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科研費 KAKENHI

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研究課題名（和文）DOCK11ノックダウンによりcccDNA排除の新規抗HBV治療応用への基礎研究

研究課題名（英文）Basic research on developing new anti-HBV therapeutic strategy for cccDNA elimination by DOCK11 knockdown in HBV infected hepatocytes

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研究成果の概要（和文）：cccDNAは感染肝細胞核内にプールされるため，HBVを体内から完全に排除することが難しい。我々は単細胞遺伝子解析により，HBV複製の維持に係る遺伝子はDOCK11であることを明らかにした。DOCK11遺伝子の発現を抑制することにより，HBVの核内輸送が抑えられるだけでなく，細胞障害性も示されなかったため，DOCK11遺伝子発現を抑制する新薬の開発はB型肝炎の画期的な治療法になると期待したい。

研究成果の学術的意義や社会的意義
エンテカビル（ETV）によってウイルス複製を継続的に抑制しても，感染肝細胞核内に容易に排除されることのない HBV cccDNAが残存して，再活性化と発癌を引起す可能性は高い。本研究では，cccDNAの維持を制御する一つの機構を初めて解明し，B型肝炎の治療に新しい治療法を提案した。

研究成果の概要（英文）：Hepatitis B virus (HBV) infection is difficult to cure owing to the persistence of cccDNA. Here, we identified DOCK11, a guanine nucleotide exchange factor (GEF), as a candidate druggable target for HBV. Interestingly, DOCK11 functionally associated with retrograde trafficking proteins in trans-Golgi network (TGN), AGAP2 and ARF1 together with HBV capsid, to open an alternative retrograde trafficking route of HBV capsid from early endosomes (EEs) to the TGN and then to the endoplasmic reticulum (ER), thereby avoiding lysosomal degradation. cccDNA levels were strongly suppressed by shDOCK11. Surprisingly, combination of ETV plus shDOCK11 further reduced HBVDNA and cccDNA levels. Clinically, DOCK11 levels in the liver of patients with chronic hepatitis B were significantly reduced by entecavir treatment, and this reduction correlated with HBs antigen levels. Thus, DOCK11 could be a potential therapeutic target to prevent persistent HBV infection.

研究分野：molecular biology

キーワード：HBV　cccDNA　DOCK11　Retrograde trafficking　Golgi

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様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Chronic hepatitis B virus (HBV) infection greatly increases the risk of liver fibrosis, cirrhosis, and hepatocellular carcinoma, and is responsible for nearly one million annual deaths worldwide. Current therapies rarely achieve a cure as they do not eliminate the cccDNA which is responsible for viral persistence. Thus, understanding the fundamental molecular mechanisms that governing cccDNA biogenesis is essential for the development of new anti-HBV drugs.

２．研究の目的

We have obtained the DOCK11 gene that associated with the maintenance of cccDNA, using single-cell transcriptome analysis, in an HCC-derived cell line (HC1) in which cccDNA and HBV DNA were detected, as well as HBV-derived transcripts. The purpose of this project is to understand the roles of DOCK11 in cccDNA maintenance and subsequently clarify the possibility of DOCK11 as a target molecule for treating chronic HBV infection.

３．研究の方法

1). HBV virions were preparation by PEG8000 and were purified using iodixanol density gradient analysis

2). Huh7-shDOCK11, HepG2-NTCP-C4-shDOCK11, and HepG2-NTCP-C4-RAB7KO, HepAD38-shDOCK11, HepG2.2.15-shDOCK11, and Dox-inducible HepG2-NTCP-C4-Halo-DOCK11 cell lines were established and were infected with HBV virions.

3). Hirt DNA extraction and Southern blot analysis were performed.

4). Super-resolution fluorescence microscopy analysis, live cell confocal imaging, and immunofluorescence analysis were completed.

5). Dynasore, Pitstop 2, Retro-2, BFA, and NAV-2729 treatment of HepG2-NTCP-C4 or RAB7KO cells infected with recombinant NL-HBV particles were investigated

6). Lysosome inhibitors treatment of HepG2-NTCP-C4 cells infected with recombinant NL-HBV particles were examined.

7). PLA (proximity ligation assay, HBc enzyme-linked immunosorbent assay, immunoblotting analysis, and immunoprecipitation assay were performed.

8). GEF and GAP activity assays in vitro were investigated.

9). Statistical analyses were completed.

４．研究成果

1) **DOCK11 promotes HBV replication in hepatocytes.**

We identified human DOCK11 as a candidate host factor for the maintenance of persistent HBV infection in an HCC-derived cell line (HC1 cells). DOCK11 overexpression increased HBVDNA and cccDNA levels compared with control cells by using Real-time PCR and Southern blot analysis. Furthermore, in PXB cells, ablation of DOCK11 expression by shDOCK11-lentivirus transduction significantly suppressed HBVDNA and cccDNA levels. Thus, DOCK11 promoted HBV replication in hepatocytes.

2) **HBV utilizes an alternative retrograde trafficking route via the EE-TGN-ER pathway**

Immunofluorescence analysis showed that DOCK11 was predominantly localized in the cytoplasm and partially localized in the nucleus of HepG2-NTCP-C4 cells that DOCK11 were overexpressed.

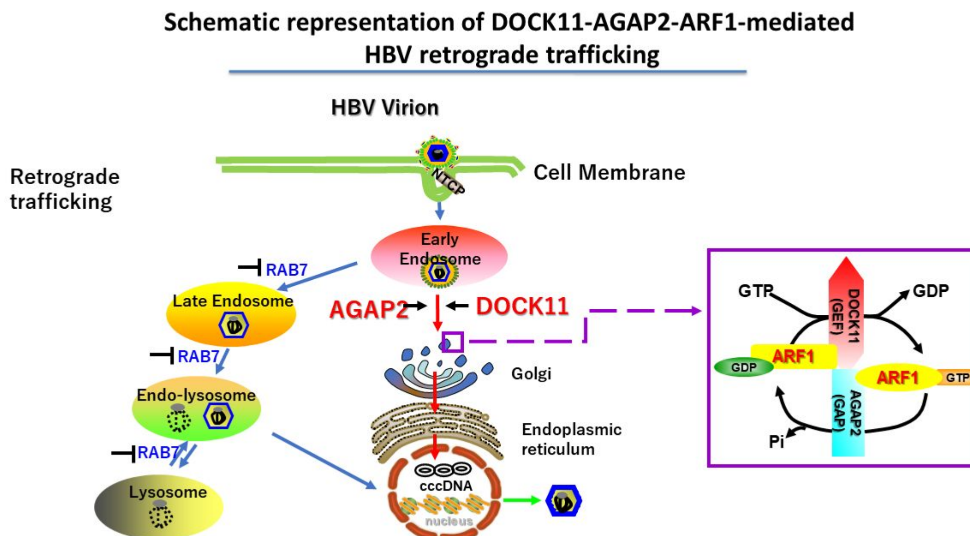
Super-resolution fluorescence microscopy in HepG2-NTCP-C4 cells infected with TC-labeled virions clearly showed that there is an alternative trafficking route from EEs to the Golgi apparatus, other than the classic trafficking route from EEs to LEs. Because Rab7 is relevance for HBV infection from EEs to LEs/Lysosome, we established the HepG2-NTCP-C4-RAB7KO cell line, in which RAB7 was stably knocked out. Surprisingly, we observed that loss of RAB7 rather increased the intracellular levels of HBVDNA and cccDNA after infection with HBV particles from HepAD38 or with NL-HBV particles. Proximity ligation assay showed that intracellular signals of localization of HBV in Rab5-positive, TGN46-positive, and PDI-positive were significantly increased in RAB7KO cells compared with ScrambleKO cells. Time-lapse images of live cells show that more HBV particles were transported to the TGN and ER in RAB7KO cells, compared with the control cells.

3) **DOCK11 promotes the alternative retrograde trafficking route of HBV via the EE-TGN-ER pathway**

We observed that, in RAB7KO cells, HBVDNA and cccDNA levels were repressed by DOCK11 suppression, but not by Scramble treatment. Immunofluorescence analysis showed that expression of DOCK11 proteins were detected within RAB5, TGN46, and PDI positive apparatus, and its expression was more obvious in DOCK11-overexpressing cells. Immunoprecipitation analysis demonstrated the physical interaction of endogenous DOCK11 with HBV capsid in HepG2-NTCP-C4 and Huh7-NTCP cells following infection with HBV virions derived from HepAD38 cells. These observations revealed that DOCK11 contributed to the retrograde trafficking of HBV via the EE-TGN-ER pathway in hepatoma cells and human primary hepatocytes.

4) **AGAP2, a member of the Arf-GAP family, is a partner of DOCK11 and contributes to the retrograde trafficking of HBV through the regulation of ARF1**

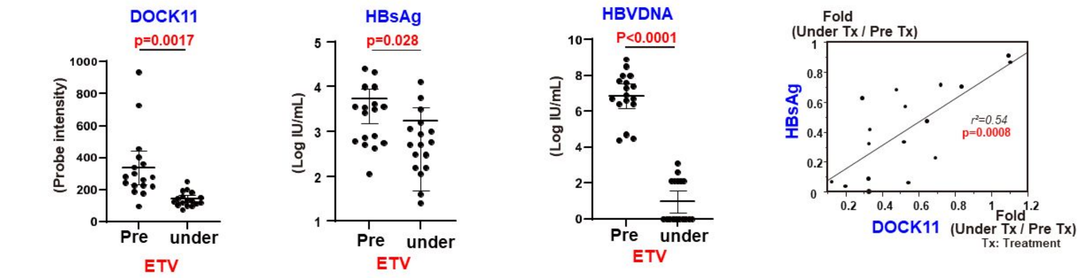
we performed liquid chromatography-tandem mass spectrometry metabolomics (LC-Ms/Ms) analysis on the Golgi fractions from Huh7-shDOCK11 cells, a DOCK11 stable Knock-down cells, and identified trafficking-associated protein AGAP2, one of the downregulated peptides compared with control. AGAP2 co-localized and co-precipitate with DOCK11 in Dox-treated HepG2-NTCP-C4-Halo-DOCK11 cells. Suppression of AGAP2 by siRNA significantly reduced HBVDNA and cccDNA levels in Dox-treated HepG2-NTCP-C4-Halo-DOCK11 cells and PXB cells. Moreover, immunoprecipitation of endogenous DOCK11 or endogenous AGAP2 could co-precipitate ARF1. immunoprecipitation of endogenous ARF1 co-precipitated DOCK11 and AGAP2. Furthermore, DOCK11 exhibited GEF activity and AGAP2 had GAP activity toward ARF1. In addition, retrograde trafficking of HBcAg could also be blocked by BFA.



5) **DOCK11 expression is associated with entecavir treatment in vivo and in vitro**

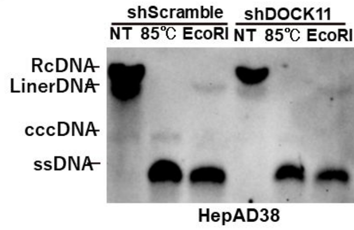
In liver biopsied samples, compared before and after Entecavir treatment, DOCK11 expression was significantly reduced after Entecavir treatment. Consistently, serum HBsAg and HBV DNA levels were significantly reduced. Interestingly, the reduction of HBsAg was significantly correlated with that of DOCK11, suggesting important role of DOCK11 in the maintenance of cccDNA in the liver of patients with Chronic Hepatitis B. In further HepAD38 cells experiment, amazingly, compared with control cells, DOCK11shRNA-lentivirus transduction suppressed cccDNA level by using southern blot and real-time PCR analysis. compared to DOCK11shRNA or Entecavir alone treatment, the combination of Entecavir plus DOCK11shRNA further reduced cccDNA levels, indicating DOCK11 might be a candidate druggable target for HBV cure.

DOCK11 might be a candidate druggable target for HBV cure

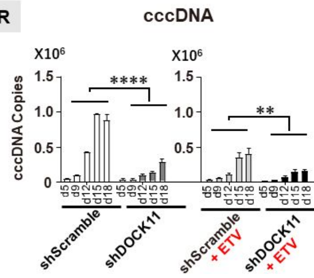


◆ The liver of patients with Chronic Hepatitis B

Southern blot



RTD-PCR



5．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　3件／うち国際共著　0件／うちオープンアクセス　3件）

1．著者名 Chen Han、Nio Kouki、Yamashita Taro、Okada Hikari、Li Ru、Suda Tsuyoshi、Li Yingyi、et al	4．巻 online publish
2．論文標題 BMP9‐ID1 signaling promotes EpCAM‐positive cancer stem cell properties in hepatocellular carcinoma	5．発行年 2021年
3．雑誌名 Molecular Oncology	6．最初と最後の頁 1-16
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/1878-0261.12963	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

1．著者名 Chen Han、Nio Kouki、Tang Hong、Yamashita Taro、Okada Hikari、Li Yingyi、Doan Phuong Thi Bich、Li Ru、Lv Junyan、Sakai Yoshio、Yamashita Tatsuya、Mizukoshi Eishiro、Honda Masao、Kaneko Shuichi	4．巻 23
2．論文標題 BMP9-ID1 Signaling Activates HIF-1α and VEGFA Expression to Promote Tumor Angiogenesis in Hepatocellular Carcinoma	5．発行年 2022年
3．雑誌名 International Journal of Molecular Sciences	6．最初と最後の頁 1475～1475
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/ijms23031475	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

1．著者名 Kazuhisa Murai, Kazuyuki Kuroki, Tomoki Nishikawa, Atsumu Yamada, Ying Wang, Atsuya Ishida, Takayoshi Shirasaki, Shotaro Kawase, Ying-Yi Li, Hikari Okada, Kazunori Kawaguchi, Taro Yamashita, Yoshio Sakai, Eishiro Mizukoshi, Masao Honda, Shuichi Kaneko	4．巻 -
2．論文標題 A Single HBV Genome with a Reporter Allows the Entire Viral Life Cycle to be Monitored in Primary Human Hepatocytes	5．発行年 2022年
3．雑誌名 Hepatology Communications	6．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） なし	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

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1．発表者名 Takayoshi shirasaki, Masao Honda, Tetsuro Shimakami, Kazunori Kawaguchi, Ying-Yi LI, Kouki Nio, Taro Yamashita, Shuichi Kaneko
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3．学会等名 2018 international HBV meeting（国際学会）
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１．発表者名 Kazunori Kawaguchi, Zijing Wang, Masao Honda, takayoshi shirasaki, Hikari Okada, Ying-Yi Li, Kazuhisa Murai, Kouki Nio, Tetsuro Shimakami, Taro Yamashita, Shinichi Hashimoto, Kazuyuki kuroki, Seishi Murakami, Shuichi Kaneko
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〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

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７．科研費を使用して開催した国際研究集会

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８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関