胞中，壓力顆粒並不會隨著 HBV 誘導天數的增加而產生，我們僅在誘導後第 4、6、8 天的少量細胞中觀察到些許壓力顆粒的產生，此外我們欲透過在誘導 HBV 產生的 HepAD38 細胞中添加偏亞砷酸鈉來觀察 HBV 核心蛋白以及壓力顆粒的共定位。然而，我們所使用



的 S162NP rabbit anti-HBc 抗體並無法在誘導的天數中有效地對 HBV 核心蛋白進行染色，因此無法觀察到其在細胞中的分布及定位；有趣的是，利用偏亞砷酸鈉所誘導的壓力顆粒並沒有因為 HBV 的產生而受到影響，這可能表明 genotype D 的 HBV 無法穩定地誘導細胞中壓力顆粒的產生，並且也不會抑制由氧化壓力所造成的壓力顆粒形成。我們接下來透過轉染 pAAV/HBV1.2 genotype A 質體後不同天數的 Huh7 觀察不同基因型的 HBV 是否在誘導壓力顆粒上具有差異，結果顯示不論是在轉染 pAAV 質體或是在轉染 pAAV/HBV1.2 genotype A 質體的 Huh7 細胞中皆沒有觀察到壓力顆粒的產生，表明 Genotype A 的 HBV 在轉染的模式上並不會誘導壓力顆粒的生成。最後我們假設 HBV 可能含有潛在的機制去抑制壓力顆粒形成，因此我們透過轉染帶有不同突變的 pAAV/HBV1.2 genotype A 質體來觀察特定的 HBV 蛋白（HBc、HBe 和 HBx）的缺失和結構改變或是 spliced RNAs 的缺失是否會誘導壓力顆粒的生成，或是利用給予細胞 core protein allosteric modulators (CpAM) 檢視 nucleocapsid 的完整性對於壓力顆粒的形成是否具有影響，結果顯示特定 HBV 蛋白及 spliced RNAs 或是 nucleocapsid 的完整性皆與壓力顆粒的抑制或形成無關聯。總結以上，我們發現 HBV 可能無法於細胞模式中普遍地誘導壓力顆粒，但特定 HBV 蛋白（HBc、HBe 和 HBx）或是 spliced RNAs 也不具有抑制細胞中的壓力顆粒生成的機制，表示 HBV 所誘導的免疫反應可能是獨立於壓力顆粒的，因此 HBV 與先天性免疫反應之間的關係仍須要進一步探討及釐清。

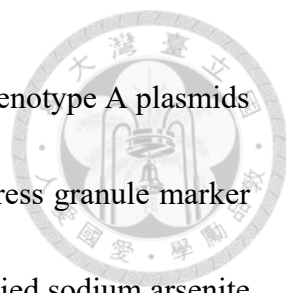
關鍵詞：先天性免疫反應、B 型肝炎病毒、壓力顆粒、HBV 核心蛋白

Abstract



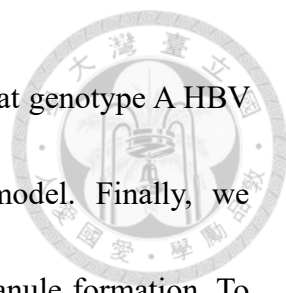
Innate immune system are crucial for initiating adaptive immune responses and the subsequent clearance of viruses. Hepatitis B virus (HBV) has long been considered an "invisible virus" as it is thought to be incapable of inducing effective innate immune responses. However, most adult patients infected with HBV exhibit adaptive immune responses that effectively clear the virus within six months, suggesting that the innate immune response, which bridges to adaptive immunity, must still be induced during HBV infection. Recent studies have shown that many viruses can induce the formation of stress granules in host cells, with various viral proteins or nucleic acids interacting with molecules within these membraneless organelles. The formation of stress granules is associated with certain virus-induced innate immune responses. Although some indirect evidence suggests that HBV may induce stress granule formation, research on the relationship between HBV and stress granules remains limited. Therefore, the aim of this study is to investigate the association between HBV, stress granule formation, and innate immune responses. We hypothesize that in the presence of HBV, host cells will form stress granules that mediate HBV-induced innate immune responses. We also propose that the highly immunogenic HBV core protein involves in the mechanism of stress granule formation.

In this study, we used the HepAD38 cell line, which stably expresses HBV genotype



D, and the Huh7 human hepatoma cell line transfected with HBV genotype A plasmids as our experimental cell models. We also utilized staining for the stress granule marker protein G3BP1 to investigate stress granule formation. First, we applied sodium arsenite (NaAsO_2) at various concentrations and durations to induce oxidative stress in cells, establishing a positive control for stable stress granule formation. We then screened for antibodies suitable for staining HBV antigens within the cells. Subsequently, we observed the formation of stress granules in HepAD38 cells at various induction days. We found that stress granules did not form with increasing HBV induction days in most cells; only a few cells showed stress granule formation on days 4, 6, and 8 post-induction. Additionally, we aimed to observe the co-localization of the HBV core protein and stress granules by adding sodium arsenite to HepAD38 cells producing HBV. However, the S162NP antibody we used did not effectively stain the HBV core protein during the induction days, making it impossible to observe its distribution and localization within the cells. Interestingly, the stress granules induced by sodium arsenite were not affected by the presence of HBV, suggesting that HBV genotype D does not stably induce stress granule formation and does not inhibit stress granules formed by oxidative stress.

Next, we observed whether different HBV genotypes differ in inducing stress granules by transfecting Huh7 cells with the pAAV/HBV1.2 genotype A plasmid. The results showed that stress granules did not form in Huh7 cells transfected with either



pAAV vectors or pAAV/HBV1.2 genotype A plasmids, indicating that genotype A HBV does not induce stress granule formation in the transfection model. Finally, we hypothesized that HBV might have mechanisms to inhibit stress granule formation. To test this, we transfected cells with pAAV/HBV1.2 genotype A plasmids containing various mutations to examine whether the deletion or structural alteration of specific HBV proteins (HBc, HBe, and HBx) or the absence of spliced RNAs would induce stress granule formation. Additionally, we assessed the impact of nucleocapsid integrity on stress granule formation by treating the cells with core protein allosteric modulators (CpAM). Our results indicated that neither specific HBV proteins, spliced RNAs, nor nucleocapsid integrity were associated with the suppression or formation of stress granules. In summary, we found that HBV may not generally induce stress granules in cellular models. However, specific HBV proteins (HBc, HBe, and HBx) or spliced RNAs also do not appear to possess mechanisms that inhibit the formation of stress granules in cells. This suggests that the immune response induced by HBV may be independent of stress granules, indicating that the relationship between HBV and the innate immune response requires further investigation and clarification.

Key words: Innate immune response, Hepatitis B virus (HBV), Stress granule (SG), HBV core protein (HBc)



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CHAPTER I: Introduction

1.1 Hepatitis B virus (HBV)

1.1.1 Introduction of HBV

The Hepatitis B virus (HBV) is a non-cytopathic DNA virus that belongs to the *Hepadnaviridae* family [1]. HBV surface antigen (HBsAg, HBs) was initially discovered in an Australian indigenous individual suffering from leukemia and was further reported by Dr. Blumberg and his colleagues in 1965 [2]. And in 1970, Dane and his colleagues observed HBV particles through electron microscopy [3]. HBV is transmitted through the blood and body fluids of infected persons, and it can either cause chronic or acute Hepatitis B. In adults, HBV infection often results in acute hepatitis, whereas when contracted by children under 5 years old, it usually leads to incurable chronic hepatitis [4, 5]. During the chronic infection period, patients generally remain asymptomatic, but as the carrier period extends, they may be at risk of developing life-threatening liver cirrhosis or even liver cancer [1, 6, 7]. Although HBV vaccine have been developed, up to 254 million people were living with chronic hepatitis B in 2022, and there were 1.2 million of new infections per year [8]. In addition, the majority of current clinical drugs and treatment strategies still aim to achieve a functional cure, and a complete cure remains a distant goal [9]. Therefore, how to treat patients with chronic infection has become an important issue.

1.2.1 The structure and genome of HBV



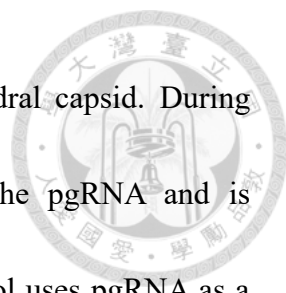
The size of HBV is about 42 nm, and the complete HBV particles are also called “Dane particles” [3]. The outer layer of the viral particle is composed of L-, M-, and S-HBsAg. Within the envelope, there is an icosahedral nucleocapsid assembled from 180-240 HBcAg proteins. Inside the capsid, the virus contains its relaxed circular partially double-stranded DNA (rcDNA) genome and DNA polymerase (pol) [10-12]. In addition to the 42 nm Dane particles, two other non-infectious sub-viral particles (SVP) have been found in the serum of patients. One type is 22 nm spherical particles, and the other type is 22 nm filamentous particles. These two types of particles are composed of HBsAg and do not contain viral DNA and capsid structures. Additionally, these particles are present at higher levels in the serum of patients than the Dane particles [3, 12-14].

The HBV genome is a 3.2 kb rcDNA, which includes a complete negative strand and an incomplete positive strand. The viral genome has 4 open reading frames (ORFs) including P, S, C, and X that encode 7 viral proteins: the P gene produces HBV DNA polymerase (pol); the S gene produces three different sizes of HBsAg; the C gene produces HBc (HBcAg) and HBe (HBeAg); and the X gene produces HBx. During infection, rcDNA undergoes DNA repair to form covalently closed circular (cccDNA). cccDNA can serve as a template and transcribes four different lengths of mRNA. The 3.5 kb pre-c/pregenomic RNA is translated into pol (which includes the functions of DNA

polymerase, reverse transcriptase, and Ribonuclease H) and core (which forms the capsid) and pre-core (which forms HBe); the 2.4 kb pre-S1 RNA produces large-HBs (which forms the envelope); the 2.1 kb pre-S2/S RNA synthesizes middle- and small-HBs (which forms the envelope); and the 0.7 kb X RNA produces X protein (which regulates virus replication and relates to carcinogenesis) [10, 12].

1.3.1 HBV lifecycle (Fig 1A)

HBV enters host hepatocytes by binding of L-HBs to Sodium taurocholate co-transporting peptide (NTCP). The binding triggers the internalization of HBV into endosome via endocytosis [15, 16]. After entry, the HBV nucleocapsid binds to the nuclear pore complex (NPC) and uncoating occurs to release HBV genome into nucleus [17]. In the nucleus, HBV rcDNA undergoes DNA repair that converts rcDNA into cccDNA [18]. As previously mentioned, cccDNA can transcribed into four different mRNAs. The pre-core proteins produced from 3.5 kb pre-core RNA further assemble into HBeAg and are secreted outside the cell. The three sizes of HBsAg translated from 2.4 kb and 2.1 kb RNAs are transported to the endoplasmic reticulum (ER) and Golgi apparatus. Some HBsAg are assembled into non-infectious sub-viral particles (SVP) and secreted outside the cell through Golgi apparatus [13]. The HBx protein, produced from the 0.7 kb RNA, has been shown to regulate HBV replication [19]. Meanwhile, the 3.5 kb pregenomic RNA is translated into HBV pol and HBcAg. HBcAg initially forms

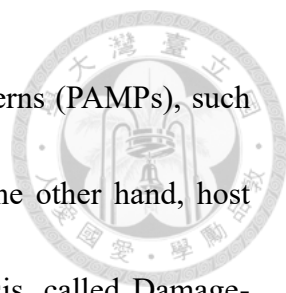


dimers, and then 90 or 120 dimers assemble to yield an icosahedral capsid. During encapsidation, pol interacts with the 5' epsilon stem loop of the pgRNA and is subsequently incorporated into the capsid [10]. Inside the capsid, pol uses pgRNA as a template to reverse transcribe negative-strand DNA, and then the RNase H activity of pol degrades pgRNA, leaving small RNA fragments attached to the DNA. These RNA fragments serve as primers to generate another segment of incomplete positive-strand DNA, forming newly synthesized rcDNA [10, 12, 20]. A portion of the capsids containing rcDNA is transported to the endoplasmic reticulum, where it assembles with HBsAg to form enveloped viral particles. The newly formed infectious virus particles are secreted outside the cell via Multivesicular bodies (MVB) [21]. Another portion of the capsids that are not enveloped and secreted can transport the HBV rcDNA back to the cell nucleus and generate cccDNA again [22, 23].

1.2 HBV and Innate immune response

1.2.1 Innate immune response

The innate immune response is an evolutionarily ancient aspect of host defense and plays an important role in defending against pathogen infections and in triggering adaptive immune responses [24, 25]. Unlike adaptive immune responses, innate immune responses are found in all multicellular organisms, such as plants, invertebrates, and mammals. The activation of the innate immune response begins with Pattern Recognition



Receptors (PRRs) recognizing Pathogen-Associated Molecular Patterns (PAMPs), such as microbial DNA, RNA, or bacterial cell wall components. On the other hand, host proteins and metabolites produced by tissue damage or cell necrosis, called Damage-Associated Molecular Patterns (DAMPs), can also activate the innate immune response [26, 27]. After initiating activation, the downstream signaling pathways activate immune cells or induce the release of antiviral molecules. Most PRRs in vertebrates can be divided into several types: (1) Toll-like receptors (TLR), which recognize several different PAMPs, such as viral nucleic acids, fungal cell walls, or gram-negative bacterial lipopolysaccharides (LPS); (2) nucleotide oligomerization domain (NOD)-like receptors (NLRs), which detect γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP) of Gram negative bacteria or muramyl dipeptide (MDP) of Gram positive and negative bacteria; (3) retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), which mainly sense viral RNA in cytoplasm (4) C-type lectin receptors (CLRs), which recognize β -Glucan or α -Mannan of fungus, and (5) Intracellular DNA sensor like Absent in melanoma-2-like receptors (ALRs) and cyclic GMP-AMP synthase (cGAS) [24]. After recognizing specific PAMPs, these PRRs trigger the production of several cytokines and chemokines. For example, the activation of RIG-I causes downstream mitochondrial antiviral-signaling proteins (MAVS) aggregation, which results in Type-I interferon production [28]. The activation of the innate immune response then triggers an adaptive immune reaction that

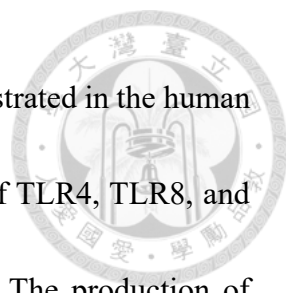
kills infected cells to limit pathogen replication.

1.2.2 Innate immune response of HBV

The host immune response induced by HBV infection is the main cause of liver damage. However, clearing HBV also relies on the activation of the immune response [1, 29, 30]. Thus, the immune response in HBV infection can be considered a double-edged sword. In the HBV-associated immune response, the adaptive immune response is typically considered the more detectable immune response during HBV infection [30-32]. However, the innate immune response, which is a key factor in initiating the adaptive immune response, has been challenging to detect in HBV infection [33-35]. Therefore, in the context of HBV-associated immune responses, there likely remain numerous unexplored and unknown aspects that warrant further investigation.

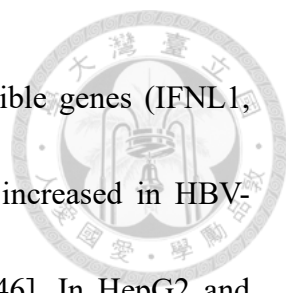
HBV has long been regarded as a stealth and invisible virus due to the inability of early studies in animal models to detect innate immune responses triggered by HBV infection [33, 34]. For example, HBV infection in chimpanzees showed the absence of activation of genes associated with innate immunity, unlike previous observations in an HCV infection model [33, 36]. Similar results were observed in the woodchuck model [34]. Moreover, the production of cytokines such as IFN- α and IL-15 was impaired in early acute HBV-infected patients [35]. Other studies also demonstrated that HBV infection did not induce type I and type III IFN responses in dHepaRG-NTCP, HepG2-





NTCP, dHepaRG, HLC, or PHH cells; these results were also demonstrated in the human liver chimeric mouse model [37, 38]. Additionally, the expression of TLR4, TLR8, and TLR9 was found to decrease in peripheral dendritic cells (pDCs). The production of IFN λ 1, TNF α , and IL-12p40/70 by conventional dendritic cells 1 (cDC1), the production of IL-12p40/70 and TNF α by conventional dendritic cells 2 (cDC2), and the production of IFN α , TNF α , and IFN λ 1 by pDCs were impaired in chronic hepatitis B (CHB) patients [39]. Suslov et al. revealed that HBV does not impede the activation of the innate immune response and the IFN pathway in the liver cells of HBV patients, suggesting that HBV evades the innate immune response by escaping recognition by PRRs [40]. However, some studies reported that HBV would suppress the innate immune response and several proteins encoded by HBV have been shown to exert suppressive functions on innate immune response [41-43]. That is, these studies suggested that HBV may not trigger typical innate immune response, but the certain mechanism is still unknown.

Conversely, Hösel et al. discovered that HBV could be recognized by nonparenchymal cells (mainly are Kupffer cells), then results in NF κ B activation and IL-6, IL-8, TNF- α and IL-1 β production [44]. A recent study showed that HBV could be sensed by primary human hepatocytes via TLR-2, leading to downstream NF κ B nuclear translocation in vitro, and this recognition caused the secretion of IL-1 β , IL-6, and TNF. However, they also found that this immune induction was only slightly observed in

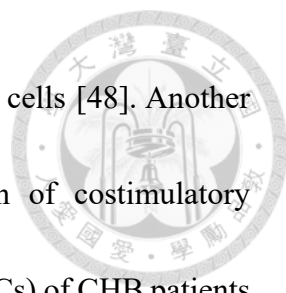


humanized mice models [45]. Another study found that IFN-inducible genes (IFNL1, IFNL2, IFNL3, CXCL10, OAS2) and RSAD2 were significantly increased in HBV-infected HepG2-hNTCP-C4 cells or human liver chimeric mice [46]. In HepG2 and HepaRG cells, transduction of Bac-HBV-WT showed an increase in IFN- β mRNA and the production of biologically active type I IFNs [47].

In summary, the conflicting studies indicate that these findings require further validation using appropriate and realistic in vivo infection models and the relationship between HBV and the innate immune response remains controversial.

1.2.3 HBV surface antigen and innate immune response

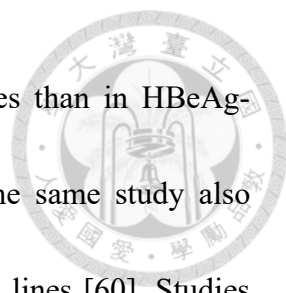
As mentioned before, HBsAg is the component of HBV envelopes and SVP, and HBsAg is considered to play the role of an immune suppressor in most of studies. Several studies have shown that HBsAg inhibits the activation of monocytes and suppresses the ability of both monocytes and macrophages to produce cytokines [48-52]. In addition, Wu et al. found that HBsAg and HBeAg could suppress the innate immune response induced by TLR agonist stimulation in murine parenchymal and non-parenchymal cells [53]. In the research of CHB patients, the production of IL-6, IL-8, and TNF- α induced by TLR-2 and TLR-4 signaling were inversely correlated with the level of HBsAg in the peripheral blood mononuclear cells (PBMCs) of CHB patients [54]. Recombinant HBsAg (rHBsAg) suppressed LPS-induced IL-18 and IL-12 production in a dose-dependent



manner and inhibited NF κ B and ERK pathway activation in THP-1 cells [48]. Another ex vivo study revealed that rHBsAg inhibited the up-regulation of costimulatory molecules (CD86, CD40, and CD80) on myeloid dendritic cells (mDCs) of CHB patients [55]. The transfection of L-HBsAg dose-dependently decreased the expression of IFN α , IFN β , IFN λ 1, IL6, and TNF α in SeV-infected Huh7 and HepG2 cells, and the activation of NF- κ B, ISRE, IFN β and IFN λ 1 promoters was also suppressed by HBsAg [56]. In an HBV transgenic mouse model, chronic HBsAg carriers exhibited a decrease in NK cells in the liver, reduced natural activation of hepatic NK cells, and attenuated cytotoxicity [57]. However, some studies have also indicated that HBsAg has the ability to activate the innate immune response. A study indicated that co-culturing PBMCs with HBsAg resulted in the production of more IL-6 and TNF-positive monocytes compared to the control group [58]. Additionally, co-culturing HBsAg with naive dendritic cells (DCs) significantly increased the number of CD83⁺CD40⁺ DCs and increased the secretion of IL-6 and IL-12 in a dose-dependent manner [59]. Together, HBsAg may acts as both an activator and a suppressor in the host immune response.

1.2.4 HBV e antigen and innate immune response

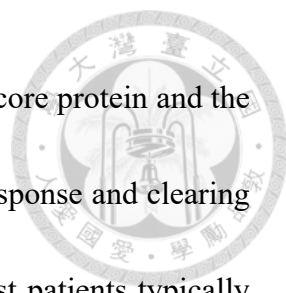
HBeAg is a secretory protein in the HBV life cycle. Many studies have demonstrated that HBeAg has immunoregulatory functions and helps the virus evade the host immune response. The liver biopsy of HBeAg-positive CHB patients shows lower TLR2



expression on hepatocytes, Kupffer cells, and peripheral monocytes than in HBeAg-negative CHB patients and steatosis patients (disease control). The same study also suggested that HBeAg suppresses TNF- α production in hepatic cell lines [60]. Studies from the same research group later revealed that HBeAg could bind to Toll/IL-1 receptor (TIR)-containing proteins Mal and TRAM and suppressed TLR2 downstream NF- κ B activation and IFN- β promoter activity [61]. Wang et al. also found that HBeAg could interact with the NF- κ B essential modulator (NEMO) and attenuate NF- κ B activation by disrupting TRAF6-dependent K63-linked ubiquitination of NEMO [62]. In Kupffer cells, HBeAg suppressed LPS-induced NLRP3 expression and IL-1 β maturation by inhibiting the NF- κ B signaling pathway and the production of reactive oxygen species (ROS) [63]. Major vault protein (MVP) is a host factor that upregulates type I IFN production and plays a role in the antiviral response. HBeAg has been reported to suppress the interaction between MVP and MyD88, thereby decreasing downstream IFN production [64]. In summary, the current understanding suggests that HBeAg predominantly plays an inhibitory role in the regulation of the innate immune response.

1.2.5 HBV core antigen and innate immune response

Among HBV proteins, HBcAg is considered to be highly immunogenic. HBcAg has been reported to have approximately 1000 times higher immunogenicity than HBeAg [65]. Additionally, during natural infection, HBcAg also exhibits higher immunogenicity



compared to HBsAg [66]. Previous studies have revealed that HBV core protein and the complete capsid structure may be important for inducing immune response and clearing the virus in mice [67, 68]. Additionally, during acute infection, most patients typically produce a high level of anti-HBc antibodies [69]. Cooper et al. found that full-length HBc capsids rather than HBc-144 capsids (C terminal domain-truncated) bind to THP-1 macrophage and induced TNF- α , IL-6, and IL-12p40 through TLR2 signaling pathway [70]. Another ex vivo study suggested that HBcAg can bind to M2 macrophages, thereby impairing their polarization. Additionally, this binding increases the production of IL-6 and TNF- α via TLR2 [71]. Moreover, compared to HBsAg, HBcAg could induce the production of IgG and IgM anti-HBc antibodies in athymic mice [72]. Another study also suggested that B cells (B1a and B1b cells), rather than dendritic cells, serve as the primary antigen-presenting cells (APCs) for HBcAg [73]. The same research team later demonstrated that B1a and B1b B cells preferentially bind HBcAg and present it to CD4⁺ T cells [74]. Together, these studies indicate that HBcAg may play a role in activating the immune response and that the capsid structure or C-terminal domain is important for these effects.

1.2.6 HBV polymerase and innate immune response

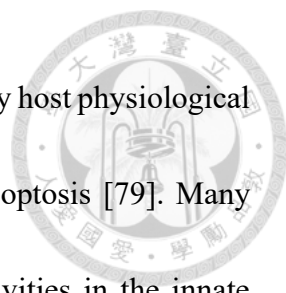
HBV polymerase is a multifunctional protein in the HBV life cycle that plays a role in reverse transcribing pgRNA into dsDNA within the viral nucleocapsid. This protein



also involves in HBV-mediated immune regulation. HBV polymerase has been reported to inhibit poly (I:C)-induced RIG-I/MDA5 and TLR3 downstream IFN signaling by disrupting the IKK ϵ -DDX3 interaction [75]. An in vitro study revealed that HBV polymerase impaired the interaction between IKK/NEMO and Hsp90/Cdc37, thereby suppressing IKK complex assembly. The same study also found that HBV polymerase inhibited NF- κ B activity and downstream IL-6 and IL-8 production [76]. In the Huh7 cell line, HBV polymerase binds to the stimulator of interferon genes (STING, a downstream protein in cytosolic DNA-sensing pathways) through its RT/RH domain, leading to a reduction in K63-linked polyubiquitination of STING and suppression of its downstream signaling pathway [77]. Lei et al. transfected plasmid vectors overexpressing the four ORFs of HBV into HepG2 cells, and then treated with or without LPS and ATP. They found that mature IL-1 β expression in HBV-pol group was significantly lower than control group and the mRNA expression of NLRP-3, NLRC-4, AIM2 and IL-1 β was also inhibited. Moreover, they observed that the expression of NLRP3 and the aggregation of ASC in the HBV polymerase group were significantly weaker than in the control group [78]. These findings suggest that HBV polymerase suppresses the host innate immune response.

1.2.7 HBV x antigen and immune response

HBV X antigen (HBx) is a nonstructural protein that plays a role in initiating and



maintaining HBV replication. Moreover, HBx is also involved in many host physiological mechanisms, such as signal transduction, cell cycle control, and apoptosis [79]. Many studies have indicated that HBx may have negative regulatory activities in the innate immune response. HBx can impair cytosolic nucleic acid sensors such as RIG-I and MDA5 [80-82]. Mitochondrial membrane protein virus-induced signaling adaptor (VISA) is the central adaptor protein in the RIG-I and MDA5 signaling pathway [80]. A study showed that HBx would interact with VISA to disrupt its associated complex and further suppressed the activation of IRF3 and the production of IFN- β [83]. Wei et al. indicated that HBx promotes MAVS degradation by increasing the ubiquitination of Lys136 in the MAVS protein, and they also found that MAVS expression in the liver of HBV-induced HCC patients is lower than in the liver of healthy donors [82]. Parkin is an RBR E3 ligase which negatively regulate MAVS downstream signaling. HBx induced Parkin expression, which then recruited the linear ubiquitin assembly complex (LUBAC) to the mitochondria, resulting in MAVS linear ubiquitination and impaired antiviral response [84]. A recent study revealed that HBx could upregulate adenosine deaminases acting on RNA 1 (ADAR1) expression and interact with HBV RNA, causing adenosine (A) to be deaminated to form inosine (I). This change inhibited the immune recognition of HBV RNA in hepatocytes [85]. "Thus, the HBx protein primarily plays a role in the suppression of the host innate immune response.



1.2.8 HBV nucleic acids and immune response

Some studies also revealed that the nucleic acids of HBV are involved in the activation or suppression of the innate immune response. A study found that RIG-I could recognize and bound to 5'-ε region of HBV pgRNA then activated Type III IFN production, moreover, this binding inhibited HBV replication by counteracting the interaction between HBV pol and pgRNA [46]. In contrast, another study indicated that HBV may evade RIG-I recognition via N6-methyladenosine modification of its RNA by m6A writer enzymes such as METTL3 and METTL14 [86]. However, Lu et al. indicated that HBV infection may trigger MDA5 signaling rather than RIG-I. By co-transfecting HBV replicative plasmid and MDA5 or RIG-I expression plasmid into Huh7 cells, they found that MDA5 not only significantly suppressed HBV replication but also induced IRF3 and NF-κB activation. In a hydrodynamic injection mouse model, the levels of serum HBsAg, HBV RNAs, and intracapsid HBV DNAs were significantly increased in the MDA5^{+/-} and MDA5^{-/-} groups but not in the RIG-I^{+/-} group at 3 days post-injection [87]. These studies suggest a possibility that the nucleic acids of HBV could be sensed by the innate immune response, but the mechanism depends on different PRRs or cell types.

In conclusion, all of the above studies and experiments indicate that the mechanisms of how HBV activates or suppresses the innate immune response are controversial and remain poorly understood. In the previous study of our laboratory, we constructed

pAAV/HBV plasmids with premature stop codons in different regions and administered them via hydrodynamic injection into C57BL/6J mice and monitored serum HBsAg titer.

The results indicated that compared to WT group, HBV clearance was inhibited in the HBcAg-null group [67]. Therefore, we suggest that HBcAg may be a key antigen in eliciting the host immune response, and this response might differ from canonical immune response pathways.

1.3 Stress granules (SGs)

1.3.1 Introduction of stress granule

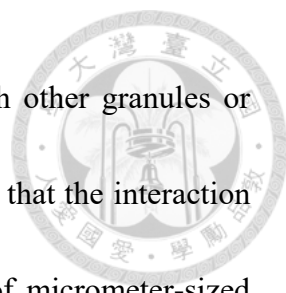
In this study, we focus on a type of membraneless organelle called stress granules (SGs), which have been shown to be associated with various viral infections and the immune responses [88]. Stress granule is a biomolecular condensates triggered by liquid-liquid phase separation (LLPS), which protect important cellular molecules from environmental stress [89]. In the canonical pathway, when cells face different stresses such as heat shock, UV light, or oxidative stress, specific kinases (e.g., GCN2, PEK, PKR, or HRI) sense these stresses and then phosphorylate eIF2 α that results in transcription initiation complex disassembly [90, 91]. The released RNA acts as a scaffold for SGs and recruits several RNA-binding proteins (RBPs) such as ras GTPase-activating protein-binding protein 1 (G3BP1), T-cell intracellular antigen-1 (TIA-1), TIA-1-related (TIAR) protein, fragile X mental retardation protein (FMRP), tristetraprolin (TTP), and cell cycle-



associated protein 1 (CAPRIN1) [91] . This process is called primary aggregation and causes the concentration of RNA and RBPs to form the stable core of SGs. After primary aggregation, these RBPs recruit other heterogeneous nuclear ribonucleoproteins or RNA-binding proteins, resulting in secondary aggregation and forming a shell of SGs that is less stable and more dynamic than the core [92]. Once the stress is relieved, SGs rapidly disassemble, and their components can be recycled for immediate cellular use [93]. Wheeler et al. indicated that SG disassembly is a stepwise process, beginning with the dissipation of the shell, and followed by the clearance of the cores [94]. On the other hand, some studies revealed that SG assembly can also start from the inhibition of the eIF4F complex, which is associated with the mammalian target of rapamycin (mTOR) and independently from eIF2 α [95].

1.3.2 Liquid-liquid phase separation (LLPS)

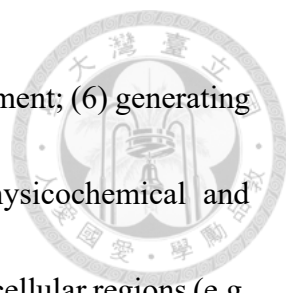
The diverse organelles in eukaryotic cells can be classified into membrane-bound organelles (e.g., endoplasmic reticulum) and membraneless organelles (e.g., P body) that both of which play important roles in biological activity [96]. Although membraneless organelles still have many unknown aspects that require further research, LLPS has been considered in recent years as a potential mechanism for the formation of these organelles [97, 98]. The discovery of LLPS in eukaryotes can be traced back to 2009 when Tony Hyman and Cliff Brangwynne observed that P granules in *C. elegans* exhibited liquid-



like behaviors, such as forming larger structures upon contact with other granules or rapidly dissolving and aggregating [99]. In 2012, a study also found that the interaction between multivalent macromolecules could induce the formation of micrometer-sized liquid droplets in an aqueous solution. These studies indicated the significance of LLPS in biological systems [100]. Therefore, an increasing number of research teams start to study LLPS.

There are two types of multivalent interactions to driving LLPS. The first one is the conventional multivalent interactions between protein-RNA, RNA-RNA, and protein-protein. The second is contributed by intrinsically disordered regions (IDRs) of proteins [96]. IDRs are relatively unstructured regions within proteins, which lack sufficient hydrophobic amino acids to fold into stable conformations and therefore do not form stable tertiary structures [101]. Thus, they have a higher potential for interacting with other proteins or molecules. The interaction between IDRs would form a weak, transient, multivalent interactions such as cation-anion interactions, π -cation interactions, π - π interactions, and dipole-dipole interactions [96].

LLPS has several potential functions within biological systems: (1) sensing environmental stress and protecting crucial intracellular molecules (e.g., RNA); (2) buffering protein concentrations within cells; (3) concentrating specific molecules to activate their reactions or signaling; (4) sequestering specific molecules to inhibit certain



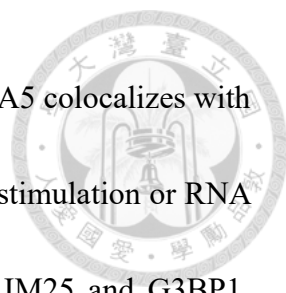
reactions; (5) rapidly exchanging molecules with the cellular environment; (6) generating mechanical forces to regulate cell structure; and (7) forming physicochemical and mechanical filters, allowing molecules to move in and out of specific cellular regions (e.g., nuclear pores) [102, 103]. Moreover, LLPS has also been reported to be associated with various diseases and biological function such as cancer, inflammatory disease, neurodegenerative disease, transcription and autophagy [104-109]. LLPS is also involved in the interactions between host and virus, for examples, LLPS mediated stress granule formation and immune responses [110, 111].

1.3.3 The interaction between stress granule and virus

Stress granules may involve in immune recognition and the regulation of immune responses. Components of stress granules such as G3BP1 and PKR have been reported to play a role in the positive regulation of the immune response [112-115]. In addition, several PRRs or antiviral proteins are also believed to be included in the composition of SGs [116]. Yang et al. found that overexpression of stress granule protein G3BP1 not only led to increase the SeV or poly (I:C) induced-IFNB1, IP-10, TNF α , IL8, RANTES, ISG56, and IL6 genes expression but also inhibit SeV and VSV replication in HEK293T cell lines [115]. On the other hand, the RGG domain of G3BP1 can bind to both viral dsRNA and RIG-I potentially facilitating RIG-I recognition of viral RNA and initiating downstream immune responses [113]. Conversely, a recent study has indicated that SGs recruit certain

innate immune molecules to mitigate excessive activation of immune responses induced by dsRNA and prevent cell death [117]. Therefore, SGs play a significant role in immune responses activation and regulation, but the detailed mechanisms remain unclear.

SGs have also been shown to be associated with antiviral responses. Onomoto et al. discovered that infection of NS1-deficient Influenza A virus (IAV) could induce RIG-I containing granules production in HeLa cells. These granules also contain SG markers but exhibit distinct properties from NaAsO₂-induced SGs. Therefore, they termed them anti-viral stress granules (avSG). By immunostaining, they found that several PRRs and antiviral proteins colocalize with avSGs. Moreover, when avSG formation was suppressed by knockdown G3BP1, the activation of type I IFN was inhibited [118]. Yoo et al. discovered that RNA virus infection would promote dsRNA/RIG-I/DHX36/PKR complex formation that facilitating PKR activation and further inducing avSG formation [112]. The infection of WT vaccinia virus also induced the formation of small amounts of avSGs. Mutating its E3L gene (which encodes a protein involved in evading host immune responses) enhanced the formation of avSG. And the VACV replication is markedly inhibited in the cells which contain avSG [119]. Another study found that rabies virus infection induced stress granule formation in U373-MG cells. They later used siRNA to knock down PKR expression in U373-MG cells and observed that SG formation and IFN- β gene expression were both inhibited, accompanied by an increase in RABV P



protein levels and viral production. Additionally, they found that MDA5 colocalizes with the SG marker TIA-1 [120]. A recent study indicated that poly (I:C) stimulation or RNA virus infection led to co-condensation of the antiviral proteins TRIM25 and G3BP1, promoting the localization of TRIM25 to avSGs. Additionally, the interaction between TRIM25 and G3BP1 regulates the localization of various innate immune-related proteins, such as ZC3HAV1, ZCCHC3, and IGF2BP2, and enhances the RIG-I signaling pathway while inhibiting replication of Sendai virus [121].

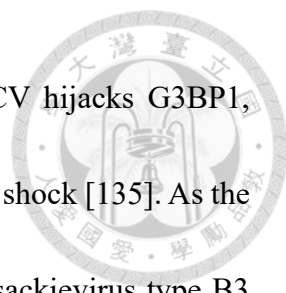
Moreover, several viruses have been reported to inhibit stress granules formation or mediates SG disassembly. The 4a accessory protein of MERS-CoV would bind to dsRNA that decrease PKR phosphorylation and results in SG formation [122]. Human immunodeficiency virus type 1 (HIV-1) inhibited the assembly of SGs by preferentially forming Staufen1-containing HIV-1-dependent ribonucleoproteins (SHRNPs) [123]. Zhang et al. found that SARS-CoV-2 nucleocapsid protein (NP) can suppress SG formation through impaired PKR activation and interacted with G3BP1 [124]. Another study indicated that SARS-CoV-2 NSP5 and N protein both play roles in inhibiting SG formation and suppressing type I and type III IFN responses [125]. In addition, there are a lots of viruses could interfere SG formation by different mechanisms [126-129]. In conclusion, the relationship between viruses and stress granules may be variable, depending on the type of virus and the cell type. That is, SGs play a significant role in

viral survival, replication, and the immune response triggered by virus infection.

1.3.4 Ras GTPase-activating protein-binding protein 1 (G3BP1)

In this study, we used G3BP1 as a marker for SGs. G3BP1 was first discovered by Parker and colleagues in 1996 [130]. In 2003, Tourrière et al. found that G3BP1 is recruited into SG after NaAsO₂ exposure, thus considering this protein a stress granule marker [131]. G3BP1 is a 68 kDa proteins that contains 466 amino acids. G3BP1 contains three IDR domains that regulate its ability in LLPS [132]. The importance of G3BP1 and its paralog G3BP2 in the formation of SGs has been demonstrated. Yang et al. found that the formation of stress granules is significantly disrupted in G3BP1 and G3BP2 double knockout U2OS cells after NaAsO₂ treatment (oxidative stress), but there is no effect on SGs induced by osmotic stress or heat shock [133]. Another study also demonstrated that single knockdown of G3BP1 or G3BP2, and double knockdown of G3BP1/G3BP2, have varying levels of impact on SGs induced by different types of stresses [134]. Thus, these studies suggested that the necessity of G3BP for stress granule formation may depend on the type of initiating stress.

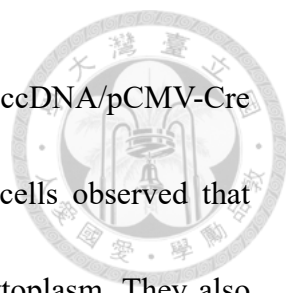
In addition to SG formation, G3BP1 is also involved in various regulatory functions within the organism such as ribosomal quality control, axonal mRNA translation, structure-mediated decay, antiviral function, and innate immune recognition [113, 115, 132]. Some studies indicated that G3BP1 is also a primary target for some viruses to



inhibit stress granule (SG) formation. Ariumi et al. found that HCV hijacks G3BP1, leading to the inhibition of SG formation induced by NaAsO₂ or heat shock [135]. As the infection progresses, the 3C proteinase of poliovirus (PV) and Coxsackievirus type B3 (CVB3) cleaves G3BP1, disrupting the SGs induced early during infection [136]. The N protein of SARS-CoV-2 could interact with G3BP1 to sequester G3BP1 and inhibits SG formation [124, 125, 137]. In conclusion, G3BP1 is a well-known SG marker that may play a crucial role in mediating the interactions between viruses and stress granules (SGs).

1.4 HBV and stress granule

There is limited research indicating a relationship between HBV and stress granules. In a proteomic study about HBV transfected HepG2 cells, the research group found that the expression level of G3BP1 was significantly upregulated in HepG2 cells transfected with the WT HBV plasmids compared to cell transfected with vector control [138]. Tsai et al. observed the aggregation of G3BP1 in Huh7 cells transfected with either the 2.2DS-RNA or the HBV genotype A expression plasmid (2.2DS-RNA⁺). However, this aggregation was not observed in cells transfected with B-gal RNA or the HBV genotype A^{G2335} (2.2DS-RNA⁻). Thus, they suggested that 2.2 double spliced-RNA (2.2DS-RNA) of HBV may triggered stress granules formation [139]. TIAR is another SG marker that relocates from the nucleus to the cytoplasm when cells are exposed to stress. A recent



study using HepAD38 (a Tet-off HBV induction cell line), prcccDNA/pCMV-Cre plasmid-transfected Huh7 cells, and HBV-infected HepG2-NTCP cells observed that HBV induces the translocation of TIAR from the nucleus to the cytoplasm. They also found an increase in the phosphorylation of eIF2 α , which mediates SG assembly initiation, in both prcccDNA/pCMV-Cre plasmid-transfected Huh7 cells and -DOX HepAD38 cells. Moreover, they compared the distribution of TIAR in Huh7 cells transfected with plasmids expressing different HBV proteins and found that only HBc induced the translocation of TIAR. Additionally, transfecting Huh7 cells with pCMV-Cp was also increase the phosphorylation of eIF2 α [140]. These data provide indirect evidence that HBV may induce SG formation.

1.5 Hypothesis and Aim

Based on the research above, we hypothesize that HBV may induce the formation of stress granules, and this phenomenon could further activate the innate immune response. The highly immunogenic HBV core protein may play a crucial role in driving the formation of stress granules. To test this hypothesis, we plan to use HepAD38 and Huh7 cells, utilizing induction and transfection methods, respectively, to investigate the relationship between HBV and stress granules, as well as whether different HBV genotypes influence stress granule formation. Additionally, we will use plasmids with different mutation sites or core protein allosteric modulators (CpAM) to verify whether

HBc or other viral proteins or nucleic acids are associated with this phenomenon. We aim to identify the key mechanisms by which HBV drives the innate immune response.



CHAPTER II: Materials and methods



2.1 Cell culture

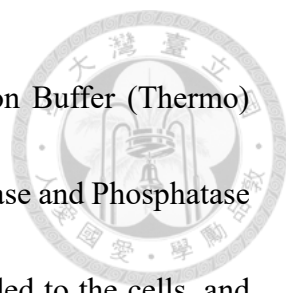
HepAD38 cells were cultured with Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, GlutaMAX™ supplement, Gibco) containing 10% fetal bovine serum (FBS, Hyclone), 10mM HEPES Buffer Solution (Biological Industries), 1% Penicillin-streptomycin-amphotericin (PSA, Capricorn Scientific), and Geneticin™ Selective Antibiotic (G418 Sulfate, Gibco). The cells were cultured with 1µg/ml doxycycline during passage to inhibit HBV expression, and doxycycline would be removed from culture medium when inducing HBV expression. The HBeAg titer in culture supernatant would be measured to assess the effect of doxycycline. Huh7 cells were cultured with Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% FBS, 10mM HEPES Buffer Solution and 1% PSA.

2.2 Measurement of HBeAg and HBsAg in culture supernatant

Approximately 200-250 µl of cell culture supernatant were collected and measured extracellular HBeAg and HBsAg titer by Elecsys HBeAg and Elecsys HBsAgII of Electrochemiluminescence immunoassay (ECLLA, Roche) in National Taiwan University Hospital Hepatitis Research Center.

2.3 Protein analysis

2.3.1 Protein extraction



To extract protein from cells, RIPA Lysis buffer and Extraction Buffer (Thermo) were mixed with Protease and Phosphatase Inhibitors (Halt™ Protease and Phosphatase Inhibitor Cocktail) at a ratio of 100:1. The lysis buffer was then added to the cells, and the cells were collected using cell scrapers. The collected cells were transferred to a new Eppendorf tube and incubated on ice for 20 minutes. After 20 minutes, the cell lysates were centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was then collected into a new Eppendorf tube for further experiments.

2.3.2 Protein quantitative

To quantify protein concentration, Bicinchoninic acid assay (BCA assay) was performed with Pierce BCA Protein Assay Kit (Thermo). The Reagent A and Reagent B were mixed as the ratio of 50:1 to prepare reagent buffer. The different concentrations (2000, 1000, 500, 250, 125, 62.5 and 31.25) of BSA standard were prepared by serial dilution, and the protein were diluted about 10 times. Then, 200 µl reagent buffer, 25 µl of diluted protein samples or BSA standards were added to 96 wells in duplicate or triplicate, incubated at 37°C for 30 min. After incubation, the concentrations of protein were measured by spectrophotometer at the absorbance at 562 nm.

2.3.3 Protein samples preparation

After quantitative, proteins were further prepared for western blotting. First, 4x protein loading dye were mixed with 2-Mercaptoethanol as the ratio of 9:1 to make

protein loading dye, and the protein were further mixed with protein loading dye as the ratio of 4:1. Then the protein samples were heated at 95°C for 10 min to denature protein 3°structure and cool down on ice for 1-2min. The protein samples were stored at -20°C.

2.3.4 Electrophoresis

To investigate the protein expression, the protein sample were separated by SDS-PAGE electrophoresis. 20-30 µg of protein samples were loaded into 12% SDS-PAGE gel, and start electrophoresis at 90V for stacking gel and 110V for separating gel. After electrophoresis, protein would be transfer to Immobilon-E PVDF membrane (Immobilon) at 100V for 90 min. Then, blocked the membrane with 5% non-fat milk in 0.1%TBST at 50 rpm, room temperature for at least 1hour. The primary antibodies were diluted in the 5% non-fat milk in 0.1% TBST with the following ration: 2A7 anti-HBcAg antibody (mouse mAb, kindly provided by Dr. Shiou-Hwei Yeh, 1:2000), E11E4 anti-HBsAg antibody (mouse mAb, kindly provided by Xiamen University, 1:500), anti-G3BP1 antibody (mouse mAb, Santa Cruz, 1:500) and anti-β-actin antibody (Merck, 1:100000). The membrane were incubated with primary antibody at 4°C overnight or at room temperature for 1 hour, and washed by 0.1% TBST 3 times at room temperature in 100 rpm. For preparing secondary antibodies, Anti-mouse antibody was diluted as 1:20000 for detected HBcAg, HBsAg, and G3BP1 and 1:30000 for detected β-actin. The membranes were next incubated with these antibodies at room temperature in 50 rpm for

at least 1 hour, then washed by 0.1% TBST 0.1% TBST 3 times at room temperature in 100 rpm. Finally, the protein signals on the membrane would be visualized and detected by using Clarity Western ECL Substrate and Amersham Hyperfilm ECL (GE Healthcare).

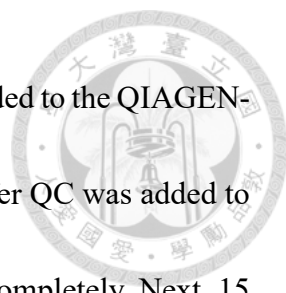
2.5 Plasmid extraction (Mini)

The High-speed plasmid mini kit (Geneaid) was used for plasmid extraction (mini). A single colony of successfully transformed cells was picked and transferred to 2 ml of Lysogeny Broth (LB) containing 0.1% Ampicillin. The culture was incubated at 37°C for 16 hours. The following day, 1.5 ml of the bacterial culture was collected into an Eppendorf tube and centrifuged at 16,000 x g for 1 minute. The supernatant was discarded. The cell pellet was resuspended in 200 µl of PD1 Buffer. Subsequently, 200 µl of PD2 Buffer was added, and the mixture was inverted 10 times and incubated at room temperature for 2 minutes. Then, 300 µl of PD3 Buffer was added, and the mixture was inverted another 10 times. The mixture was centrifuged at 16,000 x g for 3 minutes. The supernatant was transferred to the PD column placed in a collection tube and centrifuged at 16,000 x g for 30 seconds. The flow-through was discarded. Next, 400 µl of W1 Buffer was added to the PD column, followed by centrifugation at 16,000 x g for 30 seconds. After removing the flow-through, 600 µl of Wash Buffer was added to the PD column and centrifuged at 16,000 x g for 30 seconds. The flow-through was discarded again, and the PD column was centrifuged once more at 16,000 x g for 30 seconds to remove any

residual buffer. Finally, the PD column was transferred to a new Eppendorf tube. To elute the plasmid DNA, 50 μ l of Elution Buffer was added to the column, incubated at room temperature for 2 minutes, and then centrifuged at 16,000 x g for 2 minutes. The extracted plasmid DNA was stored at -20°C.

2.6 Plasmid extraction (Maxi)

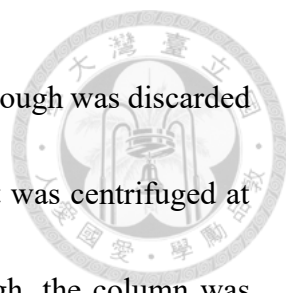
The EndoFree Plasmid Maxi Kit (Qiagen) was used for plasmid extraction (maxi). First, 1 ml of the bacterial culture containing successfully transformed bacteria was added to 200 ml of LB broth containing 0.1% Ampicillin. The culture was incubated at 37°C and 100 rpm for at least 16 hours. The following day, the cultured bacteria were transferred to a centrifuge bottle and centrifuged at 6,000 x g and 4°C for 15 minutes. The resulting cell pellet was resuspended in 10 ml of Buffer P1 by vortexing. Then, 10 ml of Buffer P2 was added and the mixture was gently inverted until mixed thoroughly, followed by incubation at room temperature for 5 minutes. After 5 minutes, 10 ml of Buffer P3 was added, and the mixture was gently inverted until the blue solution turned colorless. The mixture was then centrifuged at 6,000 x g and 4°C for 10 minutes. The supernatant was transferred to a QIAfilter Cartridge, and the plunger was used to filter the lysate. After filtration, 2.5 ml of Buffer ER was added to the filtered lysate, and the mixture was gently inverted 10 times to mix. The mixture was then incubated on ice for 30 minutes. During the incubation, 10 ml of Buffer QBT was added to a QIAGEN-tip 500



to equilibrate it. After 30 minutes, the incubated filtered lysate was added to the QIAGEN-tip 500 and allowed to flow through completely. Then, 30 ml of Buffer QC was added to the QIAGEN-tip 500 twice, allowing each volume to flow through completely. Next, 15 ml of Buffer QN was added to elute the DNA. The eluted DNA was collected into a 50 ml tube, and 10.5 ml of isopropanol was added, gently inverting the mixture 10 times to mix. The mixture was centrifuged at 12,000 x g and 4°C for 30 minutes. The resulting DNA pellet was washed with 500 µl of endotoxin-free ethanol and transferred to a new Eppendorf tube, followed by centrifugation at 12,000 x g and 4°C for 10 minutes, repeated twice. Finally, the DNA pellet was air-dried and resuspended in Water For Injection (WFI). The purified DNA was stored at -20°C for further experiments.

2.7 Virus DNA extraction

The QIAmp DNA Blood Mini Kit (Qiagen) was used for the extraction of viral DNA from cell culture supernatants. First, 100 µl of the culture supernatant was transferred to a new Eppendorf tube, and 200 µl of Buffer AL (lysis buffer) along with 20 µl of Proteinase K were added. The mixture was vortexed and briefly spun down. The mixture was then incubated at 56°C for 10 minutes. After 10 minutes, 200 µl of 100% ethanol was added, and the mixture was vortexed and spun down. The mixture was transferred to a QIAmp Mini spin column and centrifuged at 8,000 rpm and 25°C for 1 minute. The flow-through was discarded, and 500 µl of Buffer AW1 was added to the column. The column



was centrifuged at 8,000 rpm and 25°C for 1 minute, and the flow-through was discarded again. Next, 500 µl of Buffer AW2 was added to the column, and it was centrifuged at 8,000 rpm and 25°C for 1 minute. After discarding the flow-through, the column was centrifuged again at 13,000 rpm and 25°C for 3 minutes to dry the membrane. After centrifugation, the QIAmp Mini spin column was placed in a new Eppendorf tube, and 100 µl of Buffer AE was added to elute the DNA. The column was incubated at room temperature for 3 minutes, followed by centrifugation at 8,000 rpm and 25°C for 1 minute. The eluted DNA was then stored at -20°C.

2.8 Quantitative PCR (qPCR) detect HBV DNA detection

Quantification of viral DNA in cell culture supernatant was performed using the QuantiNova SYBR Green PCR Kit (Qiagen). First, a standard was prepared using the pCMV/HBV WT (genotype D) plasmid. The master mix was then prepared according to the reaction setup. Subsequently, 15 µl of the master mix and 5 µl of either the standard or purified viral DNA were added to each well of an 8-strip PCR tube. The qPCR was then carried out using the following program on the next page.

Master mix reaction setup (per reaction)

Component	Volume
2x SYBR Green PCR Master Mix	10 µL
QN ROX Reference Dye	2 µL
Primer (HBV 1827) 10 mM	1.4 µL
Primer (HBV 2016) 10 mM	1.4 µL
H ₂ O	0.2 µL
Sample	5 µL
Total	20 µL

PCR program

1	95°C	2 minutes
2	95°C	15 seconds
3	60°C	10 seconds
4	72°C	25 seconds
5	95°C	15 seconds
6	60°C	1 minute
7	95°C	15 seconds

* Step 2-4: 40 Cycles

* Step 6: Melting curve

2.9 Transfection

Huh7 cells were seed on a 15 mm coverslip in a 24-well plate in the density of 2×10^5 .

The next day, perform transfection of the specific plasmid into Huh7 cells using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). First, replace the culture medium with 300 µl of Opti-MEM™ I Reduced Serum Medium (Opti-MEM, Gibco). Next, mix 0.5 µg of the plasmid to be transfected and 1 µl of Lipofectamine™ 2000 reagent with 50 µl of Opti-MEM (each well). Then, combine the mixtures and gently pipette to mix. Incubate the mixture at room temperature for 10 minutes. After incubation, add the mixture to the corresponding wells and incubate at 37°C for 6 hours. After 6 hours,

replace the Opti-MEM with fresh culture medium to complete the transfection.



2.10 Fluorescence microscope

HepAD38 and Huh7 were seeded on 10 mm or 15 mm coverslip in 24 well (HepAD38: 1.2×10^5 to 2×10^5 /well; Huh7: 2×10^5 /well) and incubated at 37 °C, 5% CO₂ (HepAD38 for 2 days; Huh7 for 1 days). After induction or transfection, Culture medium would be changed into fresh culture medium prior 1 hour to stress induction. Then, cells were stressed with 250 μ M sodium arsenite (NaAsO₂) for 30 min (Huh7) or 1 hour (HepAD38) at 37°C, 5% CO₂. After sodium arsenite stress, medium were discarded and cell were washed with 0.05% TBST two times. Cells would be fixed and permeabilized by incubated with 500 μ l of Methanol/Acetone (1:1 mixed) at -20°C for 10 min. Following this step, cells were washed with PBS two times and blocked in blocking solution (10% FBS and 1% Sea block in diluent buffer) for 1 hour at room temperature, and then incubated with following primary antibody diluted in blocking buffer (Mouse Anti-G3BP1 antibody (H-10) 1:400; S162NP rabbit anti-HBc 1:400; Goat anti-HBs 1:500) at 4°C in 25 rpm overnight.

On the next day, Cells were washed with PBS two times and incubated with secondary antibody (Donkey anti-mouse Alexa Fluor-594; Goat anti-rabbit Alexa Fluor 488; and Donkey anti-goat Alexa Flour 647, 1:1000 diluent in blocking buffer) at room temperature in 25 rpm for 1hour. After washed with PBS two times, cells were

subsequently incubated with Hoechst 33342 (1:5000 diluent in PBS) at room temperature for 10 min. Finally, cells were washed with PBS two times and air dried before mounted with mounting buffer. The fluorescence image were examined by Leica TCS SP8 X White Light Confocal Microscope (NTUH Department of Medical Research, The second core laboratory) equipped with 20x objective len or 63x oil objective len. The images were processed using Leica LAS AF Lite screenshots software.



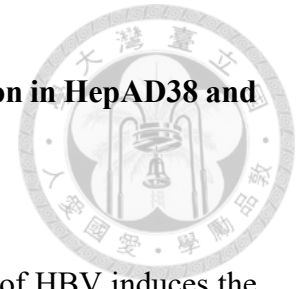
CHAPTER III: Results

3.1 The C-terminal domain (CTD) of HBc exhibits characteristics of intrinsically disordered regions (IDRs)



Some viral proteins have been demonstrated to have highly disordered sequences and exhibit the characteristics of LLPS [141-143]. Moreover, several studies found that HBc can interact with various RNA-binding proteins, similar to many proteins capable of undergoing liquid-liquid phase separation (LLPS) [144, 145]. Therefore, to determine whether HBc has potential intrinsically disordered regions (IDRs) and might be involved in LLPS, we utilized a computational tool called Predictor of Natural Disordered Regions (PONDR) to analyze the amino acid sequence of HBc. This software can analyze and predict segments of a protein's amino acid sequence that are likely to exhibit IDR characteristics. The prediction results indicated that the C-terminal domain, which contains a high number of positively charged amino acids, is the region of HBc most likely to exhibit IDR characteristics (Fig 2). Additionally, this region is also the primary site for RNA interaction. Therefore, HBc may have the ability to undergo LLPS and form membrane-less biological condensates, such as stress granules, through interactions with other proteins and RNA. The C-terminal domain (CTD) is likely the main region of HBc that participates in and regulates LLPS.

3.2 Establishment of a positive control for stress granule formation in HepAD38 and Huh7 cell lines



In this experiment, we aimed to determine whether the presence of HBV induces the formation of stress granules (SGs) in HepAD38 and Huh7 cells. HepAD38 is a cell line containing a CMVtet promoter and an HBV genotype D gene plasmid construct. In the culture medium with tetracycline (tet), HepAD38 does not produce HBV, but upon removal of tet, HBV production is stably induced. Initially, we sought to establish conditions that induce SG formation in cells as a positive control for SGs. Therefore, we applied different concentrations of sodium arsenite (NaAsO₂) to induce oxidative stress in HepAD38 and Huh7 cells. The duration of NaAsO₂ exposure was divided into 30-minute and 1-hour groups. After exposure, we stained the SG marker G3BP1 and the cell nucleus. The results showed that, compared to the 0 μ M group, all concentrations of NaAsO₂ induced significant SG formation in HepAD38 cells (ranging from 89.8% to 100% in the 30-minute group; and from 98.5% to 100% in the 1-hour group) (Fig 3A, C-D). In Huh7 cells, however, treatment with 100 μ M NaAsO₂ for 30 minutes induced stress granules in only a small fraction of cells (9.1%). Therefore, in Huh7 cells, NaAsO₂ requires either a longer treatment duration or concentrations of 250 μ M or higher to consistently induce SG formation (Fig 3B, E-F). We also noted that high concentrations of NaAsO₂ appeared to change cell morphology and adversely affect cell viability, leading

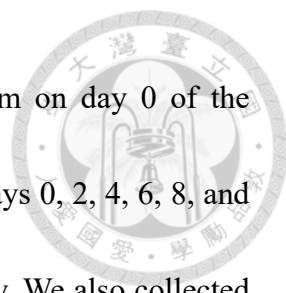
to cell death. Therefore, we chose to use 250 μ M NaAsO₂ to treat HepAD38 for one hour and Huh7 for 30 minutes as a positive control for SGs formation.



3.3 HBV genotype D does not generally induce or affect stress granule formation in HepAD38 cells

Next, in order to observe the formation of stress granules in HepAD38 cells at different days post-HBV induction, we first tested which antibodies could effectively stain HBV antigens in HepAD38 cells. We used two primary antibodies, S162NP rabbit anti-HBc antibody and goat anti-HBs antibody, to stain HepAD38 cells continuously cultured in doxycycline-free medium. Additionally, we performed double staining with anti-G3BP1 antibody (H-10). The results showed that the goat anti-HBs antibody failed to effectively detect HBs signals in HepAD38 cells at any of the three dilution concentrations (1:200, 1:300, and 1:400) (Fig 4A). However, the S162NP rabbit anti-HBc antibody could effectively detect HBc signals in HepAD38 cells at all three dilution concentrations (1:300, 1:400, and 1:500) (Fig 4B). Therefore, we decided to use the S162NP rabbit anti-HBc primary antibody at a 1:500 dilution concentration in subsequent experiments to stain HBc in the cells, aiming to investigate the relationship between HBV and stress granules formation.

Next, we observed the formation of stress granules in HepAD38 cells after different days of HBV induction. We seeded HepAD38 cells in a culture medium containing



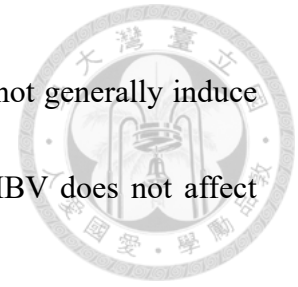
doxycycline (dox) and then switched to a dox-free culture medium on day 0 of the experiment. We then stained for G3BP1, HBc, and the nucleus on days 0, 2, 4, 6, 8, and 10 after induction and analyzed their signals by confocal microscopy. We also collected the culture supernatant and cells for HBV DNA titer and virus protein detection. The results showed that the HBeAg titer in both the +Dox and -Dox groups consistently increased from day 0 to day 6 post-induction. However, after day 6, the HBeAg titer in the -Dox group gradually diverged from that in the +Dox group. The HBeAg titer in the -Dox group continued to rise significantly, while the titer in the +Dox group remained relatively stable, with values similar to those observed on day 6. (Fig 5A). In contrast, the HBsAg levels in both the +Dox and -Dox groups rapidly increased from day 0 to day 4 post-induction and then slightly decreased after day 4. (Fig 5B). The HBV DNA titer continuously increased with the days of induction (Fig 5C). We also detected intracellular HBsAg, HBcAg, and G3BP1 expression by Western blotting. The results showed that the expression level of intracellular S-HBsAg gradually increased, while the expression of L-HBsAg slightly decreased with the days of induction. HBcAg signals were undetectable on days 0 and 2 of induction but became detectable from day 4 onwards, with expression levels increasing with the days of induction. The expression level of G3BP1 did not change with the days of HBV induction (Fig 5D). These observations indicated that the removal of doxycycline successfully induced HBV production in HepAD38 cells and that

HBV did not affect the expression of G3BP1.



In the staining results from HepAD38 cells cultured in medium containing doxycycline, we observed the formation of stress granules in only a very small number of cells on day 6 post-induction (3.2%) and on days 10 (0% and 0.9%) (Fig 6D and F-G, +Dox group). However, significant stress granule formation (92.3-100%) was observed in HepAD38 cells treated with 250 μ M NaAsO₂ for one hour (positive control for SG formation) (Fig 6A-H, +Dox+NaAsO₂ group). In doxycycline-removed cells, most cells did not exhibit stress granule formation with increasing days of HBV induction (Fig 6A-H, -Dox group). Nevertheless, we observed the formation of stress granules in a small number of cells on days 4 (2.1% and 0%), 6, (8.7% and 3.5%) and 8 (8.9% and 6.8%) post-induction (Fig 6C-E, -Dox group). We also treated cells with NaAsO₂ at different days post-induction to observe the co-localization of HBc and stress granules (Fig 6A-F, -Dox+NaAsO₂ group). However, compared to the stained positive control group, S162NP rabbit anti-HBc antibody failed to detect HBc signals on all days post-induction. Therefore, we were unable to observe the co-localization of HBc and G3BP1 in the cells. Surprisingly, in the group where HBV was induced and cells were simultaneously treated with NaAsO₂, all cells showed significant stress granule formation across all induction days (98.9%-100%) (Fig 6A-H, -Dox+NaAsO₂ group). This indicates that HBV does not inhibit the formation of typical stress granules.

In summary, these results suggested that HBV genotype D does not generally induce stress granule formation and also demonstrates that genotype D HBV does not affect stress granule formation induced by oxidative stress.



3.4 HBV genotype A does not generally induce stress granule formation in Huh7 cells

Previous studies have indicated that genotype A, rather than genotype D, of HBV can induce stress granule formation in cells [139]. Therefore, we aimed to observe whether different genotypes of HBV exhibit differences in inducing stress granules by transfecting pAAV/HBV1.2 genotype A (Fig 7A) into Huh7 cells. First, we screened antibodies suitable for staining HBV genotype A antigens. We transfected Huh7 cells with the pAAV vector only plasmid as the control group and pAAV/HBV1.2 (genotype A) plasmids in another group. Culture medium was collected on days 1 to 3 post-transfection to measure HBeAg and HBsAg levels to verify successful transfection.

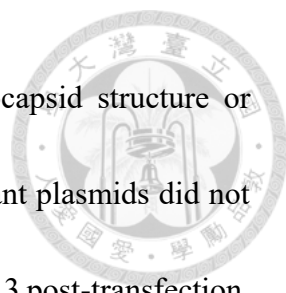
The antibodies we tested in this experiment included S162NP rabbit anti-HBc antibody, 155P rabbit anti-HBc antibody, CS170 rabbit anti-HBc antibody, and goat anti-HBs antibody, with double staining performed using mouse anti-G3BP1 antibody (H-10). The results showed that S162NP rabbit anti-HBc antibody at a 1:500 dilution, and the 155P rabbit anti-HBc antibody and CS170 rabbit anti-HBc antibody at 1:300 or 1:500 dilutions, failed to effectively stain HBc in cells transfected with pAAV/HBV1.2 (genotype A) plasmids, regardless of the post-transfection day (Fig 7B-D). Only goat anti-HBs



antibody staining could detect HBs expression in cells on days 1, 2, and 3 post-transfection (Fig 7E). Therefore, we selected this antibody for subsequent experiments. However, we also noticed that the nuclear signals in the stained cells were blurry and lacked distinct boundaries, suggesting potential microbial contamination. To verify this, we performed PCR on the culture supernatant and confirmed *Mycoplasma* infection (Fig 7F, Huh7 vector and Huh7 HBV group, red arrow). Therefore, we once again transfected Huh7 cells with either the pAAV vector or pAAV/HBV1.2 plasmid and observed the formation of stress granules in cells expressing HBs. In the results, we did not discover stress granule formation in HBs-positive cells on any day post-transfection (Fig 8A-C, HBV group). Thus, these results suggested that HBV genotype A may not induce stress granule formation in Huh7 cells.

3.5 HBV proteins and splice RNA are not related to stress granules

In the previous results, we demonstrated that the presence of HBV may not induce the formation of stress granules. This indicates two possibilities: (1) HBV itself does not induce the production of stress granules, or (2) HBV has mechanisms that inhibit the formation of stress granules. Therefore, we aimed to test whether HBV proteins or splice RNAs could inhibit the formation of stress granules. Thus, we transfected Huh7 cells with pAAV/HBV1.2 plasmids containing various mutation sites and stained for G3BP1 and HBs in the cells (Fig 9A). These mutations prevent the production of specific HBV

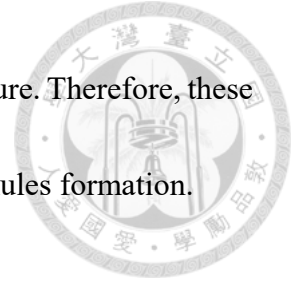


proteins (HBc, HBe, or HBx) and spliced RNA, or affect nucleocapsid structure or formation. The results showed that transfection with any of the mutant plasmids did not result in the formation of stress granules in the cells from day 1 to day 3 post-transfection. (Fig 9B-D); stress granules only formed in the positive control group for stress granules formation (Fig 9B-D, Mock+NaAsO₂ group). Therefore, this experiment indicated that the specific HBV proteins or spliced RNA do not have the ability to inhibit stress granule formation, suggesting that HBV genotype A may not induce stress granule formation inherently, rather than by inhibiting their formation by HBV proteins or spliced RNAs investigated in this study.

3.6 The capsid structure is not related to stress granules

Finally, to further investigate whether the complete capsid structure assembled by HBc is related to stress granules, we administered GLS4 to Huh7 cells one day after transfection with either the pAAV vector or pAAV/HBV1.2 plasmid (Fig 10A). GLS4 is a core protein allosteric modulator (CpAM) reported to have anti-HBV activity and to induce the assembly of HBV core protein into aberrant capsid structures [146]. After treatment, we observed no stress granule formation in both the DMSO control group and the GLS4-treated Huh7 cells (Fig 10C and D). This was consistent with observations in the group transfected with the plasmid containing the HBc Y132A mutation (Fig 9B-D,

HBc Y132A group), which restricts HBc assembly to a dimeric structure. Therefore, these results suggested that the capsid structure is not related to stress granules formation.



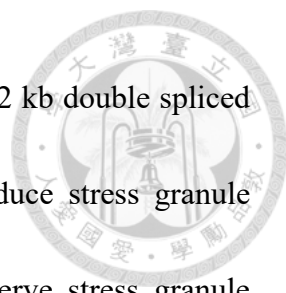
CHAPTER IV: Conclusion and Discussion



The relationship between HBV and the innate immune system has long been a challenging enigma. In our previous studies, we found that the presence of HBc is crucial for the clearance of HBV and the induction of adaptive immune responses in mice [67]. However, the mechanism of HBc activates the innate immune response prior to initiating adaptive immunity remains unclear. Thus, we hypothesized that HBc might have the ability to undergo liquid-liquid phase separation (LLPS) and involve in the formation of stress granules, thereby activating the innate immune response.

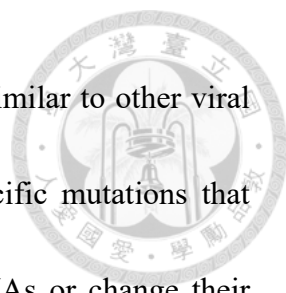
In this study, we first utilized an induction system to observe whether HepAD38 cells form stress granules under different HBV titers. During the 10-days induction of HBV production, we found that despite the gradual increase in HBV levels, most cells did not exhibit significant stress granule formation. Nevertheless, we observed a minimal number of stress granules in HepAD38 cells between days 4, 6 and 8 post-induction. This results suggested that even under the same stress conditions, the formation of stress granules might depend on cellular heterogeneity and the formation of stress granules induced by HBV is not a typical phenomenon. In summary, due to the rarity of stress granule-forming cells, we conclude that HBV genotype D cannot generally induce stress granule formation in cells.

Many viral RNAs have been shown to induce stress granule formation and colocalize



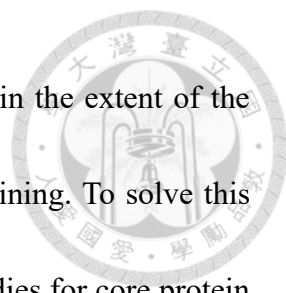
with stress granules [119, 124]. Previous studies reported that the 2.2 kb double spliced RNA (2.2DS-RNA, Sp10) produced by HBV genotype A can induce stress granule formation in cells [139]. However, in our study, we did not observe stress granule formation in Huh7 cells transfected with pAAV/HBV1.2 genotype A plasmids. Thus, we believe that this phenomenon likely depends on the amount of 2.2 kb double-spliced RNA present in the cells, this might explain why we did not observe the same phenomenon in our model. Thus, we concluded that HBV genotype A may not induce stress granule formation in transfection model.

Viruses may inhibit stress granule formation through different mechanisms. For example, poly (I:C)-induced stress granule formation occurs via the PKR pathway, while NaAsO₂-induced stress granule formation occurs via the HRI pathway. The N proteins of SARS-CoV-2 and SARS-CoV can inhibit stress granule formation induced by poly (I:C) and NaAsO₂ by interacting with G3BP1 and inhibiting eIF2 α phosphorylation caused by PKR. However, MERS-CoV only inhibit PKR pathway but not interact with G3BP1 and thus only inhibits stress granule formation induced by poly (I:C) [124]. Therefore, although in our experiments, HBV did not inhibit stress granule formation induced by NaAsO₂, we cannot exclude the possibility that HBV might inhibit stress granule formation via other pathways. Thus, we subsequently hypothesized that the inability of cells to form stress granules in the presence of HBV might be due to the capability of



HBV proteins or nucleic acids to inhibit stress granule formation, similar to other viral proteins. To test this, we used pAAV/HBV1.2 plasmids with specific mutations that prevent the production of particular HBV proteins and spliced-RNAs or change their structure. We also administered core protein allosteric modulators (CpAM) to the cells to observe whether aberrant capsid structures would affect the formation of stress granules. However, we did not find that the absence of any specific protein or double-spliced RNAs led to the formation of stress granules, and the cells treated with CpAM (GLS4) also showed no stress granule formation. That is, the lack of stress granule formation in HBV-infected cells is not due to HBV inhibiting their formation but rather because HBV itself may not be capable of inducing stress granule formation. In this experiment, we used NaAsO₂ to induce stress granule formation. However, it is important to note that anti-viral stress granules differ from typical stress granules, and many viruses induce stress granule formation by inducing PKR activation [88, 118]. Therefore, in future experiments, we could mimic virus-induced stress granules by transfecting poly (I:C) into cells. This approach will help us to better observe the relationship between HBV and stress granule formation.

Our study still has several limitations. First, we were unable to stain HBc in cells using the S162NP rabbit anti-HBc antibody in this study; therefore, we could not determine the exact distribution of the HBV core protein in the cells and observe the co-



localization of HBc and G3BP1. Additionally, we could not ascertain the extent of the impact on the core protein after treatment with CpAMs through staining. To solve this limitation, we may need to screen for more sensitive primary antibodies for core protein staining. Thus, we can use cells directly transfected with HBV core protein expression plasmids for antibody screening and as a positive control for antibody staining. By using this method, we can increase the expression of HBV core protein in cells, allowing antibodies to bind more effectively and directly observe the relationship between HBc and stress granule formation. Alternatively, we could analyze the effects of CpAM on the core protein using methods such as Western blotting after treating the cells with CpAM. Moreover, in this study, the duration of GLS4 treatment was only two days, whereas other studies have applied the treatment for over a week [146]. Therefore, we cannot confirm whether the dosage and treatment duration we used were sufficient to inhibit the core protein in the cells. Consequently, if we continue using this drug in future experiments, we may need to reevaluate the treatment duration and dosage. In this experiment, regardless of whether the drug was applied, we did not observe the formation of stress granules. This indicates that the structure of the core protein does not affect the formation of stress granules. Additionally, we only conducted experiments in transfected and induced cell models, which may differ from natural infection and animal models. Thus, we cannot conclude whether HBV would similarly fail to induce noticeable stress granule

formation in natural infection or animal models. Therefore, we could use cell lines expressing NTCP for infection experiments or employ animal models to further verify our observations.



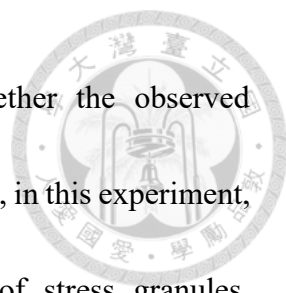
In the study by Zhang et al., it was suggested that the HBV core protein might be a key factor in regulating the distribution of the stress granule marker TIAR within the cell. They also found that TIAR binds to the 5' ϵ element of pgRNA, subsequently regulating viral replication [140]. This suggests that HBV may interfere with the formation of stress granules by hijacking with their components, similar to the observation with ZIKV or HCV [129, 135]. The regulatory factor in this process may be the pgRNA of HBV. However, in our study, the mutant plasmids we used did not include a group with the pgRNA removed. To address this, we could employ plasmids that only express specific HBV proteins without producing pgRNA. This approach would allow us to observe whether specific HBV proteins alone can induce the formation of stress granules within the cell and whether pgRNA inhibits the formation of stress granules. Furthermore, we cannot rule out the possibility that HBV may utilize HBs or HBV pol (which were not examined in our mutant plasmids) to inhibit the formation of stress granules.

The induction of stress granules largely depends on the RNA content within the cell. Recently, it has been noted that the RNA of some viruses can also induce the formation of stress granules, with viral RNA being found as part of the stress granule composition



[119, 124]. Therefore, another point of discussion is the difference between RNA and DNA viruses. Previous research has shown that viruses capable of inducing stress granules are predominantly RNA viruses [88]. These viruses have RNA genomes, and when these viral genomes are released into the cell, there is a significant increase in the RNA content of cell. This may lead to stress granule-associated proteins sensing the viral RNA, resulting in the formation of stress granules. However, as HBV is a DNA virus, the amount of RNA it produces in the cell may not be sufficient to induce the formation of stress granules. This might explain why we did not observe stress granules in cell which HBV is presence.

Recent studies have indicated that G3BP1 aggregation is also found in another RNP complex known as the ribonuclease L (RNase L)-induced body (RLB), which may also participate in antiviral responses [147]. Additionally, Burke et al. noted that during SARS-CoV-2 infection, cells might produce another complex of viral RNA and G3BP1 called "viral aggregated RNA condensates (VARCs)." The formation of VARCs inhibits viral replication, highlighting an alternative antiviral function of G3BP1 aggregation [148]. These findings suggest that G3BP1 aggregation may not solely represent stress granule formation. Therefore, when observing G3BP1 aggregation in cells, it is essential to consider the potential formation of these antiviral complexes. Consequently, future observations and experiments on stress granules should include several stress granule



markers in conjunction with G3BP1 to accurately identify whether the observed aggregates are stress granules or other condensates. On the other hand, in this experiment, only fluorescent methods were used to observe the formation of stress granules. Incorporating other methods, such as FRAP, FRET, or live-cell imaging, could help us more directly observe the formation and characteristics of stress granules in future.

In summary, our current results suggest that there may be no direct correlation between HBV and stress granules. However, we cannot completely rule out the possibility that HBV might influence the formation or disassembly of stress granules through other mechanisms. Additionally, HBV may have associations with other antiviral granules. Our study provides new insights into the relationship between HBV, stress granule formation, and the innate immune response, indicating that the innate immune response induced by HBV might be independent of stress granules.

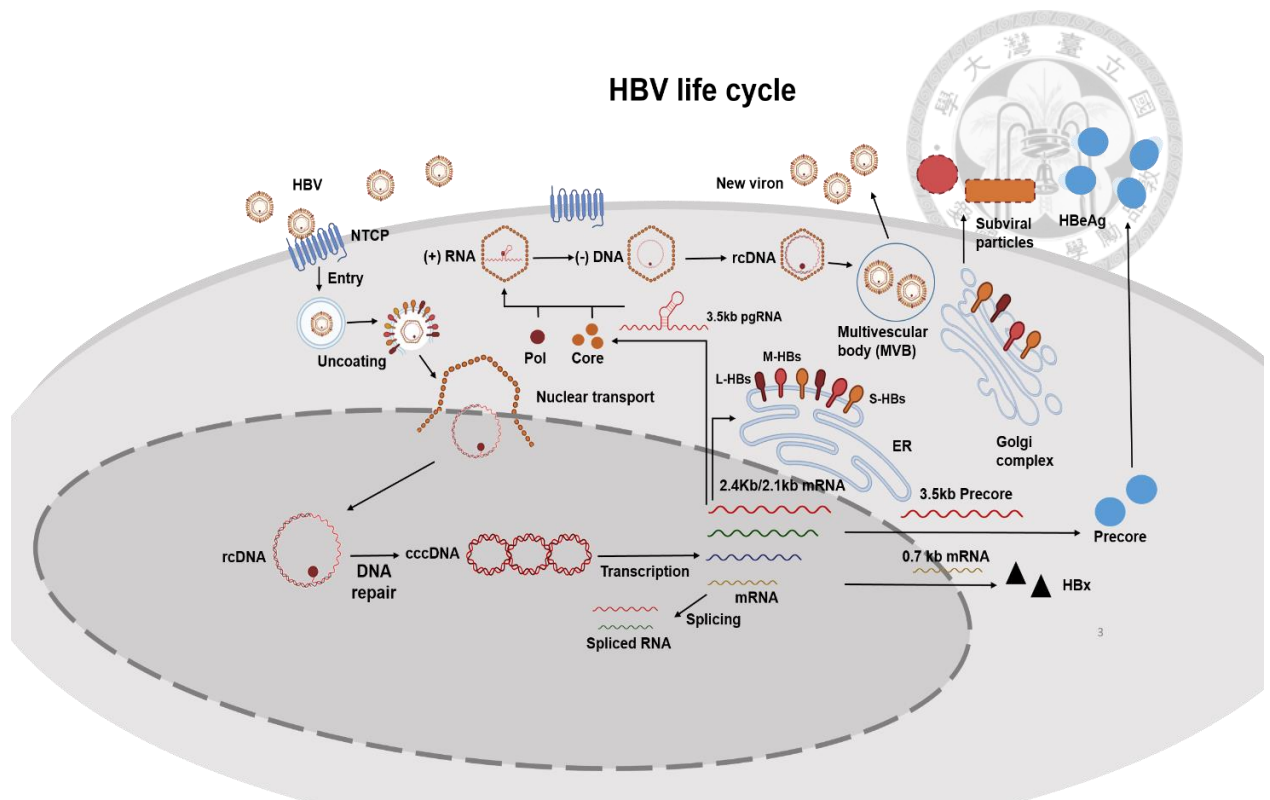


Figure 1. The life cycle of HBV.

After HBV interaction with NTCP and entry host hepatocytes, it undergoes uncoating to release its genome into the nucleus for replication. During replication, cccDNA is produced and serves as a template for transcribing mRNA. Among the mRNAs, 3.5 kb RNA translates into HBeAg, which is released extracellularly; 0.7 kb RNA produces HBx protein; and 2.1 kb and 2.4 kb RNAs forms HBsAg. Additionally, the 3.5 kb RNA, also known as pgRNA, is used to translate the HBcAg and polymerase (pol). Subsequently, HBcAg encapsulates the pol and pgRNA to form the nucleocapsid. Inside the nucleocapsid, pgRNA undergoes reverse transcription to form genomic DNA, finally, the nucleocapsid is assemble with other viral proteins to form new viral particles.

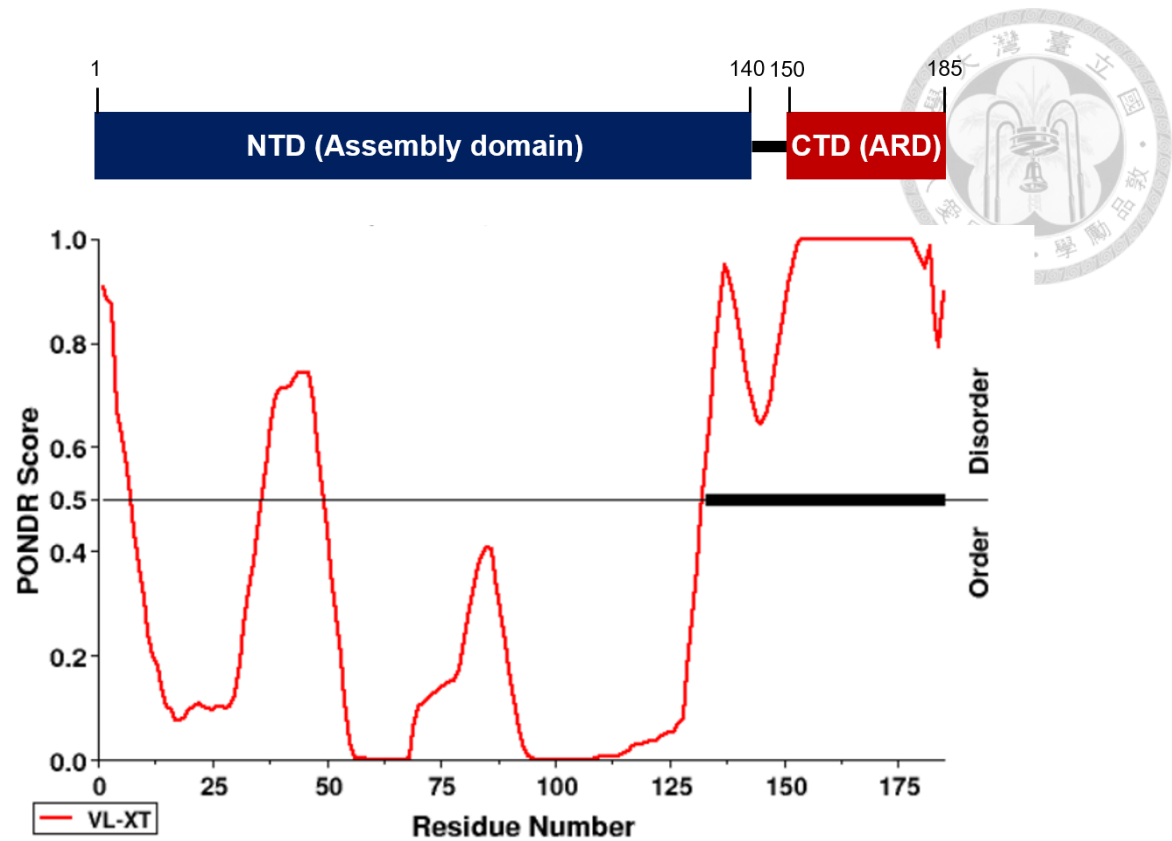
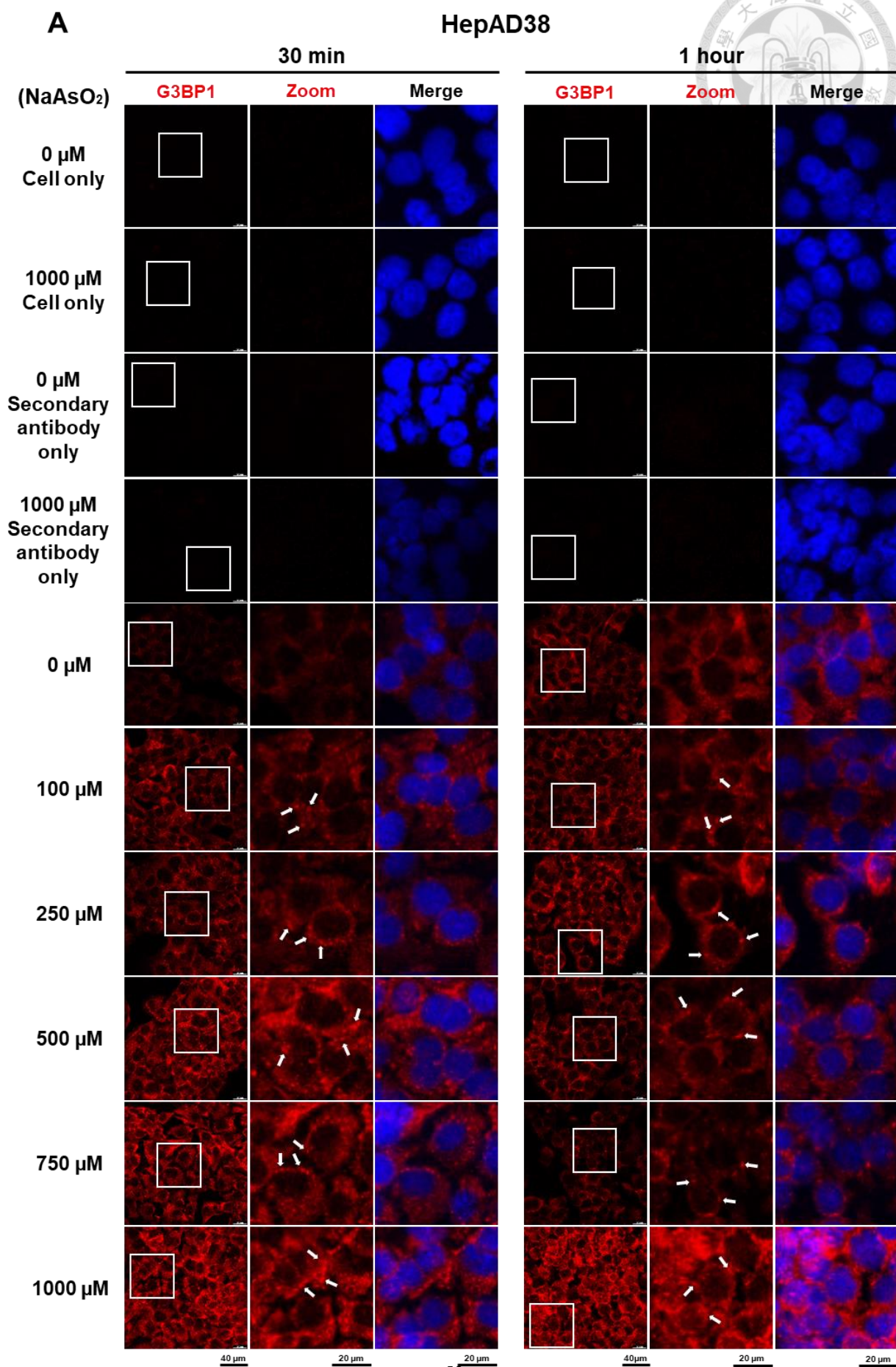
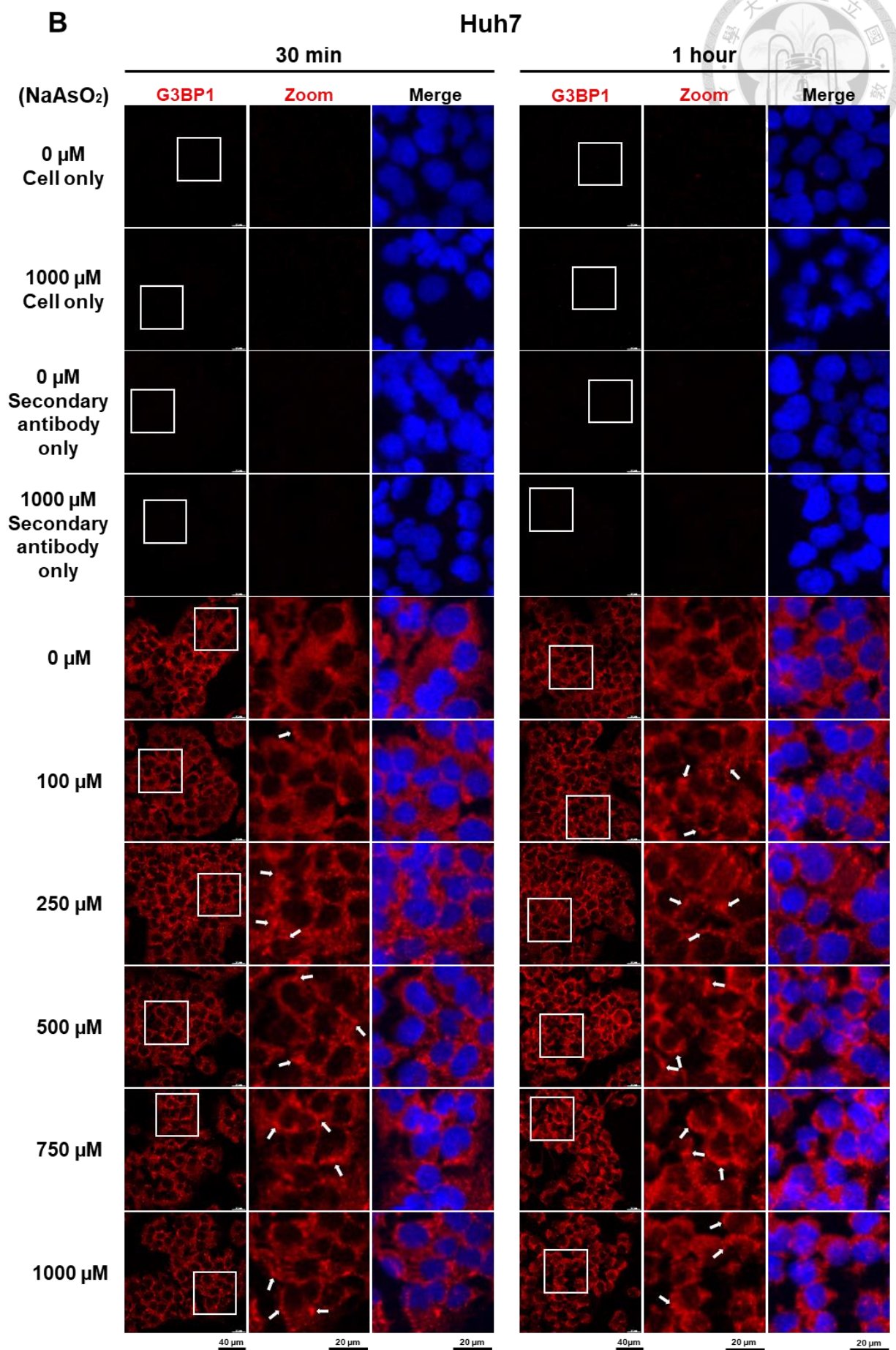


Figure 2. PONDR prediction of HBV core protein

The PONDR VL-XT prediction is given in the plot. In this figure, the score greater than 0.5 suggest disordered residues, while scores below 0.5 suggest ordered residues.





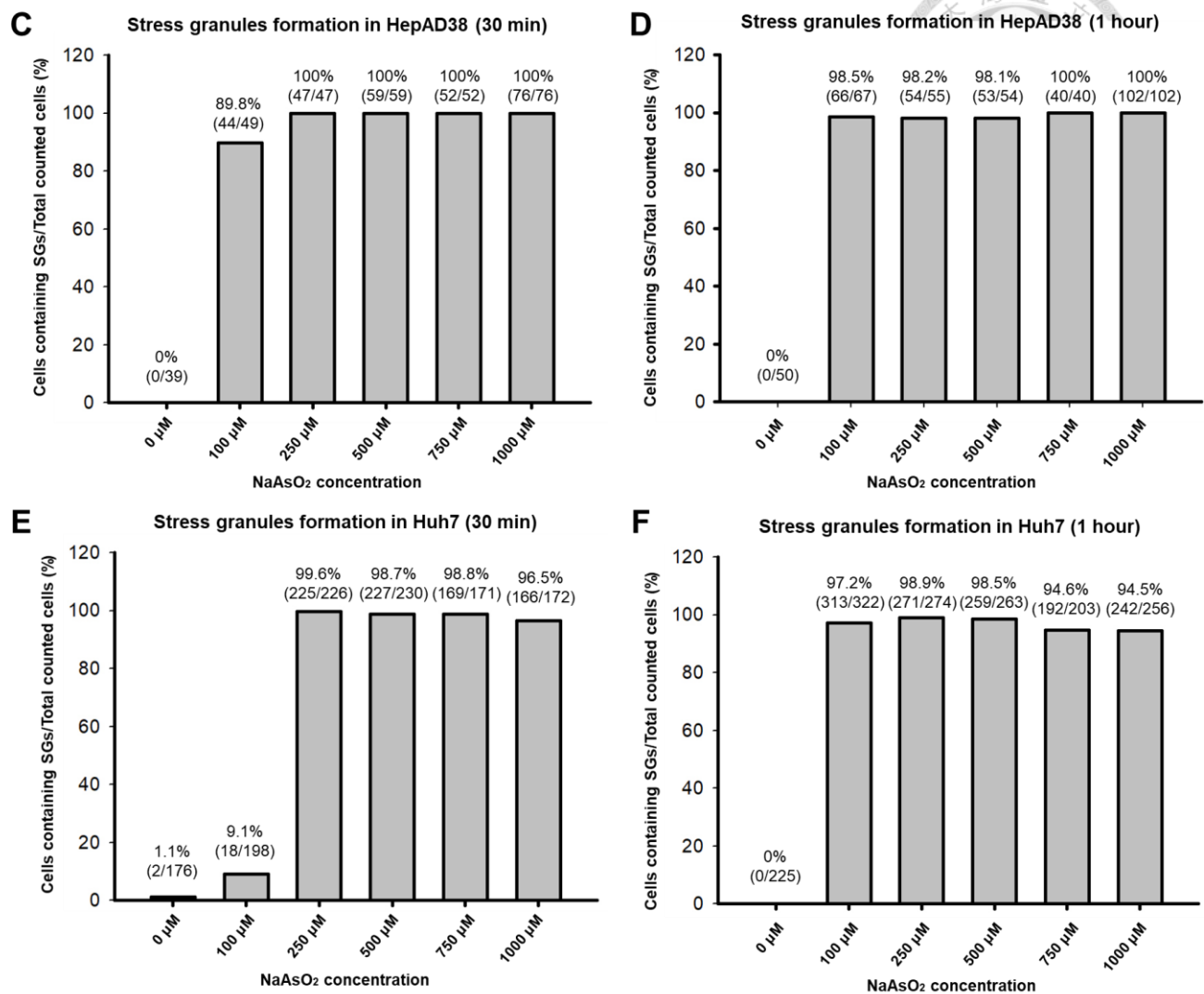
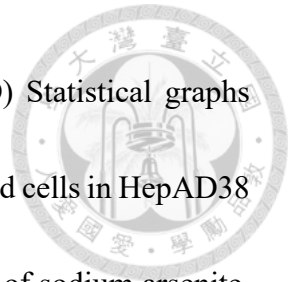


Figure 3. Stress granules formation in different concentration treated HepAD38 cell and Huh7 cell

(A-B) Confocal images of HepAD38 (A) and Huh7 (B) cells after treatment with different concentrations of sodium arsenite. HepAD38 and Huh7 cells were seeded on 15 mm (HepAD38) or 10 mm (Huh7) coverslips in a 24-well plate (2×10^5 cells/well) and treated with 0, 100, 250, 500, 750, or 1000 μ M sodium arsenite for 30 minutes or 1 hour, respectively. G3BP1 (red) was used to indicate stress granule formation (white arrow). Nuclei were stained with Hoechst 33342 (blue). Each group in this experiment was

captured in a single field of view. Scale bar, 40 and 20 μm . (C-D) Statistical graphs showing the ratio of cells containing stress granules to the total counted cells in HepAD38 cells after 30 minutes (C) and 1 hour (D) at different concentrations of sodium arsenite. The counted cells include those from one field of view. (E-F) Statistical graphs showing the ratio of cells containing stress granules to the total counted cells in Huh7 cells after 30 minutes (E) and 1 hour (F) at different concentrations of sodium arsenite. The counted cells include the sum of cells from three fields of view. Cell counting was performed manually, and graphs were generated using SigmaPlot 10.0.



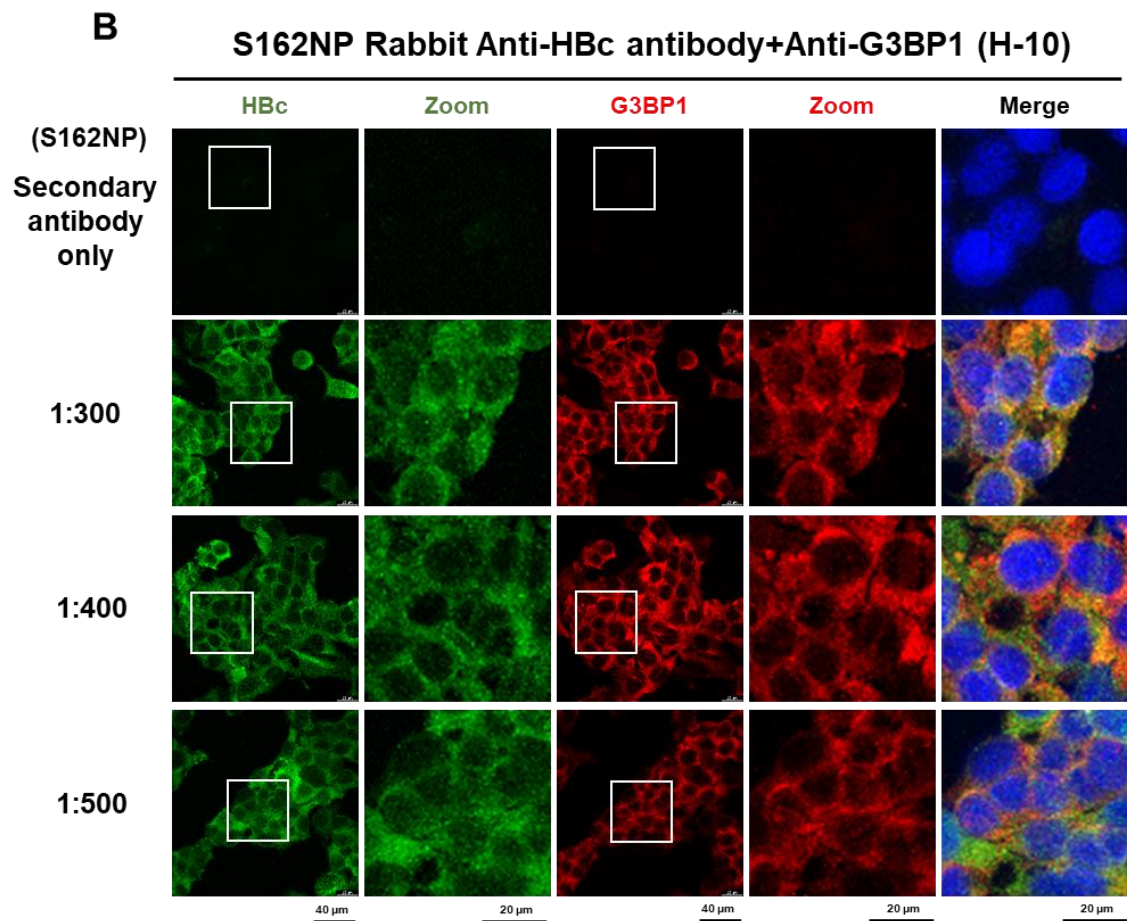
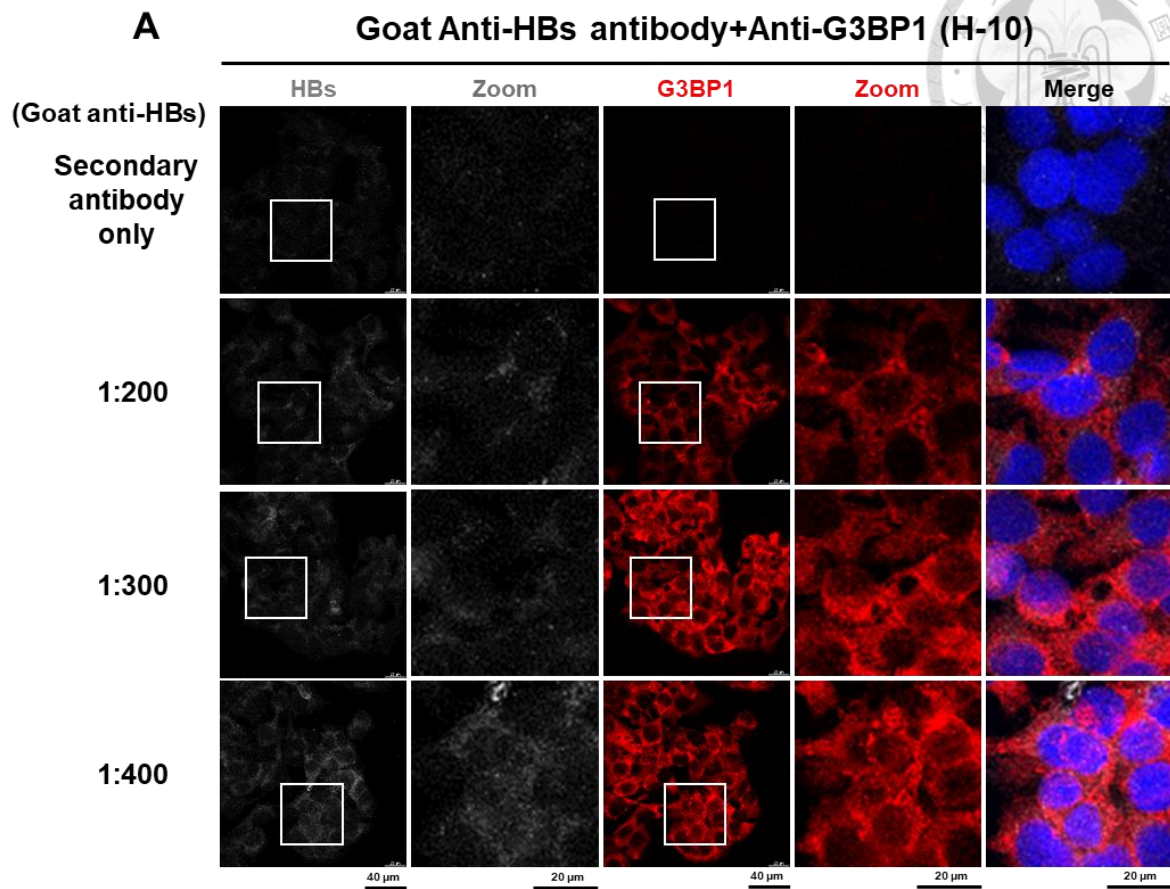
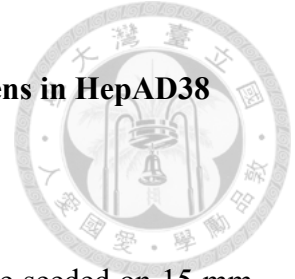


Figure 4. Staining efficacy of different antibodies for HBV antigens in HepAD38 cells



(A-B) HepAD38 cells cultured in medium without doxycycline were seeded on 15 mm coverslips in a 24-well plate (2×10^5 cells/well) for 2 days. The cells were double-stained with mouse anti-G3BP1 antibody (red) and goat anti-HBs antibody (gray, A) or S162NP rabbit anti-HBc antibody (green, B) at different dilution concentrations. Nuclei were stained with Hoechst 33342 (blue). Each group in this experiment was captured in a single field of view. Scale bar, 40 and 20 μm .

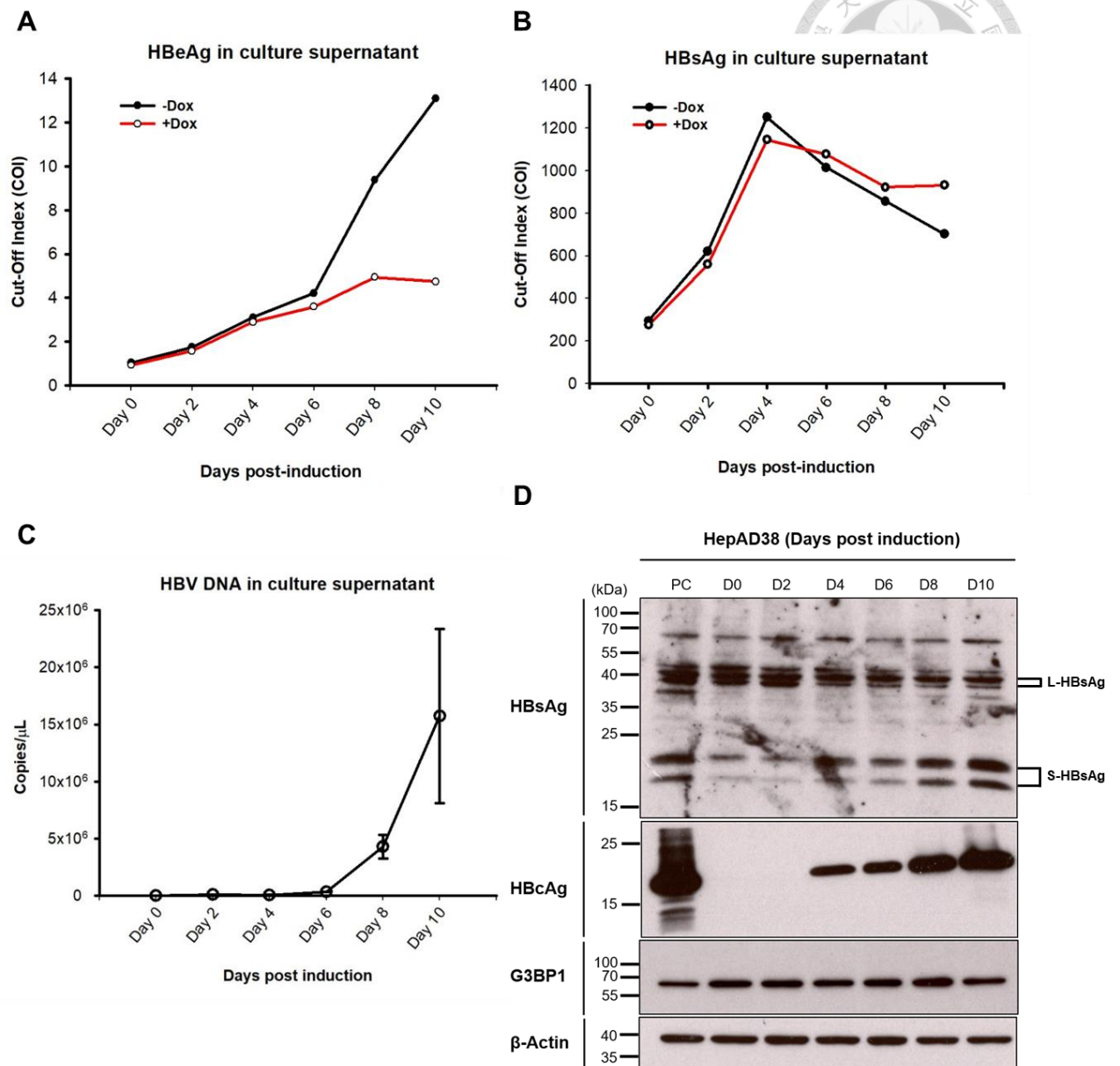
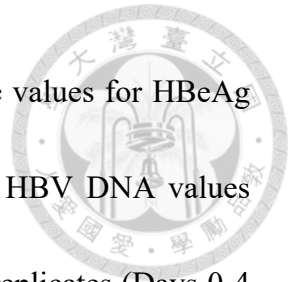


Figure 5. The expression of HBV antigens and DNA from HepAD38 cells at different induction days

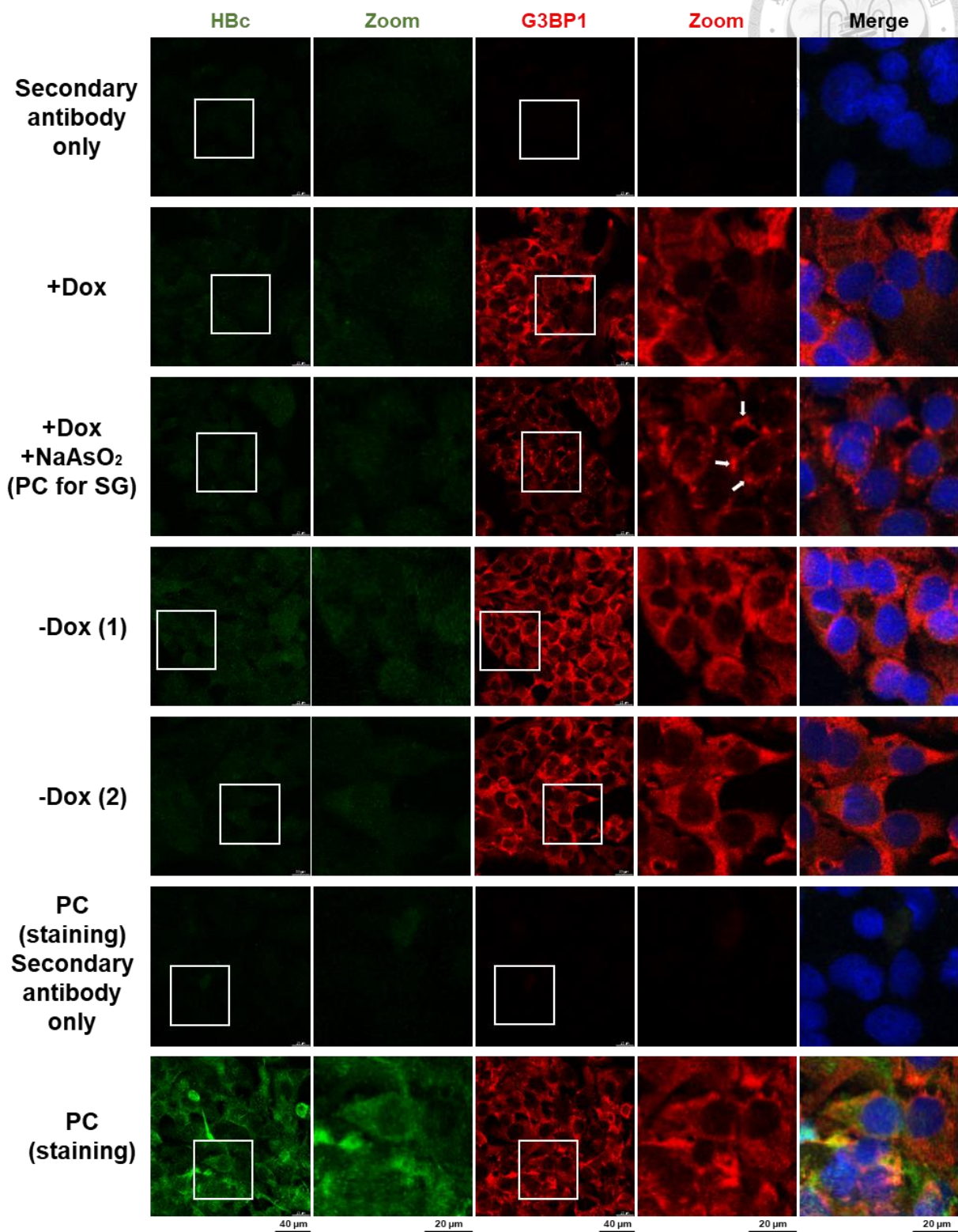
(A-D) HepAD38 cells cultured in medium containing doxycycline were seeded in 6-well plates (5×10^5 cells/well) or on 15 mm coverslips in 24-well plates (1.2×10^5 cells/well) for 2 days. Doxycycline was then removed to start the induction of HBV. At 0, 2, 4, 6, 8, and 10 day post-induction, the culture supernatant was collected to measure HBeAg (A) and

HBsAg (B) by ECLIA, and to detect HBV DNA (C) by qPCR. The values for HBeAg and HBsAg represent the average of 2 biological replicates. The HBV DNA values represent the average $\pm$ s.d. of 3 biological replicates $\times$ 2 technical replicates (Days 0-4 and 8-10 post-induction) or 2 biological replicates $\times$ 2 technical replicates (Day 6 post-induction). The cells were lysed to detect intracellular HBV antigen and G3BP1 expression by Western blotting (D). The positive control is HepAD38 cells kept incubated in culture medium without doxycycline. The graphs were generated using SigmaPlot 10.0.



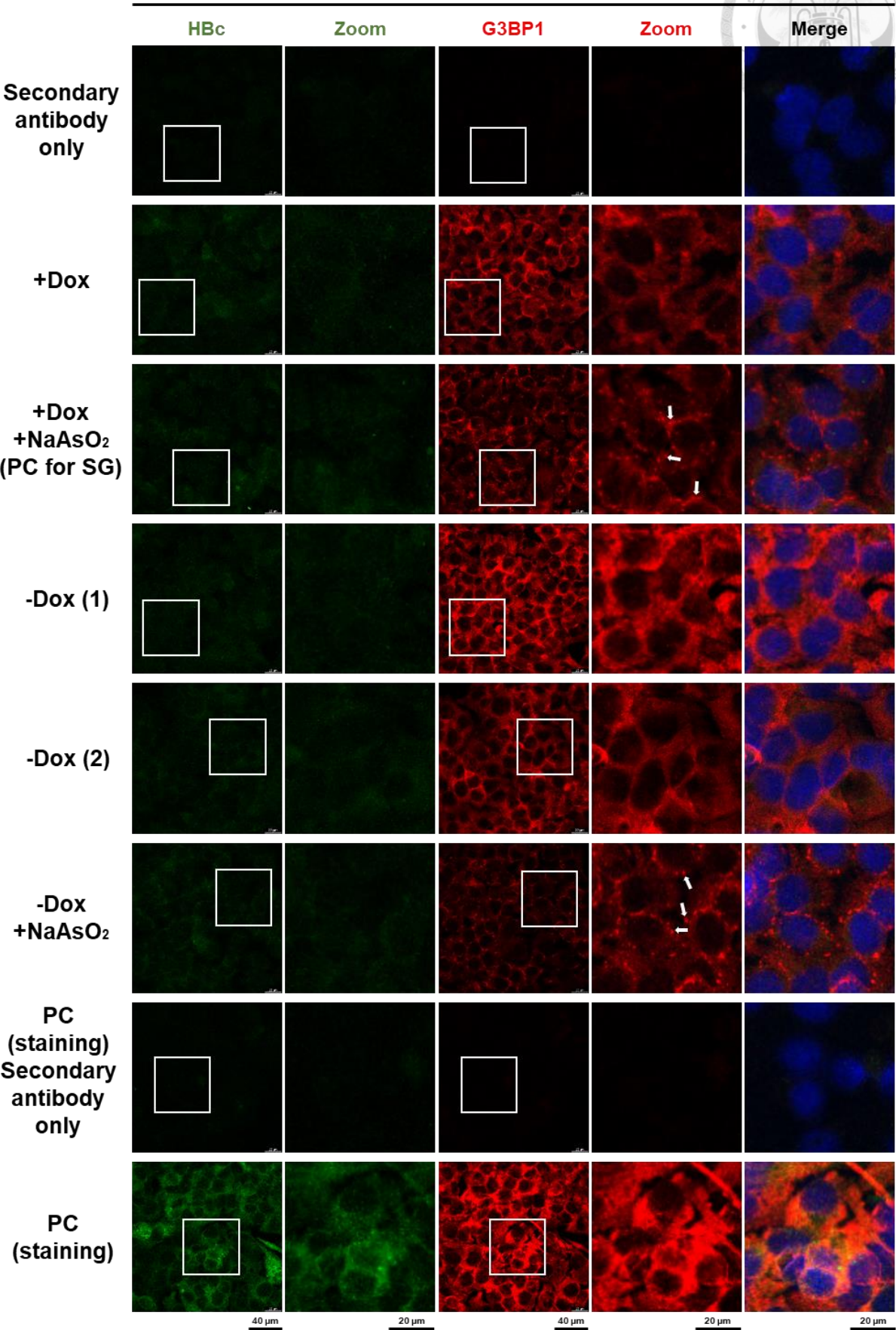
A

HepAD38 Day 0 post-induction



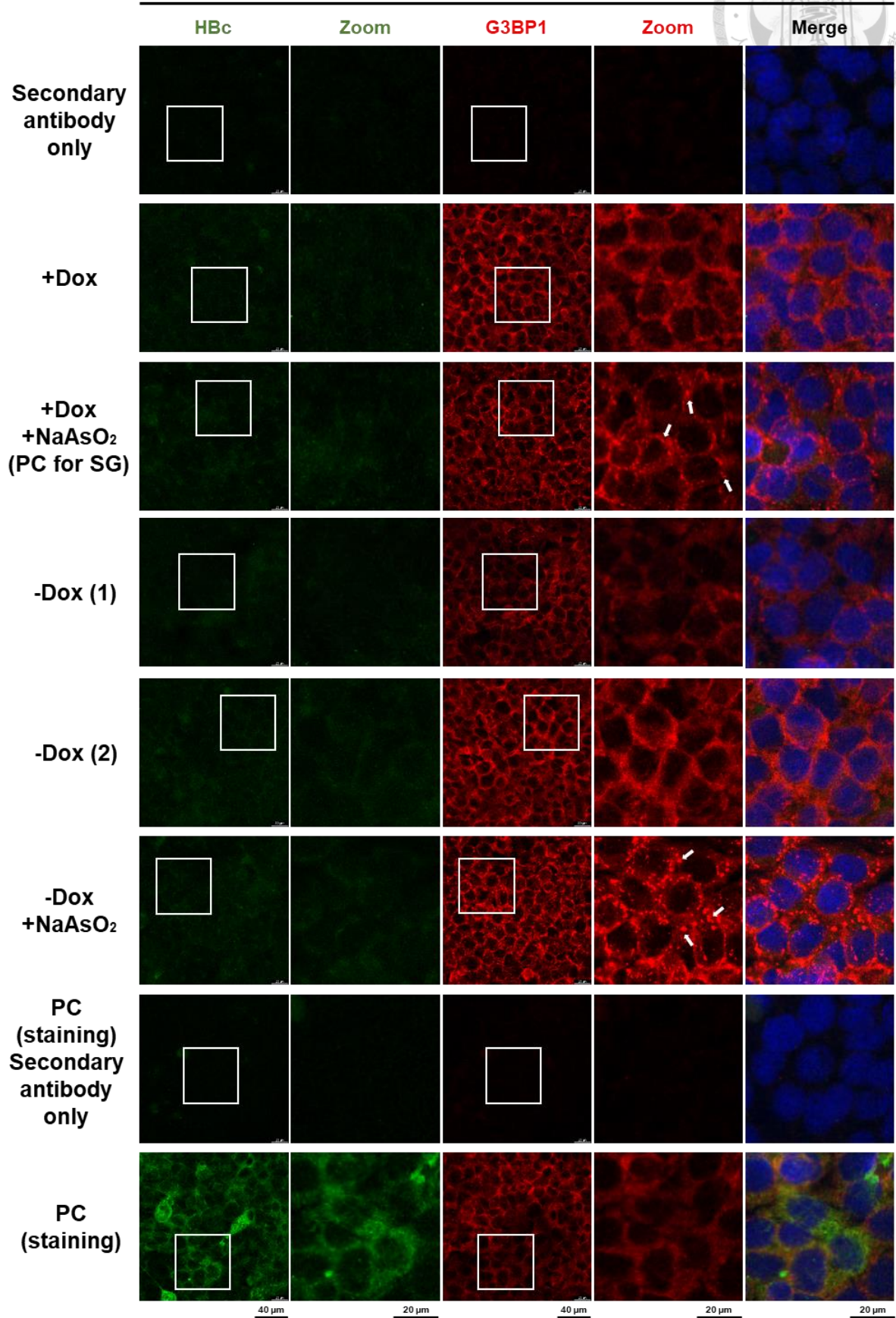
B

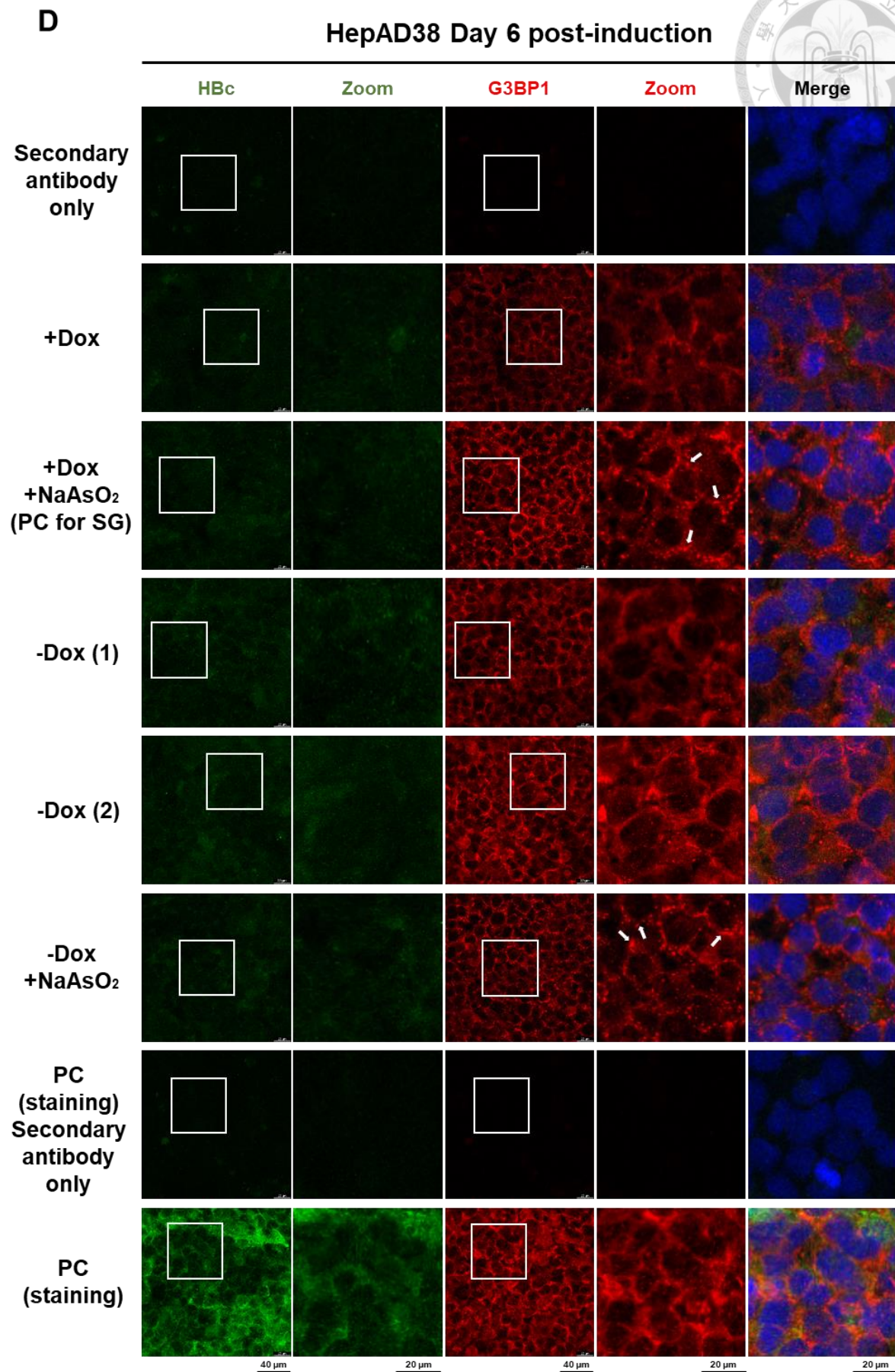
HepAD38 Day 2 post-induction

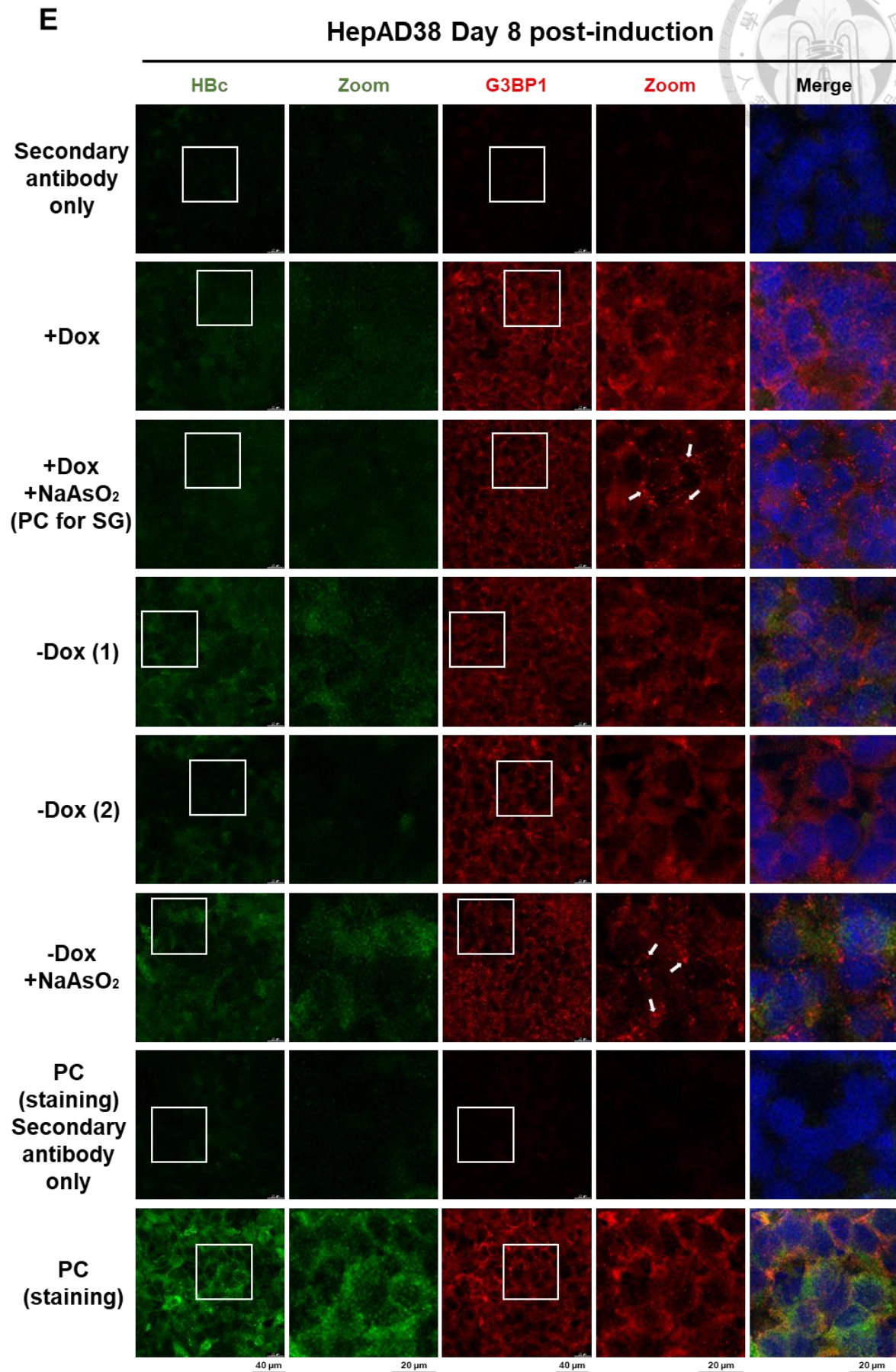


C

HepAD38 Day 4 post-induction

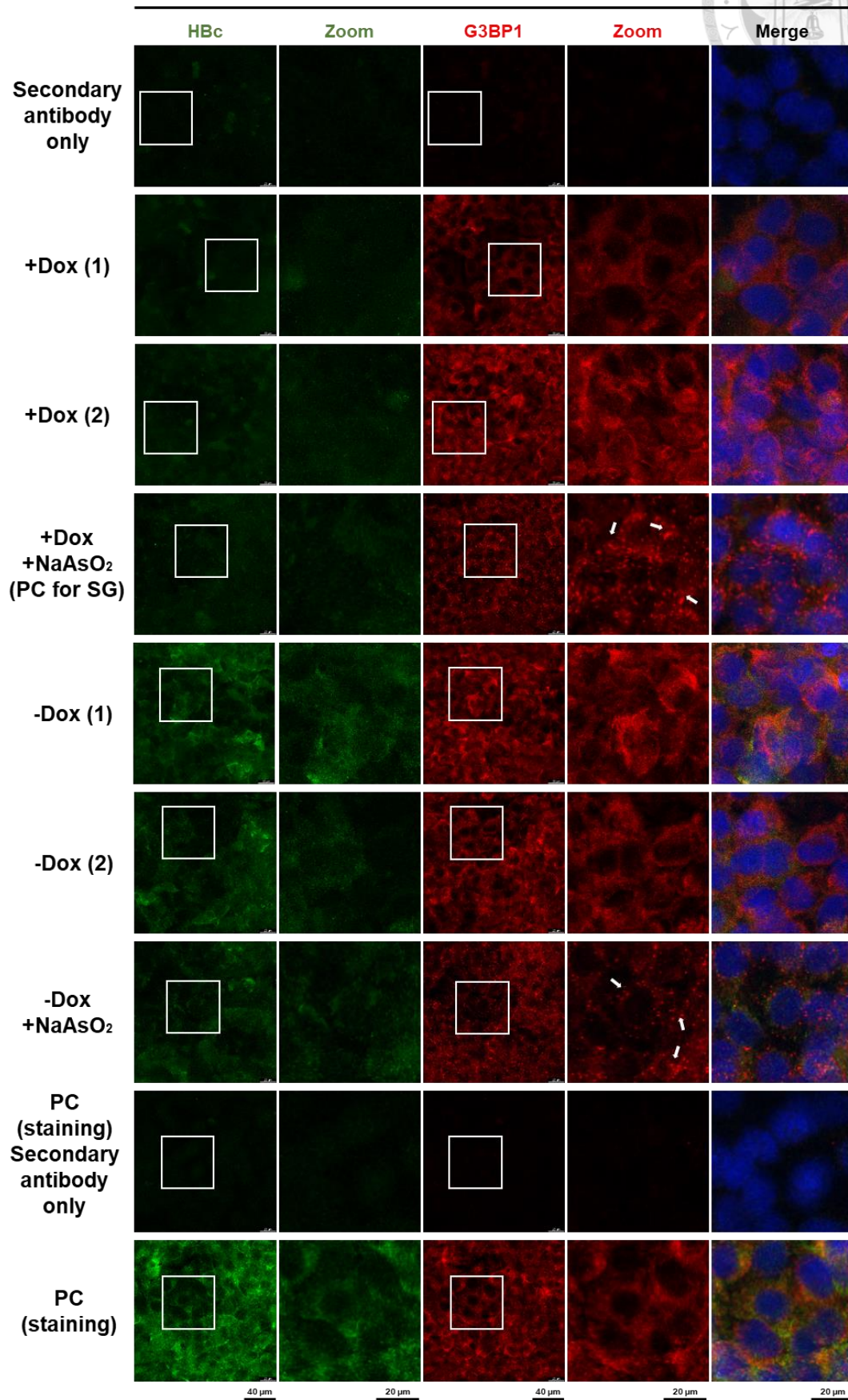






F

HepAD38 Day 10 post-induction



G

The ratio of stress granules formation in HepAD38 after different days induction

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
+Dox (1)	0/38 (0%)	0/63 (0%)	0/97 (0%)	3/94 (3.2%)	0/81 (0%)	0/57 (0%)
+Dox (2)	None	None	None	None	None	1/113 (0.9%)
-Dox (1)	0/64 (0%)	0/65 (0%)	2/94 (2.1%)	10/115 (8.7%)	11/124 (8.9%)	5/108 (4.6%)
-Dox (2)	0/47 (0%)	0/60 (0%)	0/111 (0%)	4/113 (3.5%)	5/74 (6.8%)	5/97 (5.2%)
-Dox+NaAsO₂	None	61/61 (100%)	94/94 (100%)	107/107 (100%)	111/111 (100%)	86/87 (98.9%)
+Dox+NaAsO₂	39/40 (97.5%)	50/54 (92.6%)	109/109 (100%)	105/105 (100%)	143/143 (100%)	92/92 (100%)

*Table header row: Days post-induction of HepAD38.

*Table row header: Treatment applied to HepAD38.

*The data are shown as: Cells containing SGs/Total counted cells (percentage).

*None: This group was not subjected to the experiment.

H

Stress granules formation in HepAD38

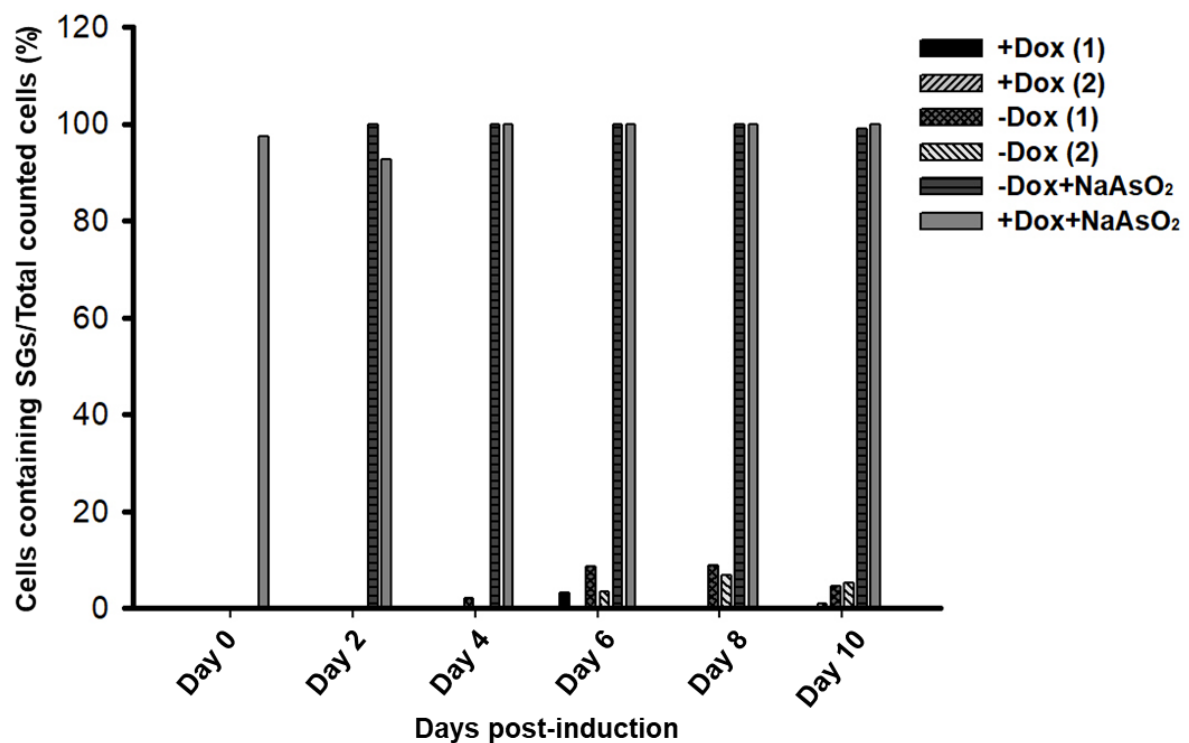
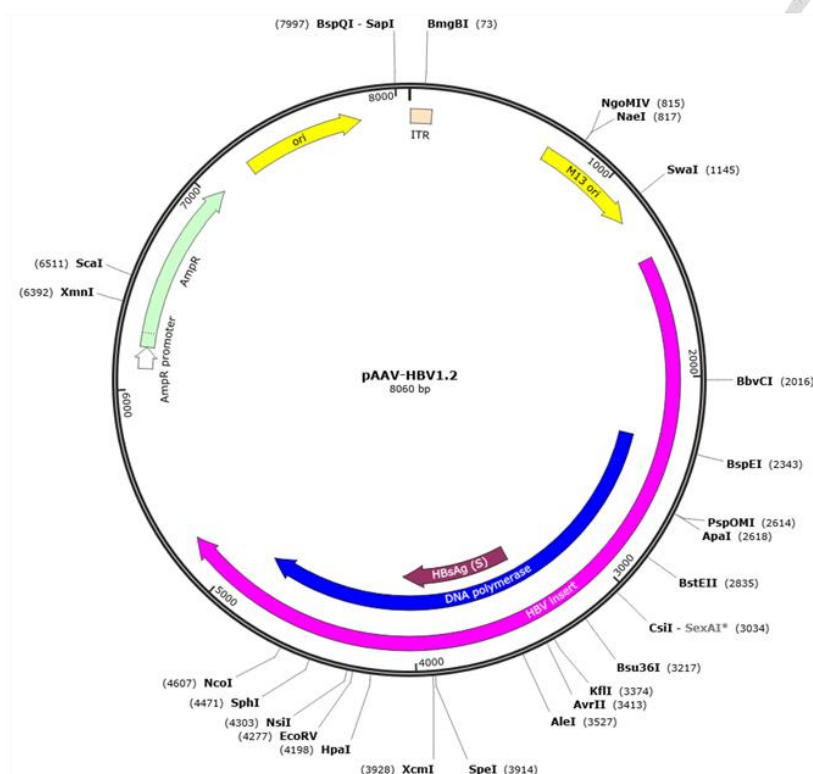


Figure 6. Stress granules formation in HepAD38 cells at different induction days

(A-F) Confocal images showing double-staining with mouse anti-G3BP1 antibody (red) and S162NP rabbit anti-HBc antibody (green) in HepAD38 cells on day 0 (A), day 2 (B), day 4 (C), day 6 (D), day 8 (E), and day 10 (F) post-induction. Nuclei were stained with Hoechst 33342 (blue). The induced HepAD38 cells at days 0-10 post-induction and HepAD38 treated with sodium arsenite at day 10 post-induction were subjected to two biological replicates of the experiment (A-H, -Dox (1) and -Dox (2); F-H, +Dox (1) and +Dox (2)). The induced HepAD38 cells treated with NaAsO₂ were used as the group for the secondary antibody only. HepAD38 cells cultured in medium containing doxycycline and treated with sodium arsenite (+Dox +NaAsO₂) were used as a positive control for stress granule formation. The HepAD38 cells kept in culture medium without doxycycline (PC) were used as a positive control for HBc staining. The white arrow represents the stress granules observed in cells. Each group in this experiment was captured in a single field of view. Scale bar, 40 and 20 μ m. (G-H) The statistical results (G) and graphs (H) showed the ratio of cells containing stress granules to the total counted cells in HepAD38 cells at different day post-induction. The counted cells include those from one field of view. Cell counting was performed manually, and graphs were generated using SigmaPlot 10.0.

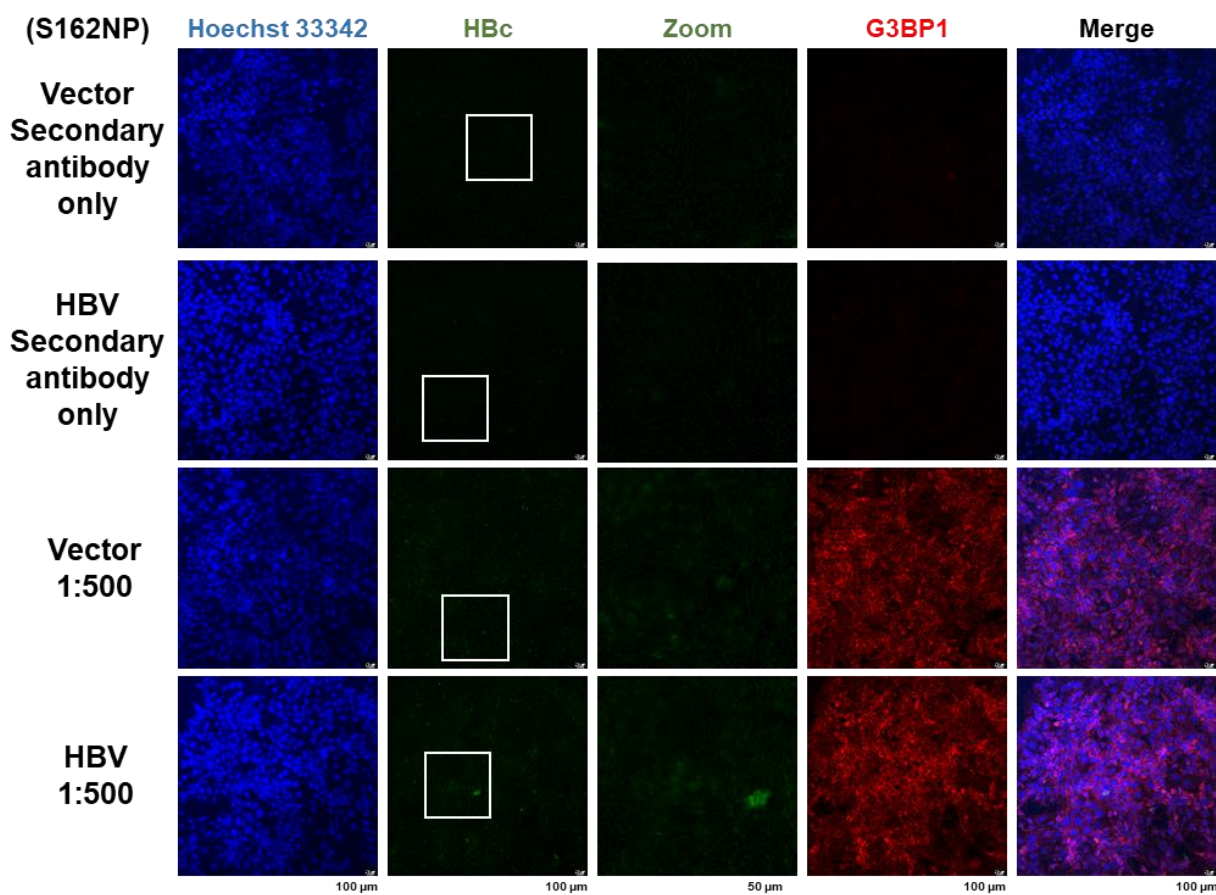


A

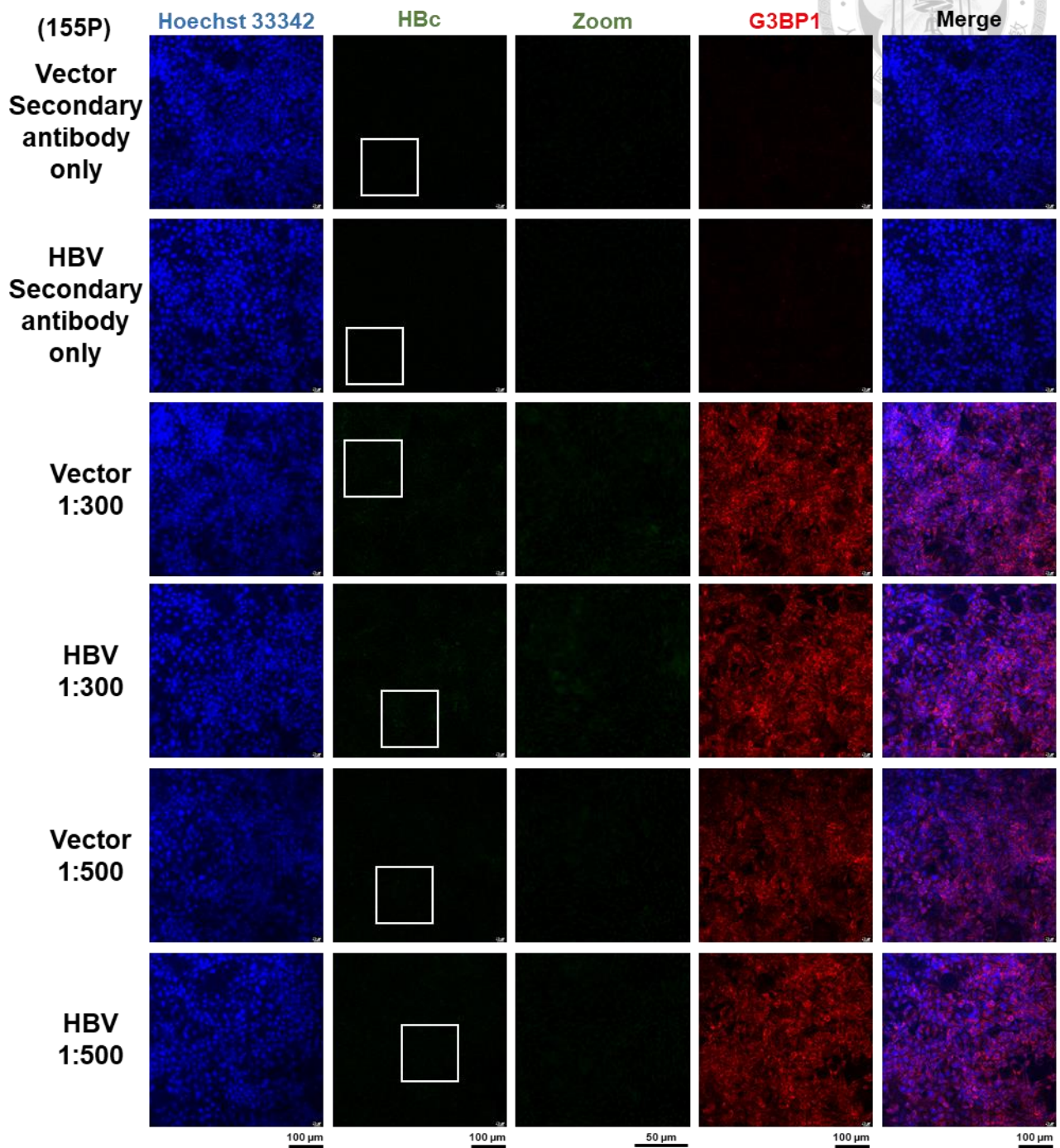


B

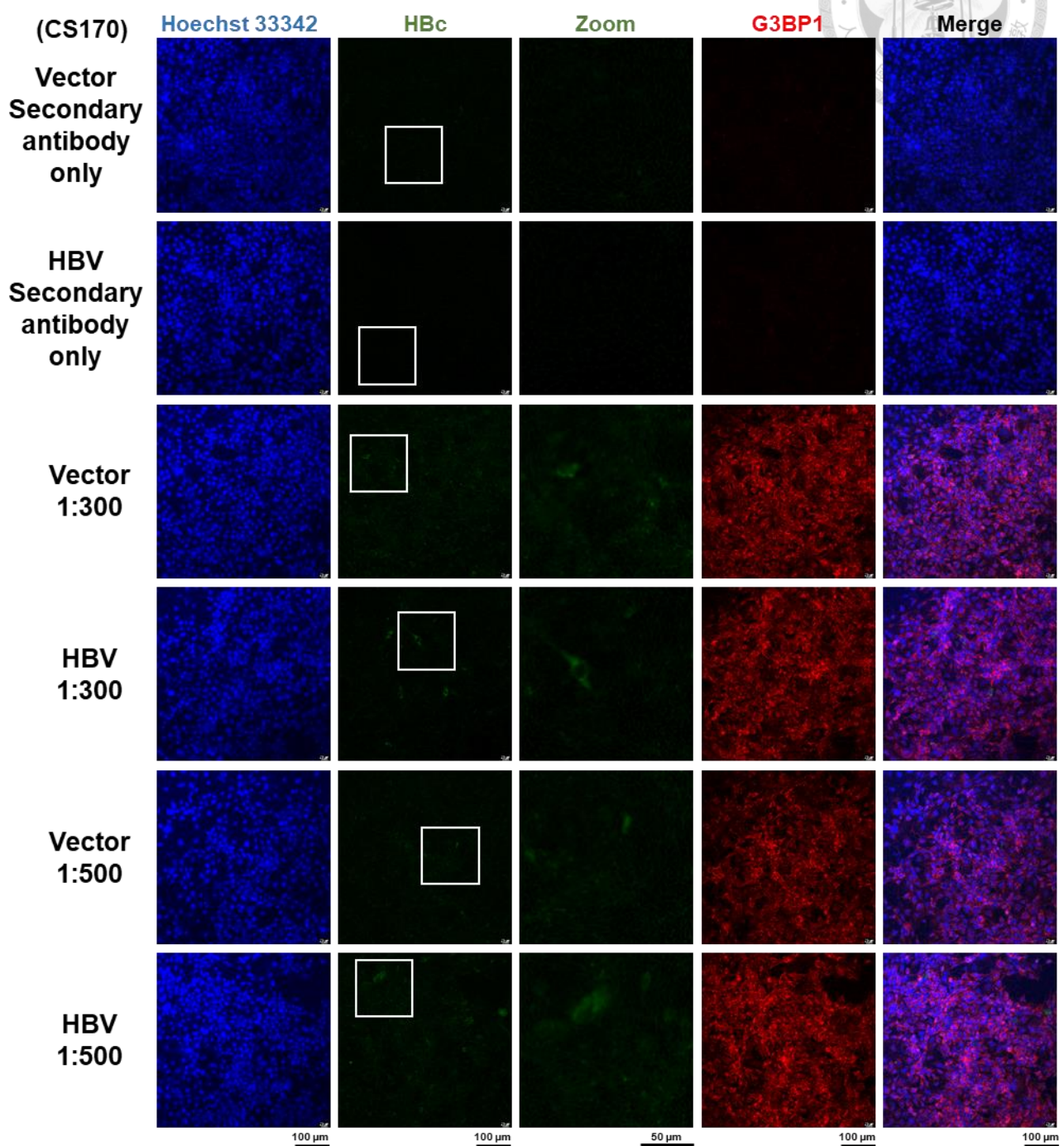
S162NP Rabbit Anti-HBc antibody+Anti-G3BP1 (H-10)



C 155P Rabbit Anti-HBc antibody+Anti-G3BP1 (H-10)

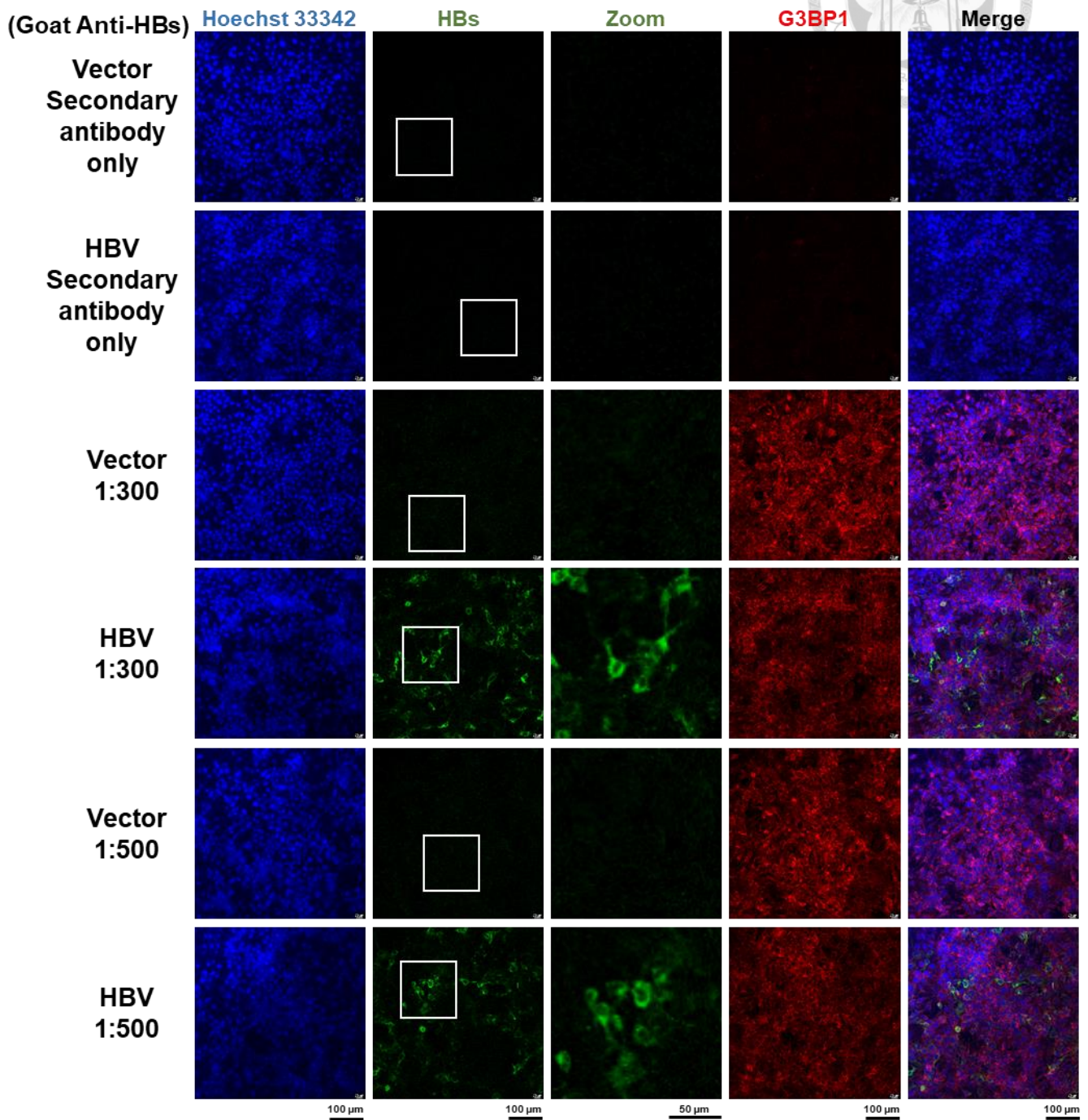


D CS170 Rabbit Anti-HBc antibody+Anti-G3BP1 (H-10)



E

Goat Anti-HBs antibody+Anti-G3BP1 (H-10)



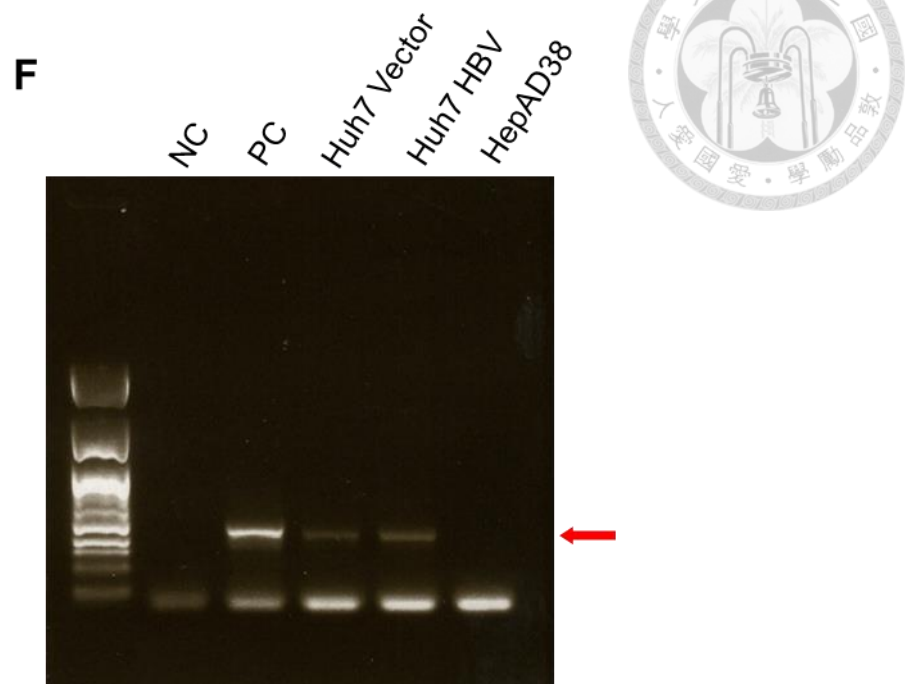
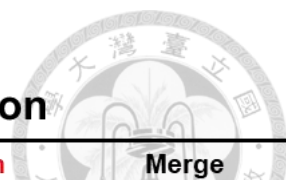


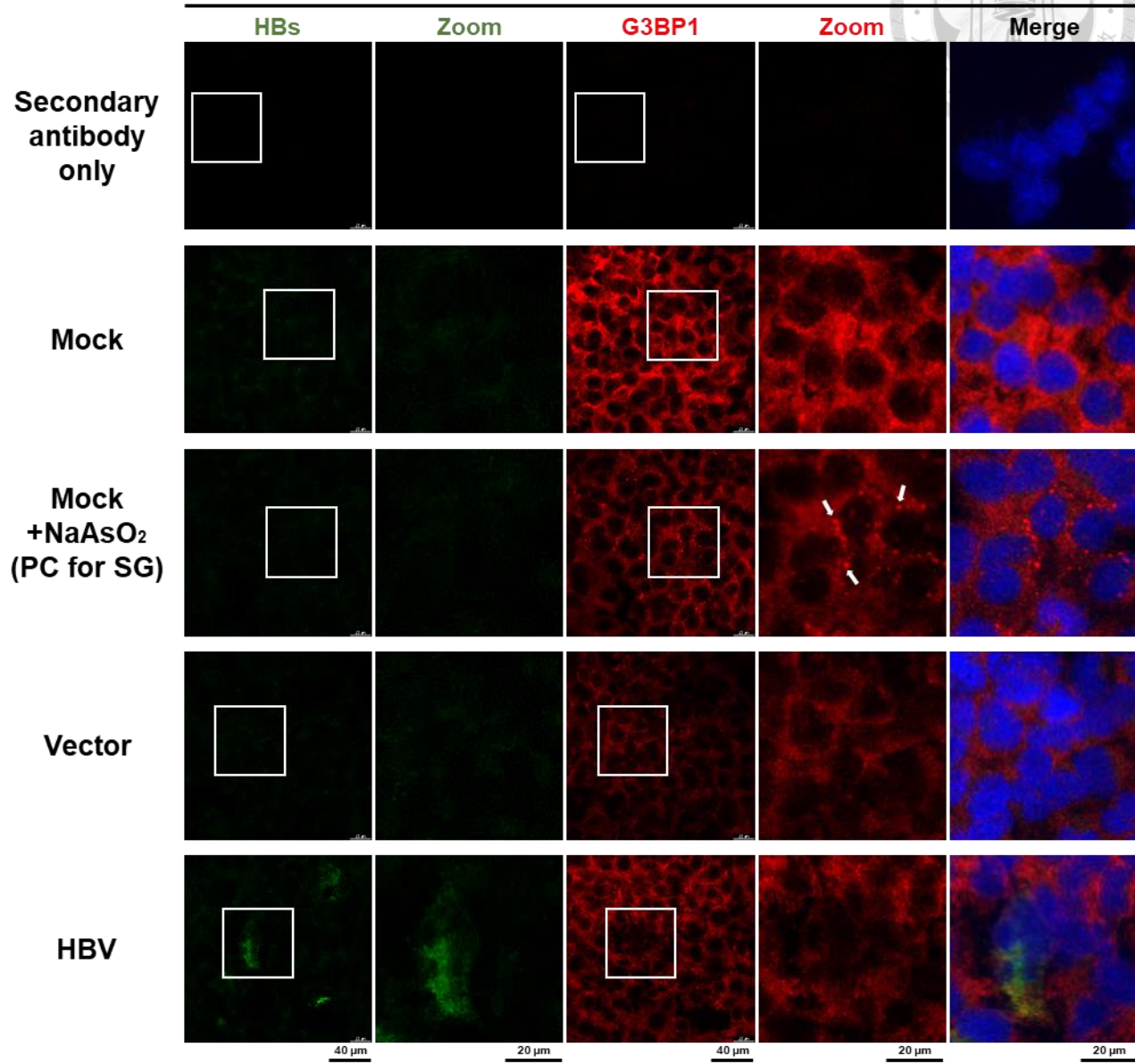
Figure 7. Staining efficacy of different antibodies for HBV antigens in Huh7 cells transfected with pAAV vector only or pAAV/HBV1.2 (genotype A) plasmids

(A) The genome map of the pAAV/HBV1.2 plasmid. (B-E) Huh7 cells were transfected with 0.5 μg of pAAV vector or pAAV/HBV1.2 (genotype A) plasmids using Lipofectamine 2000. The cells were double-stained with mouse anti-G3BP1 antibody (red, 1:400) and S162NP rabbit anti-HBc antibody (green, B), 155P rabbit anti-HBc antibody (green, C), CS170 rabbit anti-HBc antibody (green, D), or goat anti-HBs antibody (green, E). Nuclei were stained with Hoechst 33342 (blue). Each group in this experiment was captured in a single field of view. Scale bar, 100 and 50 μm . The dilution ratio of the above antibodies is shown on the left side of figures A-E. (F) Mycoplasma DNA detection by TOOLS Mycoplasma Detection Kit (PC: positive control, NC: negative control).



A

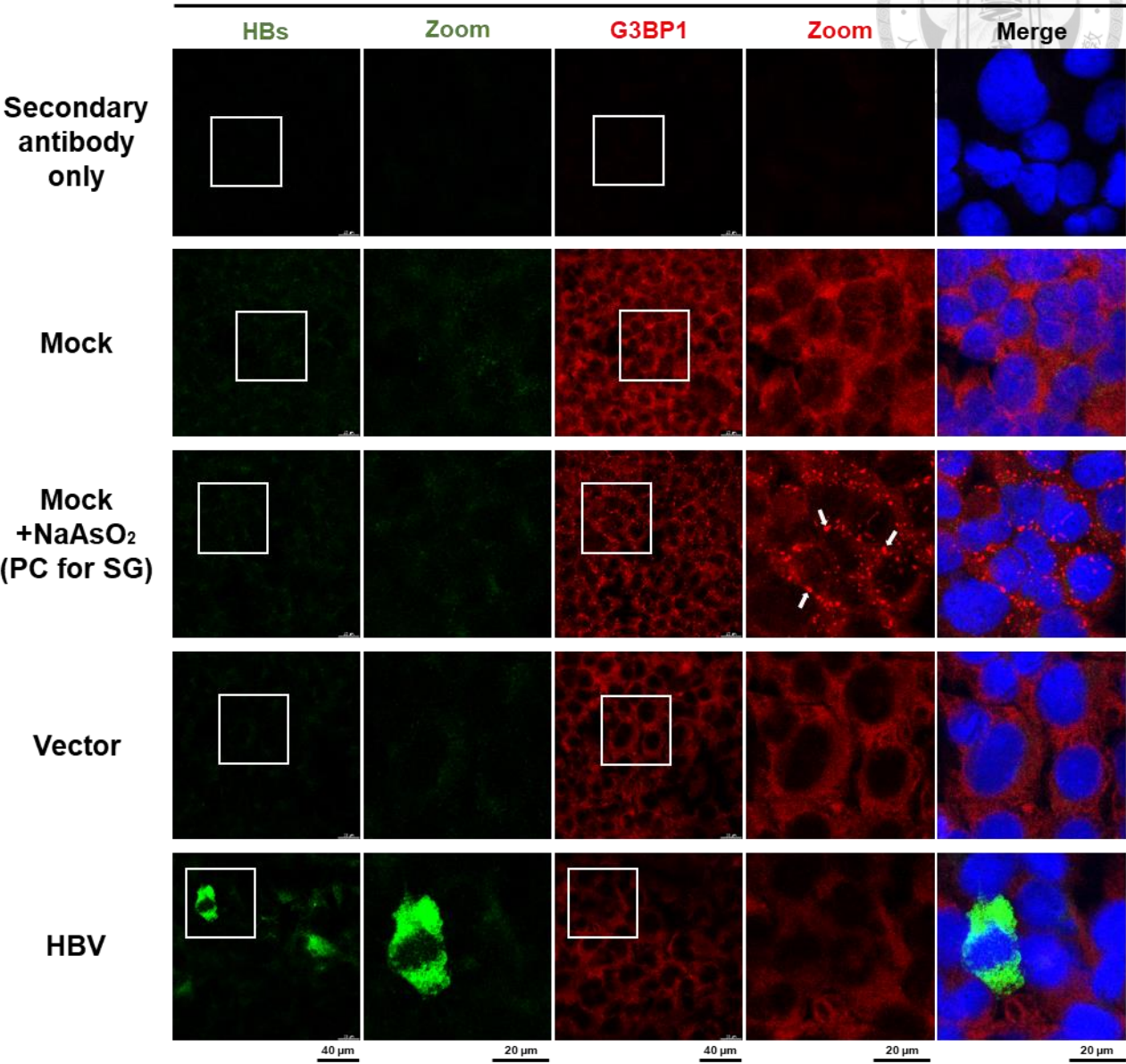
Huh7 Day 1 post-transfection





B

Huh7 Day 2 post-transfection



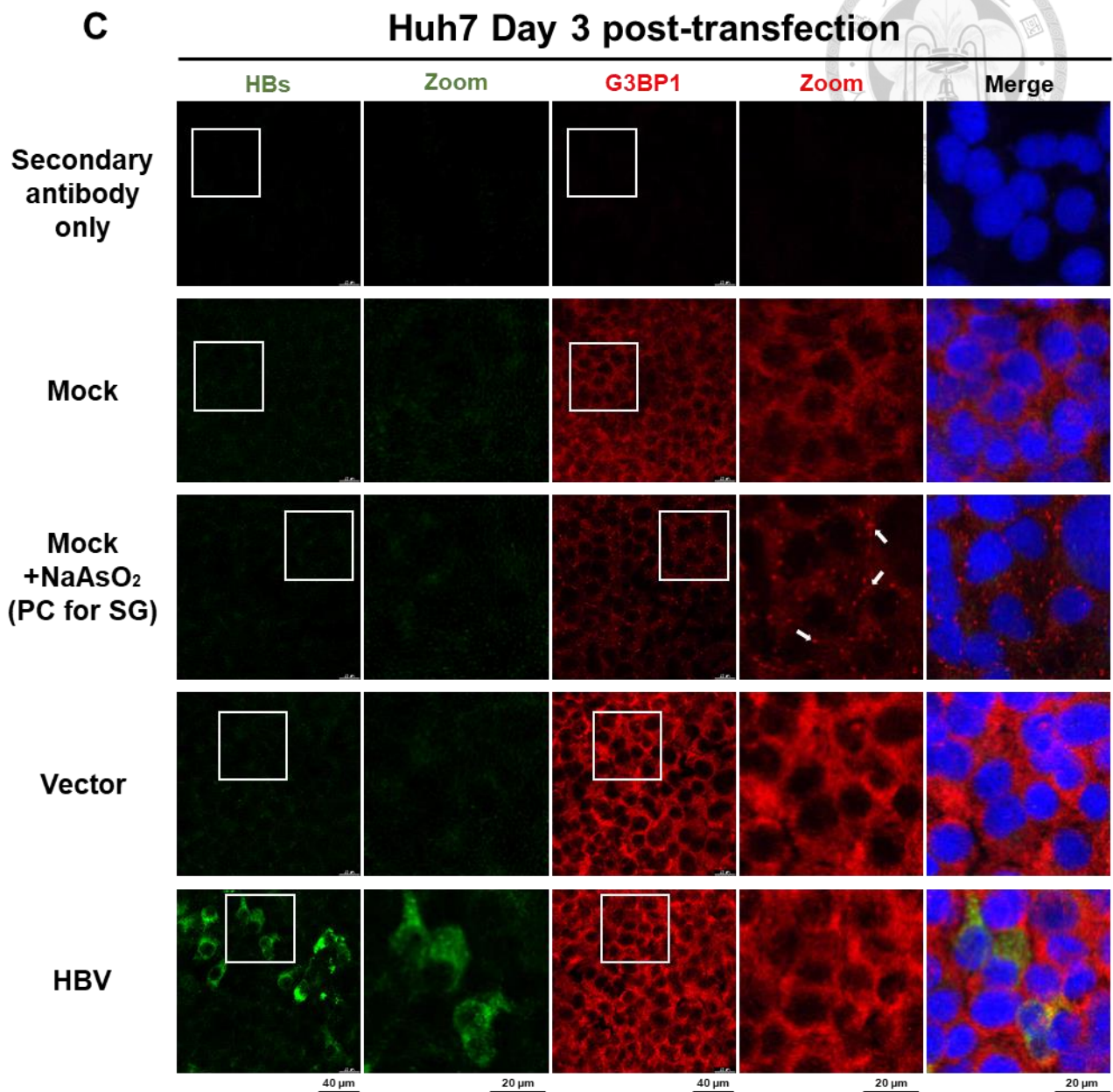
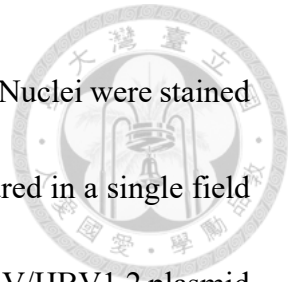


Figure 8. Stress granules formation in Huh7 cells transfected with pAAV vector only or pAAV/HBV1.2 (genotype A) plasmids

(A-C) Confocal images of Huh7 cells transfected with pAAV vector or pAAV/HBV1.2 plasmids on day 1 (A), day 2 (B), or day 3 (C) post-transfection. Huh7 cells were transfected with 0.5 μg of pAAV vector or pAAV/HBV1.2 (genotype A) plasmids using Lipofectamine 2000. The cells were double-stained with mouse anti-G3BP1 antibody (H-

10) and goat anti-HBs antibody on days 1, 2, and 3 post-transfection. Nuclei were stained with Hoechst 33342 (blue). Each group in this experiment was captured in a single field of view. Scale bar, 40 and 20 μm . Huh7 cells transfected with the pAAV/HBV1.2 plasmid and treated with NaAsO₂ were used as the group for the secondary antibody only. Mock-transfected cells treated with NaAsO₂ (Mock+NaAsO₂) were used as a positive control for stress granule formation. The white arrow represents the stress granules observed in cells.

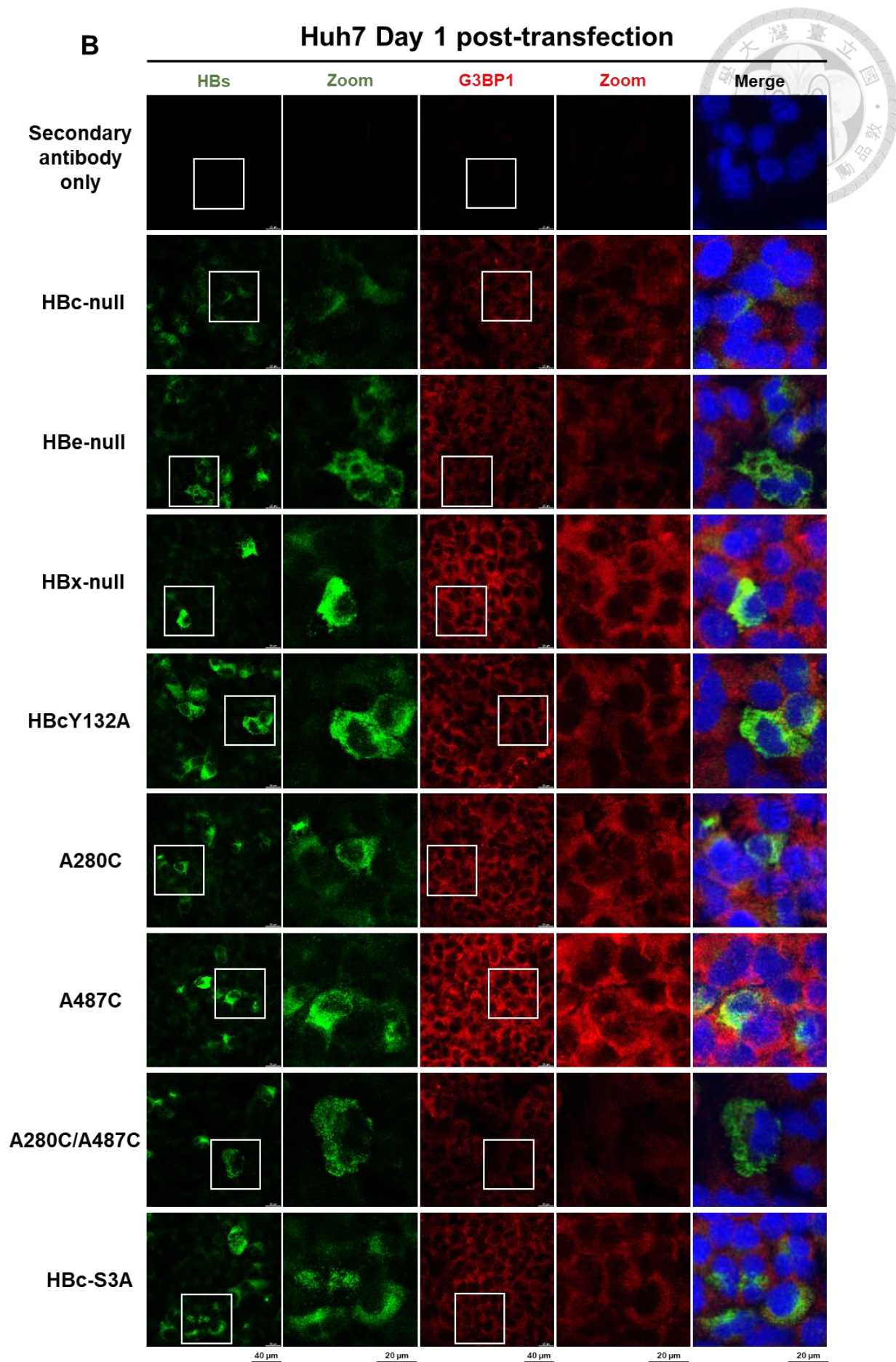




A

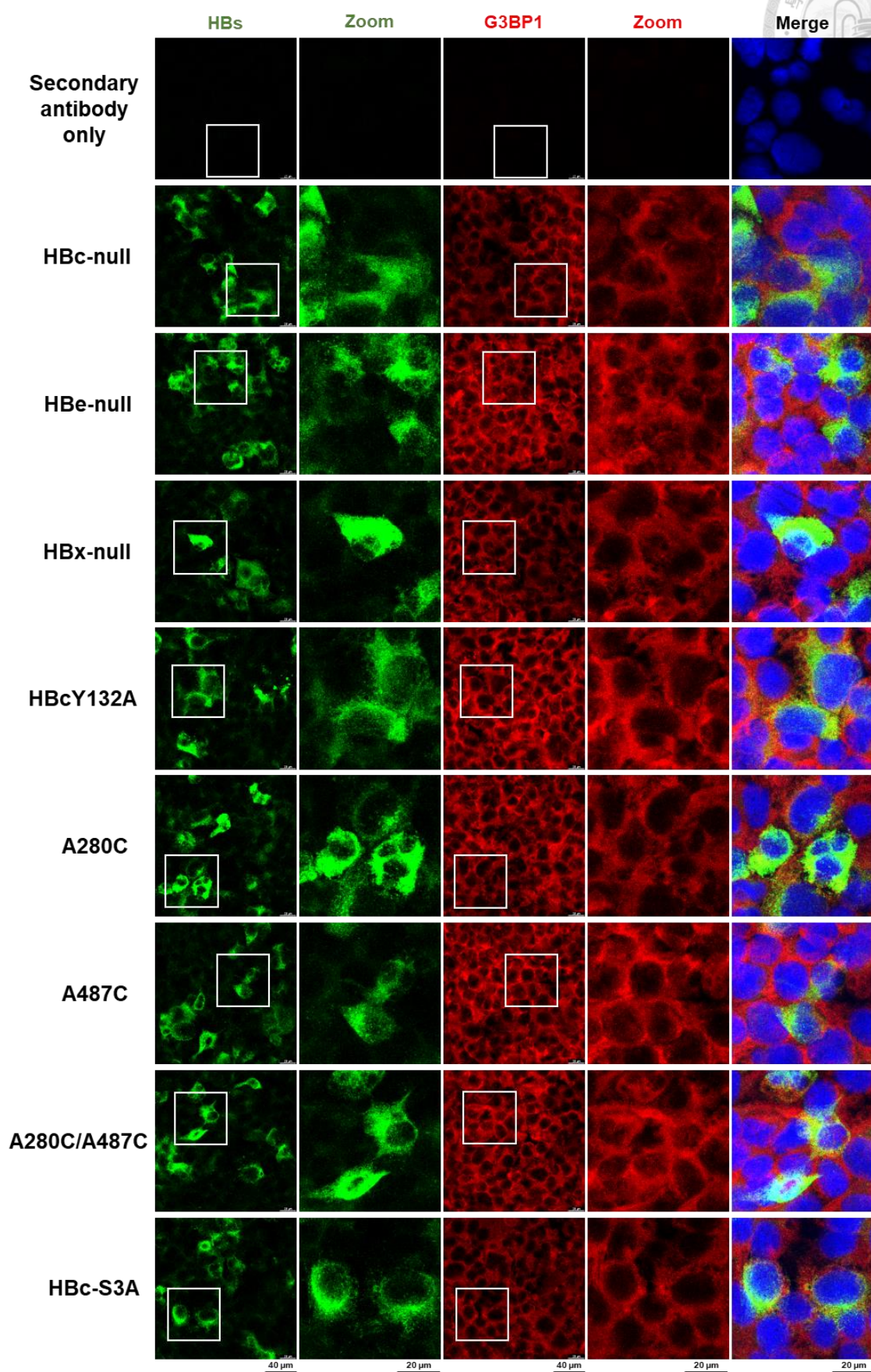
Mutant plasmid (pAAV/HBV 1.2 mutants)

Plasmid	Mutant codon	Nonsense Mutation site
HBe-null	1990 GTGGCTTTAAGGGCAT 1916	28 th aa of HBeAg
HBc-null	2019 AGCTCTGTAGCGAGA 2033	38 th aa of HBcAg
HBx-null	4623 GTACTGCTAACTGGA 4637	8 th aa of HBx
HBcY132A	2301 TCCAGCCGCTAGACC 2315	132 th of HBcAg
A280C	3508 ATTTTCTCGGGGGAT 3522	409 th of Pol 216 th of HBsAg
A487C	3709 TAATTCCCGGATCAA 3729	478 th aa of Pol 285 th aa of HBsAg
A280C/A487C	3512 TCTCGG...CCCGGAT 3726	409 th and 478 th aa of Pol 216 th and 285 th aa of HBsAg
HBc-S3A	2382 GGCC...CGCG...AGCG 2430	157 th , 164 th , and 172 th aa of HBcAg



C

Huh7 Day 2 post-transfection



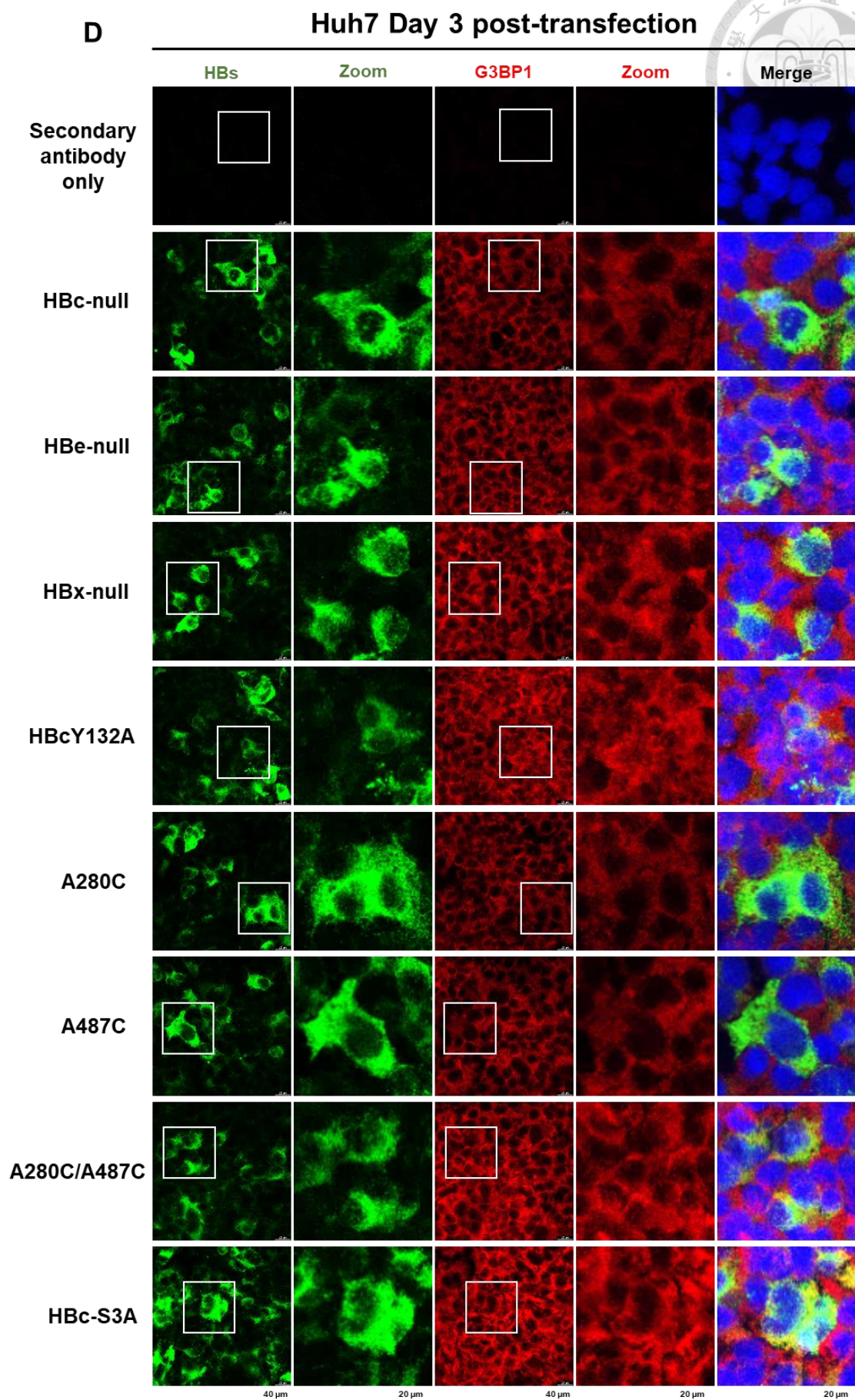
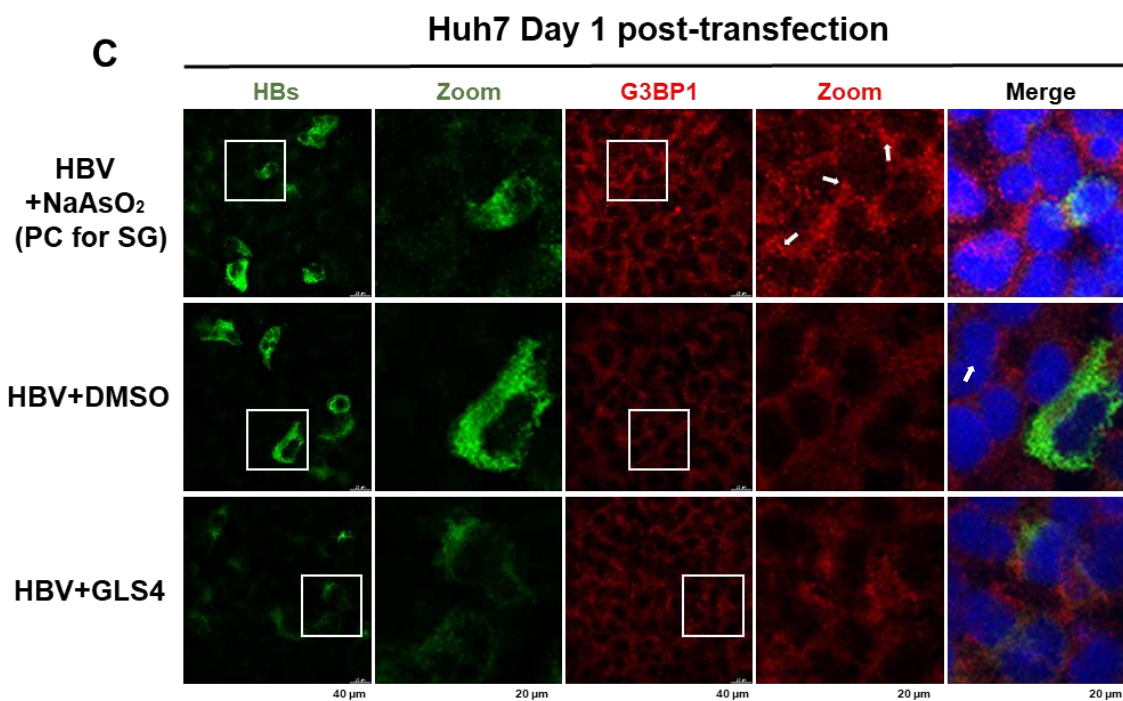
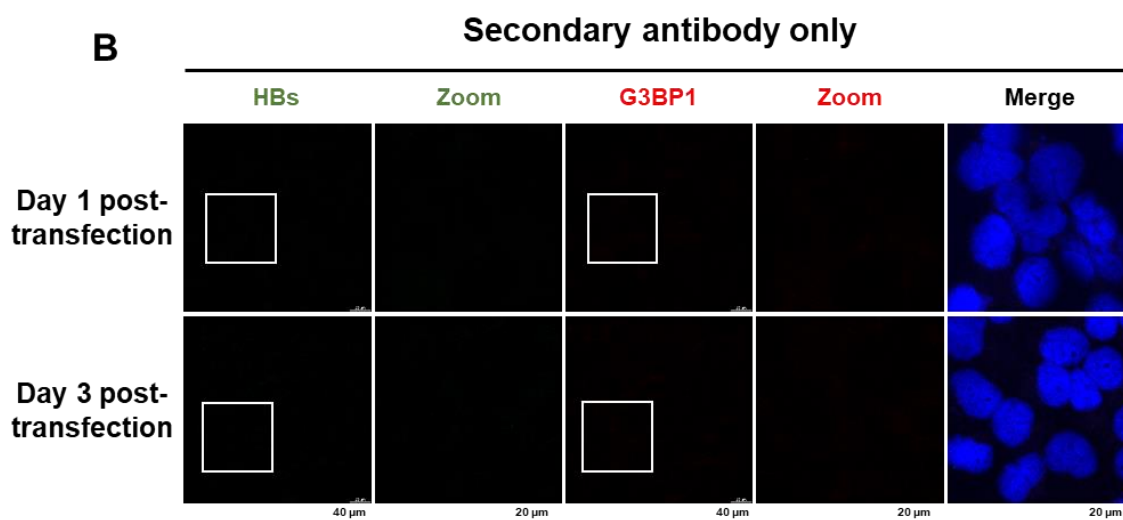


Figure 9. Stress granules formation in Huh7 cells transfected with pAAV/HBV1.2 (genotype A) plasmids containing different mutation



(A) The mutation sites of pAAV/HBV1.2 plasmids. (B-D) Confocal images of Huh7 cells transfected with different mutant pAAV/HBV1.2 plasmids as indicated on day 1 (B), day 2 (C), or day 3 (D) post-transfection. Huh7 cells were transfected with 0.5 μ g of pAAV vector as a control or pAAV/HBV1.2 (genotype A) plasmids containing different mutations. The cells were double-stained with mouse anti-G3BP1 antibody (1:400, red) and goat anti-HBs antibody (1:500, green) on days 1, 2, and 3 post-transfection. Nuclei were stained with Hoechst 33342 (blue). Each group in this experiment was captured in a single field of view. Scale bar, 40 and 20 μ m. Huh7 cells transfected with the pAAV/HBV1.2 plasmid and treated with NaAsO₂ were used as the group for the secondary antibody only.



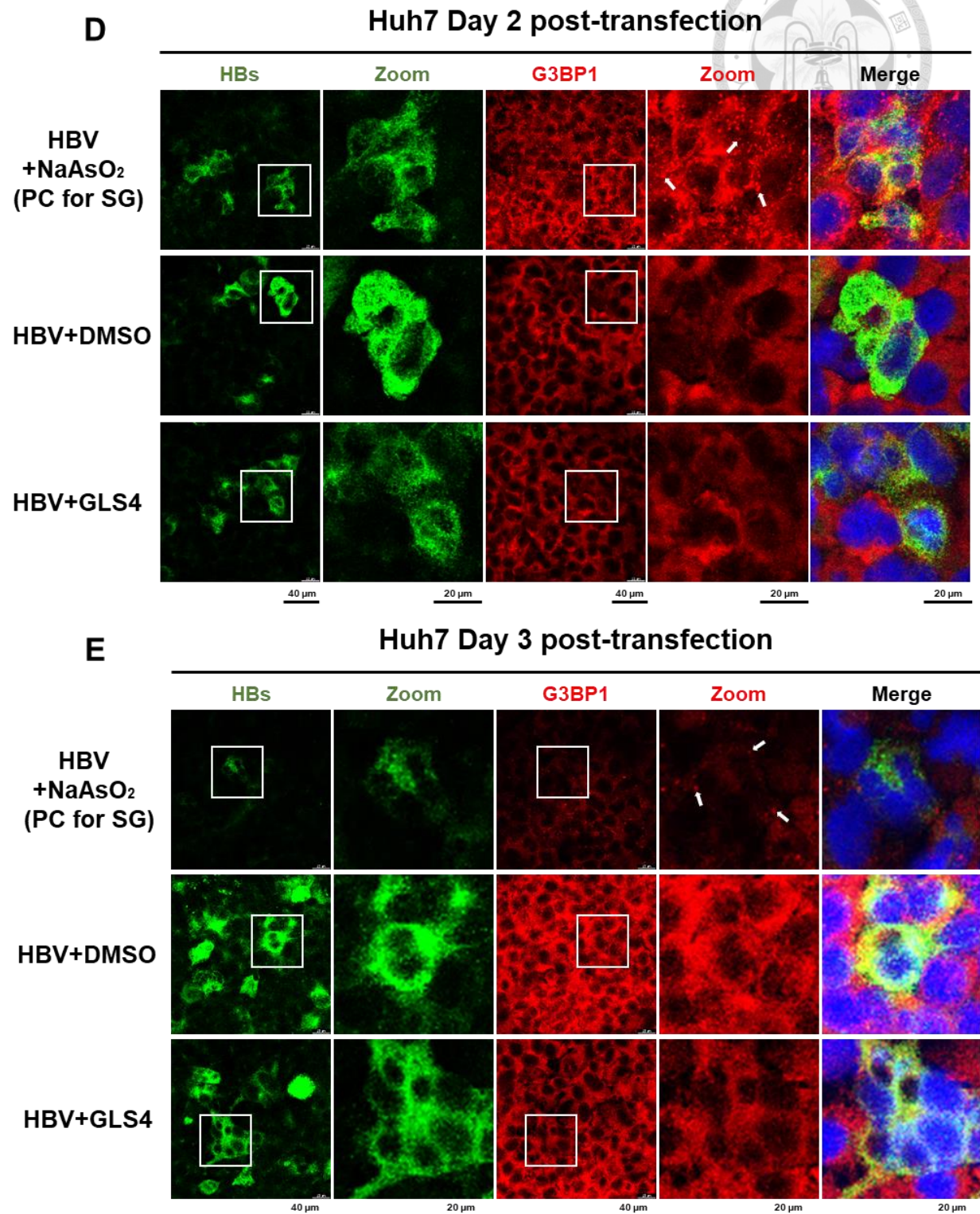


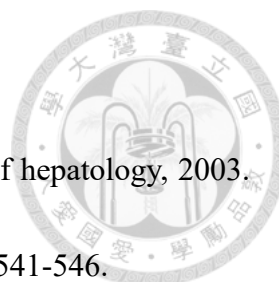
Figure 10. Stress granules formation in Huh7 cells transfected with pAAV/HBV1.2

(genotype A) plasmids and treated with GLS4

(A) Flowchart of experimental procedures. (B-D) Confocal images of Huh7 cells



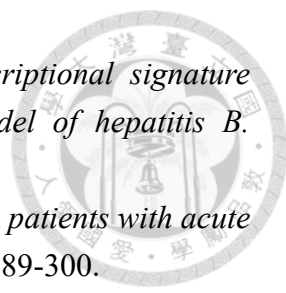
transfected with pAAV/HBV1.2 plasmids and treated with DMSO or GLS4. Huh7 cells were transfected with pAAV/HBV1.2 (genotype A) plasmids. On day 1 post-transfection, DMSO or 200 nM GLS4 was added to the culture medium and supplemented daily. The cells were double-stained with mouse anti-G3BP1 antibody (1:400, red) and goat anti-HBs antibody (1:500, green) on days 1 (B), 2 (C), and 3 (D) post-transfection. Nuclei were stained with Hoechst 33342 (blue). Each group in this experiment was captured in a single field of view. Scale bar, 40 and 20 μm . Huh7 cells transfected with the pAAV/HBV1.2 plasmid and treated with NaAsO₂, used as the group for the secondary antibody only. Huh7 cells transfected with the pAAV/HBV1.2 plasmid and treated with NaAsO₂ (HBV+NaAsO₂) were used as a positive control for stress granule formation. The white arrow represents the stress granules observed in cells.

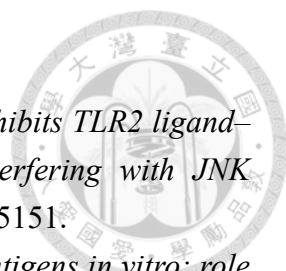


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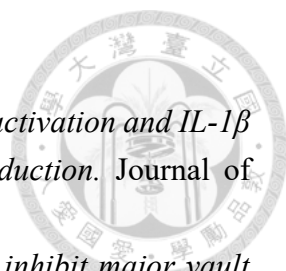
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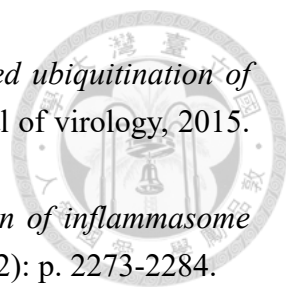
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