乙型肝炎病毒cccDNA体外实验模型研究进展

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2019-03-25 收稿, 2019-05-19 修回, 2019-05-20 接受, 2019-06-27 网络版发表

国家自然科学基金(81672034, 81871647)、上海市教育委员会科研创新计划(2017-01-07-00-07-E00057)和国家科技重大专项(2018ZX10301208)资助

摘要 乙型肝炎病毒(hepatitis B virus, HBV)是一种小的DNA包膜病毒, 属于嗜肝DNA病毒科。在细胞核内, HBV部分双链的DNA基因组修复为共价闭合环状DNA(covalently closed circular DNA, cccDNA), 后者作为HBV复制的原始模板起始病毒转录。cccDNA具有显著的稳定性, 是HBV持续感染的分子基础, 也是抗病毒药物设计的关键靶点。HBV cccDNA研究常受限于细胞内较低的拷贝数, 以及缺乏灵敏、可靠的检测方法, 因此, 建立合适的cccDNA实验模型具有重要意义。本文对近年来HBV cccDNA体外实验模型方面的主要研究进展进行综述, 以期为深入研究HBV病毒学和抗病毒药物研发提供帮助。

关键词 乙型肝炎病毒, 共价闭合环状DNA, 病毒复制, 持续性感染, 实验模型

乙型肝炎病毒(hepatitis B virus, HBV)慢性感染迄今仍然是世界性公共健康问题。我国是HBV感染高发区, 目前仍有约9300万的人口为乙肝表面抗原(HBV surface antigen, HBsAg)阳性的病毒携带者^[1]。慢性乙型肝炎(chronic hepatitis B, CHB)可以持续终生, 引起不同层级的肝脏损伤, 并最终导致肝硬化和肝肿瘤的发生。因此, 研究CHB病理机制、发展有效的抗病毒治疗策略具有重大的科学意义和社会意义。

HBV属于嗜肝DNA病毒科, 成熟粒子中含有约3.2 kb松弛环状DNA(relaxed circular DNA, rcDNA)基因组。在病毒感染的肝细胞核内, rcDNA修复为共价闭合环状DNA(covalently closed circular DNA, cccDNA), 是HBV转录复制的原始模板; 以cccDNA为模板转录的前基因组RNA(pregenomic RNA, pgRNA)转运至细胞浆后, 由病毒多聚酶蛋白识别反转录生成负链DNA, 并合成不完全的正链DNA; 部分双链的rcDNA通过成熟

的核衣壳颗粒再次转运至细胞核, 完成HBV的细胞内复制周期^[2]。另一方面, 病毒核衣壳颗粒通过内体分选转运复合体机制经由多泡体结构完成包膜化和出芽^[3]。rcDNA修复生成cccDNA的分子机制仍然不是很清楚, 但至少包括以下的步骤^[4,5]: (1) 合成并补齐不完整正链DNA序列; (2) 移除负链DNA 5'-末端共价连接的病毒多聚酶蛋白; (3) 移除负链DNA在反转录合成过程中产生的一段约10个碱基的冗余序列; (4) 移除引导正链DNA起始合成的具有5'-帽结构的小RNA片段; (5) DNA末端的连接。修复生成的cccDNA具有超螺旋结构, 同时能够结合组蛋白等形成串珠样核小体结构的微小染色体^[6], 具有显著的表现遗传学修饰特征^[7]。cccDNA在HBV感染肝细胞中仅维持数个拷贝。Laras等人^[8]基于特异性定量PCR的方法检测CHB病人中, 每10⁶肝细胞中约含有1.5×10⁶拷贝cccDNA(3.2×10³~4.0×10⁷)。其他类似研究显示, HBV慢性感染的肝组织样本

引用格式: 朱园飞, 苏瑜, 邓强. 乙型肝炎病毒cccDNA体外实验模型研究进展. 科学通报, 2019, 64: 3061–3069

Zhu Y F, Su Y, Deng Q. *In vitro* experimental models for the study of hepatitis B virus cccDNA (in Chinese). Chin Sci Bull, 2019, 64: 3061–3069, doi: 10.1360/N972019-00254

中, 每个肝细胞仅含有0.01~1.4拷贝^[9], 或者0.1~9.0拷贝的cccDNA^[10]. 尽管细胞内拷贝数较低, cccDNA的稳定性特征却很显著, 被认为是HBV慢性感染的分子基础. cccDNA在抗病毒药物抑制条件下仍具有较长的代谢周期, 能通过细胞分裂传递至子代细胞, 细胞凋亡或坏死是机体清除cccDNA的主要机制. 现有的抗病毒药物能有效抑制病毒复制和rcDNA形成, 但难以清除cccDNA, 这是CHB患者难以彻底治愈的根本原因^[11]. 另一方面, 迄今仍然缺乏简单、高效的技术方法检测cccDNA, 经典的Southern杂交技术具有可靠的特异性, 但操作复杂不易实现; 实时荧光定量PCR检测非常灵敏, 但由于难以区分rcDNA及其他病毒复制中间体, 极易导致假阳性的结果.

HBV易感宿主仅限于人和黑猩猩等灵长类动物, 小鼠是理想的实验模式动物, 然而HBV并不感染小鼠. 早期对HBV免疫病理机制的研究多依赖于转基因小鼠模型. 近年来, 很多研究者尝试在非转基因小鼠中建立病毒感染, 例如通过腺病毒或腺相关病毒载体携带HBV基因组^[12,13], 或利用尾静脉高压注射策略直接注射HBV编码质粒^[14,15], 诱导病毒持续感染和可能的肝脏免疫病理损伤. HBV实验动物模型在本期的其他论文中有详细描述. 在人类疾病发病机制和新药评价中, 除了动物疾病模型, 体外实验模型也发挥了极其重要的作用, 也是动物福利与伦理的重要体现. 本文将围绕近年HBV cccDNA体外细胞模型的重要进展进行综述和分析.

1 病毒感染细胞模型

HBV自然感染过程包括病毒粒子与细胞接触、受体介导的细胞进入、病毒核衣壳粒子释放、核衣壳粒子入核, 以及cccDNA形成等步骤. 感染细胞模型对于研究HBV如何建立感染及cccDNA复制有着极其重要的作用.

1.1 肝肿瘤细胞模型

HepG2和Huh7细胞系是最常用的肝癌细胞系, 易于遗传学操作, 在HBV DNA转染后能够支持病毒复制. 然而由于缺乏进入受体, 它们都不被HBV感染. Gripon等人^[16]2002年首次报道HepaRG细胞, HepaRG随后成为HBV研究领域最为重要的研究工具之一. HepaRG最初由肝脏肿瘤样本中筛选和发展获得, 能够表达肝细胞特异性基因并且易受HBV感染. 但这些细胞只有在皮质激素和二甲基亚砷诱导分化条件下才能获得感

染能力, 因此实验操作相对复杂. 应用HepaRG感染体系的研究显示, HBV感染动力学过程缓慢, rcDNA修复为cccDNA的效率较低, 细胞建立感染后并没有cccDNA的扩增^[17].

牛磺胆酸钠协同转运多肽(sodium taurocholate co-transporting polypeptide, NTCP)已被证明是HBV和HDV的高亲和力受体, 稳定表达外源性NTCP使得HepG2和Huh7细胞能够被HBV感染^[18,19]. 近期报道的HepG2-NTCP-K7细胞^[20]显示对HBV感染非常敏感, 病毒感染12 h即能在细胞核中检测到去蛋白形式的rcDNA(PF-rcDNA^[4,5]); cccDNA形成相对滞后, 24 h可被检测并逐渐增加, 随后(>3 d)进入平台期. 在病毒/细胞感染复数(moi)1000的条件下, 每个感染细胞中约有10.7~12.5拷贝cccDNA形成. 应用核苷类似物恩替卡韦抑制病毒复制, 测定HBV cccDNA半衰期约为40 d. HBV感染的HepG2-NTCP-K7细胞具有强大的病毒复制能力, 子代病毒颗粒能够实现和培养细胞的二次感染, 后者与细胞内病毒复制机制共同维持cccDNA池的稳定和扩增. Watashi课题组^[21]的研究表明, 表皮生长因子(EGF)能够刺激HBV对HepG2^{NTCP}细胞的感染, 应用siRNA敲低EGF受体表达显著抑制病毒感染及cccDNA形成, 进一步研究则显示EGF受体的细胞内转位可能介导了病毒粒子的内化. Qi等人^[22]应用siRNA筛选并证实, 宿主DNA多聚酶 κ 可能参与了cccDNA修复过程中正链DNA缺口补齐; 特异性敲低DNA连接酶1或3的表达, 能够显著抑制HBV cccDNA的形成^[23], 提示宿主DNA修复机制在HBV cccDNA生物合成过程扮演了重要角色. 由于尚不清楚原因, 小鼠肝脏细胞并不支持cccDNA形成^[24,25]. 表达人NTCP的小鼠肝肿瘤细胞系^[26,27], 或表达人NTCP分子的转基因小鼠^[28], 并不能支持HBV的感染. 有意思的是, Cui等人^[29]最近报道在HBV转基因诱导表达的小鼠AML12HBV10细胞系中鉴定到cccDNA, 并发现AML12HBV10细胞中成熟的病毒核衣壳粒具有显著的不稳定性.

1.2 鸭乙型肝炎病毒(duck hepatitis B virus, DHBV)/原代鸭肝细胞(primary duck hepatocytes, PDH)感染模型

HBV、土拨鼠肝炎病毒(WHV)、地松鼠肝炎病毒(GSHV)以及DHBV等同属于嗜肝DNA病毒家族, 基因组结构和功能等方面具有显著的相似性. 相比于WHV和GSHV, DHBV模型系统具有良好的经济性和可操作

性,因此经常作为替代模型应用于HBV病毒学研究。Summers实验室^[30]早在1986年即系统性报道了DHBV/PDH体外感染实验模型。病毒感染细胞后第2天即能够检测到cccDNA,后者在感染后第8~12天进入扩增期(约50~100倍)^[30,31]。PDH模型中DHBV cccDNA形成非常丰富,Tuttleman等人^[31]基于生化分离纯化的方法证实cccDNA并不采取所谓的半保留复制方式,rcDNA则是其生物合成的主要前体。Yang等人^[32]应用DHBV突变体(正链DNA引物转位缺陷)感染PDH的研究表明,病毒双链线性DNA(dsIDNA)复制中间体则可能通过非同源末端连接(NHEJ)方式形成非功能性的cccDNA。

在新鲜分离培养的PDH中,平均1~10病毒基因组当量(vge)/细胞的moi条件下即能实现可检测的病毒感染^[33]。由于易实现从头感染以及高效的病毒复制,DHBV/PDH模型成为抗病毒药物评价的重要平台^[34,35]。Kock和Schlicht等人^[36]报道,病毒多聚酶蛋白抑制剂2',3'-二脱氧鸟苷并不能抑制DHBV cccDNA修复,推测宿主机制参与了病毒正链DNA的延伸;抗病毒药物阿德福韦的临床前研究同样显示,后者能够显著抑制DHBV在PDH中复制和cccDNA扩增,但并不影响基于病毒从头感染的cccDNA形成^[35]。PDH细胞体外感染系统具有一定时限性,原代细胞体外培养7 d后即不能再为病毒感染。胚胎来源的PDH同样能够支持DHBV的从头感染,同时,这些细胞具有一定增殖潜力。有意思的是,细胞生长因子刺激的细胞增殖可能促进HBV复制和cccDNA扩增^[37],这与前文中提及有丝分裂可能导致cccDNA丢失的观点并不一致。

1.3 人原代肝细胞模型

尽管具有一定相似性,DHBV和HBV仍是不同生物特性的两种病毒,HBV也不能跨种属感染PDH。分化的原代人肝细胞(primary human hepatocytes, PHH)是体外研究HBV感染最贴近自然的细胞模型。应用PHH模型对HBV cccDNA的研究近期获得一系列重要发现。例如,HBV x(HBx)蛋白介导宿主细胞限制因子——染色体结构维持复合物5/6(Smc5/6)的泛素化降解,因此上调cccDNA在转录水平的活化^[38];Lucifora等人^[39]证实 α -干扰素能够诱导APOBEC3A和3B在肝细胞中的表达,后者通过胞嘧啶脱氨基作用实现对HBV cccDNA的编辑和清除。表观遗传学研究则显示 α -干扰素显著抑制病毒感染PHH细胞中cccDNA的转录活性^[40]。有意思的是,沉默的染色质结构特征似乎更提示cccDNA稳

定性的增加,应用PHH感染模型的研究进一步表明, α -干扰素显著抑制HBV复制中间体和病毒抗原的表达,但并不影响cccDNA水平^[41]。

然而,PHH不易获得,增殖缓慢,且难以在体外维持其分化状态,在接种后不久就会失去病毒易感性^[42]。Walter等人^[43]从树鼩中分离原代肝细胞(primary tupaia hepatocytes, PTH),体外培养体系证实PTH能够支持HBV的感染,因此可作为PHH的替代模型。李文辉实验室^[44]则应用PTH体外感染系统鉴定了HBV进入细胞的受体NTCP。另一方面,PHH体外感染模型近期获得重要进展,中国科学家^[45,46]先后报道应用特定培养基诱导PHH去分化,使其获得体外大量增殖的能力;基于3D细胞培养重新诱导分化的细胞具有PHH的主要特征,并支持HBV感染和cccDNA形成^[46]。这些模型对于研究HBV感染及cccDNA的特征有着极其重要的作用。HBV感染的PHH去分化扩增后不能检测到cccDNA和病毒抗原表达。有意思的是,重新诱导分化的PHH样细胞出现HBV病毒学反弹,cccDNA水平显著增高,提示cccDNA能够通过细胞分裂大量遗失,却并不能完全清除^[46]。这一发现在细胞培养水平重现了Dandri M研究组基于人嵌合肝小鼠模型的研究结果^[47],值得更为细致量化和深入的分析。

2 cccDNA生物合成的转染细胞模型

HBV cccDNA的生物合成指病毒复制中间体rcDNA修复成为cccDNA的过程。应用携带HBV基因组复制子的质粒转染肝肿瘤细胞系HepG2或Huh-7,能够在细胞中产生病毒RNA和DNA复制中间体,后者在细胞核内进一步修复成为cccDNA。瞬时转染的优势在于质粒DNA自身可看作是一种cccDNA替代分子,但这一模型新产生的野生cccDNA拷贝数极低。DHBV能够在宿主细胞中产生较多的cccDNA拷贝,应用敲除病毒包膜蛋白的DHBV复制子编码质粒转染鸡肝肿瘤细胞系LMH,每单个细胞中平均能够产生约200~400分子的cccDNA^[48,49]。有意思的是,在HBV表达质粒中敲除包膜蛋白基因并不能产生类似效应^[4]。进一步的研究显示,HBV和DHBV自身病毒学因素决定了cccDNA生物合成的效率,而宿主细胞种系特征并不显著^[50]。

HBV稳定转染细胞系中最为著名的是HepG2.2.15^[51]和HepAD38^[52],前者在宿主细胞基因组中整合并稳定表达HBV基因组,后者则应用四环素(tetracycline, tet)表达调控体系诱导(tet-off)HBV pgRNA和HBV核心抗

原(HBV core antigen, HBcAg)的表达. 相对于HepG2.2.15细胞系, tet-off诱导表达的HepAD38细胞支持更为显著的细胞内病毒复制和cccDNA形成, 易为Southern杂交等方法检测, 是HBV cccDNA研究的重要工具之一. Guo等人^[53]则基于LMH细胞系发展了一种tet调控的HBV基因组整合细胞系Dstet5, 通过应用工具药物抑制病毒复制等设计不同时间方案, 能够精确指示病毒复制以及cccDNA形成的动态特征, 并证实 α -干扰素能够通过表观遗传修饰抑制cccDNA的转录, 并加速后者的降解^[54].

可诱导HepAD38和类似细胞模型极大地推动了HBV病毒学研究. 为实现抗病毒药物高通量筛选, Guo H实验室^[5,55]非常巧妙地设计发展了HepDE19细胞模型: HBV复制过程中, pgRNA残余的帽结构RNA引物起始正链DNA的合成, 并转位至负链DNA 5'端的DR2区, 实现模板转换. 研究者设计构建tet-CMV启动子控制的HBV基因组编码质粒, 其5'端删除HBcAg的翻译起始码; 当正链DNA合成并修复为cccDNA后, HBcAg则获得完整蛋白的读码框, 其分泌产物因此可指示cccDNA的从头合成^[55]. 由于HBcAg蛋白质序列与HBcAg大部分重合, Cai等人^[56]进一步在其N端(epsilon信号后)设计融合了一段9aa的人类流感血凝素(HA)标签序列, 证实并不影响HBV复制.

3 病毒复制子转染细胞模型

HBV研究常基于细胞转染的方法, 应用病毒DNA复制子来模拟细胞内已建立的cccDNA. HBV包含3.2 kb单拷贝病毒基因组遗传信息, 环绕转录的4种主要病毒RNA, 分别为3.5, 2.4, 2.1, 0.7 kb. 为实现功能性转录, 研究者多应用首尾部分重复或多拷贝串联重复的病毒基因组, 即所谓的线性病毒复制子系统, 通过质粒转染或病毒载体转导方式起始HBV细胞内复制周期. 超螺旋质粒具有cccDNA的定义特征. Bock等人^[6]利用HBV编码质粒证实HBcAg参与cccDNA的染色质化过程. Lin等人^[57]则应用HBV编码质粒作为cccDNA的替代靶标, 首次概念性验证了基于CRISPR/Cas9技术的抗病毒治疗策略. 然而, 由于具有较大的基因组长度, 以及携带大量的外源性片段(例如原核特征的质粒复制和抗性筛选元件等), DNA质粒或重组病毒载体可能具有与3.2 kb HBV cccDNA显著不同的病毒学特征和宿主反应性. 例如, 在著名的高压水动力注射小鼠模型中, Huang等人^[15]构建pAAV/HBV1.2质粒, 在HBV线性基

因组两侧设计倒转的腺相关病毒末端重复序列, 使得HBV转基因在小鼠肝脏中的免疫反应显著下降.

为避免引入外源序列, 研究者常采用HBV单拷贝基因组DNA(PCR或多拷贝串联基因组的酶切产物)转染细胞, 具有黏性末端的线性DNA通过细胞内机制环化, 成为HBV转录和复制的替代模板^[58]. 这一模型极为简便, 被广泛应用于临床病毒株和突变体的克隆和分析. 早期HBV cccDNA表观遗传学的一些重要研究也正是基于这一模型展开的^[59-61]. 需要指出的是, 上述的表观遗传学研究中, 需评价相关因素(例如 α -干扰素)对线性DNA细胞内环化的影响; 此外, 环化的DNA模板与基于病毒复制从头合成的cccDNA的区分也比较模糊. 有意思的是, 狄弘玮等人^[62]报道, 在HBV基因组DR1区nt1821位置背靠背设计引物, 方向相反的两个引物不包含重叠或其他外源序列, 应用PCR产物直接转染细胞或经由高压水动力注射小鼠后, 能够显著表达HBV的两种主要病毒抗原. 与病毒dsDNA复制中间体类似, 线性化PCR产物可能通过NHEJ机制形成cccDNA模板^[32], 其形成效率和保真性值得深入分析.

4 HBV重组cccDNA(rcccDNA)模型

具有黏性末端的病毒DNA基因组可在体外由T4连接酶连接, 通过琼脂糖电泳和胶纯化获得具有超螺旋结构特征的cccDNA替代分子^[29]. 然而上述方法操作相对复杂, 且不易于大量制备. 我们和其他一些研究组^[63-65]先后报道了一种通过位点特异性重组获得rcccDNA的研究策略. rcccDNA与野生病毒cccDNA具有很大相似性, 能够在细胞内或体外系统大量诱导产生, 是HBV病毒学研究的一类重要工具. HBV基因组高度压缩, 难以容纳外源性序列. 我们在HBV基因组两侧引入同向loxP位点, 由重组酶Cre引发loxP位点之间的DNA重组和环化, 生成含有单拷贝loxP位点的rcccDNA; loxP位点则位于一段90 bp的外源性内含子中, 可在RNA转录过程中高效移除(图1)^[63]. 另一方面, 我们基于大肠杆菌PhiC31重组酶诱导表达系统, 建立了一种体外诱导rcccDNA^{attR}微环产生和纯化的策略. 纯化的rcccDNA^{attR}微环具超螺旋结构, 能够支持功能性的HBV病毒复制和抗原表达^[66]. 由于rcccDNA编码的外源性内含子序列, 应用引物特异性PCR方法易于与HBV DNA复制中间体、以及从头合成的新生cccDNA区分.

尽管并非本文主要的范畴, 由于重要的关联性, 我们仍希望对rcccDNA分子的体内代谢特征进行一些讨

图 1 (网络版彩色) Cre/loxP介导的DNA位点特异性重组诱导rcccDNA产生。rcccDNA前体质粒(prcccDNA)含有一段单拷贝的HBV基因组, 在HBsAg编码序列中设计插入一段嵌合内含子结构, 包括内含子5'端供体序列、loxP-1、质粒复制骨架、真核基因polyA加尾序列、loxP-2以及内含子分枝信号和3'端受体序列。在转染细胞中, Cre/loxP介导的DNA重组可剪除prcccDNA中的质粒复制骨架, 诱导rcccDNA产生。3.3 kb的rcccDNA含有功能性的loxP杂合内含子序列, 在转录过程中通过RNA拼接机制去除, 因此并不干扰HBV病毒复制周期

Figure 1 (Color online) Schematic illustration of Cre/loxP-mediated rcccDNA production. The precursor plasmid prcccDNA encodes a monomeric HBV genome with a DNA fragment inserted into the early open reading frame of the HBV surface antigen (HBsAg). The insert comprises, in turn, a 5' intronic donor site, a floxed (*loxP*-1 and *loxP*-2) plasmid backbone bearing a eukaryotic polyadenylation signal, and a 3' intronic branch/acceptor site. In transfected cells, a 3.3-kbrcccDNA bearing a *loxP*-chimeric intron can be produced from prcccDNA by Cre/*loxP*-mediated DNA excision. The *loxP*-chimeric intron can be spliced from viral transcripts during RNA processing, without disturbing the replication cycle of HBV

论。与普通的线性HBV复制子编码质粒相比, 应用rcccDNA^{loxP}或rcccDNA^{attR}能够在高压水动力注射小鼠模型中诱导延长的病毒抗原血症, 因此能够作为一种原型策略发展HBV持续感染的小鼠模型。由于转染效率(4%~6%)和病毒持续时间(5~7周)的限制, 应用rcccDNA的尾静脉高压注射并不能在小鼠肝脏中积累显著的慢性病理改变。在研究过程中, 我们希望在模拟体内慢性肝炎的条件下分析cccDNA稳定性特征。(1) 通过引入shRNA, 敲低rcccDNA共转染肝细胞表面的MHC-I分子, 因而抑制病毒特异性T细胞免疫应答;(2) 通过敲除HBV病毒X基因, 或通过具有DNA结合能力

的Cre-kox1融合蛋白, 实现rcccDNA的表观遗传学沉默, 诱导HBV持续表达;(3) 进一步利用重组腺病毒载体投递rcccDNA, 能够感染小鼠模型中大多数肝细胞, 诱导功能损伤的T细胞应答, 使得rcccDNA持续表达而不被清除, 并伴随慢性肝炎、肝脏纤维化和肝细胞异常增生等病理变化^[67]。该研究将有助于对HBV持续感染和相关疾病过程的理解, 同时也代表了一种重要的体内实验模型系统进行抗病毒药物筛选和评价。

5 总结

cccDNA是HBV慢性感染的分子基础和重要治疗

靶点, 病毒学研究中通常聚焦于cccDNA生物合成和已形成cccDNA的生物代谢两个方面. 在常用的体外模型中, PHH是天然的HBV宿主细胞, 易于感染HBV, 但其扩增性差, 难以在体外维持分化状态, 而近期干细胞领域的进展可能具有重要的研究价值和应用前景^[45,46]. 肝肿瘤细胞系无疑是应用最为广泛和有效的工具, 能支持HBV复制, 易于分子和细胞生物学的各种操作, 表达病毒受体蛋白NTCP的HepG2细胞能够支持HBV从

头感染和cccDNA生物合成, 代表了中国科学家近期在HBV领域最为杰出的研究工作. 重组cccDNA策略为抗病毒药物研究和高通量筛选提供了便利和高效的平台, 同时, 小鼠肝脏中投递rcccDNA代表了一种重要的体内实验模型系统, 可用于HBV持续性感染和宿主病理机制的研究. 合理地使用这些模型, 将会有助于更快、更有效地解决cccDNA带来的难题, 为人类攻克HBV奠定基础.

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Summary for “乙型肝炎病毒cccDNA体外实验模型研究进展”

***In vitro* experimental models for the study of hepatitis B virus cccDNA**

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Chronic infection of hepatitis B virus (HBV) remains a global public health problem and affects at least 240 million people worldwide. HBV is a small enveloped DNA virus belonging to the Hepadnaviridae family. The virus has a narrow host range, limited to humans and chimpanzees. In the nucleus of infected hepatocytes, the partially double-stranded DNA (rcDNA) HBV genome is converted to covalent closed circular DNA (cccDNA), which serves as the sole template for HBV transcription. HBV cccDNA exists as an episomal minichromosome in the nucleus, with a turnover rate that correlates with the mitosis or death of virus-infected hepatocytes. It is thus regarded as a primary molecular mechanism for viral persistence.

In the past decade, significant progress has been made in HBV research and the related antiviral development. Sodium taurocholate co-transporting polypeptide (NTCP) has been identified as a receptor for HBV entry into hepatocytes. It was recently shown that HBV x (HBx), an enigmatic viral protein, achieves its regulatory function by hijacking the cellular DDB1-containing E3 ubiquitin ligase to target the Smc5/6 complex for degradation. Although still controversial, type I interferon has been implicated to either affect the epigenetic control of cccDNA minichromosome, or induce cytidine deaminases-mediated specific degradation of the episomal cccDNA. Of particular note, core protein allosteric modulator (CpAM) has been most widely investigated. These studies represent a new frontier in the development of antivirals against HBV infection.

Despite continuing progress in HBV research, a complete cure of HBV infection is still not achievable. Current antiviral therapies with nucleos(t)ide analogs (NUC) and pegylated IFN- α can efficiently inhibit HBV replication, but the treatments fail to eliminate the preexisting cccDNA pool that is responsible for a viral rebound after therapy cessation. Therefore, it is widely held that cccDNA elimination is a prerequisite for complete cure of chronic HBV infection. To this end, it is essential to understand the mechanisms involved in cccDNA metabolism and transcriptional regulation. Studies of HBV cccDNA have often been hampered by the low copy number of cccDNA per cell and the lack of convenient techniques with high sensitivity and specificity for cccDNA detection. Development of appropriate experimental models is thus fundamental for HBV cccDNA studies. Herein, we reviewed recent advances in cell culture models for the study of HBV cccDNA, including models of HBV *de novo* infection and cccDNA biogenesis, cccDNA formation (rcDNA repair-related molecular events), HBV replicon-based transfection models to study established cccDNA, and the surrogate model of cccDNA produced from site-specific DNA recombination. Animal models to study HBV cccDNA will be further discussed by other authors in this issue. We hope this review may help clinicians and researchers who are interested in the study of cccDNA and antiviral development against HBV.

hepatitis B virus, covalently closed circular DNA, viral replication, viral persistence, experimental model

doi: [10.1360/N972019-00254](https://doi.org/10.1360/N972019-00254)