Next question was whether our designed HBVpp would be formed and secreted into medium from the packaging cells transfected with the HBV envelopes expression vector (pCEP4 LS-S). Immunoprecipitates with anti-HBs antibodies from the supernatant of pCEP4 LS-S transfected and non-transfected gfp/GP2 cells were analyzed by Western blotting analysis to detect HBV envelope proteins. Fig. 2C showed that all HBV envelopes were expressed and actually LS, MS and SS protein expression were detected. Thus, expressed HBV envelope proteins were secreted into medium.

Secreted envelope proteins contains the designed retroviral genome.

In order to check whether HBV membrane proteins can envelope retroviral capsids, culture medium of pCEP4 LS-S transfected gfp/GP2 cells was harvested and immunoprecipitated either with anti-HBs antibodies or anti-VSV-G antibodies in case that VSV-G was expressed. Reverse transcription (RT) was performed on the RNA extracted from the immunoprecipitates. VSV-G pseudotype was also tested as a

positive control. As shown in Fig. 3, a correct size of amplified band was observed in LS-S expressed samples as in VSV-G. This result (about ~320bp) suggests that HBV envelope proteins wrapped retroviral capsids containing retroviral genomes, in which *GFP* and *hygR* genes were encoded in this case.

HBVpp shows near identical density profiles to HBV particles (Dane particles) in CsCl density gradient ultracentrifugation.

Next, we checked the density profiles of HBVp particles in CsCl density gradient untracentrifugation. Peak fractions for HBs and preS1 were seen in fraction 8 whose density was around 1.17 g/ml. Furthermore, amplified *EGFP*-corresponding DNA by RT-PCR was detected around the fractions positive for HBsAg. The main RT-PCR products were detected in fraction 9 with higher density (1.18 g/ml) than those where the most S and preS1 antigens were detected (fraction 8). The produced pseudotyped HBV particles were a little bit smaller than the native HBV, since it was reported that Dane particles had the heavier density (around 1.25 g/ml) than small S

316 particles (1.20~1.22 g/ml) [29]. Taken together, these observations suggest that
317 retroviral capsids surrounded by HBV membrane proteins were generated and secreted
318 into the medium from the packaging cells expressing HBV membranes and thus the
319 HBV pp was produced.
320
321 **Observation of HBVpp by electron microscopy.**
322
323 Finally, we tried to observe HBVpp from the packaging cells transfected
324 HBV membrane protein expression vectors by electron microscopy. Unfortunately,
325 we were not able to observe the complete particles on the secretion process but
326 viral-like-particles in the endoplasmic reticulum of the packaging cells expressing HBV
327 membrane proteins (Fig. 5A). Most of them might be subviral particles ie., HBs
328 particles. We also observed some typical Dane-like particles in the medium (Fig. 5B),
329 though infectious viral particle formation might not be efficient with this method.

330 **Discussion**

331

332 HBV is a small DNA virus with 3.2 kb genome involved in acute and chronic
333 liver diseases, persistence of which is predisposed to in a sense incurable liver cirrhosis
334 and cancer. It is assumed that there are more than a few hundred million infected
335 people and thus this is one of the biggest infectious diseases in the world [2]. Since
336 vaccine was developed, protection from the virus infection has been enabled except
337 some vaccine escape mutants [30; 31]. And it is certain that several nucleotide analogs
338 effectively inhibit the viral replication and then disease progression [32; 33].
339 Nevertheless, the real virus life cycle has remained to be elucidated. The critical
340 reason why there are a lot of mysteries about HBV is mainly due to no convenient *in*
341 *vitro* and/or *in vivo* infection system for HBV, though there are several animal
342 hepadnavirus models such avian (duck and heron) and rodent (ground tree squirrel and
343 woodchuck) hepatitis B viruses. Hatching and raising such animals and preparation of
344 primary cultured hepatocytes from the liver of them are not so easy for daily research

345 activities and animal hepadnaviruses are quite divergent and unlikely to be good models
346 for HBV.

347 Recent progress of tissue culture system from a hepatocellular carcinoma
348 called HepaRG [7; 34] and from primary hepatocytes of Tupaia belangeri [35; 36]
349 enable us to propagate HBV in *in vitro* infection system to some extent. By
350 differentiation of the cells to differentiated parenchymal hepatocytes with 2 % DMSO
351 as well as hydrocortisone, HBV can infect the HepaRG up to 20 % or so at viral
352 genome equivalent (V.G.E.) to a cell of over 200 [7; 34; 37]. The infected HBV,
353 however, did not produce infectious daughter viruses in spite of observation of
354 Dane-like particle formation [34]. Furthermore, it takes more than two weeks to
355 differentiate the HepaRG to susceptible for HBV infection [7]. Thus, HepaRG cells
356 are useful but it would be still less efficient for HBV infection and time-consuming
357 system to analyze the HBV life cycle.

358 It is reasonable to find out an HBV receptor in order to overcome this
359 situation followed by establishing a HBV infection system. Several candidates for
360 HBV receptors have been reported. Starting from polymerized human serum albumin

361 [38; 39], several kinds of molecules; endonexin II [40], asialoglycoprotein receptor

362 (ASGPR) [41; 42], lipoprotein lipase (LPL) [43], gp120 and gp180 (carboxypeptidase

363 D) [17; 44] and so on were reported for HBV receptors, including those for DHBV.

364 None of them, however, has been utilized for establishing an *in vitro* HBV infection

365 system as an HBV receptor molecule. And most recently, NTCP (sodium-taurocholate

366 co-transporting protein) has been nominated as a plausible HBV receptor molecule,

367 which has been under evaluation [45; 46; 47].

368 On the other hand, the ligand, i.e., HBV membrane proteins have been

369 characterized as well. There are three kinds of HBV membrane proteins; large S (LS),

370 middle S (MS) and small S (SS) and seems to be no doubt that preS1 region in the

371 N-terminal end of LS has a key role for receptor binding [48]. Especially, a

372 well-conserved region around preS1 9-23 amino acids (aa) might be critically important

373 and might function as a fusogenic peptide, since antibodies against preS1 (2-48 aa)

374 could neutralize HBV infection but not preS1 (1-21 aa) in PTH system [49; 50; 51].

375 And also, experiments using hepatitis delta virus (HDV) provided us with similar

376 important information on the HBV entry [52; 53; 54].

Nevertheless, we have yet obtained an easy and convenient *in vitro* cell culture system for HBV infection. Thus, identifying an HBV receptor seems to be very hard, because it has not been achieved for a half century since HBV was found. We do not understand why classical methods such as a phage screening system expressing human liver cDNA library and so on do not work well and therefore, we may need to design a new revolutionary assay system.

We here would like to propose a new biological assay using HBV pseudotype particles (HBVpp) in which retroviral core particles were enveloped by HBV membrane proteins. Successful production of HBVpp would allow us to test HBVpp infectivity to cell culture systems introduced some cDNA library from human hepatocytes and/or differentiated HepaRG cells. In this report, we tested whether such HBVpp could be generated. The immunoprecipitation with anti-HBs antibodies followed by RT-PCR and the physicochemical study using ultracentrifugation followed by RT-PCR revealed that murine leukemia virus based core/capsid, which contained recombinant retroviral genomes with *EGFP* and Hyg^R, were enveloped by HBV membrane particles. Thus, HBVpp should be generated and next question would be whether this HBVpp really

393 could infect well-differentiated hepatocytes at suitable condition. It could be a weak
394 point that the HBVpp was based on murine leukemia virus, which demanded cell
395 growth for efficient viral genome integration into the host genome, compared to the
396 same kind system based on a lentivirus. The HBV receptor could be a complex
397 consisted of several molecules. In such a case, it should be very difficult to clone the
398 HBV receptor by cDNA library introduction to ordinary cells, since two hits or more
399 might be required. Nevertheless, it could possibly work if a cDNA encoding an HBV
400 receptor or a gene affecting HBV attachment and entry from a human liver source was
401 tested for HBV infectivity in various hepatoma cell culture systems and thus this
402 HBVpp will be a powerful tool for separation and identification of an HBV receptor
403 with infectivity as a polestar.
404

405 **References**

406

407 [1] Blumberg, B.S., Alter, H.J., and Visnich, S., A "New" Antigen in Leukemia
408 Sera. JAMA 191 (1965) 541-546.

409 [2] Seeger, C., Zoulim, F., and Mason, W.S., Hepadnaviruses, Lippincott
410 Williams and Wilkins, Philadelphia, 2007.

411 [3] Funk, A., Mhamdi, M., Will, H., and Sirma, H., Avian hepatitis B viruses:
412 molecular and cellular biology, phylogenesis, and host tropism. World
413 J Gastroenterol 13 (2007) 91-103.

414 [4] Sureau, C., Eichberg, J.W., Hubbard, G.B., Romet-Lemonne, J.L., and
415 Essex, M., A molecularly cloned hepatitis B virus produced in vitro is
416 infectious in a chimpanzee. J Virol 62 (1988) 3064-3067.

417 [5] Bchini, R., Capel, F., Dauguet, C., Dubanchet, S., and Petit, M.A., In vitro
418 infection of human hepatoma (HepG2) cells with hepatitis B virus. J
419 Virol 64 (1990) 3025-3032.

420 [6] Paran, N., Geiger, B., and Shaul, Y., HBV infection of cell culture:

421 evidence for multivalent and cooperative attachment. EMBO J 20
422 (2001) 4443-4453.
423 [7] Gripon, P., Rumin, S., Urban, S., Le Seyec, J., Glaise, D., Cannie, I.,
424 Guyomard, C., Lucas, J., Trepo, C., and Guguen-Guillouzo, C.,
425 Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl
426 Acad Sci U S A 99 (2002) 15655-15660.
427 [8] Marion, M.J., Hantz, O., and Durantel, D., The HepaRG cell line:
428 biological properties and relevance as a tool for cell biology, drug
429 metabolism, and virology studies. Methods Mol Biol 640 (2010)
430 261-272.
431 [9] Bissig, K.D., Wieland, S.F., Tran, P., Isogawa, M., Le, T.T., Chisari, F.V.,
432 and Verma, I.M., Human liver chimeric mice provide a model for
433 hepatitis B and C virus infection and treatment. J Clin Invest 120
434 (2010) 924-930.
435 [10] Meuleman, P., and Leroux-Roels, G., The human liver-uPA-SCID mouse:
436 a model for the evaluation of antiviral compounds against HBV and

437 HCV. Antiviral Res 80 (2008) 231-238.

438 [11] Dandri, M., Burda, M.R., Torok, E., Pollok, J.M., Iwanska, A., Sommer,

439 G., Rogiers, X., Rogler, C.E., Gupta, S., Will, H., Greten, H., and

440 Petersen, J., Repopulation of mouse liver with human hepatocytes and

441 in vivo infection with hepatitis B virus. Hepatology 33 (2001) 981-988.

442 [12] Llovet, J.M., Burroughs, A., and Bruix, J., Hepatocellular carcinoma.

443 Lancet 362 (2003) 1907-1917.

444 [13] Kremsdorf, D., Soussan, P., Paterlini-Brechot, P., and Brechot, C.,

445 Hepatitis B virus-related hepatocellular carcinoma: paradigms for

446 viral-related human carcinogenesis. Oncogene 25 (2006) 3823-3833.

447 [14] Lupberger, J., and Hildt, E., Hepatitis B virus-induced oncogenesis.

448 World J Gastroenterol 13 (2007) 74-81.

449 [15] Baumert, T.F., Thimme, R., and von Weizsacker, F., Pathogenesis of

450 hepatitis B virus infection. World J Gastroenterol 13 (2007) 82-90.

451 [16] Li, J.S., Tong, S.P., and Wands, J.R., Characterization of a

452 120-Kilodalton pre-S-binding protein as a candidate duck hepatitis B

453 virus receptor. J Virol 70 (1996) 6029-6035.

454 [17] Kuroki, K., Cheung, R., Marion, P.L., and Ganem, D., A cell surface

455 protein that binds avian hepatitis B virus particles. J Virol 68 (1994)

456 2091-2096.

457 [18] Breiner, K.M., Urban, S., and Schaller, H., Carboxypeptidase D (gp180),

458 a Golgi-resident protein, functions in the attachment and entry of

459 avian hepatitis B viruses. J Virol 72 (1998) 8098-8104.

460 [19] Cerec, V., Glaise, D., Garnier, D., Morosan, S., Turlin, B., Drenou, B.,

461 Gripon, P., Kremsdorf, D., Guguen-Guillouzo, C., and Corlu, A.,

462 Transdifferentiation of hepatocyte-like cells from the human

463 hepatoma HepaRG cell line through bipotent progenitor. Hepatology

464 45 (2007) 957-967.

465 [20] Parent, R., Marion, M.J., Furio, L., Trepo, C., and Petit, M.A., Origin

466 and characterization of a human bipotent liver progenitor cell line.

467 Gastroenterology 126 (2004) 1147-1156.

468 [21] Dropulic, B., Lentiviral vectors: their molecular design, safety, and use

469 in laboratory and preclinical research. Hum Gene Ther 22 (2011)
470 649-657.
471 [22] Sung, V.M., and Lai, M.M., Murine retroviral pseudotype virus
472 containing hepatitis B virus large and small surface antigens confers
473 specific tropism for primary human hepatocytes: a potential
474 liver-specific targeting system. J Virol 76 (2002) 912-917.
475 [23] Saha, M.N., Tanaka, A., Jinno-Oue, A., Shimizu, N., Tamura, K.,
476 Shinagawa, M., Chiba, J., and Hoshino, H., Formation of vesicular
477 stomatitis virus pseudotypes bearing surface proteins of hepatitis B
478 virus. J Virol 79 (2005) 12566-12574.
479 [24] Chai, N., Chang, H.E., Nicolas, E., Gudima, S., Chang, J., and Taylor, J.,
480 Assembly of hepatitis B virus envelope proteins onto a lentivirus
481 pseudotype that infects primary human hepatocytes. J Virol 81 (2007)
482 10897-10904.
483 [25] Evans, M.J., von Hahn, T., Tscherne, D.M., Syder, A.J., Panis, M., Wolk,
484 B., Hatziioannou, T., McKeating, J.A., Bieniasz, P.D., and Rice, C.M.,

485 Claudin-1 is a hepatitis C virus co-receptor required for a late step in
486 entry. Nature 446 (2007) 801-805.
487 [26] Glebe, D., and Urban, S., Viral and cellular determinants involved in
488 hepadnaviral entry. World J Gastroenterol 13 (2007) 22-38.
489 [27] Ueda, K., Tsurimoto, T., and Matsubara, K., Three envelope proteins of
490 hepatitis B virus: large S, middle S, and major S proteins needed for
491 the formation of Dane particles. J Virol 65 (1991) 3521-3529.
492 [28] Bruss, V., and Ganem, D., The role of envelope proteins in hepatitis B
493 virus assembly. Proc Natl Acad Sci U S A 88 (1991) 1059-1063.
494 [29] Chairez, R., Hollinger, F.B., Melnick, J.L., and Dreesman, G.R.,
495 Biophysical properties of purified morphologic forms of hepatitis B
496 antigen. Intervirology 3 (1974) 129-140.
497 [30] Lacombe, K., Boyd, A., Gozlan, J., Lavocat, F., Girard, P.M., and Zoulim,
498 F., Drug-resistant and immune-escape HBV mutants in HIV-infected
499 hosts. Antivir Ther 15 (2010) 493-497.
500 [31] Sheldon, J., and Soriano, V., Hepatitis B virus escape mutants induced

501 by antiviral therapy. J Antimicrob Chemother 61 (2008) 766-768.

502 [32] Zoulim, F., Antiviral therapy of chronic hepatitis B. Antiviral Res 71

503 (2006) 206-215.

504 [33] Locarnini, S.A., and Yuen, L., Molecular genesis of drug-resistant and

505 vaccine-escape HBV mutants. Antivir Ther 15 (2010) 451-461.

506 [34] Gripon, P., Diot, C., and Guguen-Guillouzo, C., Reproducible high level

507 infection of cultured adult human hepatocytes by hepatitis B virus:

508 effect of polyethylene glycol on adsorption and penetration. Virology

509 192 (1993) 534-540.

510 [35] Kock, J., Nassal, M., MacNelly, S., Baumert, T.F., Blum, H.E., and von

511 Weizsacker, F., Efficient infection of primary tupaia hepatocytes with

512 purified human and woolly monkey hepatitis B virus. J Virol 75 (2001)

513 5084-5089.

514 [36] Lanford, R.E., Chavez, D., Brasky, K.M., Burns, R.B., 3rd, and

515 Rico-Hesse, R., Isolation of a hepadnavirus from the woolly monkey, a

516 New World primate. Proc Natl Acad Sci U S A 95 (1998) 5757-5761.

517 [37] Hantz, O., Parent, R., Durantel, D., Gripon, P., Guguen-Guillouzo, C.,
518 and Zoulim, F., Persistence of the hepatitis B virus covalently closed
519 circular DNA in HepaRG human hepatocyte-like cells. J Gen Virol 90
520 (2009) 127-135.
521 [38] Yu, M.W., Finlayson, J.S., and Shih, J.W., Interaction between various
522 polymerized human albumins and hepatitis B surface antigen. J Virol
523 55 (1985) 736-743.
524 [39] Pontisso, P., Petit, M.A., Bankowski, M.J., and Peeples, M.E., Human
525 liver plasma membranes contain receptors for the hepatitis B virus
526 pre-S1 region and, via polymerized human serum albumin, for the
527 pre-S2 region. J Virol 63 (1989) 1981-1988.
528 [40] Hertogs, K., Leenders, W.P., Depla, E., De Bruin, W.C., Meheus, L.,
529 Raymackers, J., Moshage, H., and Yap, S.H., Endonexin II, present on
530 human liver plasma membranes, is a specific binding protein of small
531 hepatitis B virus (HBV) envelope protein. Virology 197 (1993) 549-557.
532 [41] Treichel, U., Meyer zum Buschenfelde, K.H., Stockert, R.J., Poralla, T.,

533 and Gerken, G., The asialoglycoprotein receptor mediates hepatic
534 binding and uptake of natural hepatitis B virus particles derived from
535 viraemic carriers. J Gen Virol 75 (Pt 11) (1994) 3021-3029.
536 [42] Zhang, X., Lin, S.M., Chen, T.Y., Liu, M., Ye, F., Chen, Y.R., Shi, L., He,
537 Y.L., Wu, L.X., Zheng, S.Q., Zhao, Y.R., and Zhang, S.L.,
538 Asialoglycoprotein receptor interacts with the preS1 domain of
539 hepatitis B virus in vivo and in vitro. Arch Virol 156 (2011) 637-645.
540 [43] Deng, Q., Zhai, J.W., Michel, M.L., Zhang, J., Qin, J., Kong, Y.Y., Zhang,
541 X.X., Budkowska, A., Tiollais, P., Wang, Y., and Xie, Y.H.,
542 Identification and characterization of peptides that interact with
543 hepatitis B virus via the putative receptor binding site. J Virol 81
544 (2007) 4244-4254.
545 [44] Urban, S., Breiner, K.M., Fehler, F., Klingmuller, U., and Schaller, H.,
546 Avian hepatitis B virus infection is initiated by the interaction of a
547 distinct pre-S subdomain with the cellular receptor gp180. J Virol 72
548 (1998) 8089-8097.

549 [45] Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y.,
550 Peng, B., Wang, H., Fu, L., Song, M., Chen, P., Gao, W., Ren, B., Sun,
551 Y., Cai, T., Feng, X., Sui, J., and Li, W., Sodium taurocholate
552 cotransporting polypeptide is a functional receptor for human
553 hepatitis B and D virus. elife 1 (2012) e00049.
554 [46] Ueda, K., Start or end ? One of the Biggest mysteries is finally solved?
555 J Infect Dis Ther 1 (2013) e101.
556 [47] Ueda, K., One year passed since a bile acid transporter (sodium
557 taurocholate cotransporting peptide [NTCP]) was nominated as a
558 hepatitis B virus (HBV) entry receptor; has the NTCP been as a ral
559 HBV receptor? J. Infect Dis Ther 1 (2013) e102.
560 [48] Neurath, A.R., Kent, S.B., Strick, N., and Parker, K., Identification and
561 chemical synthesis of a host cell receptor binding site on hepatitis B
562 virus. Cell 46 (1986) 429-436.
563 [49] Pontisso, P., Ruvoletto, M.G., Gerlich, W.H., Heermann, K.H., Bardini,
564 R., and Alberti, A., Identification of an attachment site for human

565 liver plasma membranes on hepatitis B virus particles. Virology 173
566 (1989) 522-530.
567 [50] Glebe, D., Urban, S., Knoop, E.V., Cag, N., Krass, P., Grun, S., Bulavaite,
568 A., Sasnauskas, K., and Gerlich, W.H., Mapping of the hepatitis B
569 virus attachment site by use of infection-inhibiting preS1 lipopeptides
570 and tupaia hepatocytes. Gastroenterology 129 (2005) 234-245.
571 [52] Engelke, M., Mills, K., Seitz, S., Simon, P., Gripon, P., Schnolzer, M., and
572 Urban, S., Characterization of a hepatitis B and hepatitis delta virus
573 receptor binding site. Hepatology 43 (2006) 750-760.
574 [51] Bremer, C.M., Sominskaya, I., Skrastina, D., Pumpens, P., El Wahed,
575 A.A., Beutling, U., Frank, R., Fritz, H.J., Hunsmann, G., Gerlich, W.H.,
576 and Glebe, D., N-terminal myristoylation-dependent masking of
577 neutralizing epitopes in the preS1 attachment site of hepatitis B virus.
578 J Hepatol 55 (2011) 29-37.
579 [52] Engelke, M., Mills, K., Seitz, S., Simon, P., Gripon, P., Schnolzer, M., and
580 Urban, S., Characterization of a hepatitis B and hepatitis delta virus

receptor binding site. Hepatology 43 (2006) 750-760.

[53] Blanchet, M., and Sureau, C., Analysis of the cytosolic domains of the hepatitis B virus envelope proteins for their function in viral particle assembly and infectivity. J Virol 80 (2006) 11935-11945.

[54] Abou-Jaoude, G., and Sureau, C., Entry of hepatitis delta virus requires the conserved cysteine residues of the hepatitis B virus envelope protein antigenic loop and is blocked by inhibitors of thiol-disulfide exchange. J Virol 81 (2007) 13057-13066.