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## The role of a novel CAR-induced gene TUBA8 in hepatocellular carcinoma cell lines

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### Abstract

Phenobarbital (PB), a non-genotoxic carcinogen, activates the nuclear constitutive active/androstane receptor (CAR), resulting in the transcriptional induction or repression of various hepatic genes. We previously demonstrated that liver tumors developed following chronic PB treatment only when CAR is present. In order to understand the molecular mechanism of tumor promotion, cDNA microarray analysis was performed and we identified the Tubulin 8 (TUBA8) as the one of the candidate genes that may be involved in liver tumor promotion. TUBA8 mRNA was induced with PB treatment in mouse livers before tumor development as well as in tumor tissues. Since the functions of TUBA8 are unknown in liver, we investigated the effects of TUBA8 gene expression on cell growth, proliferation and cell migration. Sense- or anti-sense cDNA for mouse TUBA8 (mTUBA8) was stably transfected into Huh7 and HepG2 cells. Exogenous over-expression of mTUBA8 inhibited cell growth and proliferation in Huh7, but not in HepG2 cells, while cell migration was increased in HepG2 cells, but not Huh7 cells. These results indicate that TUBA8 can play a role in the regulation of cell growth, proliferation and cell migration in a cell specific manner in vitro, suggesting that TUBA8 may contribute to mouse liver tumorigenesis through these functions.

### Keywords

CAR; Nuclear receptor; TUBA8; liver cancer; cell migration; cell growth

### 1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers in the world, and the high rate of mortality is observed because there are no visible symptoms until the late stages of development. Various environmental risk factors, including hepatitis B virus or C virus infection, heavy alcohol consumption, dietary aflatoxin and exposure to oncogenic chemicals have been identified as causes of HCC [1, 2]. Such a wide range of causes makes it difficult to elucidate the molecular pathogenesis of HCC. The progression to HCC is usually promoted by a long-term period of virus infection or exposure to chemicals, which causes chronic inflammation. The resulting accumulation of genetic and epigenetic

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alterations in liver cells is thought to play an important role in liver tumorigenesis [3–5]; however the details of the molecular-based mechanism(s) remain unknown.

Rodent models have been used to clarify the underlying mechanism of HCC development, in particular the promotion of liver tumors that occurs without gene mutations. Liver tumors including HCC in rodents are usually produced by the chronic treatment of nongenotoxic agents such as phenobarbital (PB), dichlorodiphenyltrichloroethane (DDT) or ethinyl estradiol after initiation with genotoxic carcinogens (e.g., nitrosamines) [6–8]. Using this two-step mouse model of HCC development, we have reported that CAR, which is the nuclear receptor that is activated by PB in liver, was essential for liver tumor development. CAR is a member of the thyroid/steroid hormone nuclear receptor superfamily and plays an important role in regulating the expression of drug-metabolizing enzymes and transporters such as cytochrome P450s (CYPs) and multidrug resistance-associated proteins (MRPs) [9–11]. CAR is also an essential factor regulating the metabolism of bile acids and bilirubin, which cause liver injury [12, 13]. While CAR regulates these normal liver functions, the relationship between CAR and the development of liver diseases such as steatohepatitis, cholestasis and liver tumors has recently been shown [14]. These findings suggest that CAR is an important regulator of various liver functions. We have previously found that *Car*<sup>-/-</sup> mice did not develop liver tumors after chronic PB treatment for over 34 weeks compared to *Car*<sup>+/+</sup> mice [15]. Based on this observation, we performed cDNA microarray analysis to identify the expression of candidate gene(s) correlating with liver tumor development. Over 90 genes were identified as being CAR-regulated, and TUBA8 was selected as a candidate that may be involved in CAR mediated liver tumor development.

TUBA8 was identified as a novel gene belonging to  $\alpha$ -tubulin superfamily in 2000 [16], but its physiological function in vivo is still unknown. In general, the heterodimer complex of  $\alpha$ - and  $\beta$ -tubulin assemble into microtubules (MTs) which constitute one of the major components of the cytoskeleton of eukaryotic cells and are involved in many essential processes including cell division, motility and intracellular transport [17]. Post-translational modification of  $\alpha$ - or  $\beta$ -tubulin is important for controlling microtubule dynamics [18], and the mechanism of these modifications has been examined in detail in brain and neuronal cells [19]. However, TUBA8 is an atypical  $\alpha$ -tubulin and does not have specific amino sequences involved in these modifications, suggesting that TUBA8 might have a unique function compared to other  $\alpha$ -tubulins during the promotion of liver tumors by PB treatment.

Here we show the up-regulation of TUBA8 mRNA expression during the process of mouse liver tumor development in a CAR dependent manner. To examine the differences in malignant phenotypes including proliferation potency, colony-forming ability and cell migration, ectopic over-expression of TUBA8 in Huh7 and HepG2 cells (both human hepatocellular carcinoma cell lines) was tested. Interestingly, inhibition of cell growth and proliferation was observed in Huh7 cells, while up-regulation of cell migration was observed in HepG2 cells. Our results contribute to the understanding of TUBA8 function in the development of liver tumors.

## 2. Materials and methods

### 2.1. Animals

*Car*<sup>+/+</sup> and *Car*<sup>-/-</sup> mice were previously generated in a C3H/HeNCR1BR (C3He) background [15]. C3H/HeNCR1BR (C3He) and C57BL/6 mice (5–7 weeks-old) were obtained from Charles River Laboratories International Inc. (MA, USA), housed in a specific pathogen-free rodent facility under a standard 12 h-light/12 h-dark cycle and were fed a standard rodent chow and water *ad libitum*. PB (100 mg/kg) was administered

intraperitoneally. All animal procedures were approved by the Animal Ethics Committee of National Institute of Environmental Health Sciences.

## 2.2. Real-Time PCR

Total RNAs were extracted from mouse livers or culture cells using TRIzol reagent (Invitrogen, CA, USA), and used to synthesize cDNAs using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems Inc. CA, USA). Real-Time PCR was performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems Inc. CA, USA). Forward and reverse primes for mouse TUBA8 cDNA were: 5'-CCGCCTTGACCATAAGTTTGA-3' and 5'-TTCTTCCATACCCTCTCCAACATAC-3', for human TUBA8 cDNA were: 5'-CATACGGAAGCTGACAGATGCT-3' and CAGCAGAGAAGTGAAGCCG-3', and for human TUBA1A cDNA were: 5'-GCCCGAGGGCACTACACCAT-3' and 5'-CAGTCCCCCGCCAAAGCT-3'. The TaqMan rodent GAPDH or human B-actin (Applied Biosystems Inc. CA, USA) was used as the reference housekeeping gene, and the expression level of each mRNA was normalized to that of mouse GAPDH or human B-actin mRNA.

## 2.3. cDNA cloning and construction of expression plasmids

A full-length cDNA of mouse TUBA8 (mTUBA8) was amplified using a set of primers, 5'-ACCATGAGGGAATGCATATCG-3' and 5'-TTAAAATTCCTCCCCCTCATTC-3', and was subsequently cloned into pcDNA3.1-His-V5-TOPO plasmid (Invitrogen, CA, USA). Sense-mTUBA8 and anti-sense-mTUBA8 expression plasmids were selected after sequencing of their insert DNA. The expression plasmid of pEYFP-mTUBA8 fusion protein was generated using mTUBA8 cDNA, which was cloned into pcDNA3.1-His-V5-TOPO plasmid. The mTUBA8 cDNA was digested by using EcoRI and BamHI, and then cloned into the pEYFP-C1 vector (Clontech, CA, USA). The pECFP-hTUBA1 plasmid was purchased from Clontech (CA, USA).

## 2.4. Cell culture

HepG2 and Huh7 cells were cultured in 10% MEM (minimal essential medium supplement with 10% (v/v) fetal bovine serum, antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) and 2mM glutamine) in a 5% CO<sub>2</sub> atmosphere at 37°C.

## 2.5. Colony formation assay

Huh7 cells were seeded in 10-cm culture dishes and 24 h later expression vector (sense- or anti-sense-mTUBA8 expression plasmid) was transfected using FuGENE 6 transfection reagent (Roche Diagnostics, IN, USA). 24 h after transfection, the harvested cells were diluted and reseeded on 10-cm culture dishes in triplicate. The transfected cells were grown in the presence of 0.8 mg/ml Geneticin (Invitrogen, CA, USA) for 2 weeks, after which the colonies formed from each cell were fixed with 10% formaldehyde, stained with 0.125% crystal violet and counted.

## 2.6. Selection of cell strains with stable expression of mTUBA8

HepG2 or Huh7 cells were pre-seeded on 10-cm culture dishes at a density of  $1.0 \times 10^6$  cells/dish. 5 µg of either sense-mTUBA8, anti-sense-mTUBA8 or empty expression plasmid was transfected into the cells using FuGENE 6 transfection reagent (Roche Diagnostics, IN, USA); 24 h after transfection, cells were diluted and reseeded on 10-cm culture dishes in triplicate. Cells were grown in the presence of 0.8 mg/ml Geneticin (Invitrogen, CA, USA) for 2~3 weeks for stable cell selection. The expression of mTUBA8 mRNA or the presence of integrated plasmid DNA in each stably-transfected cells was confirmed by RT-PCR. Total RNA was extracted from culture cells using TRIzol reagent, and total genomic DNA

was extracted from culture cells using DNeasy Blood & Tissue kit (QIAGEN Inc., CA, USA) as described in the manufacturer's protocol. Integrated sense- or anti-sense mTUBA8 plasmid DNA was amplified using these primers, T7 primer: 5'-TAATACGACTCACTATAGGG-3', BGH reverse primer: 5'-TAGAAGGCACAGTCGAGG-3' and R1 primer: 5'-ACTCTGTGAGGTCCACATTGA-3'.

### 2.7. MTT assay

Cell proliferation was determined by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Stably transfected Huh7 or HepG2 cells were seeded on 96 well plates at densities of  $2.0 \times 10^3$  cells/well (Huh7 stable cells) or  $4.0 \times 10^3$  cells/well (HepG2 stable cells) in 10% MEM medium. At selected time intervals, 10  $\mu$ l of MTT working solution (5 mg/ml in ddH<sub>2</sub>O) was added and incubated at 37°C for 4 h. At the end of the incubation, the media was discarded and 100  $\mu$ l of DMSO was added to each well for dissolving the dye. After incubation at room temperature for 1 h with gently shaking, the absorbance of the converted dye was measured using a microplate spectrophotometer (SpectraMax Plus 384; Molecular Device Inc., CA, USA) at 495 nm.

### 2.8. Analysis of cell cycle by flow cytometry

Stably transfected Huh7 cells were seeded on 6 cm dishes at a density of  $5.0 \times 10^5$  cells/dish in 10% MEM medium. After 48 h, the cultured cells were trypsinized and harvested in PBS followed by two washes with PBS. Then the cells were resuspended in 70% ethanol overnight at 4°C. The fixed cells were centrifuged, resuspended in RNaseA solution (0.2 mg/ml RNaseA in PBS) and incubated at 37°C for 30 min. The cells were pelleted, incubated in Propidium Iodide (PI) staining solution (20  $\mu$ g/ml PI in PBS) for overnight at 4°C. The cells were analyzed by flow cytometer (BD FACSCalibur), and Cell Quest software (BD Biosciences) was used for data acquisition and analysis.

### 2.9. Recombinant adenoviruses

Based on the mTUBA8 expression plasmid, an Ad-mTUBA8 expression adenovirus vector was constructed with the AdEasy Vector System kit (Q-BIO gene, Montreal Canada) as described in the manufacturer's protocol. Adenoviruses were propagated in HEK293 cells. Ad- $\beta$ -gal encoding  $\beta$ -galactosidase previously constructed in our laboratory was used as control. Virus titers were measured and calculated using the 50% tissue culture infectious dose assay (TCID<sub>50</sub> method) as described in the manufacturer's application manual.

### 2.10. Cell migration assay

Transwell Chambers (8- $\mu$ m pore size; Corning, MA, USA) were used for the *in vitro* cell migration assay. Stably transfected HepG2 cells were seeded into the upper part of chamber at a density of  $5.0 \times 10^4$  cells in 300  $\mu$ l of 10% MEM medium, and the lower compartment was filled with 600  $\mu$ l of 10% MEM medium. In parallel experiments, HepG2 cells were infected with Ad- $\beta$ -gal or Ad-mTUBA8 at 10 MOI (Multiplicity Of Infection) 24 h post-seeding, and cells were seeded into the chamber with the same method described above. After incubation for 72 h, cells were fixed with 10% formaldehyde. Non-migrating cells were gently removed from the upper chamber with cotton, and cells on the underside of the membrane were stained with 0.1% crystal violet. Migrating cells were counted in four microscopic fields at  $\times 100$  magnification.

### 2.11. Confocal microscopy

C3He male mice were euthanized at 8 h after tail-vein injection of pEYFP-mTUBA8 plasmid or pECFP-hTUBA1 plasmid using TransIT *In Vivo* Gene Delivery System (Mirus, WI, USA) with described protocol. The liver was then removed, embedded in Tissue-Tek

OCT embedding compound (SAKURA Finetechnical Co., Ltd., Tokyo, Japan) and frozen on dry ice. Frozen liver sections of 30 $\mu$ m thickness were made using a cryostat by a routine procedure.

HepG2 or Huh7 cells were seeded on a 2-well chamber slide (Nalge Nunc International Corp., IL, USA) at a density of  $1.2 \times 10^5$  cells or  $0.8 \times 10^5$  cells per well in 10% MEM medium. The pEGFP-mTUBA8 plasmid was transfected into cells on the chamber slide using FuGENE 6 transfection reagent. After 48 hrs of transfection, cells were fixed with 100% cold methanol and permeabilized with 0.1% Triton-X100. The slides of liver sections or transfected cells were embedded with Hoechst 33258 solution (0.5  $\mu$ g/ml in 80% glycerol), followed by confocal microscopic observation.

## 2.12. Statistical analysis

The statistical significance between two groups of data sets was determined by using the Student's *t* test (two-tailed) with  $P < 0.05$  regarded as statistically significant.

## Results

### 3.1. CAR-regulated induction of the mTUBA8 gene by PB in mouse liver

Our previous study demonstrated that CAR is an important factor in promoting the development of liver tumors following chronic PB treatment [15]. *Car*<sup>+/+</sup> C3He mice treated with DEN followed by PB treatment for 23–32 weeks developed lesions and tumors, while DEN/32 weeks PB-treated *Car*<sup>-/-</sup> C3He mice didn't show any liver tumors. To screen for genes that are induced during the development of mouse liver tumors in a CAR dependent manner, cDNA microarray analysis was first performed (unpublished data) and a number of candidate genes were detected. We focused on the *mTUBA8* gene for further investigation, because there have been no previous reports on mTUBA8 in liver. We also found other tubulin family genes in the microarray analysis data, but most of them did not show any change in mRNA expression in PB treated liver. A slight induction of mTUBB2 mRNA was observed in tumor samples, although not as high compared with mTUBA8 (Table 1).

The mRNA for mTUBA8 was already induced after 24 h of PB treatment in the livers of *Car*<sup>+/+</sup> but not in *Car*<sup>-/-</sup> C3He mice, and continued to be induced in the DEN/32 weeks PB-treated *Car*<sup>+/+</sup> C3He mice (Fig. 1A). Interestingly, the induction level of mTUBA8 mRNA was clearly higher in liver tumor tissues with more than 100-fold induction compared to control samples (Fig. 1A). These results indicated that the mTUBA8 gene undergoes CAR-mediated induction in the liver prior to tumor development and continues to be induced in tumor tissues.

### 3.2. Strain-dependent expressions

The C3He mouse is known to be more susceptible to developing liver tumors than the C57BL6 mouse [20, 21]. To examine the relationship between the induction level of mTUBA8 mRNA and liver tumor susceptibility, male mice were treated with PB for 6 weeks and liver RNA was prepared for real-time PCR to measure mTUBA8 mRNA. Compared with an over 6-fold increase of mTUBA8 mRNA in the PB-treated C3He males, the corresponding PB-treated C57BL6 mice showed a lower but still significant increase (2-fold) of mTUBA8 mRNA (Fig. 1B). This difference in induction rate between the two mouse strains suggests that mTUBA8 may correlate with the susceptibility of developing liver tumors.

### 3.3. TUBA8 Expression in human hepatocellular carcinoma cell lines

For further *in vitro* experiments using human hepatocellular carcinoma cell lines HepG2 or Huh7, we examined the basal expression level of human TUBA8 mRNA by real-time PCR. In addition to TUBA8, TUBA1A mRNA was also expressed in these cells (Fig. 2). Therefore, these cell lines can be useful for ectopic over-expression experiments to examine the function of mTUBA8.

### 3.4. Suppression of colony formation ability by over-expression of mTUBA8

In order to explore the function of mTUBA8 in cell growth, we carried out colony formation assays after transfecting the sense-mTUBA8 and anti-sense-mTUBA8 plasmid vectors into Huh7 cells. There were  $109.3 \pm 4.5$  (mean  $\pm$  SD) colonies formed with the anti-sense-mTUBA8 plasmid transfected Huh7 cells and  $46.0 \pm 11.4$  colonies formed with the sense-mTUBA8 expression plasmid transfected Huh7 cells (Fig. 3). This indicates that mTUBA8 over-expression in Huh7 cells inhibits cell growth.

### 3.5. Expression of mTUBA8 mRNA in transfected stable cells

We next established Huh7 and HepG2 cells that were stably expressing mTUBA8. We transfected empty, anti-sense-mTUBA8, and sense-mTUBA8 expression plasmids into Huh7 or HepG2 cells, and after G418 selection culture the expression of mTUBA8 mRNA was examined in three independent experiments by RT-PCR. A strong expression of mTUBA8 mRNA was detected in the sense-mTUBA8 plasmid stably transfected Huh7 cells, but not in empty-plasmid or anti-sense-mTUBA8 plasmid stably transfected Huh7 cells (Fig. 4A). Similar results were observed in stably transfected HepG2 cells, and a weak signal was detected in anti-sense-mTUBA8 plasmid transfected HepG2 cells. To confirm that the sense-mTUBA8 or anti-sense-mTUBA8 expression plasmids were integrated into genomic DNA of the stably transfected HepG2 cells, genomic DNA was isolated and PCR was carried out. As a result, we confirmed that the slight expression of mTUBA8 was derived from the anti-sense mTUBA8 expression plasmid (Fig. 4B). Finally, we compared the sense-mTUBA8 plasmid transfected Huh7 and HepG2 cells to empty or anti-sense-mTUBA8 plasmid transfectants selected at random as experimental controls. We could not see any morphological changes in stably transfected Huh7 or HepG2 cells (data not shown).

### 3.6. Influence of mTUBA8 gene on the growth of Huh7 cells

In order to confirm the results of the colony formation assay which suggested that mTUBA8 plays a role in growth of Huh7 cells, the proliferation rate of transfected Huh7 cells was determined by MTT assay. A slight inhibition of cell proliferation was observed in sense-mTUBA8 transfected Huh7 cells after 72 hrs of culture compared with empty or anti-sense-mTUBA8 transfected Huh7 cells, and this became statistically significant after 96 hrs ( $P < 0.01$ ; Fig. 5A). We also examined the cell cycle distribution of mTUBA8-transfected cells by flow cytometry after 48 hrs culture. Although not statistically significant, an increase in the percentage of the cell population at the G0/G1 phase and decrease in the percentage of the cell population at the S phase and the G2/M phase was observed (Fig. 5B). These results suggest that mTUBA8 over-expression in Huh7 cells leads to an inhibition of cell growth.

### 3.7. Enhancement of cell migration in HepG2 cells by mTUBA8 over-expression

We also examined the effect of mTUBA8 over-expression in HepG2 cells. Stably transfected HepG2 cells were first examined for their rate of cell proliferation by MTT assay; however there was no important difference (Fig. 6A). This result suggested that mTUBA8 over-expression did not affect the proliferation of HepG2 cells. We next examined the effects of mTUBA8 expression on cell migration, since individual cell

migration is an important characteristic of invasive tumor cells. Interestingly, sense-mTUBA8 transfected HepG2 cells had enhanced migration ability compared with other transfected cells ( $P < 0.01$ ; Fig. 6B), with the migration rates of the empty, anti-sense-mTUBA8, and sense-mTUBA8 transfected HepG2 cells at  $181.3 \pm 26.8$  (mean  $\pm$  SD),  $180.3 \pm 43.7$ , and  $434.3 \pm 39.0$  cells, respectively (Fig. 6C).

A similar induction of cell migration by ectopic mTUBA8 over-expression was observed in adenovirus-based infection experiments. The migration rates of Ad- $\beta$ -gal and Ad-mTUBA8 infected HepG2 cells were statistically significant ( $P < 0.01$ ) at  $117.5 \pm 28.6$  (mean  $\pm$  SD) and  $307.3 \pm 68.6$  respectively (Fig. 6D). This indicates that expression of mTUBA8 induced cell migration of HepG2 cells. We also examined cell migration rates in adenovirus based mTUBA8 over-expressed Huh7 cells, but there was no difference between Ad-mTUBA8 and Ad- $\beta$ -gal infected (Supplemental Fig. 1). In addition to Huh7 and HepG2 cells, the neuroblastoma U373MG cells were utilized in the similar experiments, from which any changes in cell migration and cell proliferation were observed (data not shown).

### 3.8. Localization of mTUBA8 protein in hepatocytes and culture cells

We constructed a pEYFP-mTUBA8 expression plasmid to examine the localization of the fusion protein in liver cells since there is no commercially available mTUBA8 specific antibody. We co-injected the pEYFP-mTUBA8 and pECFP-hTUBA1 expression plasmids via the tail vein, and examined the localization of the fusion proteins in mouse liver. Most of the expressed pEYFP-mTUBA8 fusion protein was observed in the cytoplasm with several expression patterns described as fiber, smear or dotted (Fig. 7A). Moreover, the pEYFP-mTUBA8 and pECFP-hTUBA1 fusion proteins were co-localized in mouse liver (Fig. 7A). We also examined the localization of pEYFP-mTUBA8 fusion protein in Huh7 and HepG2 cells. Expressed pEYFP-mTUBA8 fusion protein were mainly observed in cytoplasm with a fiber-like structure (Fig. 7B), indicating that mTUBA8 may contribute to the construction or the maintenance of the cytoskeleton.

## Discussion

Here, we demonstrate that mTUBA8 mRNA is induced in mouse liver by PB treatment in a CAR dependent manner. This gene, which is barely expressed in normal mouse livers, showed the highest expression in mouse liver tumors, suggesting that mTUBA8 has an important role in PB promoted liver tumorigenesis. CAR is a known transcriptional factor that regulates target genes, but we were unable to find candidate binding site(s) for CAR on the mTUBA8 promoter. Therefore CAR may not regulate mTUBA8 directly and how CAR regulates mTUBA8 mRNA expression in liver is an important theme of our future work.

In mammals, six  $\alpha$ -tubulin genes have been cloned and sequenced, and human and mouse TUBA8 was identified and characterized as the 7<sup>th</sup> member of  $\alpha$ -tubulin family [16]. Although higher TUBA8 expression was demonstrated in human heart, skeletal muscle and testis compared to other tissues including liver, its function remains unknown [16]. In general,  $\alpha$ -tubulin undergoes posttranslational modifications in microtubules including: 1) acetylation of 40<sup>th</sup> lysine, 2) detyrosination or removal of the C-terminal tyrosine residue exposing glutamic acid as the new C-terminal residue, 3) phosphorylation, 4) polyglutamylolation and 5) polyglycylation [18, 19, 22]. Acetylation and detyrosination of  $\alpha$ -tubulins have been especially examined in brain tissue or neuronal cells. For example, acetylation of  $\alpha$ -tubulins at the 40<sup>th</sup> lysine by the elongator complex is an important step to control the migration and differentiation of cortical neurons in the intermediate zone [23]. Detyrosination of C-terminal residues or polyglutaminylation of  $\alpha$ -tubulins around the C-terminal region seem to be important for the stabilization of microtubules, because C-terminal residues are involved in the interaction with many proteins such as microtubule

associated proteins (MAPs) and motor proteins [19, 24]. However TUBA8 seems to be an atypical  $\alpha$ -tubulin because TUBA8 doesn't have the 40<sup>th</sup> lysine or the tyrosine residue located at the C-terminal. This suggests that TUBA8 may not be regulated by posttranslational modification of acetylation and of detyrosination, and allows for TUBA8 to regulate MAPs interactions and microtubule dynamics in a different manner from other  $\alpha$ -tubulin isoforms.

As a potential function of mTUBA8, we demonstrated that ectopic over-expression of mTUBA8 decreased the rate of cell proliferation of Huh7 cells and accelerated cell migration in HepG2 cells. We hypothesized that these characteristic functions may be due to the lack of acetylation and/or detyrosination sites, so we constructed mutants of mTUBA8 that have a 40<sup>th</sup> lysine instead of the 40<sup>th</sup> alanine (A40K mutant), a 449<sup>th</sup> tyrosine instead of a 449<sup>th</sup> phenylalanine (F449Y mutant) or both double mutations (A40K/F449Y mutant). We performed colony formation assays in Huh7 cells and cell migration assays in HepG2 cells using these materials; however we could not detect any important difference between the mutants and wild type of mTUBA8 (data not shown). As a possible explanation for these results, the regulation system of acetylation and/or detyrosination of alpha-tubulin in these cell lines may not work well; we could not detect the acetylated or detyrosinated (Glu-terminaled) alpha-tubulin in parental Huh7 and parental HepG2 by western blotting analysis using specific antibodies (data not shown). Although we could not examine the effect of acetylation / detyrosination on cell growth or cell migration using these experimental conditions, our findings support the role of mTUBA8 in the inhibition of cell growth in Huh7 cells and the induction of cell migration in HepG2 cells.

How does mTUBA8 inhibit cell growth or accelerate migration of cells? As for the inhibition of cell growth, we demonstrated a slight increase in the cell population at the G0/G1 phase and decrease in the cell population at the S phase in mTUBA8 stably transfected Huh7 cells (Fig. 5B). This result may suggest that ectopic mTUBA8 over-expression affects the process of DNA replication rather than the process of mitosis or cytokinesis. In addition, a relationship between the dynamics of microtubules (MTs) and cell migration has been suggested from several reports. For example, destruction of MTs by the treatment of colcemid inhibited cell migration via inhibition of lamellipodial protrusion on the active edge of migrating fibroblast cells [25]. Moreover, recent studies of Adenomatous Polyposis Coli (APC) or p120-catenin showed that MT dynamics is important for cell migration [26, 27]. Interestingly, over-expression of MAP2, one of the MAP families, enhanced cell motility in oral cancer cells [28], suggesting that MTs and their interaction with  $\alpha/\beta$ -tubulin are involved in the regulation of cell migration. Over-expressed mTUBA8 may interact with the MTs instead of endogenous  $\alpha$ -tubulin and enhance cell migration in liver cells. In any case, further study will be required to solve these questions.

Recently, a relationship between a splicing mutation of TUBA8 and the disease of polymicrogyria with optic nerve hypoplasia has reported [29], but this is the only report described the potential physiological function of TUBA8. The detailed function of TUBA8 or the connection to other diseases including cancer remains unknown. We found that mTUBA8 was over-expressed during the development of liver tumors using cDNA microarray analysis. Additionally, we also detected a slight induction of mTUBB2 in mouse liver tumor (Table 1). Interestingly, human TUBB2 was found to be over-expressed in various normal tissues particularly in fetal and neonatal tissues or neoplastic tissues [30] and in a variety of cancers [31], suggesting that TUBA8 may be over-expressed in tumors as well as TUBB2. In preliminary observations, we examined the expression of TUBA8 mRNA in human hepatocellular carcinoma tissues by real-time PCR, and 8 cases out of a total 31 cases showed a higher expression of TUBA8 in tumor tissues compared to normal tissues derived from same the liver (unpublished data). Although the levels were not



associated with the sex, age, etiology, multiplicity or stage of tumors, these findings suggest that TUBA8 may be linked to the development or promotion of human liver cancer.

In conclusion, we have demonstrated that mTUBA8 is up-regulated during mouse liver tumorigenesis in a CAR dependent manner. This is the first report that mTUBA8 may be involved in mouse liver tumorigenesis.

The biological process and detailed mechanism by which TUBA8 is involved in liver tumor development remains to be determined; for example, how the repression of cell growth by TUBA8 play a role in the liver during HCC development, while TCPOBOP activation of CAR increases proliferation of normal hepatocytes. Since both cell growth and death are simultaneously involved in tumor development, our findings suggest CAR-regulated repression of cell growth by TUBA8 may also be critical for tumor development and that the cell migration may be a factor for an intra-hepatic metastasis and a poor prognosis of liver cancer patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

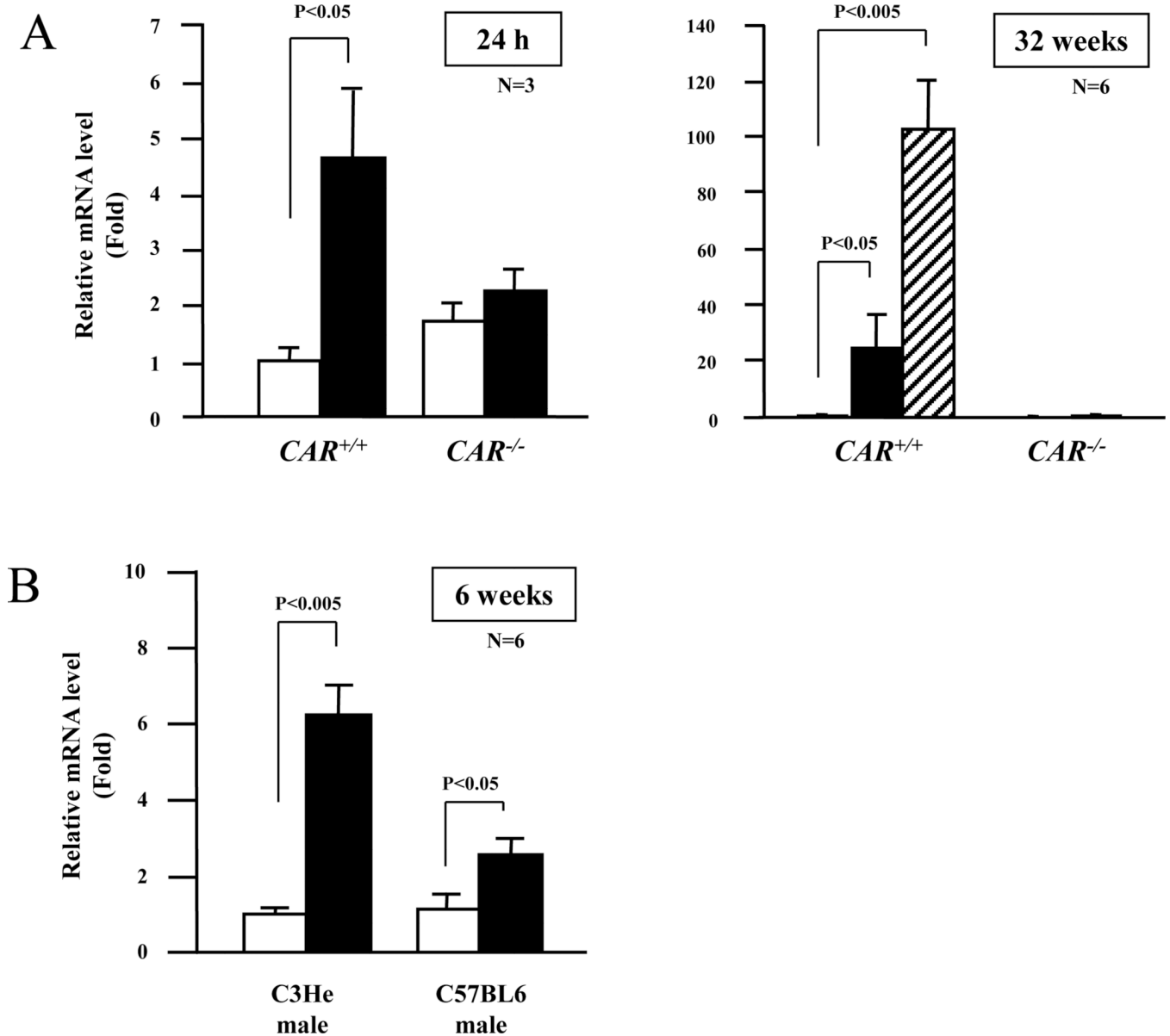
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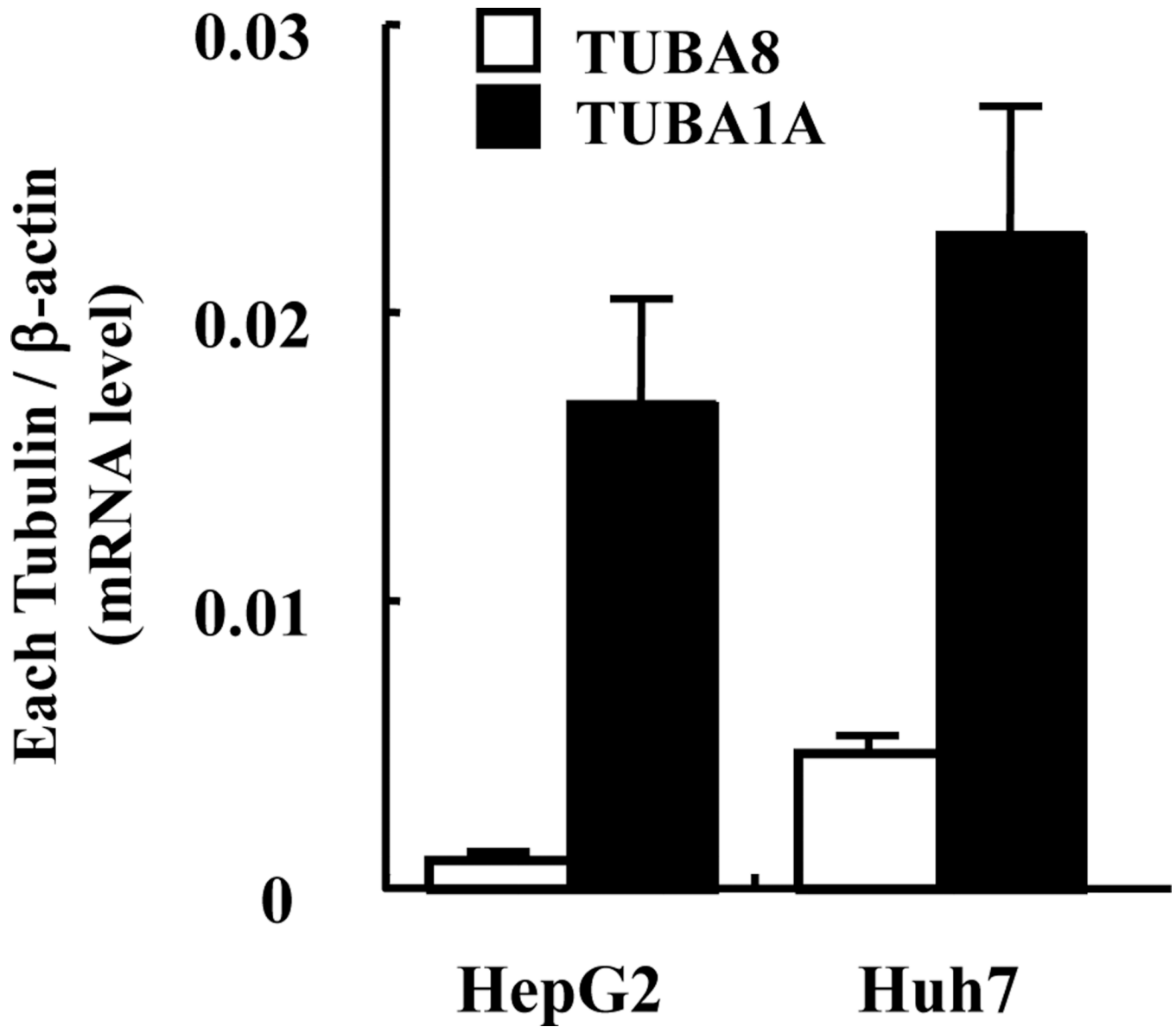
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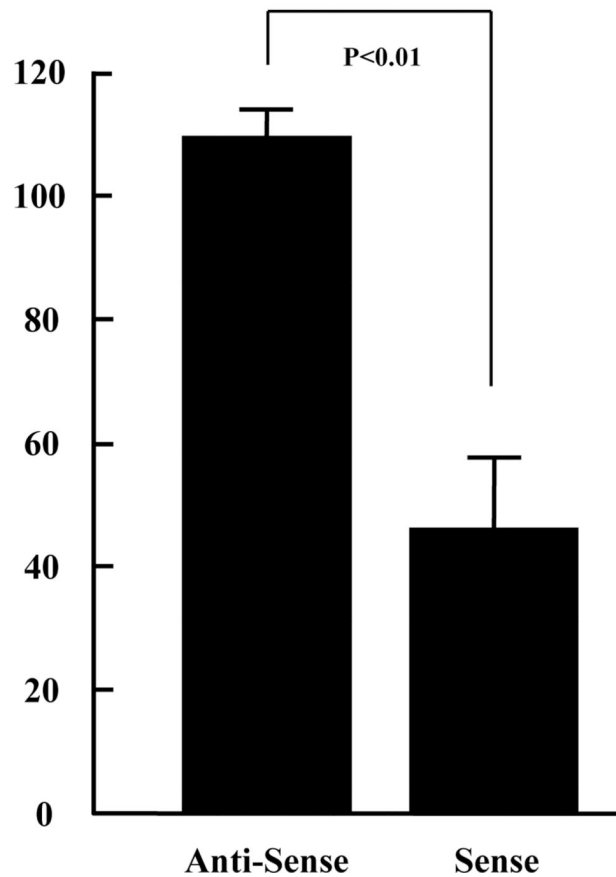
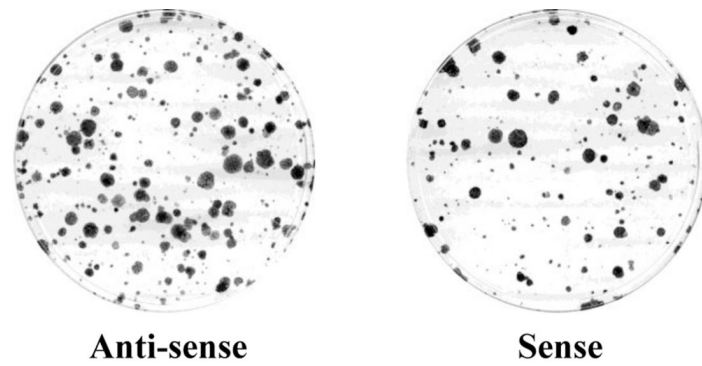
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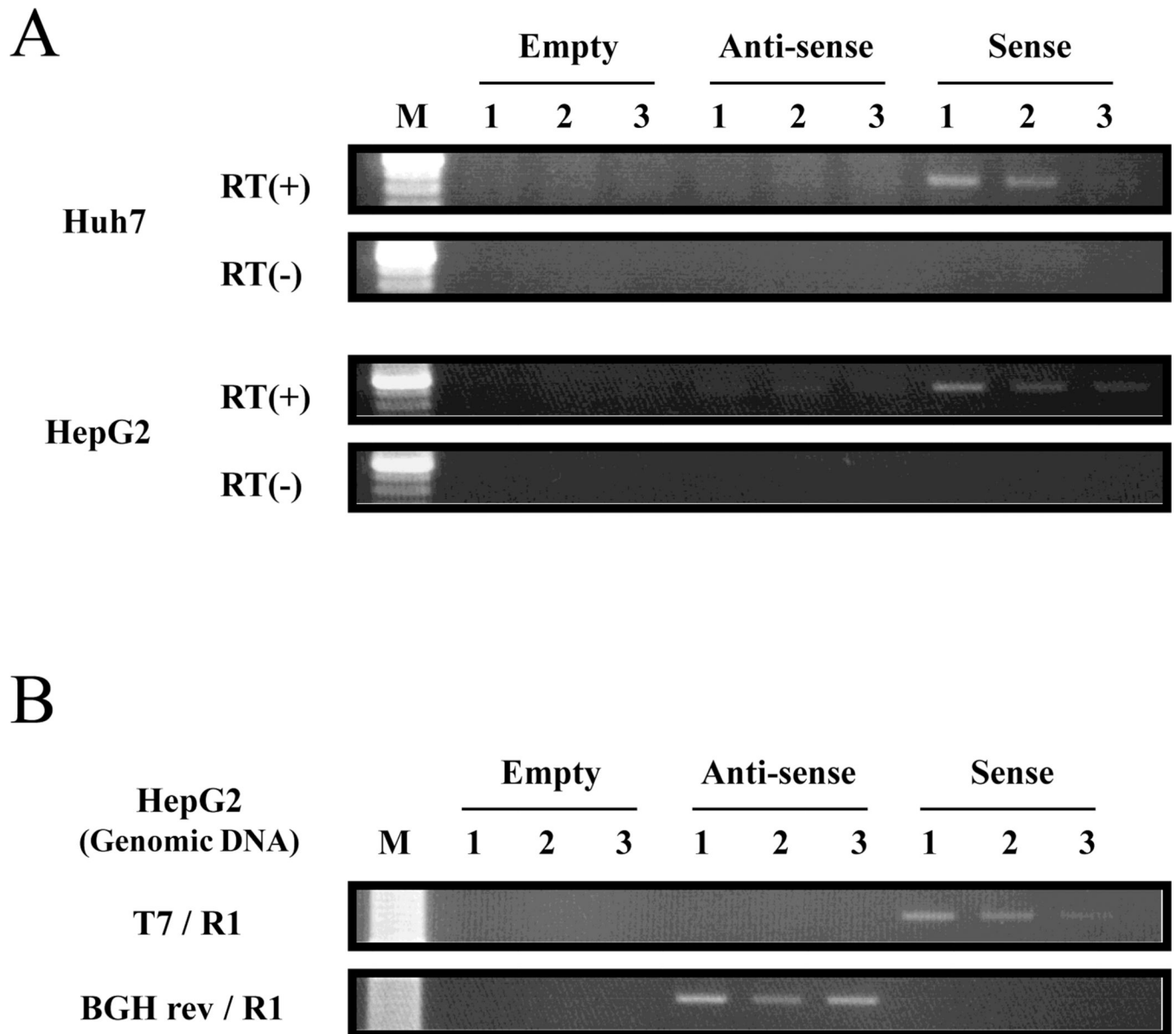
**Fig. 1.** Induction of mTUBA8 mRNA in the livers of PB treated mice. (A) The 24 h or 32 weeks indicate the period that the *Car*<sup>+/+</sup> and *Car*<sup>-/-</sup> C3He mice were treated with PB. Open and closed bars show the levels of mTUBA8 mRNA in the non-tumor tissues from the non-PB treated and PB treated mice, respectively. The hatched bar shows the level of mTUBA8 mRNA in tumor tissues. The relative levels of mTUBA8 mRNA were calculated by setting the levels in the livers of non-PB treated *Car*<sup>+/+</sup> C3He mice as one. The data are reported as mean  $\pm$  SD, n=3 (24 h) or n=6 (32 weeks). (B) The 6 weeks indicate the period that the C3He or C57BL6 male mice were treated by PB. Open and closed bars show the levels of mTUBA8 mRNA in the liver tissues from the non-PB treated and PB treated mice, respectively. The relative levels of mTUBA8 mRNA were calculated by setting the levels in the livers of PBS-treated C3He male mice as one. The data are reported as mean  $\pm$  SD, n=3.



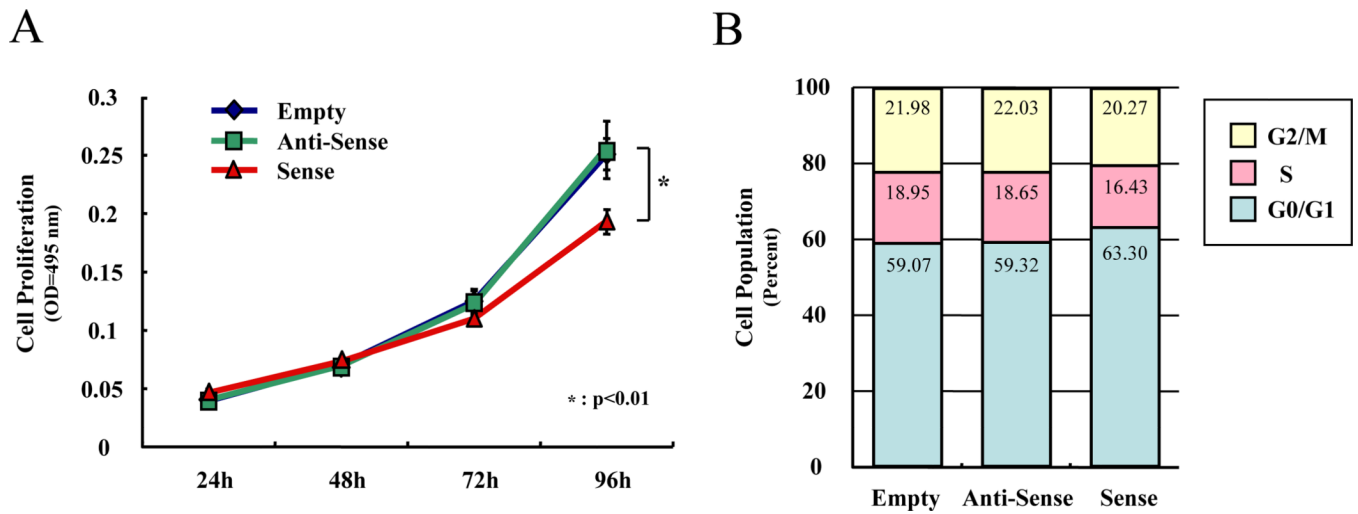
**Fig. 2.** Expression of TUBA8 in human hepatocellular carcinoma cells. Basal expression levels of human TUBA8 and TUBA1A mRNA in Huh7 or HepG2 cells were evaluated by real-time PCR, and normalized to that of human  $\beta$ -actin mRNA. Open and closed bars show the levels of human TUBA8 and TUBA1A mRNA in the cells, respectively. The data are reported as mean  $\pm$  SD, n=3.



**Fig. 3.** Inhibition of Huh7 colony formation by ectopic expression of mTUBA8. Huh7 cells were transfected with anti-sense-mTUBA8 or sense-mTUBA8 expression plasmid, and grown on triplicate culture dishes in the presence of 0.8 mg/ml Geneticin for 2 weeks. Geneticin-resistant colonies were fixed, stained and counted. The data are reported as mean  $\pm$  SD of three independent experiments.



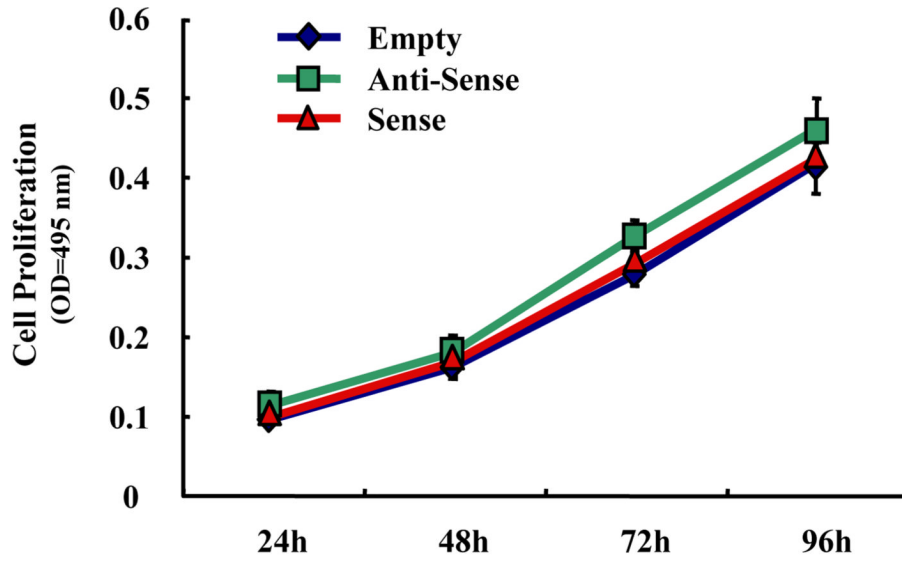
**Fig. 4.** Confirmation of exogenous mTUBA8 expression in transfected Huh7 and HepG2 cells. (A) Empty-, Anti-sense, or Sense-mTUBA8 expression plasmid was transfected into Huh7 and HepG2 cells, followed by culture in G418 containing medium to establish stable transfectant cells. Expression of mTUBA8 in transfected cells was examined by RT-PCR. RT denotes Reverse Transcriptase, and M denotes size marker. (B) Genomic DNA was isolated from each set of transfected HepG2 cells, and the integration of plasmid DNA was examined by PCR. The T7/R1 primer set detects the sense-mTUBA8 plasmid, and the BGH rev/R1 primer set detects the anti-sense-mTUBA8 plasmid in transfected HepG2 cells.



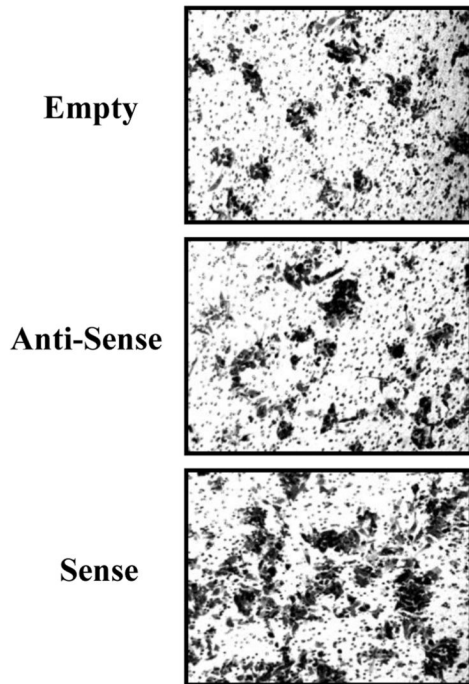
**Fig. 5.**

Cell proliferation and cell cycle analysis in transfected Huh7 cells. (A) MTT assay was carried out 24, 48, 72 and 96 h after cell seeding. The results are reported as mean  $\pm$  SD of six independent experiments. (B) Each set of stably transfected Huh7 cells was collected after 48 h of cell seeding, and the DNA content was analyzed by flow cytometry to determine the cell cycle distribution. Experiments were repeated three times independently and the average percentages are indicated in the bar graph.

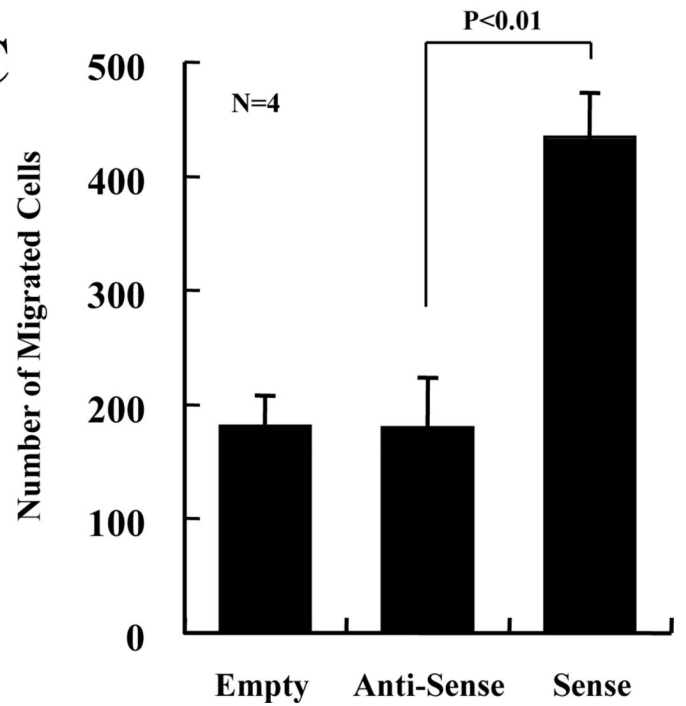
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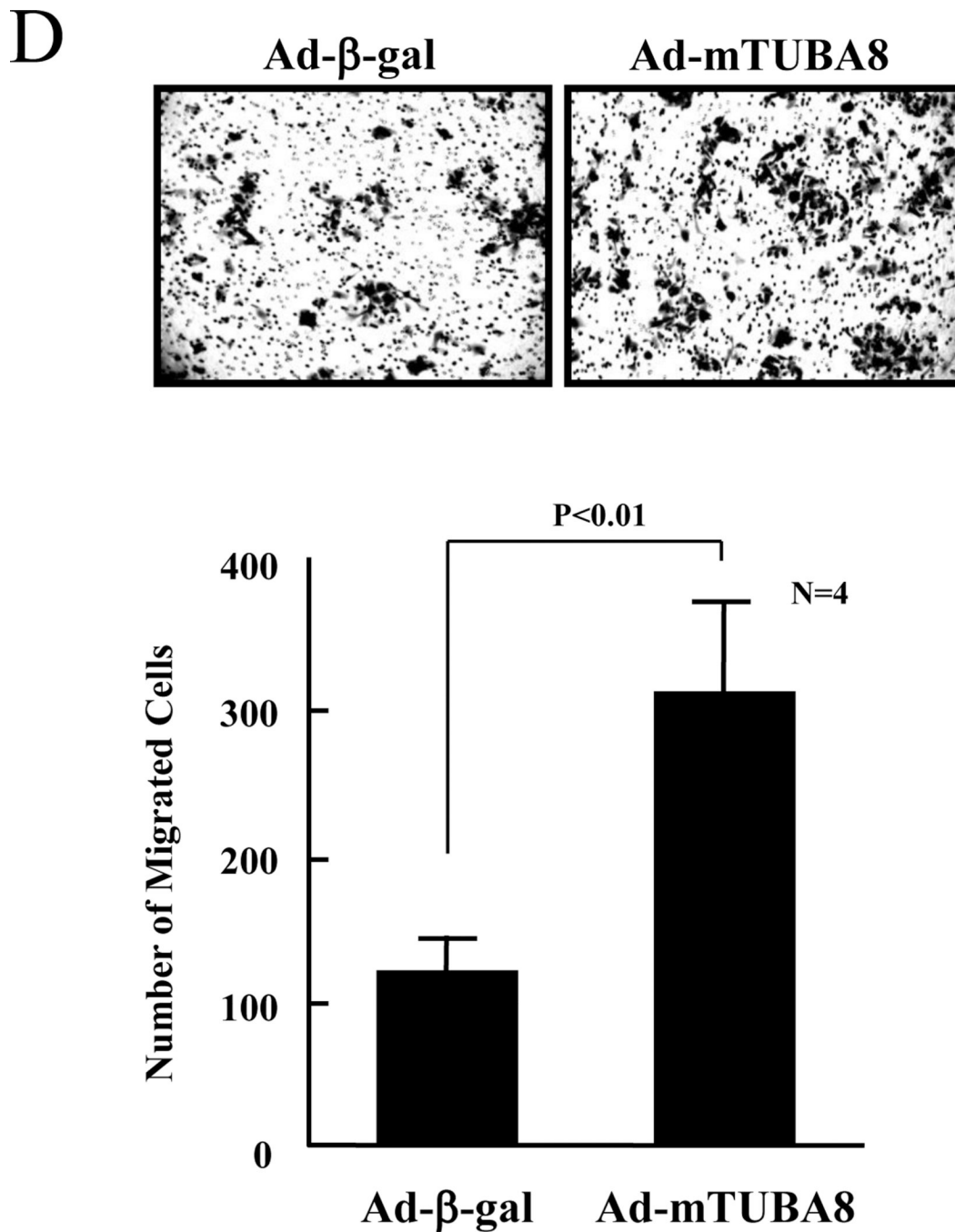
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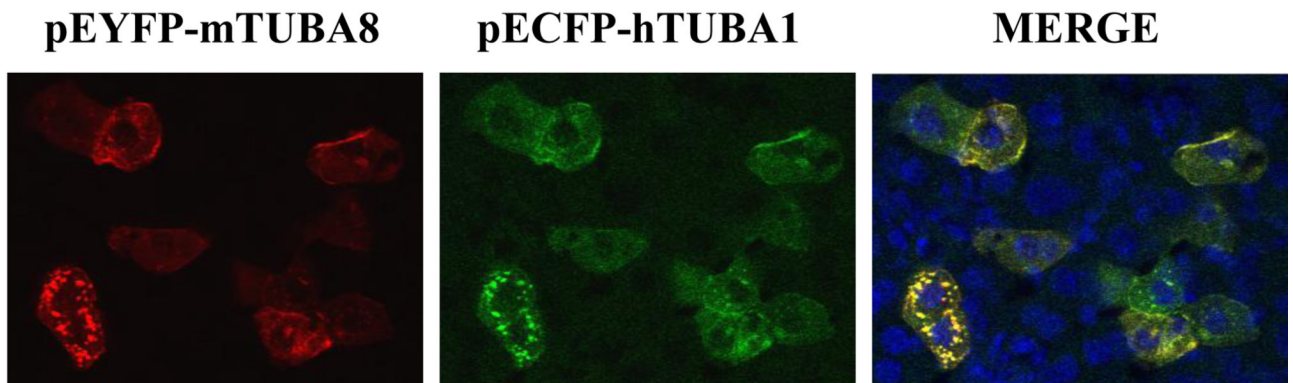
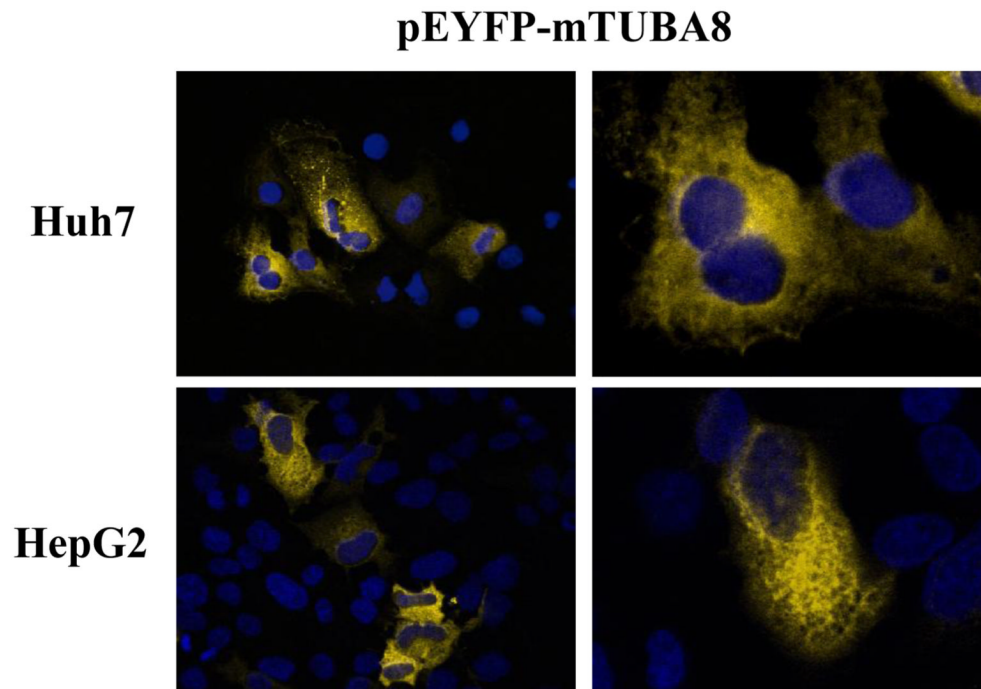






**Fig. 6.** Induction of cell migration ability in transfected HepG2 cells. (A) MTT assay was carried out 24, 48, 72 and 96 h after cell seeding. The results are reported as mean  $\pm$  SD of six independent experiments. (B) Transfected cells were seeded on transwell chambers. After 72 h incubation, the migrated cells were fixed, stained with 0.1% crystal violet and photographed. (C) The number of migrated cells was counted in four fields for each group. The results are reported as mean  $\pm$  SD of three independent experiments. (D) Parental HepG2 cells were infected with Ad- $\beta$ -gal or Ad-mTUBA8 for 24 h at 10 MOI and then seeded on transwell chambers. After 72 h incubation, the migrated cells were fixed, stained and photographed (upper panel). The number of migrated cells was counted in four fields for

each group (lower panel). The results are reported as mean  $\pm$  SD of three independent experiments.

**A****B**

**Fig. 7.** Subcellular localization of mTUBA8 in mouse liver and cultured cell lines. (A) pEYFP-mTUBA8 and pECFP-hTUBA1 expression plasmids were injected into mice via tail vein, and liver sections were prepared for examination using confocal microscopy. pEYFP-mTUBA8 expression is shown as a red signal, and pECFP-hTUBA1 expression is shown as a green signal. (B) pEYFP-mTUBA8 expression plasmids were transfected into Huh7 cells and HepG2 cells, and its expression was analyzed by confocal microscopy. pEYFP-mTUBA8 expression is shown as a yellow signal.

**Table 1**

The list of fold change about tubulin family genes that were found out by microarray analysis

Gene Name	Gene Description	CAR-KO (PB-) 32W	CAR-KO (PB+) 32W	CAR-KO (PB+) 32W
		V.S. CAR-KO (PB+) 32W	V.S. CAR-WT (PB+) 32W	V.S. CAR-WT Tumor 32W
		Fold Change	Fold Change	Fold Change
TUBB3	Tubulin, beta 3	-1.01	1.04	1.09
TUBG2	Tubulin, gamma2	1.2	-1.08	-1.05
TUBA4	Tubulin, alpha 3	1.43	1.13	1.41
TUBB4	Tubulin, beta 4	1.26	1.13	1.42
TUBA3	Tubulin, alpha 3	-1.07	1.37	1.44
TUBA1	Tubulin, alpha 1	1.11	1.14	1.23
TUBD1	Tubulin, delta 1	-1.01	1.67	-1.05
TUBB5	Tubulin, beta 5	1.26	1.24	2.05
TUBB2	Tubulin, beta 2	1.02	2.04	4.44
TUBA8	Tubulin, alpha 8	-1.01	8.01	12.36

\* Microarray data are deposited to GEO: GSE29108.