The C-terminus domain of the hepatitis B virus X protein stimulates the proliferation of mouse foetal hepatic progenitor cells, although it is not required for the formation of spheroids

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Abstract. The hepatitis B virus X (HBx) protein is an important factor in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC). The C-terminal region of HBx plays a major role in the replication of HBV. Notably, HBx promotes the expansion and tumourigenesis of hepatic progenitor cells (HPCs) in mice. However, it remains unclear as to whether the C-terminal region of HBx is required for the stimulation fo the proliferation of mouse foetal HPCs (FHPCs). In our study, we used EpCAM$^+$, CD133$^+$ and CD49f$^+$ FHPCs, which are bipotential clonogenic cells. These FHPCs transformed into mature hepatocytes and cholangiocytes when cultured under conditions that facilitate differentiation. Compared with the FHPCs grown as monolayers, spherical cell proliferation occurred more rapidly. Furthermore, spherically cultured FHPCs can grow in semi-solid agar and tend to maintain the morphology and characteristics of stem cells compared with growth in rat tail collagen. Notably, we also demonstrate that the C-terminus of HBx stimulates the proliferation of FHPCs, but is not required for the formation of spheroids, similar to hepatic cancer stem cells. These findings enhance our understanding of the HBx-induced tumourigenicity of FHPCs and may aid in the treatment of HCC.

Introduction

The hepatitis B virus (HBV) infects more than 350 million individuals worldwide and is the main cause of primary hepatocellular carcinoma (HCC). HBV-associated HCC is the fifth most common malignant disease worldwide (1). HCC is associated with a very high mortality rate, and the recurrence and metastasis of liver cancer remain a major concern. The hepatitis B virus X (HBx) protein is a low-molecular-weight (17-kDa), 154-amino-acid soluble protein that is a well-known risk factor for HBV-induced carcinogenesis (2). Although HBx, particularly the C-terminal region of HBx, is known to stimulate quiescent hepatocytes to undergo a G0/G1 transition and enter the cell cycle, thereby maximising the replication performance of HBV (3); however, the precise function of HBx in HBV-associated HCC remains unclear (4-6). It has been found that hepatic progenitor cells (HPCs) become activated in patients with chronic hepatitis C or B, HCC or other severe liver diseases, and damage occurs to liver cells and bile duct epithelial cells (7). A pluripotent progenitor cell in normal adult human liver has already been identified (8). It is well known that HCCs contain tumour-initiating stem-like cells (TISCs) (9-11), and microarray technologies utilizing integrative comparative genomic analysis of human HCC tissue samples, HCC cell lines and transgenic mouse models confirm that liver cancer stem cells and HPCs exhibit similar molecular characteristics (12). It has also been suggested that TISCs are derived from the malignant transformation of stem/progenitor cells (13). In addition, a study on the HBx-stimulated transformation of intrinsic cellular pathways to promote HPC proliferation and tumourigenicity also supports this claim (14). We thus hypothesised that the truncation of HBx is associated with the mechanism of HBx-induced proliferation.

The foetal and adult liver are common sources of HPCs. The activation of HPCs from adult liver has reportedly been induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (15,16), by choline-deficient or ethionine-supplemented diets (18), in transgenic mice [such as p53 null, methionine adenosyltransferase 1A (MAT1A)$^{-/-}$ or liver-specific phosphatase and tensin homolog deleted on chromosome 10 (Pten)$^{-/-}$ mice] (19-22), and by reprogramming fibroblasts by defined factors (23). In addition, the foetal liver contains large numbers of HPCs, such as rat embryonic day (ED)14 foetal HPCs (FHPCs), which were purified to 95% homogeneity and are expected to be HPCs (24). In mice, over 60% of cells derived from ED14.5 Dlk$^+$ cells are

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able to differentiate into both hepatocytes and biliary epithelial cells (25), and it has been shown that density gradient centrifugation can be used to purify mouse FHPCs (26). In the present study, we used ED14.5 mouse FHPCs to demonstrate that they display the characteristics of spherical growth and that the C-terminal region of HBx is required for the stimulation of cell proliferation. The findings of our study may enhance our understanding of the mechanisms through which HBx induces the tumourigenicity and proliferation of HPCs and may facilitate the development of future treatments for liver diseases.

Materials and methods

Animals. Primary hepatocytes were isolated from 6-8-week-old wild-type male C57BL/6 mice by a two-step collagenase perfusion (3), and FHPCs were isolated from ED14.5 wild-type C57BL/6 mice, as previously described (25). The animals were obtained from the Animal Experiment Center of Chongqing Medical University (Chongqing, China) and all animal experiments were performed according to the guidelines set by the National Health and Medical Research Council of China. All animal experiments were approved by the Ethics Committee of the Second Hospital of Chongqing Medical University (no. 2013-026).

Isolation of FHPCs. Pregnant C57BL/6 mice (14.5 days) were sacrificed and subjected to caesarean delivery. The livers were removed from the mouse foetuses and washed 2 times in cold phosphate-buffered saline (PBS), dissected into sections, digested with 0.1% collagenase IV and 0.05% DNAse (Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12), and incubated for 30 min at 37˚C, and a single cell-suspension was obtained via successive filtration through 150 and 75-μm cell strainers. Subsequently, the cell suspension was centrifuged at 1,000 x g for 10 min, and the cell pellet was collected and resuspended in 3 ml of PBS. Discontinuous Percoll (Amersham Pharmacia Biotech, Freiburg, Germany) gradients were prepared in 15-ml tubes by layering 3 ml each of 70% Percoll, 50% Percoll and 30% Percoll in sequence, after which the cell suspension was layered atop the Percoll solution. The tubes were centrifuged at 400 x g for 25 min. Depending on their different sizes and densities, cells aggregate at specific positions along the density gradient. The cell fraction at approximately 50% Percoll was collected and centrifuged again at 300 x g for 10 min, and the pelleted cells were collected as FHPCs. The trypan blue dye exclusion assay was used to assess cell viability. All steps were performed on ice to reduce the risk of cell damage.

Culture of FHPCs. The FHPCs were seeded onto type I collagen (Sigma-Aldrich)-coated 60-mm dishes at a density of $1x10^6$ viable cells. The cells were maintained in DMEM/F-12 containing 1% penicillin/streptomycin, 10 μg/ml insulin (Sigma-Aldrich), 10% fetal bovine serum (FBS), 20 ng/ml epidermal growth factor (EGF; Peprotech, Inc., Rocky Hill, NJ, USA), 10 ng/ml leukemia inhibitory factor (LIF; Sigma-Aldrich) and 2 mM L-glutamine at 37˚C with 5% CO_2. The medium was changed after 24 h to remove dead and non-adherent cells, and the culture medium was subsequently changed every 1-2 days. The cells were observed daily under an inverted microscope (Nikon Eclipse Ti-U). The stellate cells were selectively detached from the dish by repeated differential digestion with TrypLE™ Express (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C for 2.5 min. The purified FHPCs were first passaged at a ratio of 1:2 at 5-7 days after plating and passaged every 2-3 days after a generation. The passaged FHPCs were serially diluted to a density of one cell/100 μl of culture medium and replated onto uncoated 96-well plates. Colonies containing >50 cells were quantified after 2 weeks using a binocular inverted microscope (Nikon Eclipse Ti-U).

Establishment of spherical FHPCs clonal cells. The cells were seeded into a 25 cm^2 plastic culture bottle coated with a low concentration of type I collagen at a density of $2x10^5$ viable cells/ml in serum-free medium; spherical cells (10,27) were collected by gentle centrifugation at 30 x g after 5-7 days and replated onto type I collagen-coated 6-well plates as FHPC clonal cells.

Periodic acid-Schiff (PAS) staining. After the attachment of spheroid cells on type I collagen for 7 days, glycogen storage was detected using a PAS staining kit (3), according to the manufacturer's instructions. The cells were fixed with 4% paraformaldehyde for 5 min, washed with distilled water for 1 min and incubated in periodic acid (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) at room temperature for 10 min in the dark. The cells were then washed with distilled water for 5 min and incubated in Schiff's reagent (Nanjing Jiancheng Bioengineering Institute) for 15 min at room temperature. After a final wash in running distilled water for 1 min, the cells were dried at room temperature, incubated in methyl green solution for 30 sec, cleaned with double distilled water and subsequently observed under an inverted microscope (Nikon Eclipse Ti-U).

Alkaline phosphatase (AP) staining. AP staining is often used to identify stem cells (21) and cholangiocytes (23). Both spheroid and attached cells were fixed with 4% paraformaldehyde for 10 min and then washed 3 times with distilled water for 5 min each. BCIP/NBT working solution (Beyotime Institute of Biotechnology, Haimen, China) was prepared according to the manufacturer's instructions. The cells were incubated with BCIP/NBT solution for 30 min at room temperature in the dark, washed with distilled water for 30 sec and analysed under a light microscope.

Flow cytometry. The cells were harvested and washed twice with PBS, followed by staining in washing buffer (PBS containing 0.05% rat serum) at room temperature for 30 min. A total of $1x10^6$ cells were incubated in 100 μl PBS with chromophore-conjugated antibodies at 4˚C for 30 min. The following chromophore-conjugated antibodies were used: anti-mouse CD133 (prominin-1) PE (12-1331) (5 μl) and anti-mouse epithelial cell adhesion molecule FITC (EpCAM; 11-5791) (1 μl) (both from eBioscience, San Diego, CA, USA), anti-human and mouse CD49f PE (10 μl; 130-100-096; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Following incubation, the cells were washed twice and analysed on a flow cytometer (Becton Dickinson, San Jose, CA, USA). Isotype antibodies were used as negative controls.

Table I. Primer sequences used for real-time PCR.

Genes	Forward (5'→3')	Reverse (5'→3')
AFP	ACCTTCCTGTCTCAGTCATTCT	CCTGACATCCAGGTAGATTTCCA
CK19	GTTCAGTACGCAGGGTCAGT	GAGGACGAGGTCACGAAGC
CFTR	CTCAGCCTTCTGTGGCCGG	TCCGGGTCATTTTCAGCTCCAC
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

AFP, alpha fetoprotein; CK19, cytokeratin 19; CFTR, cystic fibrosis transmembrane conductance regulator; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Immunofluorescence. Cells grown on type I collagen-coated coverslips were fixed with 4% paraformaldehyde at 4˚C for 30 min. The cells were then washed three times for 5 min with PBS, permeabilised with 0.1% Triton X-100 in PBS for 10 min and blocked with 6% rat serum in PBS for 30 min at room temperature. The cells were then incubated with chromophore-conjugated antibodies in PBS at 37˚C in the dark for 30 min. The chromophore-conjugated antibodies were as described above. The cells were washed with PBS 3 times for 5 min in the dark. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime Institute of Biotechnology) for 3 min in the dark. The cells were then examined under a fluorescence microscope (Olympus IX83).

Soft agar clone. The spheroid cells were collected and enzymatically (TrypLE™ Express; 8 min at 37˚C) and mechanically dissociated into single cells. Agar (0.6%) in William's medium E (19) (WME; Sigma-Aldrich) supplemented as described above, kept at 40˚C and poured into 6-well plates to form the lower layer. After the agar medium had solidified, either $1x10^3$ single cells or spheroid cells in 1.0 ml of 0.36% agar at 40˚C were plated onto the lower layer. Fresh culture medium was added weekly to keep the cultures moist. The cells were incubated with 5% CO_2 at 37˚C; growth potential and character were assessed after 3 weeks.

Colony formation assay. Spheroid cells and single cells were plated onto type I collagen-coated 6-well plates at a density of $1x10^3$ viable cells, and colonies containing >50 cells were quantified after 2 weeks. The single cells were seeded onto type I collagen-coated 24-well plates at a density of $1x10^4$ viable cells, and cell numbers were quantified over 6 sequential days using a binocular inverted microscope (Nikon Eclipse Ti-U) and plotted to generate a cell proliferation curve.

Differentiation into hepatocytes and cholangiocytes in vitro. The clonally expanded cells ($5x10^3$ viable cells/cm^2) were cultured in standard medium and differentiation medium supplement with 10 ng/ml hepatocyte growth factor (HGF; Peprotech, Inc.), $1x10^{-7}$ mol/l dexamethasone and 1% dimethyl sulfoxide (DMSO) (both from Amresco LLC, Solon, OH, USA), but without LIF. After 2 weeks, the cells were collected and subjected to PAS staining as described above, as well as immunofluorescence (28). The primary antibodies used for immunofluorescence included albumin (ALB; 1:50, sc-50536), cytokeratin 19 (CK19; 1:50, sc-25724) and hepatocyte nuclear factor (HNF)4α (1:50, sc-8987) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The tetraethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit antibody (SA00007-2) was obtained from Proteintech Group, Inc. (Chicago, IL, USA). The FHPC clones were seeded onto type I collagen (29) and fibronectin (28), cultured in standard medium without LIF for 2 weeks, and observed for morphological changes using an inverted fluorescence microscope (Olympus IX83).

Relative transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed as previously described (23). Total RNA was isolated from cell lines and clinical samples using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription reactions were conducted with oligo (dT) 18 primers and random primers according to the instructions of the manufacturer of the M-MLV Reverse Transcriptase kit (Invitrogen). Quantitative PCR (qPCR) was performed with SYBR Premix Ex Taq™ (TaKaRa, Tokyo, Japan) using the Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The primer sequences used for PCR are listed in Table I. The gene expression levels were calculated relative to the expression of β-actin in tumor cell lines or clinical samples using the $2^{-\Delta\Delta Ct}$ method.

Plasmid DNA. The pGEMHBV and pGEMHBVΔx plasmids both encode a more-than-unit-length HBV genome (pay-w1.2), though the latter contains a stop codon at amino acid 7 of the HBx ORF (payw1.2*7) (30). The plasmids pSI-HA-x, pSI-HA-x^{1-101} and pSI-HA-x^{43-154} were generated by PCR cloning into the pSI vector. According to the previous studies by Hodgson *et al* (31) and Luo *et al* (3), anine-amino-acid HA epitope tag was cloned at the N-terminus of HBx and its truncation mutants.

Transfection of the FHPCs. The medium was changed 1 h prior to transfection. HPCs were transfected with PolyJet (SignaGen® Laboratories, Ijamsville, MD, USA) according to the manufacturer's instructions, and a green fluorescent protein (GFP)-expressing plasmid was co-transfected at a proportion of 1:1 in order to assess the transfection efficiency. GFP expression was observed daily, and the cells were collected at the highest efficiency of expression.

Western blot analysis and immunoprecipitation. At 4 days post-transfection, the cells were collected and lysed on ice in RIPA Lysis Buffer (Beyotime Institute of Biotechnology).

The primary antibodies included anti-HBcAg (1:400; B0586; DakoCytomation, Glostrup, Denmark) and anti-HA (1:200, sc-805; Santa Cruz Biotechnology, Inc.). The horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:5,000; ZB-2010) and the anti-β-actin antibody (1:500; TA-09) were from Beijing Zhongshan Golden Bridge Biotechnology, Co. (Beijing, China). Western blot analysis was performed as previously described (3), and protein bands were quantitated using the Quantity One Image analysis system. HBx immunoprecipitation was performed prior to western blot analysis. Equal amounts of total protein from cells transfected with pSI-HA-x, pSI-HA-x^{1-101}, pSI-HA-x^{43-154} and pSI-control were incubated with protein A/G Plus-agarose beads (Beyotime Institute of Biotechnology) and primary anti-HA antibody. Specific operations were performed according to the manufacturer's instructions.

FHPC proliferation following transfection in vitro. The FHPCs transfected with pSI-HA-x, pSI-HA-x^{1-101}, pSI-HA-x^{43-154} and pSI-control were plated at a density of 1,000 small cell clusters onto type I collagen-coated 6-well plates as described above. Colonies containing 10-50 and >50 cells were quantified after 1 week using a binocular inverted microscope (Nikon Eclipse Ti-U).

Statistical analyses. The data are presented as the means ± SD. Statistical analyses were carried out using one-way analysis of variance (ANOVA) and Tukey's test facilitated with GraphPad software version 5.0 (San Diego, CA, USA). A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Isolation and culture of FHPCs. Recently, more than half of FHPCs enriched by Percoll discontinuous gradient centrifugation (PDGC) were found to be positive for CD133, CD49f and CD90 (26) in rats. We hypothesised that PDGC may also be used to enrich HPCs in mouse foetuses at ED14.5. In agreement with our hypothesis, the enriched FHPCs were concentrated in the 50% Percoll™ layer, after plating onto type I collagen for 24 h, and the erythrocyte and anchorage-independent cell population was removed by washing and replacing the medium. Primary cultures of the purified FHPCs formed a variety of colonies after 36-48 h. Those cells derived from the upper part of 50% Percoll™ cell layer appeared larger and were arranged in a paving-stone-like pattern (Fig. 1A-C), while those from the lower part of the 50% Percoll™ cell layer formed denser colonies that were more homogenous (Fig. 1D) and proliferated rapidly. After 5 days of primary culture, the colonies mixed together (Fig. 1E and F) and presented a high nucleus/cytoplasm ratio. The cells were digested at 7-10 days in primary culture and split at a 1:2 ratio, and the cells proliferated rapidly after passaging (Fig. 1G). To obtain single clonal cell lines, we performed limiting dilution as previously described by Conigliaro *et al* (28) by seeding cells in a 96-well plate at a concentration of 1 cell/well. Small colonies were visible at 5 days and 2 weeks after plating, and 27.98% of wells contained colonies with >50 cells (data not shown).

Spheroid FHPC clonal cells. In our study, single FHPCs proliferated very slowly; changing the concentration of type I collagen coated onto the plates and culturing in serum-free medium led the HPCs to also form spheroids. When the concentration was at 4-5 $\mu g/cm^2$, over the next 3-5 days, a subpopulation of cells detached and grew in suspension as spheroid cells (Fig. 1H). When the spheroid FHPCs were replated onto 8-10 $\mu g/cm^2$ type I collagen in DMEM/F12 medium containing FBS (32,33), they attached immediately and formed dense cell colonies in one day (Fig. 1I). After 5 days, the periphery of the colonies consisted of cells forming a cordlike morphology (Fig. 1J) as previously described by Schmelzer *et al* (34). We classified the colonies as a spheroid FHPC line, given their high proliferative potential and maintenance of a dense colony morphology following continuous passage in culture (Fig. 1K).

Culture of FHPCs on collagen-coated dishes. We then performed immunophenotyping profiling of the FHPCs in spheroid cells and after attachment. We assessed the expression of EpCAM, CD133, CK19 and CD49f in our FHSCs by immunofluorescence staining. At 7 days after being seeded onto type I collagen, the spheroids and attached cells were both found to co-express CK19 and CD49f (Fig. 2A-C). EpCAM expression was high in the spheroids (Fig. 2A), but was low in the attached cells (Fig. 2B). EpCAM also became downregulated after the attachment of the spheroid FHPCs. To further verify these results, culturing was prolonged to 13 days. CD133 was expressed in both the spheroid and attached cells (Fig. 2D and E), and EpCAM expression was identical to that described above. In the single cells and extensions from the periphery of the spheroids, the expression of CD133, EpCAM, CK19 and CD49f was lower than that in the centre of the spheroids. Notably, EpCAM and CD133 were expressed not only at the surface of the attached cells, but also in the cytoplasm, though the entire sphere expressed both EpCAM and CD133 in the spheroid cells (Fig. 2D and E). FCM analyses of the colonies revealed that 98.98±0.20, 93.24±4.14 and 71.67±12.29% of the attached cells were positive for CD133, CD49f and EpCAM expression, respectively (Fig. 2F), while only 55.44±22.46% of the cells co-expressed CD133 and EpCAM, indicating an overlap between CD133-positive and EpCAM-positive cells; a subpopulation of cells was only positive for EpCAM. Thus, almost all the spheroid cells, as well as the majority of the attached cells, expressed markers of FHPCs. The spheroid FHPCs cultured on collagen-coated dishes for 7 days were negative for PAS staining (Fig. 3A), while red glycogen particles were visible in the cytoplasm of cells at the centre of the attached colonies (Fig. 3B), indicating that the spheroids had less stem cell characteristics and differentiated into hepatocytes following attachment. Consistent with these findings, spheroid human hepatic stem cells (hHpSCs) initially transition into hepatoblasts on plastic and then give rise into hepatocytic and cholangiocytic lineages (17), while induced hepatic stem cells (iHepSCs) from mouse embryonic fibroblasts form aggregates of approximately 100 mm in diameter and show significant glycogen storage staining by PAS when plated on Matrigel (23). Similarly, staining the spheroids for AP revealed that the entire sphere was stained purple brown (Fig. 3C), and only the centre of the attached cells was AP-positive (Fig. 3D). Cholangiocytes (23) and human embryonic stem cells (35) have previously been shown to stain positively for AP, and our findings with spheroid FHPCs are also consistent with these results.

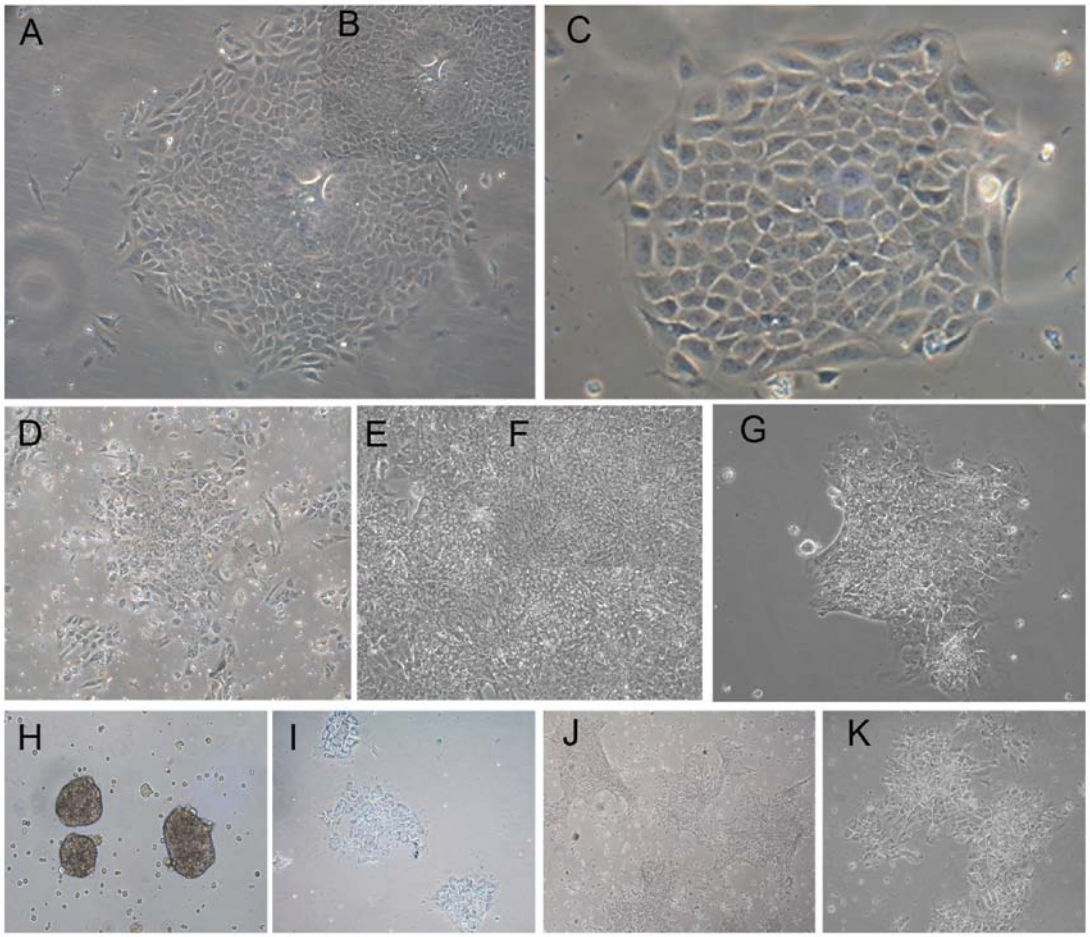

Figure 1. Foetal hepatic progenitor cell (FHPC) culture. (A-C) Phase micrograph of primary cultures of cells from the upper portion of the 50% Percoll cell layer seeded onto type I collagen. (D-G) The lower 50% Percoll cell layer seeded onto type I collagen, (D) at 2 days, (E and F) 5 days and (G) the first passage. (H-K) Spheroid FHPCs culture. (H) The sphere cells, (I) attached at 1 day, (J) 5 days and (K) the fifth passage. (B and F) show enlarged images of (A and E), respectively. (A, D, E and G-K) Original magnification, x100; (B, C and F) x200.

Self-renewal of FHPCs in collagen and soft agar. We then determined whether single cells taken from spheroids could still form spheroid colonies. Therefore, we dissociated spheroids by mechanical pipetting and enzymatic digestion to obtain single cells, which were then replated at a density of 1,000 viable cells/well onto 6-well plates coated with collagen or soft agar. Spheroids were also seeded as a control. After 3 weeks of subculture, the single cells grown in soft agar formed dense cell colonies (Fig. 3E), while no spheroid cells were observed in collagen (Fig. 3F), and spheroid cells in soft agar tended to balloon (Fig. 3G), whereas the spheroids seeded on collagen attached and expanded within 3 days (Fig. 3H). We then replated equivalent numbers of attached cells, following dissociation by enzymatic digestion into small cell clusters, and spheroids on collagen for subculturing. Over the next 2 weeks, the spheroids formed 3-fold more colonies than the attached cells (Fig. 3I). We then collected 10^4 attached cells as described above and seeded them on collagen-coated 24-well plates to generate a growth curve (Fig. 3J). Thus, we concluded that the cells were able to maintain their viability and ability to form new spheroids in soft agar, but lost this capacity on collagen. Furthermore, this colony-forming and self-renewal potential was downregulated in small cell clusters derived from attached cells, yet these small cell clusters still retained robust proliferation. Furthermore, the study by Yamashita *et al* (10) indicated that spheroid HCC cells maintained greater EpCAM expression than attached cells, and spheroid FHPCs also shared this feature.

FHPC differentiation into hepatocytes in vitro. To address whether the established spheroid FHPCs could differentiate into hepatocytes *in vitro*, we cultured the FHPCs in differentiation medium supplemented with 10 ng/ml HGF, 1% DMSO and 10^{-7} mol/l dexamethasone, as well as in standard medium. HGF, DMSO (29,36) and dexamethasone (37) have been shown to facilitate hepatocyte differentiation *in vitro*. The FHPCs were cultured in differentiation medium for 2 weeks; the cell size increased, whereas the nucleus/cytoplasm ratio decreased, and compared to culture in standard medium (Fig. 4A), glycogen particles accumulated distinctly (Fig. 4B), as shown by immunofluorescence staining (Fig. 4C). In addition, real-time PCR (Fig. 4D) revealed that the expression of FHPC marker genes (AFP), as well as the co-expression of FHPCs and cholangiocyte marker genes (CK19) (17), was distinctly downregulated, but the expression of cholangiocyte marker genes (CFTR) was upregulated. Immunofluorescence staining showed that FHPCs expressed ALB, but were negative for HNF4α. Consistent with these findings, a portion of cell aggregates that had formed after spheroid attachment disintegrated into larger-sized individual

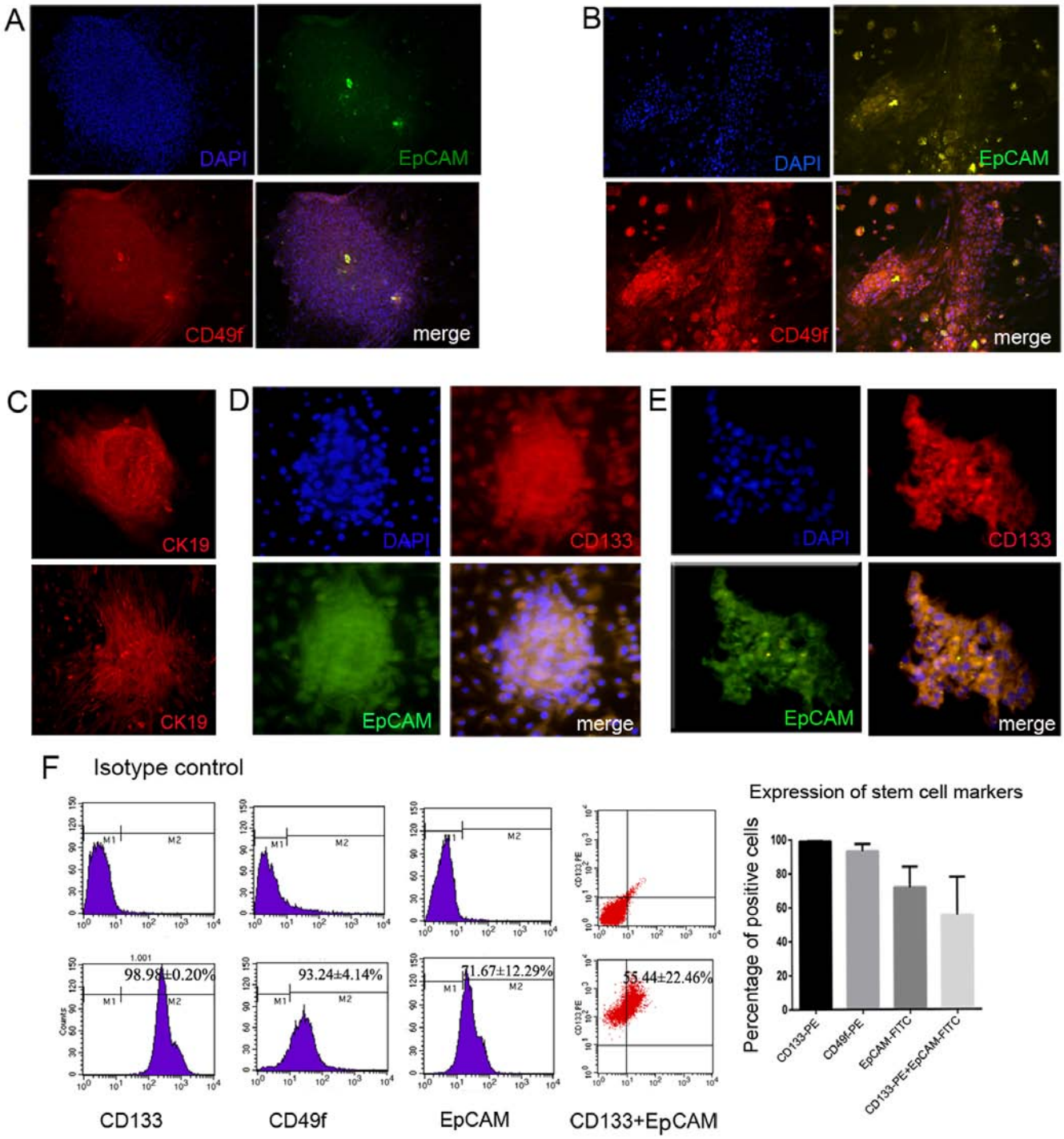

Figure 2. Characteristics of spheroid cells. (A-E) Immunofluorescence analysis of the co-positive for epithelial cell adhesion molecule (EpCAM) and CD49f (A) in spheroid cells and (B) attached cells, (C) the positive for cytokeratin 19 (CK19) in spheroid cells and attached cells after 7 days of primary culture; in 13-day primary cultures, the co-positive for EpCAM and CD133 (D) in spheroid cells and (E) attached cells. (F) Flow cytometric analysis of the expression of CD133, CD49f, EpCAM and co-expression of both CD133 and EpCAM in spheroid foetal hepatic progenitor cells (FHPCs) clones plated onto type I collagen for 7 days. The upper panel is the isotype control and data are derived from 3 independent experiments. Original magnification, x200.

cells within 24 h in standard medium (data not shown), indicating that the FHPCs colonies spontaneously differentiated in the presence of cytokine and collagen.

FHPC differentiation into cholangiocytes in vitro. Upon the addition of EGF, but without LIF, for 2 weeks, colonies with branch structures at the periphery were consistently observed (Fig. 4E). Under similar conditions using collagen, colonies with branches (Fig. 4F) and bile duct structures also formed in culture. Moreover, immunofluorescence staining revealed that the expression of FHPC markers (CD133 and EpCAM) was downregulated in these bile duct structures (Fig. 4G-I). Taken together, these results indicated that the FHPCs differentiated into cholangiocytes *in vitro*.

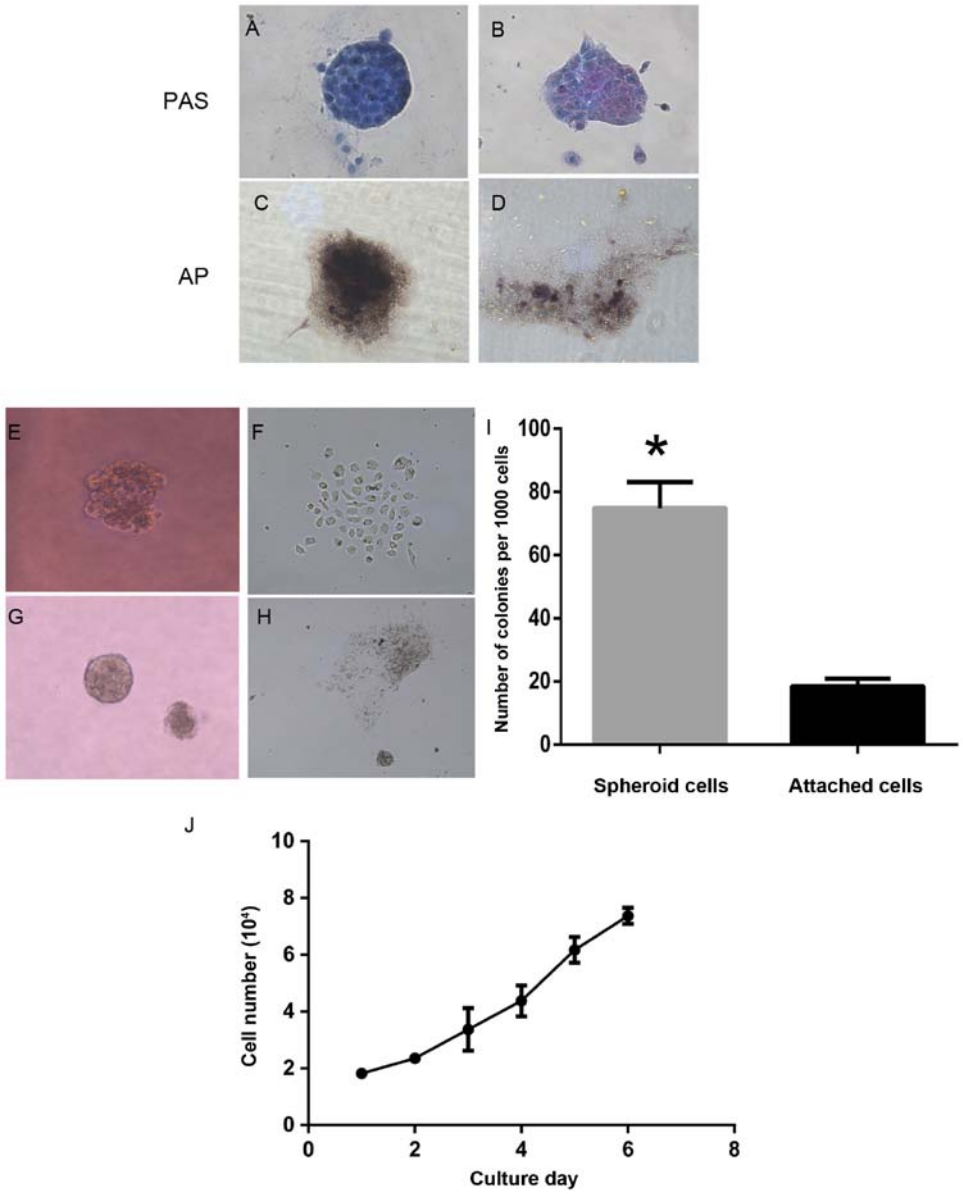

Figure 3. The colony formation and proliferation of spheroid foetal hepatic progenitor cells (FHPCs). After 7 days on type I collagen, (A and B) periodic acid-Schiff (PAS) stains the spheroid cells (A) and the (B) attached cells. (C and D) Alkaline phosphatase (AP) stains (C) the spheroid cells and (D) attached cells. (E-H) Colony formation assay of the single FHPCs (E) in soft agar culture and (F) collagen for 3 weeks; at 3 days spheroids (G) in soft agar and (H) in collagen. (I) Colony formation assay of the spheroid FHPCs and attached cells. (J) The growth curve of attached cells over a 6-day period. Original magnification, x100. All results are means ± SD. *P<0.05.

The C-terminal region of HBx is required for the HBx-induced proliferation of FHPCs. Given that full-length HBx promotes the expansion and tumourigenicity of HPCs in DDC-treated mice (14), we considered whether HBx would also promote the proliferation of FHPCs, as well as the ability of HBx truncation mutants to maintain HBx activity. FHPCs transfected with pSI-HBV, pSI-HBVΔx, pSI-HA-x, pSI-HA-x^{1-101}, pSI-HA-x^{43-154} and pSI-control were examined at 4 days post transfection (Fig. 5A). It was worth noting that in the FHPCs transfected with full-length HBx, spheroid colonies formed in culture, as observed in previous studies (14), whereas no spheroids were observed with the HBx truncation mutants. The transfected cells were collected, and HBc expression was analysed by western blot analysis. HBx expression was detected using a combination of immunoprecipitation and western blotting. We considered whether the HBx protein is possibly influenced by other HBV proteins. Of note, the HBc protein levels showed no change in the pSI-HBV-and pSI-HBVΔx-transfected HPCs (Fig. 5B) and were consistent with previous results in mouse hepatocytes (3). Notably, we found that HBx protein levels were higher after HBx transfection when the protein amount to 200 μg. The levels of HBx^{43-154} reach approximately the same level as full-length HBx, but the expression of HBx^{1-101} was lower than that of full-length HBx (Fig. 5C). These results are consistent with the findings of previous studies (3,38) on mouse hepatocytes and HepG2 cells, which showed that C-terminal deletion result in lower levels of HBx expression, whereas N-terminal deletion had no significant effects. In addition, FHPCs transfected with pSI-HA-x and pSI-HA-x^{43-154} permitted subculturing for 1 month, with a minimum of 5 passages. Collectively, these

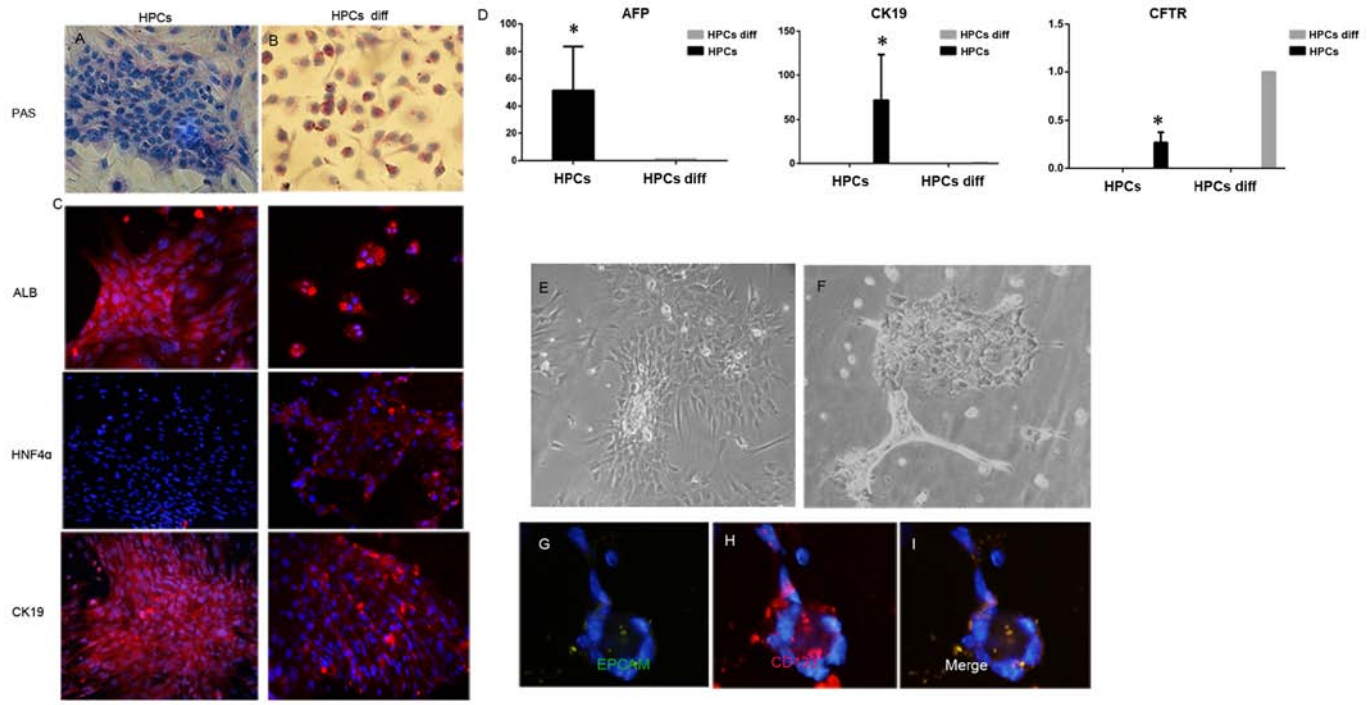

Figure 4. Foetal hepatic progenitor cells (FHPCs) form cholangiocytes and hepatocytes in culture. (A and B) Periodic acid-Schiff (PAS) stained of FHPCs. (C) Immunocytochemistry examines the expression of hepatocyte and cholangiocyte markers of FHPCs cultured in standard and differentiation medium. (D) Real-time PCR analysis of the expression of FHPCs genes (AFP and CK19) and cholangiocyte gene (CFTR). (E and F) The phase micrograph of FHPCs seeded onto (E) laminin and (F) type I collagen for 2 weeks. (G-I) Immunocytochemistry analysis of the expression of epithelial cell adhesion molecule (EpCAM) and CD133 in bile duct structures. (A, B, E and F) Original magnification, x100; (C and G-I) x200. (D) The data are derived from 3 independent experiments, and the results are the means ± SD. *P<0.05.

findings indicated that the C-terminal region of HBx was required for HBx proliferation, but was not necessary for the formation of spheroids.

Full-length and N-terminally deleted HBx promote clone formation in FHPCs. Next, 1,000 small cell clusters transfected with pSI-HA-x, pSI-HA-x$^{1-101}$, pSI-HA-x$^{43-154}$ and pSI-control were subcultured in 6-well plates. Small cell clusters attached and extended after 1 week, and compared to the pSI-control (Fig. 5D), the colonies formed by the pSI-HA-x transfected FHPCs (Fig. 5E) were larger in size and greater in number. Moreover, colonies were small and growed slowly after pSI-HA-x$^{1-101}$ (Fig. 5F) transfection; however, the colonies formed by the PSI-HA-x$^{43-154}$-transfected cells (Fig. 5G) exhibited no significant difference compared to the pSI-HA-x-transfected cells, which indicated that the C-terminal region of HBx was necessary for HBx to promote the proliferation of FHPC colonies. However, it remains unclear precisely how the C-terminal region affects the function of HBx in FHPCs and whether the C-terminal region specifically participates in signalling pathways [interleukin-6 (IL-6)/STAT3 activities, and Wnt/β-catenin] that enable the proliferation and tumourigenicity of FHPCs.

Discussion

HCC is associated with a very high mortality rate, and the recurrence and metastasis of HCC have not been targeted by specific treatments. In recent years, TISCs have been found in HCC (10,11,13,22,39), and the association between stem cells and TISCs has attracted increasing attention (10,22,38-41). Rat ED14 FHPCs exhibited 95% homogeneity, as well as the cell culture and gene expression characteristics of HPCs (24). Early studies also indicated that over 60% of cells derived from E14.5 Dlk$^+$ foetal mice were also positive for both ALB and CK19, which became downregulated during liver development (25). In our study, enriched FHPCs were concentrated in the 50% Percoll™ layer following PDGC, whereas the 20% Percoll™ layer contained the hepatic oval cells from adult mice fed a choline-deficient, ethionine-supplemented (CDE) diet (18).

Human mammary epithelial cells proliferate in suspension, forming non-adherent mammospheres (27) that are enriched in progenitor/stem cells. Hepatic spheroids suspended in serum-free Kubota's medium (KM) (34,42) and seeded onto tissue culture plastic or embryonic stromal cell feeders can form cell colonies with two distinct morphologies: hepatoblasts and hHpSCs (34), and the hHpSCs can transition to hepatoblasts (17). Notably, it has been demonstrated that EpCAM$^+$ HCCs can also form spheroids efficiently (10). Consistent with these observations, we demonstrated that the FHPCs formed spheroids at low concentrations of collagen. Furthermore, these spheroids were positive for EpCAM, CD133, CD49f, CK19 and ALB. Previous studies have shown that the hHpSC phenotypic profile includes neural cell adhesion molecule (NCAM), CD133 and CK8,/18/19 (17,34). Mouse HPCs express CD49f (18,42), CD133, Dlk/Pref-1, CK19 (25) and TROP2 (36), but are negative for Thy-1, CD11b, CD31, CD34, c-kit and CD45 (16,33). EpCAM was considered in all parenchymal cells and biliary epithelial cells, as a marker

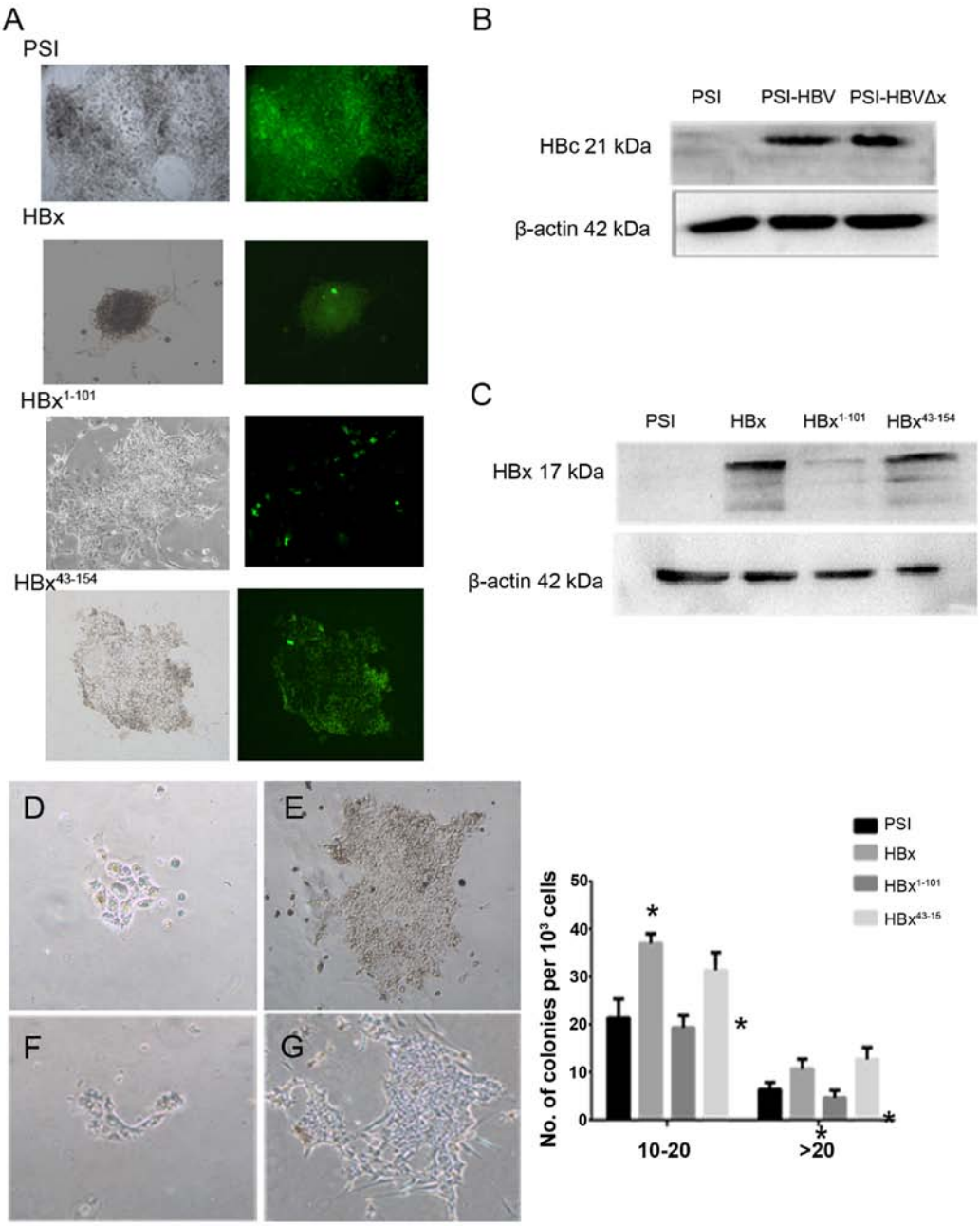

Figure 5. The C-terminal region of hepatitis B virus X (HBx) iks required for HBx to stimulation proliferation in foetal hepatic progenitor cells (FHPCs). (A) The fluorescence image showing the expression of green fluorescent protein (GFP) overlaid atop the corresponding phase-contrast micrograph 4 days after transfection. (B) Western blotting of HBc in FHPCs after 4 days of transfection with pSI-HBV, pSI-HBVΔx and pSI as a control. (C) IP/western blotting for HBx in FHPCs transfected with pSI-HA-x, pSI-HA-x^{1-101}, pSI-HA-x^{43-154} and pSI-control as described above. (D-G) Colony formation assay of FHPCs transfected with (D) pSI-control, (E) pSI-HA-x, (F) pSI-HA-x^{1-101} and (G) pSI-HA-x^{43-154}. Original magnification, x100. All data shown are from 3 independent experiments. The results are the means ± SD. $^{*}P<0.05$.

identifying HPCs in humans (17,34), TISCs in HCCs (10) and HPCs in adult mice administered a DDC diet (14,36) or a partial hepatectomy (PH) model (29).

Compared with FHPCs attached as a monolayer, the spherical cells proliferated more rapidly. The spherical FHPCs could also grow on semi-solid agar and tended to retain the morphology and characteristics of stem cells compared with growth in rat tail collagen. It is known that hepatic oval cells from adult mice stop growing on semi-solid agar (18), which seems to be in conflict with the findings of our study; there may exist some diversity in terms of the tumourigenicity of adult mice hepatic oval cells and FHPCs.

FHPCs converted into mature hepatocytes and bile duct epithelial cells when cultured under conditions that facilitate differentiation. Our data further suggested that FHPCs differentiated into both hepatocytes and cholangiocytes upon exposure to EGF, HGF and DMSO. Matrigel has been used to maintain and differentiate cholangiocytes (23,29,33,35). Of note, collagen also shared this ability, but few colonies tended to form biliary epithelial cell under these conditions.

The study by Wang *et al* (14) showed that HBx prompted intrinsic cellular transformation by stimulating the expansion and tumourigenicity of HPCs. Lizzano *et al* (41) found that the C-terminal region of HBx was crucial for the stability and

function of HBx, and Hodgson *et al* (31) demonstrated that the carboxyl region was required for maximal HBV replication in HepG2 cells both *in vitro* and *in vivo*. Notably, we confirmed that the C-terminus of HBx is essential for its capacity to stimulate the proliferation of FHPCs, but is not necessary for the formation of spheroids, similar to hepatic cancer stem cells; we conjecture that the truncation mutants of HBx downregulate its tumourigenicity in FHPCs. Collectively, our study provides preliminary evidence of the relevance between C-terminus and full-length HBx in FHPCs and lays the foundation for further studies of the exact mechanism and signalling pathways through which the C-terminus of HBx stimulates proliferation and tumourigenicity in FHPCs.

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