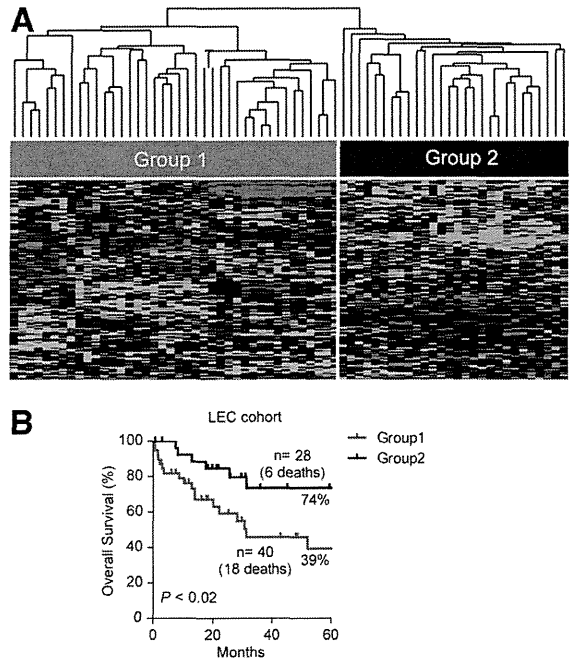

Fig. 3. Validation of the ICC-specific gene signature in an independent ICC cohort as a survival predictor. (A) Hierarchical clustering of 68 Caucasian ICC cases based on the expression of 158 overlapping genes between stem-like ICC and stem-like HCC genes using centered correlation and average linkage. The heatmap depicts high (red) and low (green expression of these genes based on a log2 scale. (B) Kaplan-Meier plot of 68 ICC patients based on the dendrogram classification from (A).

cell-like phenotype. We plotted the density distribution of Spearman correlation coefficients of 636 experimentally derived genes and 23 experimentally derived microRNAs (Fig. 4A). This analysis revealed that there was a clear enrichment of correlative mRNA-microRNA pairs derived from these signatures because a positive correlative curve shifted to the right and a negative correlative curve shifted to the left when compared to a normal distribution curve derived from a global correlation of all available mRNA and microRNA probes (Fig. 4A). A correlation coefficient of 0.5, corresponding to the 95th percentile of the 100-fold random permutations, was used as the cutoff threshold for positive correlation. These results indicated that ICC-specific mRNAs and microRNAs are enriched in the experimentally derived signatures and they are highly correlated.

To determine if there is any enrichment of affected networks associated with ICC subgroups, we combined significantly correlative mRNA-microRNA pairs and performed pathway analysis using Ingenuity Pathway Analysis (IPA, v. 9.0) that incorporates microRNA-mRNA target relationships from TargetScan. Among 1,077 mRNA-microRNA pairs identified by this analysis, 479 pairs showed negative correlation. Among the top nine networks (Table S4), five microRNAs including miR-200c and miR-141 that are encoded by the same transcript were negatively correlated with genes

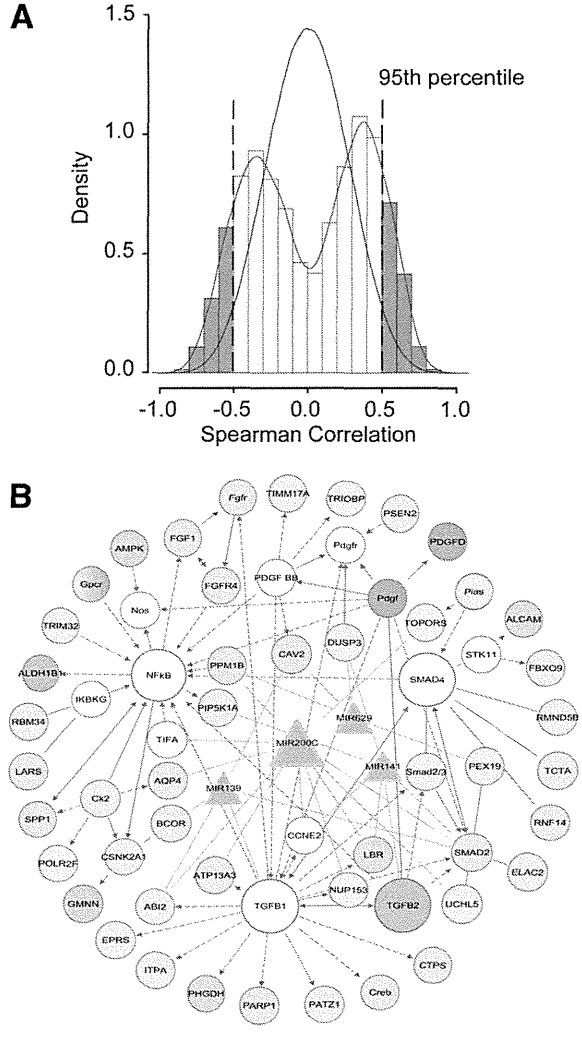

Fig. 4. Integrative analyses of ICC-specific mRNA and microRNAs based on Spearman correlation and ingenuity pathway. (A) Correlation between ICC-specific 636 mRNA and 23 microRNA signatures. (B) The top nine gene networks of signaling including TGF-β, Smad4, and NF-κB pathways activated in stem-like ICC tumors. Red shaded ovals represent up-regulated genes in HpSC-ICC tumors, and open ovals represent genes that are not on the list of significant genes but are reported to be associated with the network. Blue shaded triangles represent down-regulated microRNAs specific to HpSC-ICC tumors. The open ovals that are labeled TGF-β, Smad4, and NF-κB represent molecular nodes related to their respective signaling pathways. Arrows represent positive regulation of gene expression, with solid arrows indicating direct regulation and broken arrows indirect regulation. Blue lines connecting between microRNA and genes represent direct targeting predicted by TargetScan.

in the transforming growth factor beta (TGF-β), nuclear factor kappa B (NF-κB), and Smad signaling pathways (Fig. 4B). A common link between ICC-specific mRNA and microRNA seemed to be related to EMT, where all three pathways are known regulators. Consistently, known stem cell-related genes such as POU5F1 (Oct4), NANOG, NCAM1, and PROM1 (CD133) were much more abundantly expressed in HpSC-ICC than MH-ICC cases (Fig. S5A). TGFB1 was also significantly elevated in HpSC-ICC compared to MH-ICC. However, no difference in EpCAM expression was observed among these two subgroups. An elevated expression of NCAM1 and TGFB1 in a majority of HpSC-ICC cases was confirmed by immonohistochemistry analysis (IHC) (Fig. S5B).

Among the affected networks, it was noticeable that miR-200c appeared a common molecular note linking to EMT, as it had a direct interaction with many of the affected genes in this pathway (Fig. 4B). Consistently, the expression level of miR-200c was associated with overall survival and disease-free survival in ICC cases (Fig. S6). These data suggested that miR-200c may play an important role in maintaining HpSC-like phenotype.

To determine whether EMT was functionally linked to HpSC-ICC cells, we first analyzed representative expression levels of EMT markers in ICC specimens by qRT-PCR. Consistently, mesenchymal markers such as ZEB1, ZEB2, CDH2, and VIM were more abundantly expressed, whereas an epithelial marker, CDH1, and miR-141/miR-200c were much less abundantly expressed in HpSC-ICC cases as compared to MH-ICC cases (Fig. 5A). Next, we determined if an altered miR-200c expression could lead to EMT in ICC cells. We selected two ICC cell lines that represent two opposite ends of the EMT spectrum. A nonmalignant H69 cell line derived from normal human intrahepatic cholangiocytes was included as a control.[24] HuH28 cells had fibroblast-like cell morphology with mesenchymal appearances and expressed very low levels of miR-200c but high levels of mesenchymal markers, whereas HuCCT1 cells had cobblestone-like cell morphology with epithelial appearances and expressed high levels of miR-200c but low levels of mesenchymal markers (Fig. 5B). The miR-200c level was also relatively high in H69 cells with epithelial morphology. Transient transfection of miR-200c oligos in HuH28 cells induced a reversed EMT from a mesenchymal-like to a cobblestone-like morphology with a suppression of genes that mediate EMT (Fig. 5C). Conversely, transfection of an anti-miR-200c oligo in HuCCT1 resulted in an induction of mesenchymal markers (Fig. 5D). In addition, overexpression of miR-200c suppressed cell migration (Fig. 5E) and invasion (Fig. 5F) in HuH28 cells. However, miR-200c did not affect cell proliferation and apoptosis in HUH28 cells as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Transferase-Mediated dUTP Nick-End Labeling (TUNEL) assays (Fig. S7).

Analyses of the genomic region encoding the human miR-200c/miR-141 locus at the UCSC Genome Browser revealed that miR-200c and miR-141 are derived from a single transcript encoded by a predicted gene (ENST00000537269) (Fig. 6A). The available Chip-Seq data revealed several transcriptional factors such as c-Myc and TCF4 to be preferentially bound to the immediate 5′ upstream sequence of the predictive transcription initiation site. To determine whether c-myc directly regulates miR-200c expression, we silenced c-Myc expression with a c-myc-specific small interfering RNA (siRNA) in HuH28 cells and examined the activity of a luciferase reporter containing an upstream 0.9 kb fragment of pri-miR-200c[25] (Fig. 6B). Consistently, we found that inhibition of c-Myc resulted in an increased hmiR-200cLuc activity. Moreover, c-myc siRNA could effectively induce endogenous miR-200c expression, however suppress mesenchymal markers but induce epithelial marker (Fig. 6C).

NCAM1 as a Direct Target of MiR-200c. Because several stem/progenitor cell-related genes such as POU5F1, NANOG, MYC, TGFB1, NCAM1, and PROM1 are overexpressed in HpSC-ICC cases (Fig. S5), we reasoned that some of these genes may be targets of miR-200c. TargetScan analysis (TargetScanHuman 6.0) revealed that only NCAM1 contained a classical and evolutionarily conserved miR-200c binding site at its 3′ untranslated region (UTR) (Fig. 7A). Ectopic expression of miR-200c in HuH28 cells resulted in a reduction (Fig. 7B), whereas inhibition of miR-200c in HuCCT1 cells led to an increased expression of NCAM1 (Fig. 7C). To further determine whether NCAM1 was a bona fide target of miR-200c-mediated silencing, the miR-200c binding site was cloned into a luciferase reporter. We found that forced expression of miR-200c in HUH28 cells resulted in decreased luciferase activity when a wildtype sequence but not a mutant sequence was present (Fig. 7D). Moreover, inhibition of miR-200c in HuCCT1 cells resulted in increased luciferase activity only from a wildtype reporter (Fig. 7E). Consistently, ICC cases with high levels of NCAM1 had a worse survival compared to those with low NCAM1 expression (Fig. 7F). Moreover, a significant inverse correlation was observed between miR-200c and NCAM1 (Fig. 7G).

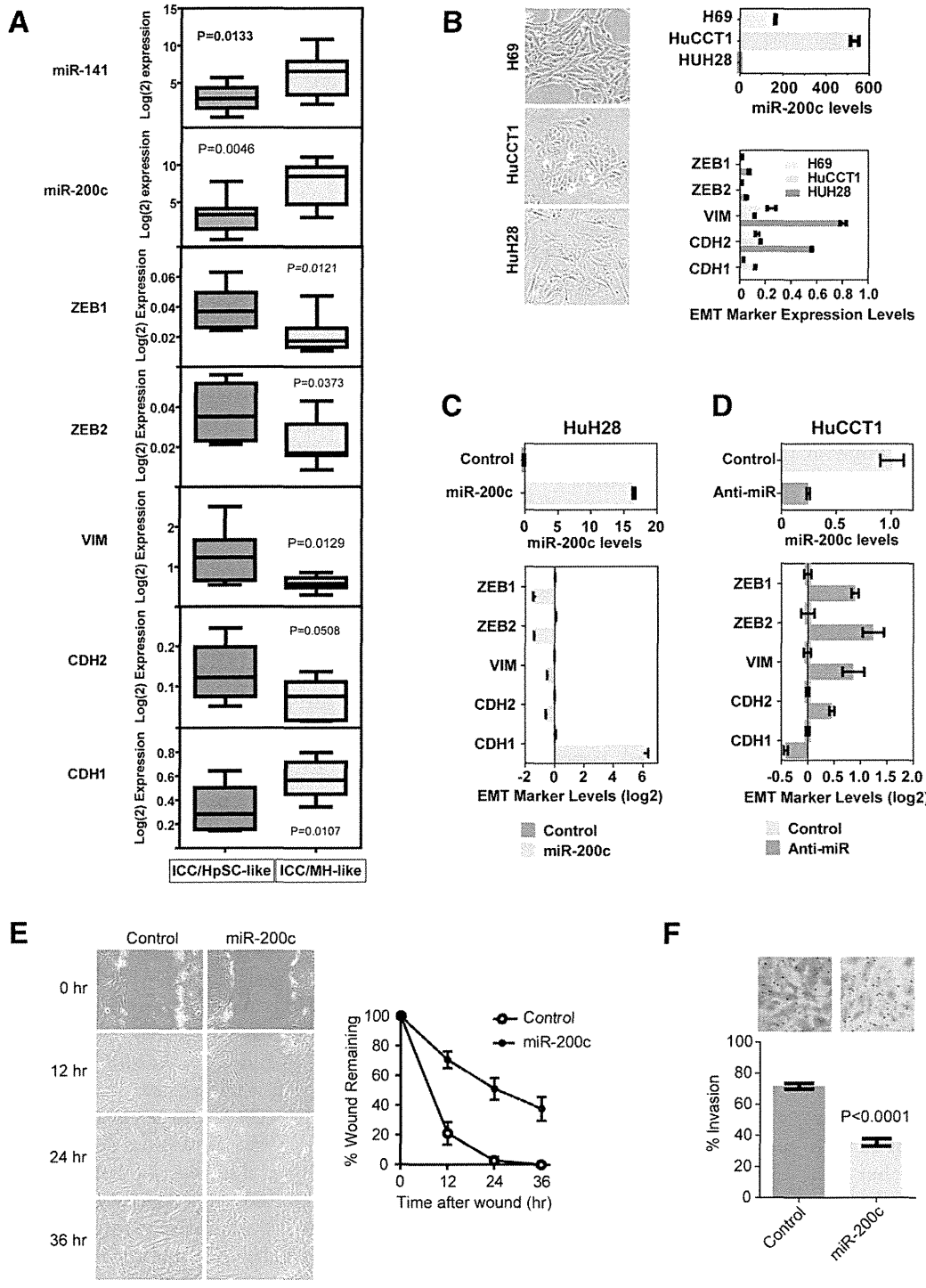

Fig. 5. Inactivation of miR-200c/miR-141 and activation of EMT-related genes are associated with stem-like ICC. (A) Expression analyses of miR-141/miR-200c transcripts and EMT-specific markers based qRT-PCR data in eight HpSC-ICC and eight MH-ICC samples classified by gene clustering from Fig. 1. The horizontal lines in the boxplots represent the median, the boxes represent the interquartile range, and the whiskers represent the 10th and 90th percentiles. A nonparametric test was used to compare the two groups and *P* values are indicated. (B) Expression of miR-200c and EMT-specific genes in HuH28, HuCCT1, and H69 cells as analyzed by qRT-PCR. (C) Expression of EMT-specific genes in HuH28 cells transduced with miR-200c as analyzed by qRT-PCR. (D) Expression of EMT-specific genes in HuCCT1 cells transduced with an anti-miR-200c oligo as analyzed by qRT-PCR. (E) Cell migration of HuH28 cells transduced with miR-200c as determined by the wound healing assay. (F) Cell invasion of HuH28 cells transduced with miR-200c as determined by the Boyden chamber cell invasion assay. Representative images are shown.

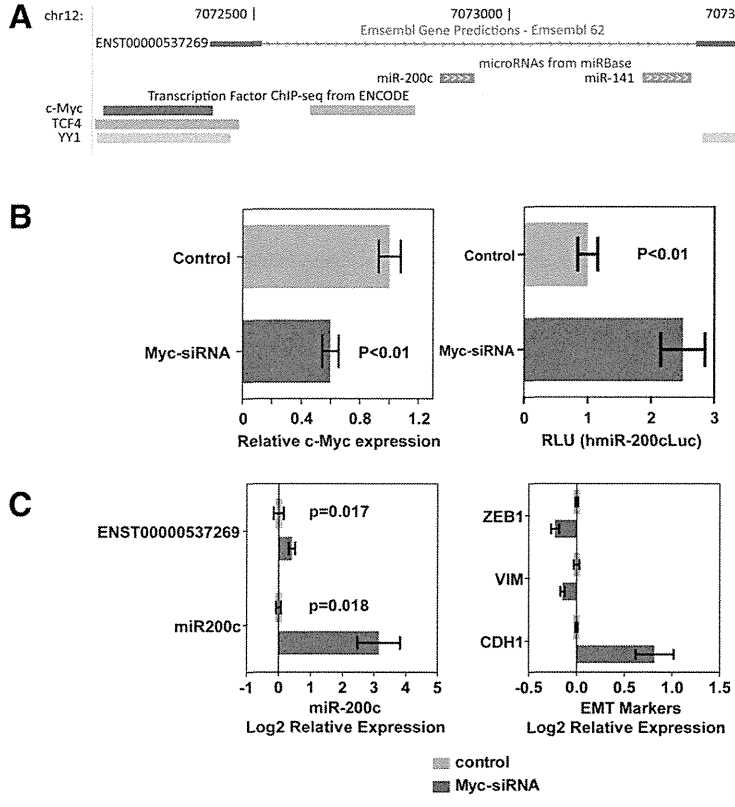

Fig. 6. c-Myc-mediated silencing of miR-200c and induction of EMT. (A) The genome position of ENST00000537269 encoding miR-200c and miR-141, based on Ensembl Gene Predictions using UCSC Genome Browser. (B) Effect of c-Myc siRNA on miR-200cc-Myc expression (left panel) and the miR-200c promoter luciferase activity (right panel) in HuCCT1 cells. (C) Effect of c-Myc siRNA on endogenous levels of miR-200c and induction of EMT-related gene expression in HuCCT1 cells.

Discussion

Similar to HCC, ICC is heterogeneous in clinical presentation, although our knowledge related to its tumor biology is limited. Several recent studies have begun dissecting the molecular pathogenesis of ICC including functional roles of microRNA in ICC cells.[27,28] Recently, we used global transcriptomic approaches to study HCC heterogeneity and identified critical genetic loci functionally linked to hepatic CSCs with gene expression profiles resembling normal hepatic stem cells.[7,8] We also used these approaches to study cholangiocarcinoma.[15,23] In this study, we examined whether ICC and HCC are distinct at the transcriptomic levels. Using two independent transcriptomics approaches, we found that ICC cases from Asian patients can be mainly divided into two subgroups with one resembling of stem-like HCC and other mature hepatocyte-like HCC. Consistently, we found that several known hepatic stem/progenitor cell-specific genes such as POU5F1 (Oct4), NANOG, MYC, TGFB1, NCAM1, and PROM1 are more abundantly expressed in stem-like ICC than mature hepatocyte-like ICC.[29] Moreover, both ICC-specific mRNA and microRNA signatures could independently predict HCC survival as well as ICC prognosis in Caucasian patients. These results are consistent with our recent finding that a subset of HCC may share an ICC-like gene expression trait.[15] Integrative pathway analyses revealed that an altered miR-200c signaling pathway linked to EMT may be responsible for the maintenance of stem-like ICC associated with poor prognosis. For example, we found that two significant microRNAs, i.e., miR-200c and miR-141, encoded by the same transcript, were negatively correlated with genes in the TGF-β, NF-κB, and Smad signaling pathways. These two microRNAs share the same seed sequences and are predicted to have similar cellular functions. EMT is an important biological process contributing to embryogenesis and organ development.[30] Recently, components of EMT have been shown to be critical in promoting cancer invasion and metastasis.[31] TGF-β is essential for the induction of EMT during various stages of embryogenesis and plays an important role in carcinoma progression into an invasive state.[32-34] Smad signaling is essential for TGF-β-induced EMT.[35] Furthermore, miR-200 family members including miR-141 and miR-200c induce epithelial differentiation, thereby suppressing EMT by inhibiting translation of mRNA for the EMT-activators ZEB1 and

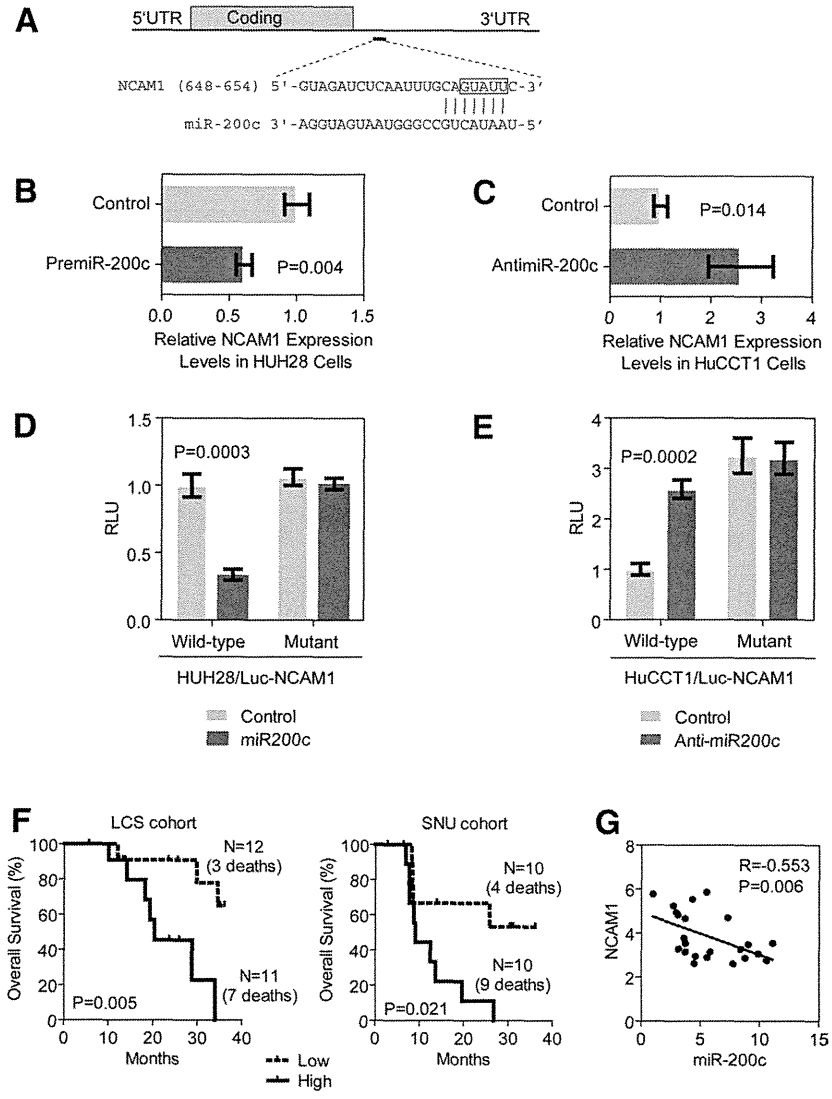

Fig. 7. Functional interactions between miR-200c and NCAM1. (A) Predicted duplex formation between the 3′UTR sequences of human NCAM1 and miR-200c where vertical bars represent the paired seed sequences. The box highlights the nucleotides that were changed to CAUAA in a mutant luciferase reporter. (B) Effect of premiR-200c oligo on NCAM1 expression in HuH28 cells as determined by qRT-PCR. (C) Effect of anti-miR-200c oligo on NCAM1 expression in HuCCT1 cells as determined by qRT-PCR. (D) Luciferase activities of wild-type and mutant reporters in HuH28 cells with or without the presence of pre-miR-200c oligo. (E) Luciferase activities of wildtype and mutant reporters in HuCCT1 cells with or without the presence of anti-miR200c oligo. Each experiment was repeated at least three times and the expression value is shown as the mean ± standard deviation. (F) Kaplan-Meier estimates of overall survival according to expression of NCAM1 in ICC cases from LCS and SNU cohorts. NCAM1 expression values were dichotomized into low and high groups using the within cohort median expression value as a cutoff. (G) Spearman correlation analysis of NCAM1 and miR-200c expression data as determined by mRNA and microRNA arrays.

ZEB2.[36,37] miR-200 family members are functionally linked to EMT, in part by way of targeting ZEB1 and ZEB2, as well as cell migration, invasion, and tumorigenicity.[36,38] These results suggest that the ZEB1-miR-200 feedback loop is critical for maintaining aggressive tumor features. In addition, we also found that miR-200c directly targets NCAM1. NCAM1 is highly expressed in hepatic stem cells and its function has been tightly linked to EMT.[29,39] Our results are consistent with the hypothesis that the miR-200-EMT gene axis may be functional critical to the development of stem-like ICC. Shared molecular activities including EMT and microRNA among HCC and ICC have been noted in recent publications.[40,41] Interestingly, abnormal regulation of EMT-related genes has been reported to be linked to HCC development.[42-44] However, no evidence has linked miR-200 to HCC development. Consistently, we found no evidence that miR-200c is silenced in stem-like HCC (data not shown). It is plausible that the miR-200c-EMT gene axis is a unique signaling pathway functionally important for stem-like ICC and could be exploited as molecular targets for ICC therapies. In addition, unlike HCC, EpCAM is not a good prognostic biomarker for ICC because its expression is highly elevated in both HpSC-ICC and MH-ICC (data not shown). Further studies involving in-depth analyses of miR-200c and EMT signaling and using relevant animal models would be needed to test the therapeutic relevance of these targets for ICC.

Human adult livers are believed to be comprised of maturational lineages of cells beginning intrahepatically

near the portal triads referred to as canals of Hering.[45] This region is close to intrahepatic bile ducts and is believed to contain liver stem cells. It is suggested that liver stem cells may give rise to bipotent progenitor cells, which have the potential to differentiate into both hepatocytic and cholangiocytic lineages. In principle, hepatic stem/progenitor cells could be the common cellular origin for both HCC and ICC. It is hypothesized that cancer progression is driven by the presence of CSC, which is also responsible for treatment resistance and tumor relapse.[46] CSCs have been demonstrated in a growing range of epithelial and other solid organ malignancies, suggesting that the majority of malignancies are dependent on such a compartment.[47] This model is attractive because it may help to address the heterogeneity of HCC and ICC, and could facilitate research strategies to define novel and effective therapies.[48] Consistently, studies on clinicopathological features of ICC suggest that some ICCs could arise from liver stem cells rather than from mature cholangiocytes.[49,50] Moreover, gene expression profiling revealed that some HCC cases contain ICC-like gene expression trait and embryonic stem cell-like traits.[15] These results suggest that certain types of ICC could be derived from the same cell origin that leads to HCC, whereas distinct mechanisms may be involved in the genesis of ICC.

CHC has been traditionally classified into three subtypes based on the histological description by Allen and Lisa in 1949.[51] These subtypes include type-A (collision or double cancer, which is referred to as separate HCC and ICC arising in the same liver), type-B (contiguous mass, which is referred to as admixed HCC/ICC such as fibrolamellar tumors), and type-C (transitional tumors, which are referred to as a tumor mass with cellular features of both HCC and ICC). In type-A tumors, the HCC and ICC lesions could be interpreted as originating separately from hepatocyte and bile duct epithelium. Type-B tumors could follow the same mechanism as type-A because it is difficult to distinguish them based on histological data. Because both HCC and ICC cellular features are intimately associated with the type-C tumors, they have been interpreted as arising from the same site and sharing the same cell origin. In our study, two Chinese and five Japanese CHC cases belong to type-B and seven Korean CHC cases[15] belong to type-C. Hierarchical clustering analyses revealed that both type-B and type-C CHC samples could be divided into stem-like ICC and mature hepatocyte-like ICC, which are also associated with survival (Fig. S8). Although these results are not conclusive due to limited cases, they appear consistent with the hypothesis that both type-B and type-C CHC could originate from the same hepatic progenitor cells shared by HCC and CHC tumors. A new histological subtyping of CHC according to the WHO Classification based on the presence of stem-cell features has been proposed.[52] Long-term follow-up of larger cohorts is needed to define the clinical and biological behavior of all CHC cases.

Our analysis dissecting the heterogeneous ICC based on the expression of stem cell-like signatures could classify ICC cases into subgroups with more uniform and prognostic phenotypes. In principle, targeting molecular pathways specific to each subpopulation would be more effective for the development of personalized clinical strategies. We suggest that the miR-200c-associated EMT pathway and stem-cell activities may contribute to the development of the HpSC-ICC tumors. The association of EMT with poor prognosis is well known in many cancer types. Moreover, recent studies have demonstrated the critical role of miR200c in the control of stem/progenitor cell renewal and differentiation. Our findings are consistent with the hypothesis supporting the pivotal role of miR-200c in the aggressive progression of stem-like ICC.

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Nucleostemin in Injury-Induced Liver Regeneration

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The high regenerative capacity of liver contributes to the maintenance of its size and function when injury occurs. Partial hepatectomy induces division of mature hepatocytes to maintain liver function, whereas severe injury stimulates expansion of undifferentiated hepatic precursor cells, which supply mature cells. Although several factors reportedly function in liver regeneration, the precise mechanisms underlying regeneration remain unclear. In this study, we analyzed expression of nucleostemin (NS) during development and in injured liver by using transgenic green fluorescent protein reporter (NS-GFP Tg) mice. In neonatal liver, the hepatic precursor cells that give rise to mature hepatocytes were enriched in a cell population expressing high levels of NS. In adult liver, NS was abundantly expressed in mature hepatocytes and rapidly upregulated by partial hepatectomy. Severe liver injury promoted by a diet containing 3,5-diethoxycarbonyl-1,4-dihydrocollidine induced the emergence of NS-expressing ductal epithelial cells as hepatic precursor cells. NS knockdown inhibited both hepatic colony formation in vitro and proliferation of hepatocytes in vivo. These data strongly suggest that NS plays a critical role in regeneration of both hepatic precursor cells and hepatocytes in response to liver injury.

Introduction

The liver is an organ with high regenerative capacity, enabling it to maintain a constant size and function following injury [1,2]. In resting liver, hepatocytes are quiescent and rarely undergo cell division. Therefore, hepatocyte replacement occurs slowly in static conditions. However, when the liver is injured, cells replicate to restore loss of tissue mass and function. Proliferation of hepatocytes and bile duct epithelial cells contributes to liver maintenance. After partial hepatectomy, the remaining lobes regenerate the entire liver mass within 5–7 days, a process accomplished primarily by division of mature cells rather than of stem/precursor cells. In mice, division of hepatocytes starts after partial hepatectomy and is maximal 24–48 h later. Several molecules, including hepatocyte growth factor (HGF), interleukin-6, tumor necrosis factor α, transforming growth factor, and epidermal growth factor (EGF), reportedly govern this process [1,2]. Termination of regeneration is also important for the maintenance of homeostasis. In severe injury associated with defects in hepatocyte proliferation, it is believed that bipotential precursors of hepatocytes and cholangiocytes contribute to liver regeneration [3,4]. Currently, it is thought that potential hepatic precursor cells emerge from smaller branches of the biliary tree. In rats, a population of small cells exhibiting large nuclei, called oval cells, emerges around portal veins following liver injury [5]. In mice, a diet supplemented with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) induces ductal proliferation and morphological changes similar to those seen in the rat oval cell response [6]. Precursor cells appear to regenerate hepatocytes and cholangiocytes through proliferation, migration, and differentiation processes. Thus, proper control of both mature cells and hepatic stem/precursor cells is critical for liver regeneration.

Nucleostemin (NS) is a GTPase that binds to p53 and was originally reported to be highly expressed in stem cells from several tissues, including embryonic stem (ES) cells, immature hematopoietic cells, and neural stem/progenitor cells [7]. NS loss results in reduced cell proliferation and increased apoptosis in both ES cells and ES cell-derived neural stem/progenitor cells [8]. Structural comparisons have been used to isolate NS homologues in *Caenorhabditis elegans* [9], newt [10], *Xenopus* [11], mouse [7], and human [12]. In the regenerating newt lens, NS protein rapidly accumulates in nucleoli of dedifferentiating pigmented epithelial cells and multinucleate muscle fibers [10], suggesting that its expression correlates with undifferentiated status in newt cells. In contrast, the NS homologue in *Caenorhabditis elegans* (*nst-1*) is expressed in both proliferating and differentiated cells. *Nst-1* mutants exhibit defects in larval growth and cell cycle

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progression in germline stem cells [9]. Because *nst-1*-mutant germ cells can still differentiate into mature sperm, nst-1 may play a critical role in germline stem cell proliferation but not in differentiation. In addition, NS is reportedly expressed at similar levels in non-proliferating muscle stem cells (satellite cells), rapidly proliferating precursor cells (myoblasts), and post-mitotic terminally differentiated cells (myotubes and myofibers) [13]. NS downregulation inhibits differentiation of myoblasts to myotubes, suggesting a role in post-mitotic terminal differentiation in this type. Thus, NS has pleiotropic effects on cellular function, and it is unclear how NS is involved in cell differentiation.

In a previous study, we generated a reporter system where the NS promoter drives green fluorescent protein (GFP) expression (termed NS-GFP) in vivo [14]. We successfully used this reporter system to identify a specific fraction of neonatal germ cells as spermatogonial stem cells with long-term repopulating capacity. We also combined the NS reporter system with a mouse brain tumor model and demonstrated the existence of an undifferentiated tumor-initiating cell (TIC) population in a highly aggressive brain tumor by analyzing GFP fluorescence intensity [15]. Consistent with our data, a recent report employing a bacterial artificial chromosome transgenic mouse line expressing GFP from the NS promoter showed that NS-enriched mammary tumor cells are highly tumorigenic in vitro and in vivo [16]. Further, another recent report showed that NS overexpression enhanced tumorigenicity of TICs, increased expression of genes that maintain undifferentiated status, and enhanced radioresistance [17]. These data suggest that NS functions to maintain stem cell properties in malignant cells.

In this study, we examined the expression and function of NS in liver. Interestingly, we found that NS contributes to the proliferation of hepatocytes after partial hepatectomy and to the regenerative capacity of hepatic precursor cells. Our data strongly suggest that NS is essential for injury-induced liver regeneration.

Materials and Methods

Animals

Mice used in this study were on a C57BL/6 background. NS-GFP Tg mice were generated as described previously [14]. Livers were collected at fetal (embryonic day 14.5: E14.5), neonatal (postnatal day 5: P5), and adult (8 weeks old) stages. For experiments involving severe liver injury, adult mice were fed a diet containing 0.1% DDC (Sigma-Aldrich, St. Louis, MO) for 2 weeks [6]. For partial hepatectomy, mice were anesthetized and 70% of the liver was resected. All procedures were performed in accordance with the animal care guidelines of Kanazawa University.

Isolation of liver cells

For digestion of fetal or neonatal liver cells, livers were minced and dissociated with enzyme-based dissociation buffer (Invitrogen Life Technologies, Carlsbad, CA) as described previously [18]. For isolation of adult liver cells, a 2-step perfusion method was utilized [19]. Briefly, perfusion collagenase solution (0.5 g/L; Sigma-Aldrich) was administered to a sacrificed mouse via the portal vein. Nonparenchymal cells were separated from parenchymal cells by centrifugation (50 *g*, 1 min), and dead cells were removed by centrifugation through 25% Percoll solution (GE Healthcare, Tokyo, Japan).

Flow cytometry

Single-cell suspensions from fetal, neonatal, or adult liver were incubated with an anti-CD16/CD32 antibody on ice for 10 min, followed by incubation with phycoerythrin (PE)-conjugated anti-TER119 and anti-CD45 antibodies (BD Pharmingen, San Diego, CA) on ice for 30 min. Cells were washed thrice in staining solution [2% fetal calf serum (FCS)/phosphate-buffered saline (PBS)] and incubated with anti-PE microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). After 3 washes, $CD45^{-}TER119^{-}$cells were collected (MACS; Miltenyi Biotech). Fetal liver cells were incubated with a biotin-conjugated anti-Dlk antibody (MBL, Nagoya, Japan), followed by incubation with allophycocyanin-conjugated streptavidin antibody (BD Pharmingen). Dead cells were stained with propidium iodide. Fluorescence-labeled cells were analyzed and sorted with JSAN (Bay Bioscience Co, Kobe, Japan).

Hepatic colony forming assay

Cells fractionated by flow cytometry were inoculated at 2,500 cells/well into six-well dishes coated with type I collagen (0.3 mg/mL; Nitta Gelatin, Osaka, Japan). The culture medium included Dulbecco's modified Eagle medium (DMEM)/F-12 supplemented with 10% fetal bovine serum, 5 mmol/L HEPES (Wako, Osaka, Japan), 200 μmol/L L-glutamine (Invitrogen Life Technologies), 50 μmol/L 2-mercaptoethanol (Sigma-Aldrich), 10 mmol/L nicotinamide (Sigma-Aldrich), 10^{-7} mol/L dexamethasone (Sigma-Aldrich), 1 mg/L insulin (Wako), 1× penicillin/streptomycin (Invitrogen Life Technologies), 50 ng/mL HGF (Peprotech, Rocky Hill, NJ), and 20 ng/mL EGF (Sigma-Aldrich).

Immunohistochemical analyses

Adult liver tissues were fixed with 4% paraformaldehyde at 4°C overnight and embedded in paraffin. Frozen sections were sliced and then fixed with 4% paraformaldehyde. The following primary antibodies were used: goat anti-NS (1:200; R&D Systems, Inc., Minneapolis, MN), rabbit anti-GFP (1:500; Invitrogen Life Technologies), and mouse anti-Ki-67 (1:200; BD Pharmingen). Sections were incubated with primary antibodies for 16 h at 4°C, followed by incubation with the appropriate Alexa Fluor dye conjugated to anti-goat IgG, anti-rabbit IgG, or anti-mouse IgG secondary antibodies (all 1:200; Molecular Probes, Inc., Eugene, OR). Staining was visualized using confocal microscopy (FV1000; Olympus, Tokyo, Japan). For some experiments, primary antibodies were detected using peroxidase-conjugated secondary antibodies (GE Healthcare, Amersham, Buckinghamshire, UK) in combination with a 3, 3'-diaminobenzidine (DAB) Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with Mayer's hematoxylin and analyzed using a microscope (Ax80; Olympus).

Immunocytochemical analyses

Hepatic colonies or cell lines were fixed with 4% paraformaldehyde for 10 min, followed by incubation with goat anti-albumin (1:100; Bethyl Laboratories, Montgomery, TX),

rabbit anti-cytokeratin 19 (1:1,000, a gift from Dr. Atsushi Miyajima), chicken anti-GFP (1:500; AVES, Tigard, OR), and/or mouse anti-Ki-67 (1:200; BD Pharmingen) at 4°C overnight and then stained with Alexa 546–conjugated and/or Alexa 488–conjugated secondary antibodies.

Western blotting analyses

Liver samples were lysed with sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sample buffer, sonicated, boiled, and used as total liver cell lysates. Protein concentrations were measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL), and equal amounts of protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skim milk in PBS containing Tween 20 for 1 h at room temperature. Membranes were then incubated with a goat anti-NS antibody (1:1,000; Neuromics, Edina, MN) for 16 h at 4°C and a mouse anti-β-actin antibody (1:1,000; Sigma-Aldrich) for 1 h at room temperature. Immune complexes were detected using peroxidase-conjugated secondary antibodies (1:1,000; GE Healthcare and DAKO, Glostrup, Denmark) for 30 min at room temperature and the ECL Prime western blotting detection system (GE Healthcare).

Reverse transcription–polymerase chain reaction

An RNeasy Mini Kit (Qiagen GmbH, Germany) was used in accordance with the manufacturer's instructions to extract total RNA from nonparenchymal cells sorted by fluorescence-activated cell sorting from adult mice treated with DDC. The primers used were as follows: GAPDH (5′-ACCA CAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTG CTGTA-3′), NS (5′-TCGGAGTCCAGCAAGCATTG-3′ and 5′-GCAGCACTTTCCACATTTGGG-3′), CK19 (5′-GTCCTAC AGATTGACATTGC-3′ and 5′-CACGCTCTGGATCTGTGA CAG-3′), EpCAM (5′-AGGGGCGATCCAGAACAACG-3′ and 5′-ATGGTCGTAGGGGCTTTCTC-3′), Prominin1 (5′-GTA CCTCAGATCCAGCCAGCAA-3′ and 5′-ATTCTTCCAGCT

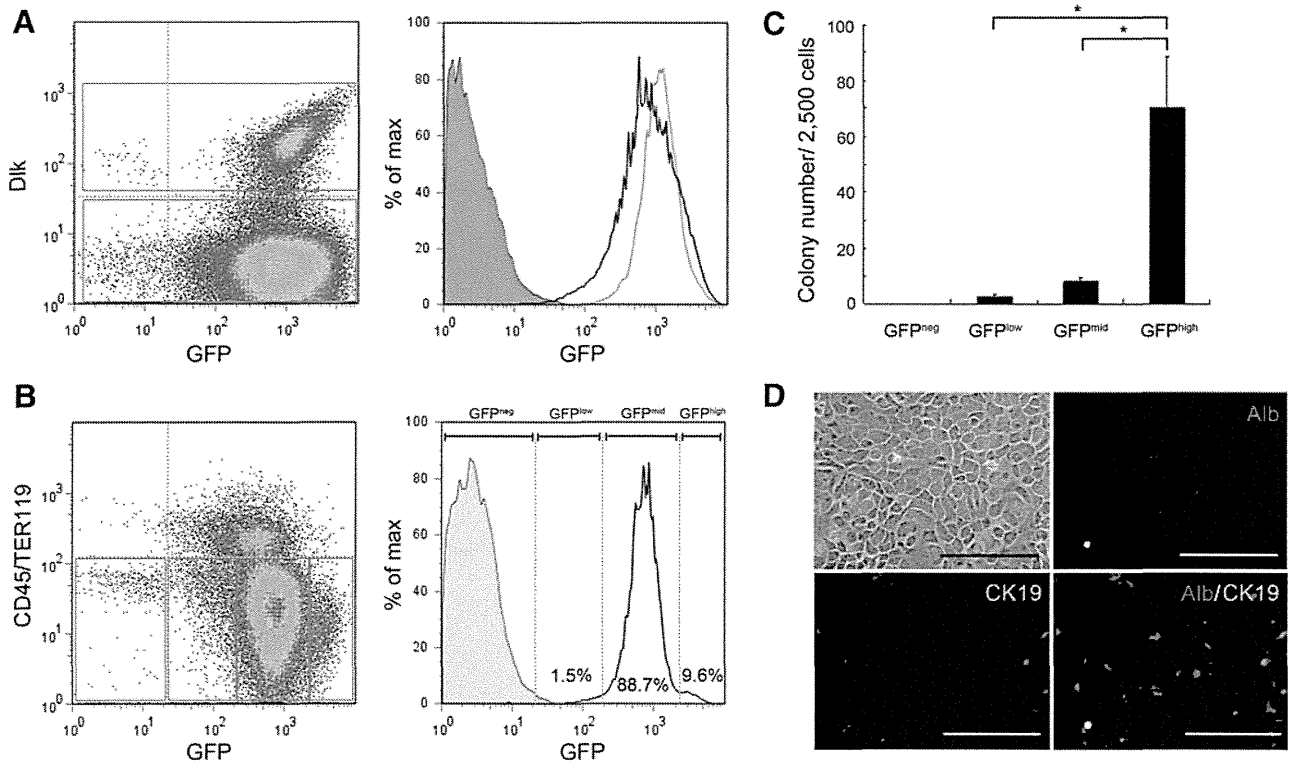

FIG. 1. Correlation of nucleostemin (NS) expression with colony-forming capacity of hepatic precursor cells in developing liver. **(A)** Flow cytometry analysis of green fluorescent protein (GFP) expression in NS-GFP Tg fetal liver. Nonhematopoietic cells from fetal liver cells at E14.5 were isolated by depletion of $CD45^+Ter119^+$ cells. *Left panel*: flow cytometry analysis of Dlk and GFP in $CD45^-Ter119^-$cells. *Right panel*: histogram of GFP expression. GFP expression in Dlk^+ cells (*gray line*) was slightly higher than that seen in Dlk^- cells (*black line*). *Gray region*: wild-type control mouse. The data shown are representative of 3 independent experiments. **(B)** Flow cytometry analysis of GFP expression in NS-GFP Tg neonatal liver. Flow cytometry analysis with CD45/Ter119 and GFP, and histogram with GFP in $CD45^-Ter119^-$ (nonhematopoietic cells) from neonatal liver (P5) are shown in the *left* and *right panels*, respectively. Nonhematopoietic cells ($CD45^-Ter119^-$) were fractionated into GFP^{neg}, GFP^{low}, GFP^{mid}, and GFP^{high} subpopulations. Values in panels are the percentage of the specified subpopulation among $CD45^-Ter119^-$ cells. The data shown are representative of 5 independent experiments. **(C)** Hepatic colony formation of subpopulations in **(B)**. Fractionated cells in **(B)** were cultured for 5 days. Data shown are the mean number ± standard deviation (SD) of colonies ($n = 3$). $^*P < 0.01$ **(D)** Characterization of hepatic colonies. Colonies (brightfield, *upper left panel*) were fixed and stained with anti-albumin (*red*) and anti-CK19 (*green*) antibodies. Most colonies in the culture express albumin or CK19. Representative data are shown. Scale bars, 100 μm.

TGGGCAGC-3'), and CD44 (5'-GGCTTTCAACAGTACC TTAC-3' and 5'-TGAAGCAATATGTGTCATAG-3').

Lentiviral transduction of short hairpin RNA

To downregulate NS in hepatic precursor cells or mouse hepatic cell lines (Hepa1-6, a mouse hepatocellular carcinoma cell line and BNL C1. 2, a mouse embryonic liver cell line), lentiviruses carrying short hairpin RNA (shRNA) against NS was prepared as previously described [14]. Oligonucleotides encoding shRNA directed against mouse NS mRNA were synthesized as follows: NS #1: sense, AGTAGA AATTTGATGGGCA; antisense, AGCAGAAACTTGATAG GCA; NS #2: sense, GAGGAAAGTTGTTTCGTTA; antisense, GAAGAAAGTTGTTCCATTA. Hepatic colonies derived from Dlk^+ fetal liver cells or cell lines were infected with lentivirus for 12 h, followed by washes with PBS, and incubation with culture medium. Cell lines were cultured with 10% FCS/DMEM (Invitrogen Life Technologies).

NS knockdown by hydrodynamic shRNA injection

In vivo transfection of shRNA plasmids into hepatocytes was performed by hydrodynamic injection using 6-week-old mice 3 days prior to partial hepatectomy, in accordance with a previous report [20]. A 27-gauge needle was used to inject 40 μg of plasmid in 2 mL PBS through the tail vein within 10 s. Three days later, the animals were sacrificed and the livers were fixed with 4% paraformaldehyde in PBS and embedded in paraffin for sectioning.

Statistical analyses

Statistical differences were determined using the unpaired Student's *t*-test for *P* values.

Results

NS expression correlates with the colony-forming capacity of hepatic precursor cells in developing liver

To investigate NS expression in developing liver, we evaluated GFP intensity in liver cells of NS-GFP Tg fetuses (E14.5) and neonates (P5). Flow cytometry analysis of fetal liver cells showed that most $CD45^-Ter119^-$ (non-hematopoietic) cells expressed high GFP levels (Fig. 1A). Although GFP levels were very high in Dlk^+ cells, in which hepatic stem/precursor cells are enriched [21], those levels were only slightly higher than those in Dlk^- cells (Fig. 1A). Thus, GFP expression was not indicative of a particular subpopulation in fetal liver. Interestingly, however, NS-GFP neonatal liver cells fell into distinct populations based on GFP

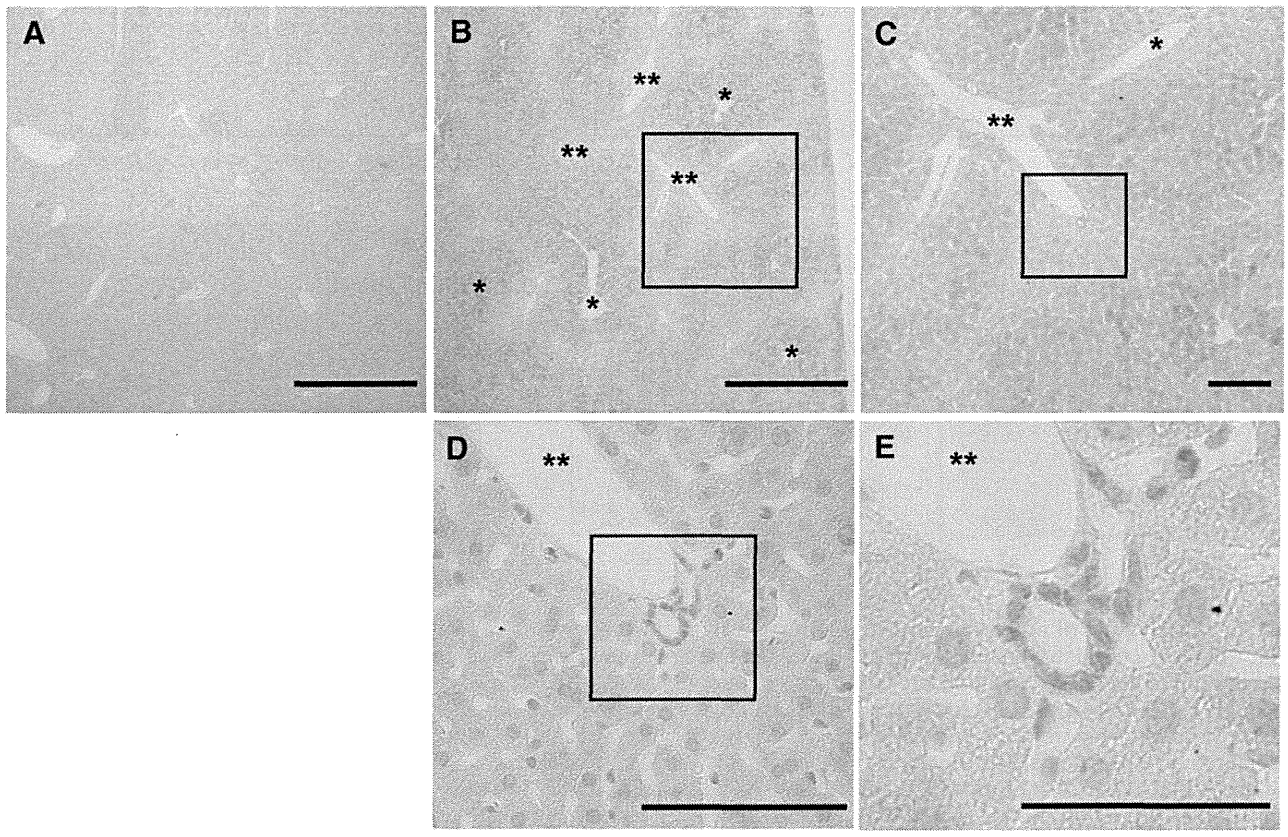

FIG. 2. Expression of NS-GFP in hepatocytes and bile duct epithelial cells of adult liver. **(A–E)** Immunohistochemical analyses of GFP in livers of adult mice (8 weeks old). Sections were stained with an anti-GFP antibody (*brown*), followed by a 3, 3'-diaminobenzidine (DAB) peroxidase reaction. **(A)** Wild-type control (C57BL/6) mice. **(B–E)** NS-GFP Tg mice. **(B)**, **(C)**, and **(D)** are lower-power views of areas shown at higher power in **(C)**, **(D)**, and **(E)**, respectively. Scale bars, 500 μm **(A, B)**, 100 μm **(C, D)**, 50 μm **(E)**, *central vein, **portal vein.

fluorescence intensity (Fig. 1B). While most non-hematopoietic cells were GFP^{mid}, we found a distinct GFP^{high} population. The proportions of GFP^{low} and GFP^{neg} cells were very small. To determine the potential functional significance of these subpopulations, we evaluated hepatic colony forming ability. GFP^{high} cells generated colonies at higher frequency than did any other cell population (Fig. 1C). Most colonies derived from GFP^{high} cells were $CK19^{+}$ or $albumin^{+}$ hepatocytes (Fig. 1D), suggesting that NS is an indicator of hepatic precursor cells in neonatal liver.

Partial hepatectomy upregulates NS expression in hepatocytes

We next examined NS-GFP expression in adult liver. We found that NS-GFP is highly expressed in hepatocytes. In

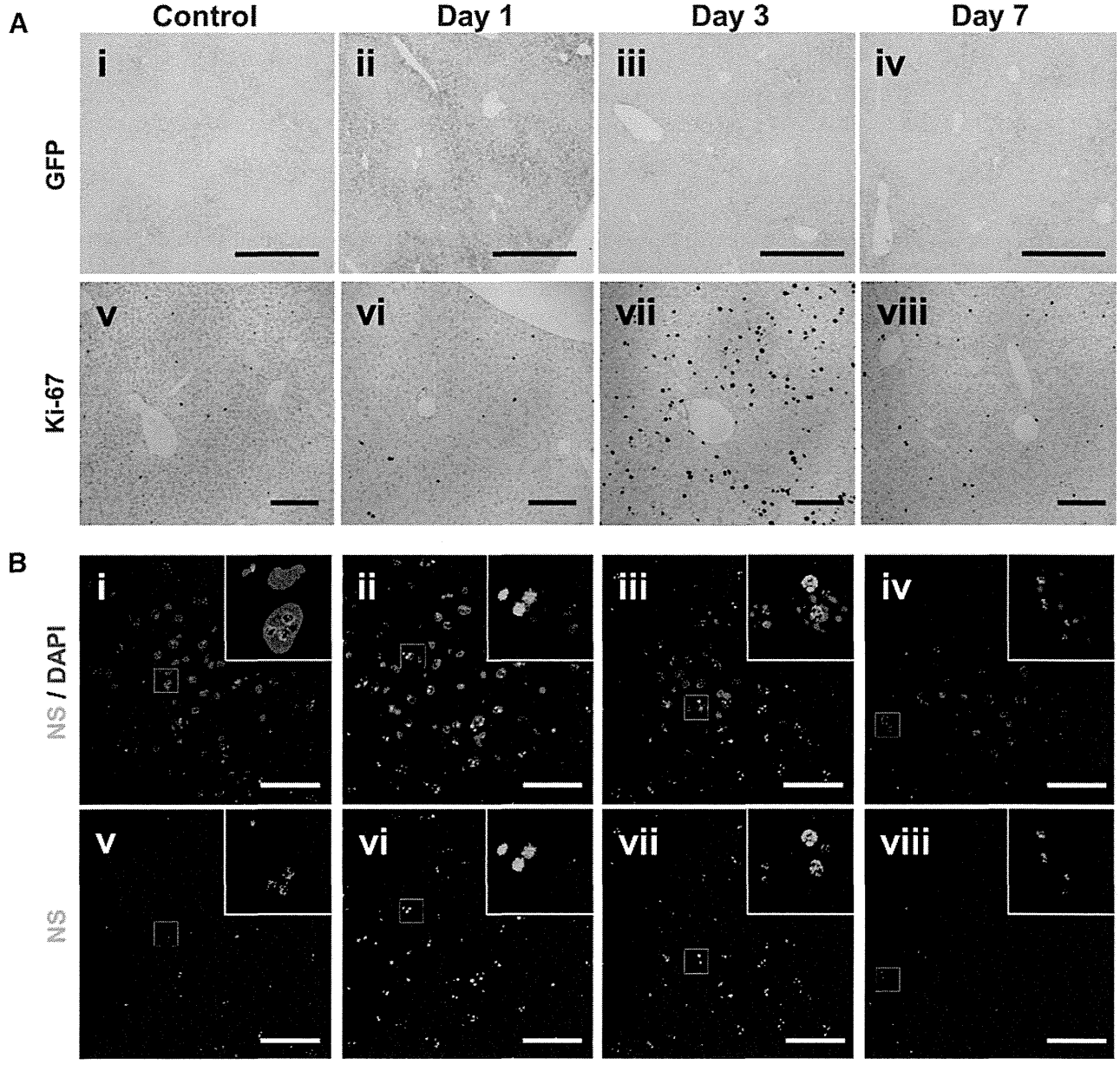

FIG. 3. Upregulation of NS-GFP in adult hepatocytes in response to partial hepatectomy. **(A)** Immunohistochemical analyses of GFP in liver of adult NS-GFP Tg mice after partial hepatectomy. Sections were stained with anti-GFP **(i–iv,** *brown***)** or anti-Ki-67 antibodies **(v–viii,** *brown***)**, followed by DAB peroxidase reactions. **(i, v)** control, **(ii, vi)** day 1, **(iii, vii)** day 3, **(iv, viii)** day 7. Scale bars, 500 μm **(i–iv)**, 100 μm **(v–viii)**. **(B)** Immunohistochemical analyses of endogenous NS in liver of adult NS-GFP Tg mice after partial hepatectomy. Liver sections were stained with an anti-NS antibody, followed by a secondary antibody conjugated to Alexa 488 **(***green***, i–viii)** plus DAPI (nuclear staining, *blue*, **i–iv)**. **(i, v)** control, **(ii, vi)** day 1, **(iii, vii)** day 3, **(iv, viii)** day 7. Scale bars, 50 μm. *Insets* are magnified views of the indicated areas. **(C)** Western blotting analyses of endogenous NS in liver of adult wild-type mice after partial hepatectomy. Lysates were prepared from liver (3 independent samples for each group) and immunoblotted to detect NS and β-actin as a loading control. Short and long exposures are shown for NS in the *upper* and *middle panels*, respectively.

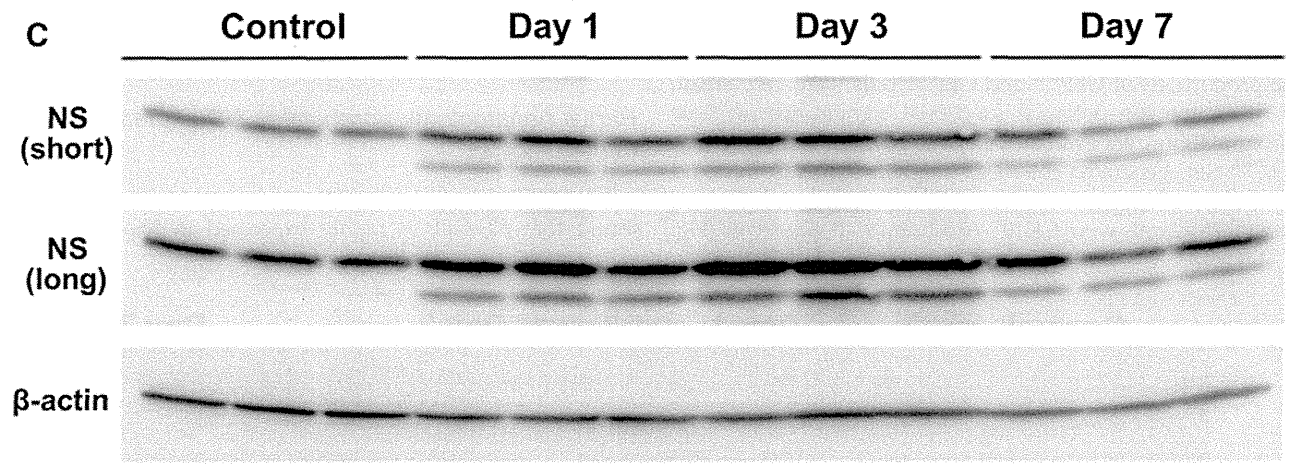

FIG. 3. (Continued).

particular, hepatocytes near central veins showed higher levels of NS-GFP expression than did those in the portal area (Fig. 2A–C). In addition, we found that NS-GFP is also highly expressed in bile duct epithelial cells (Fig. 2D, E). Next, we asked whether expression levels of NS-GFP are altered by liver injury. After partial hepatectomy, NS-GFP was upregulated within 24 h (Fig. 3A i–iv). Immunohistochemical analyses showed that NS protein expression was remarkably increased in nucleoli 24 h after hepatectomy. Although there were variations among samples, NS protein levels appeared to remain elevated at day 3 (Fig. 3B) and reverted to baseline levels at day 7, whereas NS-GFP expression was already downregulated at day 3. Western blotting analysis using an anti-NS antibody consistently showed an increase in endogenous NS protein on day 1 and day 3 after partial hepatectomy (Fig. 3C). The discrepancy between NS-GFP and endogenous NS is possibly due to differences in protein stability stemming from different post-translational modification of these molecules. Interestingly, partial hepatectomy induced a variant form of NS that is reported to be expressed in particular tissues [22,23]. Hepatocytes that had begun to proliferate showed a small increase in the expression of Ki-67, a marker of cell proliferation, at day 1, and further increases in Ki-67 expression were observed at day 3 (Fig. 3A v–viii). These data indicate that NS gene expression is rapidly upregulated before the start of cell division in response to partial hepatectomy.

A DDC diet induces emergence of ductal epithelial cells expressing NS-GFP

DDC treatment inhibits the capacity for hepatocytes to regenerate, while inducing ductal proliferation in mice in what is known as the oval cell response [6]. We found that DDC treatment reduced expression of NS-GFP in hepatocytes (Fig. 4A i, ii) compared to the expression in untreated hepatocytes. We also found that bile duct-like NS-GFP-positive cells emerged in the portal zone following DDC treatment (Fig. 4A iii, iv). Interstitial cells surrounding ductal cells did not express NS-GFP. We confirmed by immunofluorescence that NS-GFP was expressed in $CK19^+$ ductal epithelial cells (Fig. 4B). To investigate the regenerative capacity of NS-GFP-expressing cells in DDC-treated liver, we evaluated NS-GFP intensity in nonparenchymal cells, since oval cells reportedly reside in that population [6]. Flow cytometry analysis showed that most $CD45^-Ter119^-$ nonparenchymal cells were GFP-positive (Fig. 5A), although the intensity of NS-GFP expression in non-parenchymal cells in adult mice appeared lower than that seen in developing liver cells. These NS-GFP-positive cells fell into GFP^{high} and GFP^{low} populations. GFP^{high} cells were relatively rare, but only GFP^{high} cells showed hepatic colony forming ability (Fig. 5C). In contrast, no colonies were generated from GFP^{low} or GFP^{neg} cells. Severe liver injury promoted by a DDC diet increased the proportion of GFP^{high} cells relative to the proportion in untreated mice (Fig. 5B). Because we found that ductal cells express NS-GFP (Figs. 2 and 4), we assumed that the DDC diet increased the number of ductal cells, resulting in an increase in the proportion of GFP^{high} cells. Hepatic colonies were generated only from GFP^{high} cells (Fig. 5D). GFP^{high} cells expressed higher levels of NS mRNA, indicating that GFP expression corresponded with that of endogenous NS, and also expressed several genes reportedly expressed in oval cells [19] (Fig. 5E). These data indicate that hepatic precursor cells induced by severe liver injury express NS.

NS downregulation inhibits proliferation of hepatic precursor cells

Next, to address whether NS is required for regeneration of hepatic precursor cells, we downregulated NS expression in hepatic cell line and primary fetal liver cells in vitro. Previously, we successfully suppressed NS in a germ cell line by infection with a lentivirus carrying NS shRNA [14]. In this system, infected cells were identified by GFP expression driven by the lentivirus vector (GFP^+ cells). For the current study, we infected the mouse hepatocellular carcinoma cell line Hepa1-6 and the mouse embryonic liver cell line BNL C1.2 with lentiviruses carrying NS shRNA (#1 or #2) or a scrambled control shRNA and then stained the cells with an anti-NS antibody. Both shRNAs, but not the scrambled control, efficiently reduced expression of NS protein in the hepatic cell lines (Fig. 6A, data not shown). NS knockdown (GFP^+) cells in the cell lines had dramatically reduced colony-forming capacity (Fig. 6B, data not shown). We also found that NS downregulation significantly decreased the

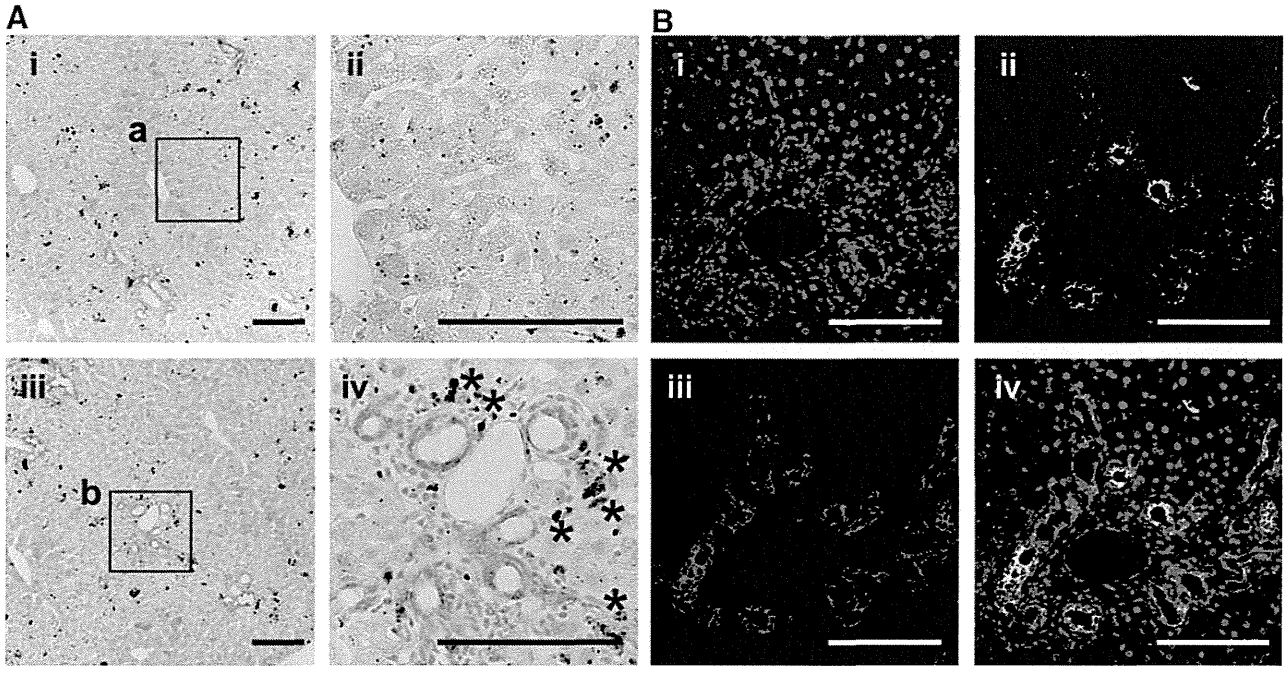

FIG. 4. Duct-like cells express NS-GFP in liver of adult mice fed a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet. **(A, B)** GFP in hepatocytes of adult NS-GFP Tg mice fed a DDC diet. **(A)** Sections were stained with an anti-GFP antibody. **a** and **b** are lower power views of the areas shown at higher power in **(ii)** and **(iv)**, respectively. *Deposition of iron hemes, visible as *brown* clots. **(B)** Sections were stained with anti-GFP (*green*) and anti-CK19 (*red*) antibodies and DAPI (nuclear staining, *blue*). **(i)** DAPI, **(ii)** GFP, **(iii)** CK19, **(iv)** merged. Scale bars, 100 μm.

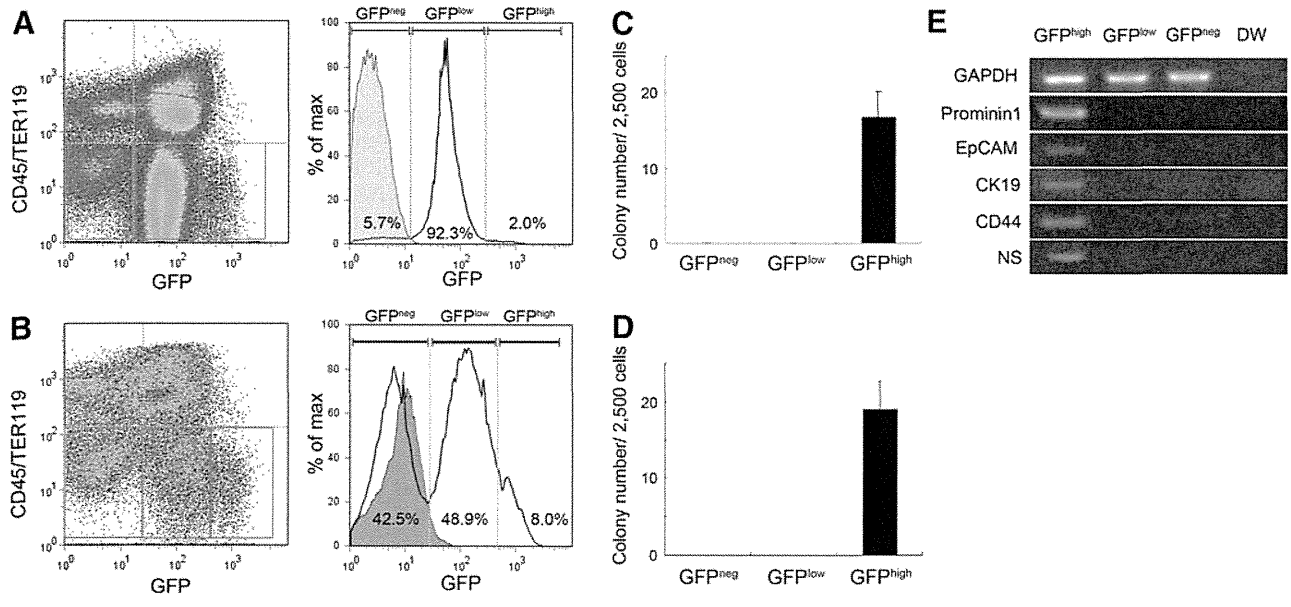

FIG. 5. Increased ratios of NS-GFP^{high} hepatic precursor cells are seen following a DDC diet. **(A, B)** Flow cytometric analyses of GFP expression in nonparenchymal cells of adult NS-GFP Tg mice without **(A)** and with **(B)** a DDC diet. Flow cytometry with CD45/Ter119 and GFP, and a histogram of GFP in CD45$^-$Ter119$^-$ cells are shown in the *left* and *right* panels, respectively. Nonhematopoietic nonparenchymal cells (CD45$^-$Ter119$^-$) were fractionated into 3 distinct subpopulations (GFP^{neg}, GFP^{low}, and GFP^{high} cells). Values in panels are the percentage of the specified subpopulation among CD45$^-$Ter119$^-$ cells. The data shown are representative of 3 independent experiments. **(C, D)** Hepatic colony formation of subpopulations. Fractionated cells were cultured for 7 days. Data shown are the mean ratio ± SD of colonies derived from mice fed a control **(C)** and DDC **(D)** diet ($n = 3$ each). **(E)** Gene expression in NS-GFP subpopulations. Total RNA was purified from the subpopulations indicated in **(B)**, and mRNA levels of the indicated molecules were evaluated by reverse transcription–polymerase chain reaction. DW, distilled water.

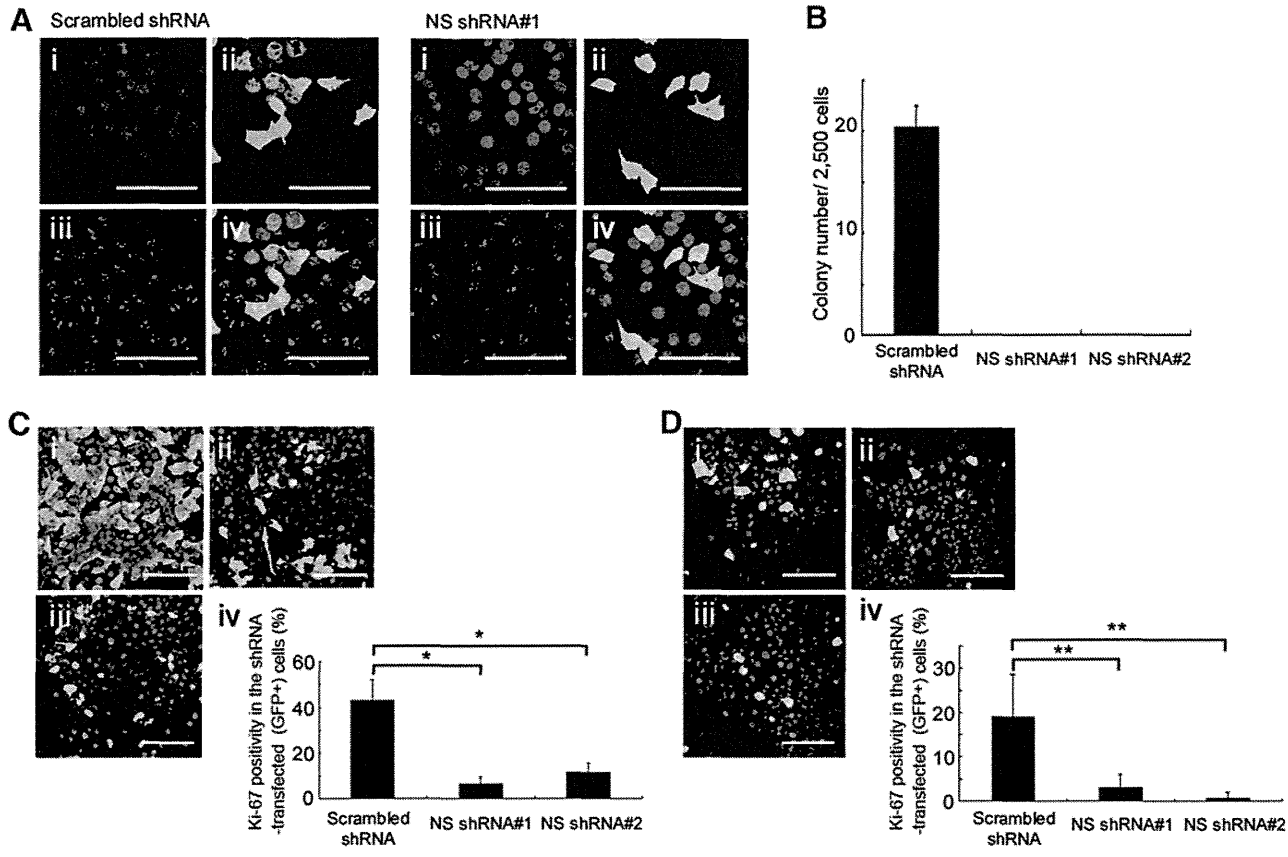

FIG. 6. Inhibition of proliferation of hepatic precursor cells following NS downregulation in vitro. **(A)** NS knockdown in a hepatic cell line. A scrambled shRNA (control, *left panels*) or NS shRNA#1 (*right panels*) was introduced into Hepa1-6 cells (a murine hepatocellular carcinoma cell line) by lentiviral infection, followed by staining with anti-GFP (*green*) and anti-NS (*red*) antibodies and DAPI (*blue*). **(i)** DAPI, **(ii)** GFP, **(iii)** NS, **(iv)** merged. NS protein expression (*red*) was reduced in GFP^+ cells treated with shRNA#1 but not the control shRNA. **(B)** Colony formation of Hepa1-6 cells following NS knockdown. GFP^+ Hepa1-6 cells, indicating those transduced with shRNA, were isolated and cultured for 10 days. Data shown are the mean number ± SD of colonies ($n = 3$). **(C, D)** Proliferation of Hepa1-6 cells **(C)** and Dlk^+ hepatic precursors from fetal liver **(D)** following NS knockdown. Hepa1-6 cells and Dlk^+ fetal liver cells were infected with the shRNA lentivirus, and then cultured for 3 **(C)** or 10 **(D)** days. Cells were stained with an anti-Ki-67 antibody (*red*), anti-GFP antibody (*green*), and DAPI (*blue*). Representative data are shown in **(i)**, **(ii)**, and **(iii)** for the scrambled control, NS shRNA#1, and NS shRNA#2, respectively. Data shown in **(iv)** are the mean ratio ± SD of Ki-67 positivity among cells transfected with the indicated shRNA plasmids (GFP^+ cells) ($n = 3$).*$P < 0.01$, **$P < 0.05$. Scale bars, 100 μm.

proportion of Ki-67-positive cells among transfected (GFP^+) cells in the hepatic cell line (Fig. 6C) and in the hepatic colonies derived from freshly isolated fetal liver precursor cells (Fig. 6D). Thus, NS downregulation inhibits proliferation of hepatic precursor cells.

NS plays an essential role in hepatocyte proliferation in response to liver injury in vivo

The observation that NS is upregulated in hepatocytes after partial hepatectomy suggested that NS is essential for hepatocyte proliferation. To examine the effect of loss NS function in hepatocytes in vivo, we introduced the shRNA plasmids into liver cells by hydrodynamic injection of plasmid DNA via the tail vein [20]. Partial hepatectomy was performed 3 days later and we found that hepatocytes were successfully transfected with shRNA plasmids by detection of GFP expression. Three days after partial hepatectomy, we found that NS knockdown in the hepatocytes significantly suppressed expression of the Ki-67 antigen (Fig. 7), indicating that NS is essential for hepatocyte proliferation in response to liver injury in vivo.

Discussion

In this study, we examined the expression and function of NS in developing and injured liver, and we evaluated the capacity of hepatic NS-expressing cells to form colonies by using an NS-GFP system. As previously reported [19], DDC treatment induced the emergence of ductal cells that express both cholangiocellular and hepatocytic markers, called "oval cells," in periportal regions. Several studies have identified markers of oval cells, including Ep-CAM and CD133 [19,24,25]. NS-GFP was not specific for oval cells, since most hepatocytes and nonparenchymal cells also expressed GFP. Therefore, NS-GFP expression alone cannot be used to purify hepatic stem/precursor cells. However, since a distinct subpopulation of cells expressing high GFP levels (GFP^{high}) showed higher clonogenic potential, combining this system with evaluation of other stem cell markers could enable

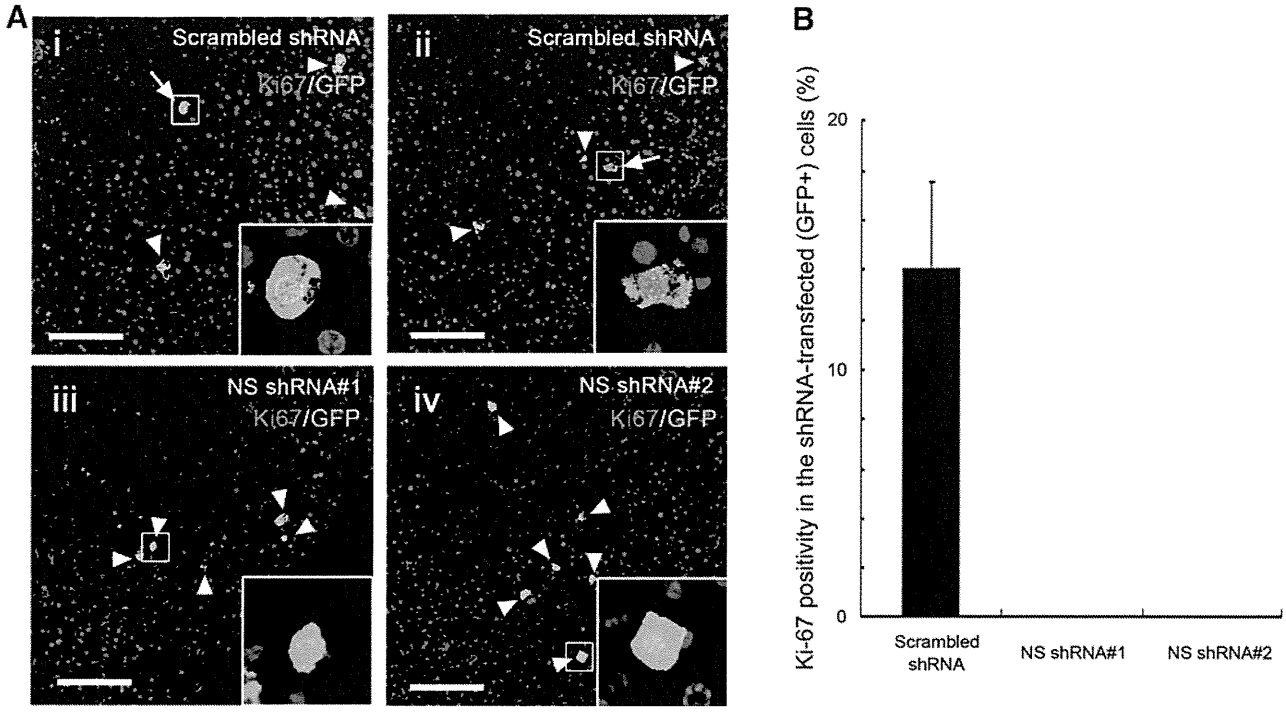

FIG. 7. Inhibition of hepatocyte proliferation following NS downregulation in vivo. NS shRNA plasmids were introduced into liver cells by hydrodynamic injection of the plasmid DNA via the tail vein, followed by partial hepatectomy 3 days after injection. Liver tissue specimens were prepared 3 days later, and sections were stained with an anti-GFP antibody (*green*), anti-Ki-67 antibody (*red*), and DAPI (*blue*). Ki-67 expression was evaluated in more than 60 GFP^+ cells for each sample. **(A)** Representative data for the scrambled control **(i, ii)**, NS shRNA#1 **(iii)**, and NS shRNA#2 **(iv)**. *Arrows*; $GFP^+Ki\text{-}67^+$ cells, *arrowheads*; $GFP^+Ki\text{-}67^-$cells. *Insets* are magnified views of the indicated areas. **(B)** Mean percentage ± SD of Ki-67 positivity in hepatocytes transfected with the indicated shRNA plasmids (GFP^+ cells) ($n=3$). Scale bars, 100 μm.

efficient enrichment of a stem/precursor cell population. In addition, NS may be particularly important for the development of liver, since the expression level of NS-GFP in developing liver cells appeared to be higher than that in adult non-parenchymal cells. NS may therefore play a critical role in expansion of the hepatic stem/precursor cells during liver development.

A previous study demonstrated that NS is required for rRNA processing [9,26], suggesting that NS expression regulates protein synthesis. Enhanced protein synthesis requires activation of ribosomal biogenesis. NS belongs to the class of nucleolar GTPases that includes yeast Nug1, which exports pre-60S ribosomal subunits out of the nucleolus [27]. In *Caenorhabditis elegans*, *nst-1* mutants exhibit reduced rRNA levels, suggesting a critical role of NS in ribosome biogenesis [9]. NS knockdown apparently delays processing of 32S pre-rRNA into 28S rRNA and is accompanied by a substantial decrease in protein synthesis and in the levels of rRNAs and some mRNAs [26]. Because protein synthesis is required for cell growth and proliferation, NS expression in both hepatic precursor cells and hepatocytes may be important for tissue regeneration. On the other hand, protein synthesis appears to be enhanced in resting hepatocytes for reasons unrelated to regeneration. Mature hepatocytes exhibit high levels of protein synthesis to maintain serum protein levels, and protein translation actively occurs even in non-dividing hepatocytes. Thus, NS expression may be controlled by several different signals.

One possible regulator of NS is Myc, which is upregulated by partial hepatectomy [28]. When Myc is overexpressed in mouse hepatocytes in vivo using recombinant adenovirus, hepatocytes enlarge in the absence of significant cell proliferation, an event associated with upregulation of large- and small-subunit ribosomal and nucleolar genes [29]. In addition, a recent study identified the NS gene as a direct transcriptional target of the Myc oncoprotein [22]. Therefore, NS may function to increase cell mass in response to Myc activation following partial hepatectomy. It has also been reported that, constitutive activation of Myc generates hepatocellular carcinoma, whereas Myc inactivation promotes differentiation of tumor cells into hepatocytes and biliary cells, which form bile duct structures [30], suggesting that Myc maintains cells in an undifferentiated status. NS may have a similar function in the case of hepatic malignancy. In addition, Myc may control post-translational regulation of NS protein. A recent study revealed that NS is a target of reactive oxygen species (ROS) [31]. In transformed hematopoietic cells, Myc activation leads to high ROS levels, resulting in impaired NS protein degradation. Therefore, Myc activation may stabilize NS protein in regenerating liver. Because GFP would not be stabilized in the same way as the NS protein, these findings suggest that NS protein levels may not be precisely correlated with NS-GFP levels. Nonetheless, both NS protein levels and NS-GFP expression are consistently upregulated by partial hepatectomy. These findings suggest that overall NS expression is likely regulated by both transcriptional and protein stability.

In conclusion, we have demonstrated that NS is essential for proliferation of both hepatic precursor cells and

hepatocytes. Understanding the mechanisms regulating NS expression and function may contribute to development of methodologies useful for enhancing liver regeneration in pathological states.

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Author Disclosure Statement

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MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Molecular mechanisms of hepatocarcinogenesis in chronic hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) infection is a major cause of hepatocellular carcinoma (HCC) and chronic liver disease worldwide. Recent developments and advances in HCV replication systems *in vitro* and *in vivo*, transgenic animal models, and gene expression profiling approaches have provided novel insights into the mechanisms of HCV replication. They have also helped elucidate host cellular responses, including activated/inactivated signaling pathways, and the relationship between innate immune responses by HCV infection and host genetic traits. However, the mechanisms of hepatocyte malignant transformation induced by HCV infection are still largely unclear, most likely due to the heterogeneity of molecular paths leading to HCC development in each individual. In this review, we summarize recent advances in knowledge about the mechanisms of hepatocarcinogenesis induced by HCV infection.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer death worldwide.[1] The majority of HCCs arise from a background of chronic liver diseases caused by infection with hepatitis B virus (HBV) or hepatitis C virus (HCV).[2] Although both viruses are hepatotropic and regarded as causative agents of HCC, the underlying mechanisms of hepatocarcinogenesis are considered to be largely different, partly due to differences in the nature of DNA viruses (with an integration capacity for the host genome) and RNA viruses (with no genome integration capacity).

Hepatitis C virus is an RNA virus that is unable to integrate into the host genome but, instead, its proteins interact with various host proteins and induce host responses that potentially contribute to the malignant transformation of cells. In addition, HCC usually develops in the setting of liver cirrhosis after long-term continuous inflammation/regeneration processes; these accelerate the turnover of hepatocytes with increased risk of replication errors and DNA damage. Furthermore, recent genome-wide association studies have suggested that the natural course of HCV infection might be modified by the genetic background of the host.[3,4] Thus, both host and virus factors are considered to affect the process of hepatocarcinogenesis in a complex manner.

In this review, we summarize the current knowledge of the mechanisms of hepatocarcinogenesis induced by HCV infection. We also focus on recent findings of transcriptomic characteristics of HCV-related HCC and summarize the potential signaling pathways that are altered in this condition.

Epidemiology

Chronic HCV infection is a major risk factor for the development of HCC worldwide. According to the World Health Organization (WHO), approximately 170 million people are chronically infected with HCV. Although epidemiological evidence has suggested a clear, close relationship between HCV infection and HCC,[5,6] the prevalence of HCV infection in HCC patients differs noticeably between geographical regions. Thus, HCV infection is found in 70–80% of HCC patients in Japan, 70% in Egypt, 40–50% in Italy and Spain, about 20% in the United States (US), and less than 10% in China.[7–9] In industrialized countries including the US, a recent increase in HCC incidence and mortality has been observed, potentially due to the rising incidence of HCV infection transmitted through contaminated blood.[10]

Hepatitis C virus increases the risk of HCC by promoting inflammation and fibrosis of the infected liver that eventually results in liver cirrhosis. Once HCV-related cirrhosis is established, HCC develops at an annual rate of about 4–7%.[11] Other factors including alcohol intake, diabetes, and obesity have also been reported to increase the risk of HCC development by about two- to fourfold, indicating a strong life-style effect on the process of hepatocarcinogenesis.[12,13] Age and male gender are also contributing risk factors for HCV-related HCC, although the detailed mechanisms are still debatable.

Virus proteins and host responses

Hepatitis C virus belongs to the Flaviviridae family. It has a positive-stranded linear RNA genome of about 9.6-kb containing a

single large open reading frame encoding three structural (core, E1, and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins.[14] The structural proteins form the HCV virions, whereas non-structural proteins are involved in the processes of viral replication, assembly, and maturation. HCV proteins are known to be processed by host and viral proteases. Both structural and non-structural proteins can interact with various host cellular proteins to potentially promote the malignant transformation of hepatocytes (see recent reviews[7,15,16]). In this review, because of space limitations, we focus on the findings of core and NS5A proteins in terms of host responses potentially evoked during the process of HCV-related hepatocarcinogenesis.

Core protein

Hepatitis C virus core is a 21-kDa nucleocapsid protein with an RNA-binding capacity. In addition to its function in regulating HCV-RNA translation and HCV particle assembly, core protein is known to be involved in mediating the alteration of various host cell signaling pathways, transcriptional activation, modulation of immune responses, apoptosis, oxidative stress, and lipid metabolism.[7] Several recent studies have indicated the statistically significant high frequency of mutations in the *core* gene in HCV-infected patients who developed HCC.[17,18] However, the functional relevance of mutant core proteins on the malignant transformation of hepatocytes or the HCV life cycle has yet to be clarified.

Evidence of core protein as a causative agent of HCC was initially obtained from the transgenic mice model in which *core* gene overexpression, under the regulation of the HBV regulatory element used as a promoter, resulted in steatosis of mouse livers in early life, with subsequent development of adenoma and HCC.[19] However, another mouse model using a different promoter and of a different strain background resulted only in steatosis or different phenotypes without HCC development.[20,21] Similar controversial findings were reported in transgenic mice expressing HCV polyprotein or structural protein with regards to the development of HCC.[22,23] Thus, the role of core protein alone in the development of HCC remains unclear in transgenic mouse models.

Although the direct role of core protein in the malignant transformation of hepatocytes is still under investigation, it seems to be related to the development of hepatic steatosis.[19,24] Indeed, steatosis is one of the risk factors for the development of HCV-related HCC,[25,26] and activation of the lipogenic pathway has been reported in a subset of HCC cases.[27] Core protein is associated with the surface of lipid droplets in infected cells and might be directly related to steatosis through several factors responsible for lipid biogenesis and degradation, including peroxisome proliferator-activated receptor alpha and sterol-regulatory element binding protein-1.[21,28–30] Furthermore, core protein is reported to interact with endoplasmic reticulum (ER) or mitochondrial outer membranes and induce ER stress by perturbation of protein folding or by the accumulation of reactive oxygen species (ROS) through mitochondrial dysfunction.[31,32] ROS produced in this way might result in DNA damage to the host genome and accelerate the process of hepatocarcinogenesis. Increased hepatic iron deposition may also induce oxidative stress and lipid peroxidation, thus increasing the risk of HCC development in HCV polyprotein transgenic mice.[33]

Since the discovery of HCV, various studies have investigated the role of core on host cells. Its effects have been demonstrated on signaling pathways responsible for the cell cycle, and apoptosis through interaction with several tumor suppressors including p53, p73, and p21[34–39] as well as apoptosis regulators such as tumor necrosis factor-α (TNF-α) signaling or Bcl-2 members.[40–42] However, the data obtained from these studies are relatively inconsistent with each other and have varied across experimental models. Core protein may influence the growth and proliferation of host cells through activation of signaling pathways such as Raf/mitogen activated protein kinase (MAPK),[43] Wnt/beta catenin,[16] and transforming growth factor-β (TGF-β).[15,44] These pathways are known to be activated in HCC.[45] The findings therefore indicate a potential role for core in cell proliferation or suppression of apoptosis during malignant transformation of hepatocytes in the liver of chronic hepatitis C, where chronic inflammation and regeneration of hepatocytes continuously occurs.

NS5A protein

NS5A is a 56–58-kDa protein phosphorylated at serine residues by serine-threonine kinase[46] and is essential for replication of the HCV genome. NS5A protein forms part of the viral replicase complex and is localized mainly in the cytoplasm of infected cells in association with the ER. NS5A can become a lower molecular weight protein through post-translational modification, after which it can undergo translocation to the nucleus where it acts as a transcriptional activator. High frequencies of wild-type *NS5A* genes were reported to be dominant in liver cirrhosis patients who finally developed HCC compared with those who did not,[47] but the mechanistic significance of the *NS5A* wild/mutant genotypes in the process of HCV-related hepatocarcinogenesis remains uncertain.

NS5A protein has been suggested to interact with various signaling pathways including cell cycle/apoptosis[48] and lipid metabolism[28,49,50] in host cells and shares some signaling targets with core protein. NS5A is recognized as a transcriptional activator for many target genes[51] including p53 and its binding protein, TATA binding protein (TBP). Transcription factor IID activities were reported to be modified by NS5A in the suppression of p53-dependent transcriptional transactivation and apoptosis.[52,53] NS5A may also interact with pathways such as Bcl2,[54] phosphatidylinositol 3-kinase (PI3-K),[55] Wnt/beta catenin signaling,[56] and mTOR[57] to activate cell proliferation signaling and inhibit apoptosis.

Taken together, intriguing data concerning the function of core and NS5A proteins on host cell signaling pathways, transcriptional activation, apoptosis, oxidative stress, and lipid metabolism described above suggest a diverse role for HCV proteins in the pathophysiology of chronic hepatitis C that leads to malignant transformation in infected hepatocytes. Key findings and present concepts are summarized in Figure 1.

Transcriptomic characteristics of HCV-related HCC

As described above, HCV proteins can evoke various host responses in infected cells at transcriptional/translational/post-translational levels. Furthermore, enhanced cell death/regeneration processes are considered to induce DNA damage and accelerate replication errors that cause frequent mutations and genomic alter-