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Hypoxia-independent overexpression of HIF-1α as an early change in mouse hepatocarcinogenesis

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Running head: HIF-1α expression in preneoplastic hepatic lesions

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Abstract

HIF-1 is involved in tumor progression/metastasis and activated in various cancers. Here we show that HIF-1α, which plays a major role in HIF-1 activation, is over-expressed in preneoplastic hepatocytic lesions from a very early stage during hepatocarcinogenesis in mice and man. Transcriptional targets of HIF-1, such as VEGF, glut-1, c-met and Igf-2, were also over-expressed in mice lesions. HIF-1α expression was independent of hypoxia, because pimonidazole immunostaining was negative in the mouse lesions after treatment with pimonidazole. On the other hand, Akt, the pathway of which can up-regulate HIF-1α expression, was activated in the mouse lesions, while HIF-1α was markedly downregulated in the mouse HCC cell lines after treatment with a PI3 kinase (PI3K) inhibitor, LY294002, indicating that HIF-1α expression is dependent on PI3K/Akt signaling. Conversely, HIF-1α knockdown by siRNA in the HCC cell line resulted in decreased expression of activated Akt together with the HIF-1 target genes, indicating that Akt activation is reversely dependent on HIF-1 activation. Treating the HCC cells with Igf-2 upregulated both phospho-Akt and HIF-1α, while inhibition of Igf-2 by the neutralizing Igf-2 antibody downregulated them both, suggesting that Igf-2 may, at least in part, mediate the activation of Akt and HIF-1α. However, Akt was not activated by Igf-2 or EGF in the HIF-1α knockdown cells, indicating that expression of the HIF-1 target genes is necessary for the Akt activation. These findings suggest that the reciprocal activation of PI3K/Akt signaling and HIF-1α may be important in progression of hepatocarcinogenesis.

Introduction

Although hepatocellular carcinomas (HCCs) are one of the leading causes of cancer death in the world, the molecular mechanism of hepatocarcinogenesis, especially in the early stage, is still not sufficiently understood. During the early stage of hepatocarcinogenesis, focal lesions called hyperplastic or dysplastic hepatocytic foci emerge (1, 2). These lesions exhibit a number of altered gene expression and higher proliferating capacity compared to the surrounding normal hepatic tissue and grow into grossly visible lesions called hepatic adenomas or dysplastic nodules in the later stage. It is thought that, during the development of these lesions, the gradual accumulation of genetic changes may occur in the preneoplastic hepatocytes, leading to the final development of HCC cells. However, how the cellular signaling enabling the early preneoplastic hepatocytes to exhibit the growth advantage occurs, is not exactly known.

Hypoxia inducible factor-1 (HIF-1) is a transcription factor that enhances many types of gene expression including those involved in angiogenesis, cell proliferation, glucose metabolism, erythropoiesis and cell survival (3). HIF-1 is composed of α and β subunits, where the β subunit is constitutively expressed, while the α subunit is degraded under normoxic conditions despite the fact that it is continuously synthesized. Under normoxic conditions, HIF-1α undergoes hydroxylation at specific proline residues by HIF hydroxylase, is immediately ubiquitinized by binding with the von Hippel-Lindau (VHL) tumor suppressor protein and then degraded by proteasomes (4-7). Under hypoxic conditions, however, HIF-1α is hydroxylated less and imported into the nucleus where it binds with HIF-1β and other transcriptional factor and co-activators to transactivate the variety of genes containing the hypoxia response element (8).

Recent studies have further shown that HIF-1 can be activated independent of hypoxia by various mechanisms such as: oncogene activation (9, 10), inactivation of tumor suppressor genes (6, 11, 12) and activation of growth factor signaling (13-15). In particular, PI3 kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling are important for HIF-1α activation (13-15). The PI3K/Akt and MAPK signaling activates mammalian target of rapamycin (mTOR) and the protein-synthesizing machinery, which in turn upregulates HIF-1α expression, leading to HIF-1 activation. Because HIF-1 transactivates growth factor genes, such as *VEGF*, *Igf-2* and transforming growth factor α (*Tgf-α*), the products of which can activate PI3K/Akt and MAPK signaling, HIF-1 can also be activated by autocrine mechanisms (3).

Although HIF-1α has been demonstrated to be frequently overexpressed in HCCs as well as various other types of cancers (16, 17), it is still not clear whether it occurs in the early stages of hepatocarcinogenesis. To clarify the role of HIF-1 in hepatocarcinogenesis, we investigated

HIF-1α expression in chemically-induced preneoplastic hepatocytic lesions in mice and dysplastic hepatocytic foci in man. Because Igf-2 and TGF-α, transcriptional targets of HIF-1 that can activate PI3K/Akt signaling, have been reported to be frequently overexpressed in preneoplastic hepatocytic lesions and HCC tissues and cell lines (18-20), we also investigated the possibility that these autocrine factors are related to the activation of HIF-1α and PI3K/Akt signaling.

Materials and Methods

Tissue samples. To induce hepatocarcinogenesis in mice, male B6C3F1 mice (Clea Japan, Tokyo, Japan) were administered diethylnitrosamine (DEN) intraperitoneally at a dose of 5 μg/g body weight at the age of 2 weeks (21) and sacrificed 5-15 months after DEN-treatment. Hepatic tumors were removed from the surrounding normal liver tissue, frozen in liquid nitrogen and stored at -80°C until use. For some mice, the livers were perfusion-fixed with 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS), further immersed in the same solution for 12 hours at 4°C and processed for paraffin-embedding. All experimental procedures were approved by the institutional committee according to the guidelines for animal protection. Paraffin blocks of human liver biopsy samples containing dysplastic hepatocytic foci were also used after obtaining the informed consent of patients.

Tissue hypoxia analysis. Mice with hepatic lesions were administered pimonidazole hydrochloride (Chemicon, Temecula, CA) intraperitoneally dissolved in PBS at a dose of 60 μg/g body weight. After 2 hours, the livers were perfusion-fixed with the paraformaldehyde solution and embedded in paraffin, followed by immunohistocehmical detection of the pimonidazole protein adducts as detailed below.

Immunohistochemistry. Serial 3.5 μm-thick paraffin sections were sequentially treated prior to application with the primary antibodies in the following way: deparaffinization, rehydration, endogenous peroxidase quenching and antigen retrieval. Antigen retrieval involved incubation with citrate buffer (0.1 M citrate pH 6.0, 0.1% NP-40) in an autocrave for HIF-1α and pimonidazole, and treatment with proteinase K (Dako, Carpinteria, CA) for phospho-Akt. In order to detect pimonidazole, the sections were incubated with the fluoresceine isothiocyanate (FITC)-conjugated monoclonal antibody against pimonidazole (Chemicon) and then with horse radish peroxidase (HRP)-conjugated secondary antibody against FITC (1:50 dilution, Chemicon), followed by colorization with diaminobenzidine. The immuhistochemistry for HIF-1α and

activated Akt was carried out using the anti-HIF-1α monoclonal antibody (1:50, Sigma-Aldrich, St. Louis, MO) and the anti-phospho-Akt (phospho-Akt Ser473) polyclonal antibody (1:50, Cell Signaling, Beverley, MA), respectively, followed by detection of antibody binding using the Catalyzed Signal Amplification System II (Dako). For all these procedures, staining without the primary antibodies was performed as a negative control.

Cell culture. HCC cell lines, which had been established previously from primary mouse HCCs in our laboratory (22), were cultured in Williams' E medium supplemented with 10% fetal bovine serum (FBS), 10^{-7} mol/l EGF (Toyobo, Japan), 10^{-7} mol/l transferrin, 10^{-7} mol/l insulin, 10^{-7} mol/l dexamethasone, 10^{-5} mol/l aprotinin, 100 units/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. For Igf-2 or EGF stimulation, the HCC cells were exposed to starvation medium (Williams' E medium containing the antibiotics alone) for 6 hours and then incubated with Williams' E medium supplemented with 5% FBS and recombinant mouse Igf-2 (R&D Systems, Minneapolis, MN) or EGF for 24 hours. Anti-mouse Igf-2 goat polyclonal antibody (R&D Systems) and non-immune goat serum were added to the medium for Igf-2 neutralization and control, respectively.

Western blot analysis. The tissue and cell samples were lysed in the SDS sample buffer, separated with 10% SDS-acrylamide gel and electro-transferred to nitrocellulose membranes. After blocking the membranes with 5% nonfat dry milk in TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20), they were probed with anti-HIF-1α (1:1000), anti-VEGF (1:500, Santa Cruz, Santa Cruz, CA), anti-glut-1 (1:500, Santa Cruz), anti-Igf-2 (1:2500, R&D Systems), anti-c-met (1:500, Santa Cruz), anti-α-tubulin (1:1000, Santa Cruz), anti-Akt (1:1000, Cell Signaling) and anti-phospho-Akt (1:1000, Ser 473, Cell Signaling) antibodies, followed by incubation with HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (1:2000, Amersham Biosciences, Buckinghamshire, UK). The antibody binding was then visualized with enhanced chemiluminescence reagent (Amersham Biosciences), and the band images detected with the LAS3000 system (Fuji Film, Japan) were densitometrically analyzed using the Image Gauge (Fuji Film).

Reverse transcription (RT)-PCR. Total RNA was isolated from each cell line using Trizol reagent (Invitrogen, Carlsbad, CA) and then reverse transcripted with the oligo dT primer and SuperScript RT (Invitrogen). The PCR primers used were as follows: for HIF-1α, 5'-TCAAGTCAGCAACGTGGAAG-3' (forward) and 5'-TATCGAGGCTGTGTCGACTG-3' (reverse); for Igf-2, 5'-GAGTTCAGAGAGGCCAAACG-3' (forward) and 5'-CCTGCTCAAGAGGAGGTCAC-3' (reverse); and for β-actin,

5'-AGCCATGTACGTAGCCATCC-3' (forward) and 5'-CTCTCAGCTGTGGTGGTGAA-3' (reverse). The thermal cycle profile consisted of a 1 minute extension at 72˚C, 1 minute denaturation at 95˚C, 1 minute annealing at 56˚C and a final 7 minutes extension. Quantitative real time RT-PCR was performed for Igf-2 mRNA using the Roche Molecular Biochemicals LightCycler with LightCycler3 Run software (version 5.10). Fluorescence was generated using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), and data were collected with LightCycler3 Data Analysis software (version 3.5.28).

Establishment of HIF-1α knockdown HCC clones. Refering to the technical information of Ambion (Austin, TX, USA), short interfering RNA (siRNA) was designed to interfere with mouse HIF-1α expression. A set of 19-mer oligonucleotides was selected from the mouse HIF-1α mRNA sequence as a target, and it was confirmed that these sequences would not interfere with other genes by analyzing the homology to other mouse genes by a BLAST search. The sense (5'-AGATGAGTTCTGAACGTCG-3') and antisense sequences (5'-CGACGTTCAGAACTCATCT-3') were linked to the 9 nucleotide spacer (5'-TTCAAGAGA-3') as a loop, and 6 T bases were added to the 3' end of the oligonucleotides. In addition, reverse oligonucleotides corresponding to the forward sequences were also designed. The BamHI (GATCC) and HindIII (AGCTT) restriction site sequences were also added to the 5' end of the forward and 3' end of the reverse oligonucleotides, respectively. The DNA was ligated with linearized pSilencer 3.1-H1 hygro siRNA expression vector (Ambion) at the BamHI and HindIII sites to construct a mouse HIF-1α siRNA vector (pSH1a-1). An unrelated control siRNA vector (pSGFP), which targets the green fluorescence protein (GFP) DNA sequence (5'-GGTTATGTACAGGAACGCA-3') with no significant match to any mouse gene, was also prepared. The HCC cells were transfected with 1 μg each of the control (pSGFP) and HIF-1α knockdown (pSH1a-1) vectors which contained the hygromycin resistance gene using Lipofectoamine (Invitrogen). The transfected cells were maintained in the culture medium containing hygromycin B (0.5 mg/ml) for 7 days, and the stable control (sGFP) and HIF-1α knockdown (sH1a-cl2 and sH1a-cl3) cell lines were established.

***In vitro* and *in vivo* growth assay.** For the *in vitro* growth assay, 2×10^5 of the original HCC cells, control cells (sGFP) or the HIF-1α knockdown cells (sH1a-cl2 and sH1a-cl3) were seeded on a 35 mm dish, harvested with trypsin-EDTA solution on days 2, 4 and 6 and finally cell numbers were microscopically examined using a hemocytometer. For the *in vivo* growth assay, 1×10^6 cells suspended in 200 μl PBS were injected into the subcutaneous tissues of the back skin of B6C3F1 mice. Tumor diameters (*d* short and *d* long) were measured with calipers, and the tumor volume was calculated using the following formula: volume=d short$^2 \times d$ long /2 (23).

Both the *in vitro* and *in vivo* growth assay was done in triplicate and repeated twice. The data was presented as the values ± standard deviations (SD).

Statistics. A statistical analysis of the data was done by the Student paired t test, and P values <0.05 were considered to be significant.

Results

Expression of HIF-1α and the HIF-1 target genes in preneoplastic lesions. In the mice treated with DEN at the age of 2 weeks, livers contained preneoplastic foci and adenomas, that were respectively smaller and larger than the hepatic lobule size (24), 5-9 months later. These lesions were found to be round in shape with weak compression against the surrounding hepatic tissue and were composed of preneoplastic hepatocytes with slightly-basophilic cytoplasm (Figure 1A-a). HIF-1α immunohistochemistry detected positive staining in the nucleus and cytoplasm of most preneoplastic hepatocytes in all the lesions of the 5 mice (Figure 1A-b). HCCs developed in the livers 12-15 months after DEN treatment (Figure 1B-a). These HCCs also showed positive HIF-1α staining in the nucleus and cytoplasm (Figure 1B-b). The human liver biopsy samples occasionally contained small lesions called dysplastic hepatocytic foci, likely to be of a preneoplastic nature as were the mouse lesions (1), which consisted of altered hepatocytes with clear cytoplasm (Figure 1C-a). Positive HIF-1α staining was also demonstrated in the cytoplasm and nucleus of the dysplastic hepatocytes in the 3 human lesions examined (Figure 1C-b). Western blot analysis revealed that HIF-1α and the products of the HIF-1 target genes, VEGF, glut-1, Igf-2 and c-met, were expressed more abundantly in the hepatic adenomas than the normal liver tissue in mice (Figure 1D).

Lack of hypoxia in the mouse preneoplastic lesions. To clarify whether HIF-1α expression was due to hypoxia, 2 hours before sacrifice, the mice bearing the hepatic lesions were administered pimonidazole, which creates adducts with thiol-containing proteins in hypoxic (PO_2<10 mmHg) cells (25), followed by immunohistochemical detection of pimonidazole. In the normal hepatic tissue, although the pimonidazole staining was weakly positive in the hepatocytes around the terminal hepatic vein as previously described (26), it was negative in other parts (Figure 2A). The staining was negative in the preneoplastic foci or adenomas (Figure 2B), while the HIF-1α was positively stained in the contiguous sections (data not shown). On the other hand, the pomonidazole staining was mostly negative within the HCC tissues (Figure 2C), but strongly positive in the focal areas (Figure 2D). These observations indicated that HIF-1

expression in the preneoplastic hepatic lesions is independent of hypoxia, whereas it may be both dependent on and independent of hypoxia in the HCC tissues.

Regulation of HIF-1α expression by PI3 kinase/Akt signaling. Because PI3K/Akt signaling can activate HIF-1α expression independent of hypoxia in a cell type specific manner (13-15), we then explored whether HIF-1α expression in the mouse preneoplastic hepatic lesions was dependent on the activation of PI3K/Akt signaling. The results shown in Figure 3A demonstrated that all mouse hepatic adenomas exhibited higher levels of the activated form of Akt (phospho-Akt Ser473) than normal livers without any change in the total amounts of Akt and α-tubulin presented as the internal control. Immunohistochemistry for activated Akt showed positive staining in the nucleus and cytoplasm of preneoplastic hepatocytes in all mouse lesions examined (Figure 3B). We then investigated whether the high HIF-1α expression correlated to the activation of PI3K/Akt signaling using the mouse HCC cell lines. These cell lines showed active cell proliferation after treatment with growth factors (EGF and insulin) plus 10% FBS (GF/FBS+), while cell growth was stopped in the absence of the growth factors and FBS (GF/FBS-) conditions (27). These HCC cells exhibited high HIF-1α and activated Akt levels under GF/FBS+ conditions, while both HIF-1α and activated Akt were downregulated under GF/FBS- conditions (Figure 3C). Moreover, both HIF-1α and activated Akt levels were markedly decreased by treatment with LY294002, a PI3K inhibitor, under GF/FBS+ conditions. These observations strongly support the hypothesis that high HIF-1α expression in the HCC cells, possibly in preneoplastic hepatic lesions as well, may be due to activated PI3K/Akt signaling.

Effects of HIF-1α knockdown in HCC cell lines. In an effort to further investigate a role for HIF-1α activation in hepatocarcinogenesis, we established two HIF-1α knockdown clones (sH1a-cl2 and sH1a-cl3) from the mouse HCC cell lines by stable expression of the mouse HIF-1α siRNA. The densitometric analysis of Western blotting revealed that the two HIF-1α knockdown clones showed about a 70% reduction of HIF-1α protein expression compared to the original HCC cells and the control cell line introduced by the GFP siRNA (Figure 4A). In accordance with the reduction of HIF-1α expression, expression of the HIF-1 target genes (VEGF, glut-1, c-met and Igf-2) was also reduced in the HIF-1α knockdown cells. Furthermore, activated Akt was also downregulated in the HIF-1α knockdown cells without any changes in the total amount of Akt (Figure 4A), suggesting that the activation of PI3K/Akt signaling is reversely dependent on the high HIF-1α expression. In the *in vitro* growth assay, proliferation of the HIF-1α knockdown cells was slower than the original HCC cells or the control GFP siRNA-introduced cells in the GF/FBS+ medium (Figure 4B). Moreover, although subcutaneous inoculation of the original HCC cells or the control cells (sGFP) resulted in tumor

formation in B6C3F1 mice, the HIF-1α knockdown cells temporally formed small tumors during the 4-6 day period after inoculation, but these tumors disappeared by day 10 (Figure 4C). These findings indicate that downregulation of HIF-1α resulted in remarkable growth retardation of HCC cells both *in vitro* and *in vivo*.

Increased expression of activated Akt and HIF-1α by growth factors. Downregulation of HIF-1α expression by inactivation of PI3K/Akt signaling and downregulation of activated Akt by HIF-1α knockdown suggests a possible cause and effect relationship between the two phenomena. Because Igf-2 is known to activate the PI3K/Akt pathway via binding to the cognate Igf-1 receptor and hybrid Igf-1/insulin receptor (28) and also because *Igf-2* has been shown to be one of the transcriptional targets of HIF-1 (29-31), it is possible that activation of HIF-1, Igf-2 and PI3K/Akt signaling may create an autocrine loop (3). Furthermore, because Igf-2 is frequently overexpressed in HCC tissue and cell lines (18, 19), it may behave like an autocrine growth factor. As shown in Figures 1D and 4A, respectively, most mouse hepatic adenomas and the HCC cell line overexpressed Igf-2 protein. In the HIF-1α knockdown HCC cells, Igf-2 protein (Figure 4A) and mRNA levels (Figure 5A-a) were markedly downregulated. Quantitative real time RT-PCR demonstrated that the Igf-2 mRNA level was decreased to about 50% in the HIF-1α knockdown cells compared to the original and control cells (sGFP) (Figure 5A-b). In order to investigate whether Igf-2 contributed to the expression of both activated Akt and HIF-1α, the HCC cells were cultured in the starvation medium (GF/FBS-) for 6 hours and then in medium containing various concentrations of mouse recombinant Igf-2 together with 5% FBS. The results shown in Figure 5B demonstrate that the expression of HIF-1α, VEGF and activated Akt proteins were upregulated by Igf-2 in a dose dependent manner in the original HCC cells. On the other hand, when the cells were treated simultaneously with Igf-2 and the neutralizing Igf-2 antibody, expression of both activated Akt and HIF-1α was reduced without any change in the total amount of Akt (Figure 5C). Surprisingly, however, when the HIF-1α knockdown cells were treated with Igf-2, Akt activation did not occur at all (Figure 5B). To address whether this phenomenon is specific to Igf-2, the cells were treated with EGF that can activate the PI3K/Akt pathway via binding to EGF receptor. The effect of EGF is thought to mimic that of TGF-α, an autocrine factor that is frequently overexpressed in preneoplastic hepatic lesions (20), because both EGF and TGF-α bind to the EGF receptor to activate the downstream signaling (32). Although Akt was activated by EGF in a dose dependent manner in the original HCC cells, it was only very weakly activated in the HIF-1α knockdown cells (Figure 5B). These observations suggest that the overexpression of activated Akt and HIF-1α in the original HCC cells may have been, at least in part, mediated by Igf-2 and possibly by TGF-α as well,

while they could no longer activate Akt in the HIF-1α knockdown cells where the HIF-1 target genes had been suppressed.

Discussion

There is increasing evidence suggesting that HIF-1 activation occurs in the early stages of carcinogenesis. It has been demonstrated that in the kidneys of patients with von Hippel-Lindau's disease, which is frequently associated with renal carcinomas, HIF-1α and the HIF-1 target genes are overexpressed in morphologically normal single renal tubular cells and epithelial cells forming multicellular foci or microcysts similar to overt renal carcinoma cells (33). HIF-1α was also shown to be expressed in a few cells in ductal hyperplastic areas adjacent to invasive breast cancer (34), dysplastic uterine cervical epithelium (35), colorectal adenomas (36) and prostate intraepithelial neoplasia (37) like their malignant counterparts. Furthermore, HIF-1α and HIF-1 target genes were shown to be overexpressed in hyperplastic and dysplastic skin lesions during multistage epidermal carcinogenesis in K14-HPV16 transgenic mice where the oncogenic proteins of human papilloma virus 16 were overexpressed in epidermal cells under the control of the keratin 14 gene promoter (38). In the present study, to the best of our knowledge, we are the first to demonstrate that HIF-1α is activated in preneoplastic hepatocytes during the early stages of hepatocarcinogenesis and long before the development of HCCs.

When the mice with the hepatic lesions were treated with pimonidazole, pimonidazole staining was not detected in the preneoplastic lesions, indicating that HIF-1α expression was independent of hypoxia. Since the early preneoplastic hepatic lesions examined were generally small in size, with dilated sinusoids covered with the endothelial cells with characteristic features such as loss of fenestrations[†] and did not show any necrosis, they may be refractory to hypoxia. In contrast, positive pimonidazole staining was seen in the focal areas within HCCs, which occasionally showed necrosis, suggesting that HIF-1α expression in HCCs may be both dependent and independent of hypoxia. HIF-1α expression independent of hypoxia has been reported for human cancer tissues (39) and cell lines (40). It has been shown that HIF-1α can be activated not only by hypoxia, but also by various other mechanisms such as the activation of oncogenes (8, 9), loss of function in tumor suppressor genes such as PTEN (11), VHL (6) and p53 (12) and activation of growth factor signaling (13-15).

In the present study, preneoplastic hepatic lesions showed increased levels of phospho-Akt

[†] Tamakawa S. et al. unpublished observation.

compared to the normal livers, although total Akt levels were unchanged. On the other hand, when HCC cells were treated with LY294002, a PI3K inhibitor, not only the levels of phospho-Akt but also those of HIF-1α were downregulated, suggesting that HIF-1α expression is dependent on PI3K-Akt signaling. Akt can activate mTOR which phosphorylates p70 S6 kinase (S6K) and the eukaryotic translation initiation factor 4E binding protein (4E-BP1) (41), leading to activation of protein synthesis (42). The increased activity of protein synthesizing machinery upregulates HIF-1α protein synthesis without affecting HIF-1α mRNA levels, HIF-1α ubiquitination and the capability of HIF-1α to interact with pVHL (13, 43). Our observations strongly suggest that activated PI3K/Akt signaling increases HIF-1α expression in HCC cell lines and presumably preneoplastic hepatic lesions as well.

Conversely, when HIF-1α was knocked-down by siRNA in the HCC cells, not only the expression of the HIF-1α target genes but also the phospho-Akt levels were downregulated without any change in total Akt, suggesting that the activation of Akt is reversely dependent on HIF-1α activation. Among HIF-1 targets, growth factors such as Igf-2, Tgf-α and VEGF and growth factor receptors such as c-met may activate PI3K/Akt signaling by activating the receptor associated tyrosine kinases (3). In the present study, Igf-2 was overexpressed in most preneoplastic lesions and the HCC cell lines. Furthermore, treatment of the HCC cells with Igf-2 in the 5% serum condition upregulated both phospho-Akt and HIF-1α, while simultaneous treatment with Igf-2 and the neutralizing anti-Igf-2 antibody downregulated them both, indicating that, at least in part, Igf-2 plays a role in the activation of PI3K/Akt signaling and subsequent HIF-1α activation in HCC cells. These observations strongly suggest that HIF-1, Igf-2 and PI3K/Akt signaling may create an autocrine loop and have an important role in the progression of hepatocarcinogenesis. However, it remains to be investigated which is the initiating change in such autocrine mechanism.

On the other hand, the capacity of Igf-2 and EGF to activate Akt was abrogated in the HIF-1α knockdown cells, suggesting that some factors directly or indirectly activated by HIF-1 may have a role in the activation of PI3K/Akt signaling. For example, HIF-1α knockdown may lead to the suppression of TGF-α, a target of HIF-1 (3), which binds to the EGF receptor to activate various intracellular signaling (32) and is frequently overexpressed in preneoplasatic and neoplastic hepatic lesions (20). Furthermore, suppression of Igf binding proteins (IGFBPs), also targets of HIF-1 (3), that can potentiate or suppress Igf-2 activity (44), may modulate the responsiveness to Igf-2 in HIF-1α knockdown cells. Recently, Calvani et al. (45) reported that basic fibroblast growth factors (bFGF), which induce the survival and sprouting of human umbilical vascular endothelial cells (HUVEC) *in vitro*, were abrogated by HIF-1α knockdown

from siRNA. Furthermore, it was demonstrated that HIF-1 enhances the responsiveness to bFGF in endothelial cells by increasing the binding of bFGF to heparan sulfate on the cell surface (46). It is then thought that HIF-1 may amplify the growth factor signaling not only by inducing the autocrine growth factors but also those factors that modulate the responsiveness to the growth factors.

C-met, a receptor of hepatocyte growth factor (HGF), which is one of the targets of HIF-1 (47, 48), was upregulated in preneoplastic hepatic lesions and HCC cell lines. HIF-1α knockdown resulted in the downregulation of c-met expression, suggesting that c-met expression is regulated by HIF-1 in HCC cell lines. Recently, Lacouter et al. (49) demonstrated that sinusoidal endothelial cells produce HGF by VEGF stimulation via the VEGF receptor 1 (Flt-1) activation in mice. It is then possible that HIF-1 activation stimulates VEGF production by preneoplastic hepatocytes, which, in turn, may stimulate the sinusoidal endothelial cells to produce HGF. On the other hand, HIF-1 may increase c-met expression in preneoplastic hepatocytes, leading to sensitization to HGF. Furthermore, it was demonstrated that HGF/c-met can activate PI3K/Akt signaling which activates HIF-1α and HIF-1 target genes in HepG2 cells (50). It is then possible that HIF-1, VEGF and c-met may create a paracrine loop involving preneoplastic hepatocytes and sinusoidal endothelial cells, although further studies are required.

In conclusion, overexpression of HIF-1α and activation of its target genes are changes in the early stages of hepatocarcinogenesis. In particular, activation of PI3K/Akt signaling and Igf-2 under the influence of HIF-1 activation may be important for proliferation of preneoplastic hepatocytes. Therefore, the HIF-1 activation may be crucial in progression of hepatocarcinogenesis by expanding preneoplastic hepatocyte populations, which in turn increases the chance for accumutation of oncogenic mutations within the populations. Intervention of the HIF-1 pathway then may be effective to prevent the development of HCCs.

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Figure Legends

Figure 1 HIF-1α expression in preneoplastic hepatic lesions and HCC. (a) H&E

staining. (b) Immunohistochemical staining for HIF-1α. **A.** Mouse preneoplastic focus. ×200. Inset: High magnification showing HIF-1α staining in the cytoplasm and nucleus. ×400. **B.** Mouse HCC. ×200. **C.** Human dysplastic hepatic focus. ×400. **D.** Western blot analysis for HIF-1α, HIF-1 target genes and α-tubulin (loading control) in normal mouse liver tissue (N) and hepatic adenomas (1-6).

Figure 2 Immunohistochemical staining for pimonidazole. **A.** Normal mouse liver. Note that the hepatocytes around the terminal hepatic vein (THV) are weakly stained. ×200. **B.** Negative staining in a mouse preneoplastic hepatic focus. ×100. **C.** Negative staining in mouse HCC. x100. **D.** Positive staining in a mouse HCC. ×200.

Figure 3 Activation of Akt in mouse hepatic adenomas and a HCC cell line. **A.** Western blot analysis for phospho- and total Akt in normal mouse liver tissue (N) and hepatic adenomas (1-6). **B.** Immunohistochemical staining for phospho-Akt in a mouse preneoplastic hepatic focus. ×400. **C.** Western blot analysis of HIF-1α and phospho- and total Akt. The effect of GF/FBS and the PI3 kinase inhibitor, LY294002, on the expression of HIF-1α and phospho-Akt in mouse HCC cells.

Figure 4 Expression of HIF-1α, HIF-1 target genes and phospho-Akt in HIF-1α knockdown cells. **A.** Western blot of HIF-1α, HIF-1 target gene products, and phospho- and total Akt in the original (WT), control (sGFP) and HIF-1α knockdown HCC cell lines (sH1a-cl2 and sH1a-cl3). **B.** Growth curve *in vitro*. Error bars represent SD of the values using 4 wells. $^{*}p<0.01$ comparing the original or control cells. **C.** Changes in tumor volume after inoculation of the cells into the mouse subcutaneous tissue. Error bars represent SD of values for 4 tumors. $^{*}p<0.01$ comparing the original or control cells.

Figure 5 Expression of Igf-2 mRNA in the HIF-1α knockdown cells, and the effect of Igf-2 on HIF-1α and phospho-Akt expression. **A**. RT-PCR (a) and real time RT-PCR analysis of Igf-2 mRNA (b) in the original (WT), control (sGFP) and HIF-1α knockdown HCC cell lines (sH1a-cl2 and sH1a-cl3). **B.** Western blot analysis of HIF-1α, VEGF, and phospho- and total Akt in the original (WT) and HIF-1α knockdown HCC cells (sH1a-cl2) after treatment with Igf-2 or EGF. **C**. Effect of neutralizing Igf-2 antibody on the expression of HIF-1α and phospho-Akt in the original HCC cells.

Figure 1

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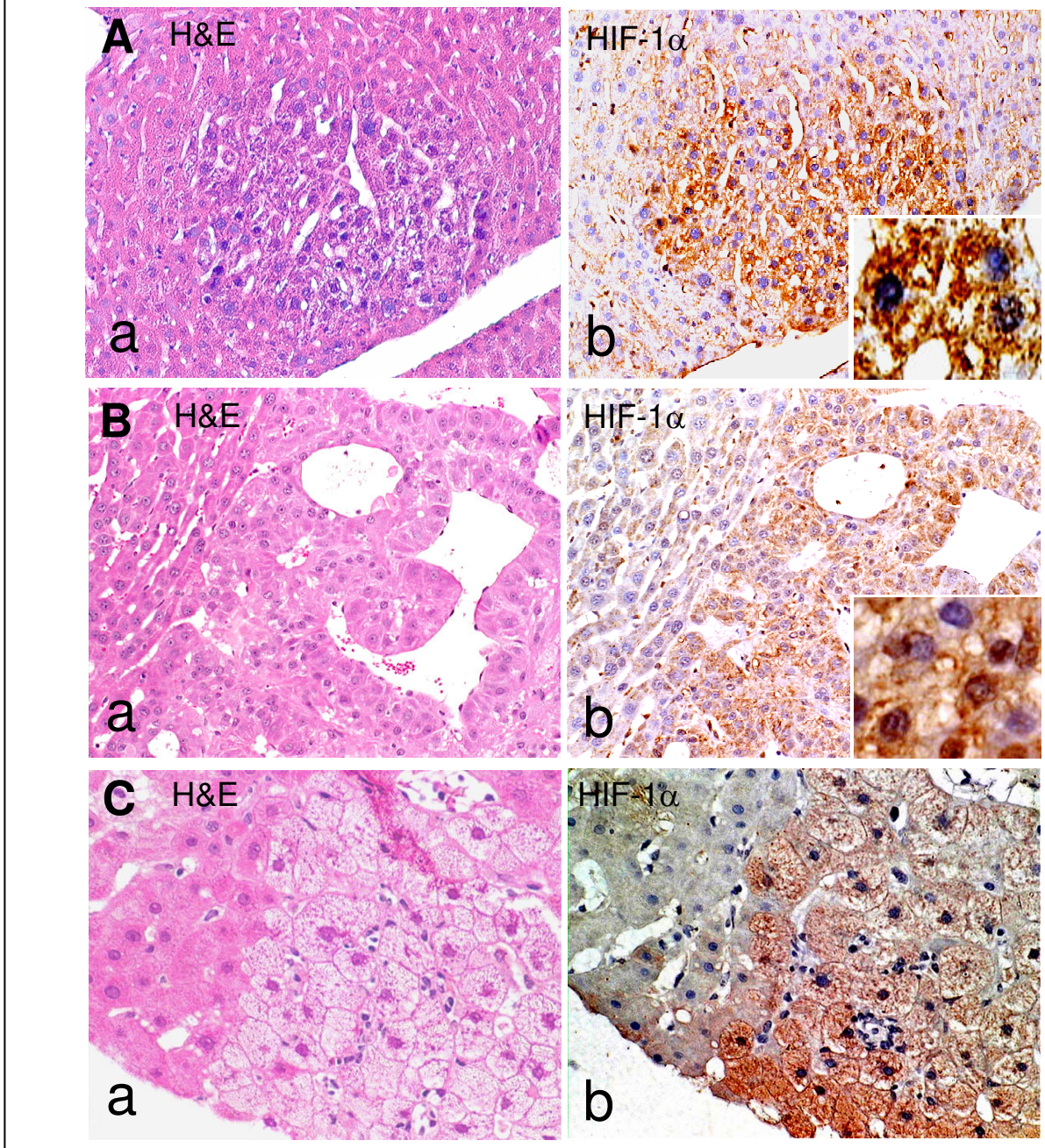

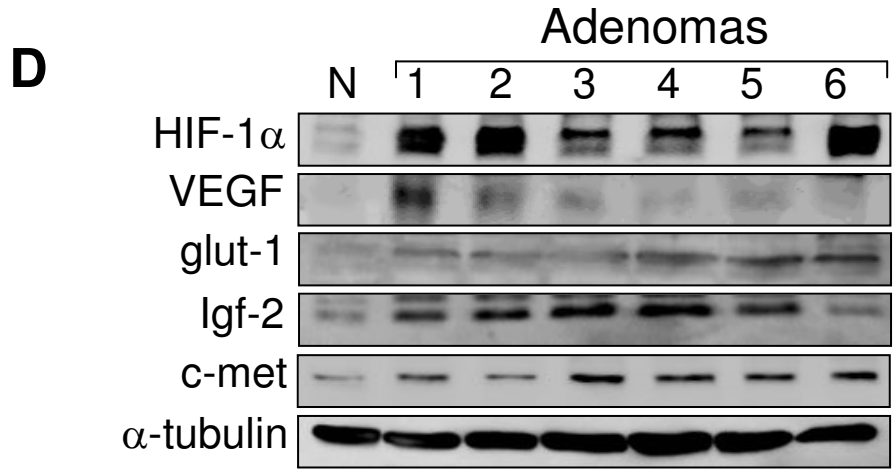

Figure 2

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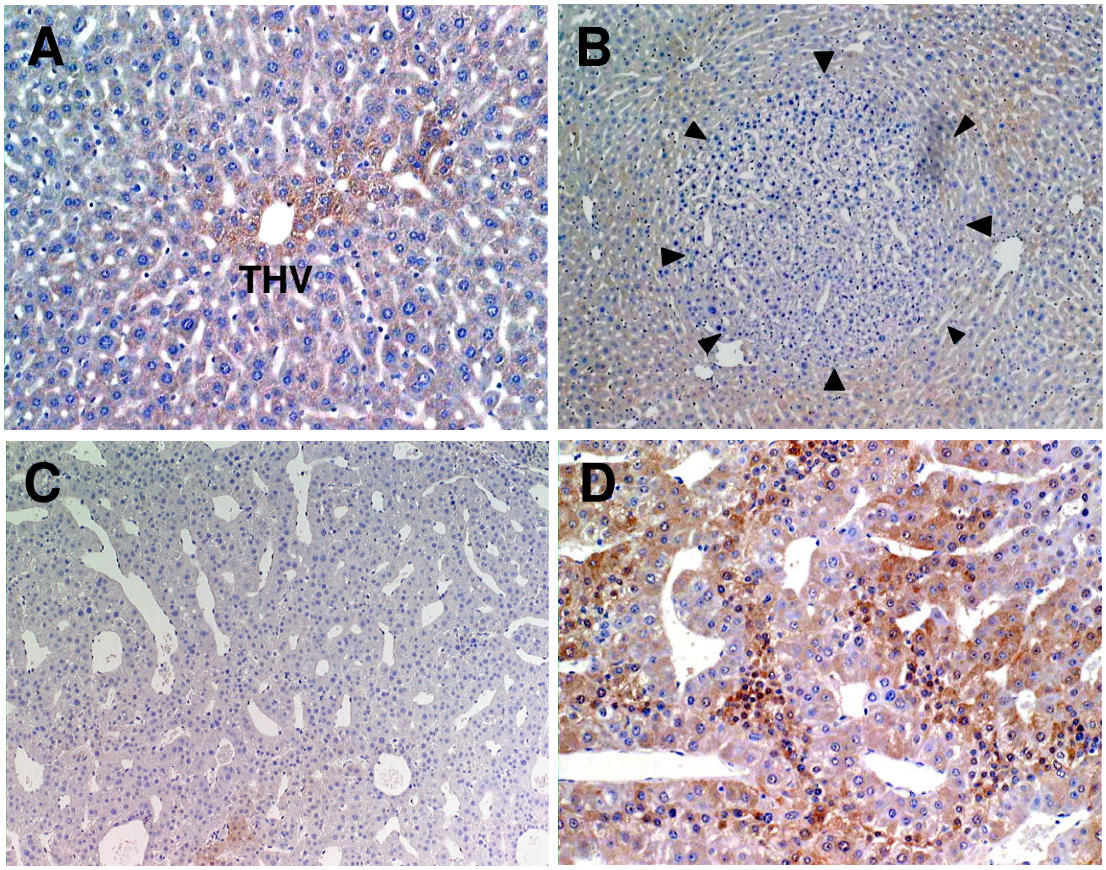

Figure 3

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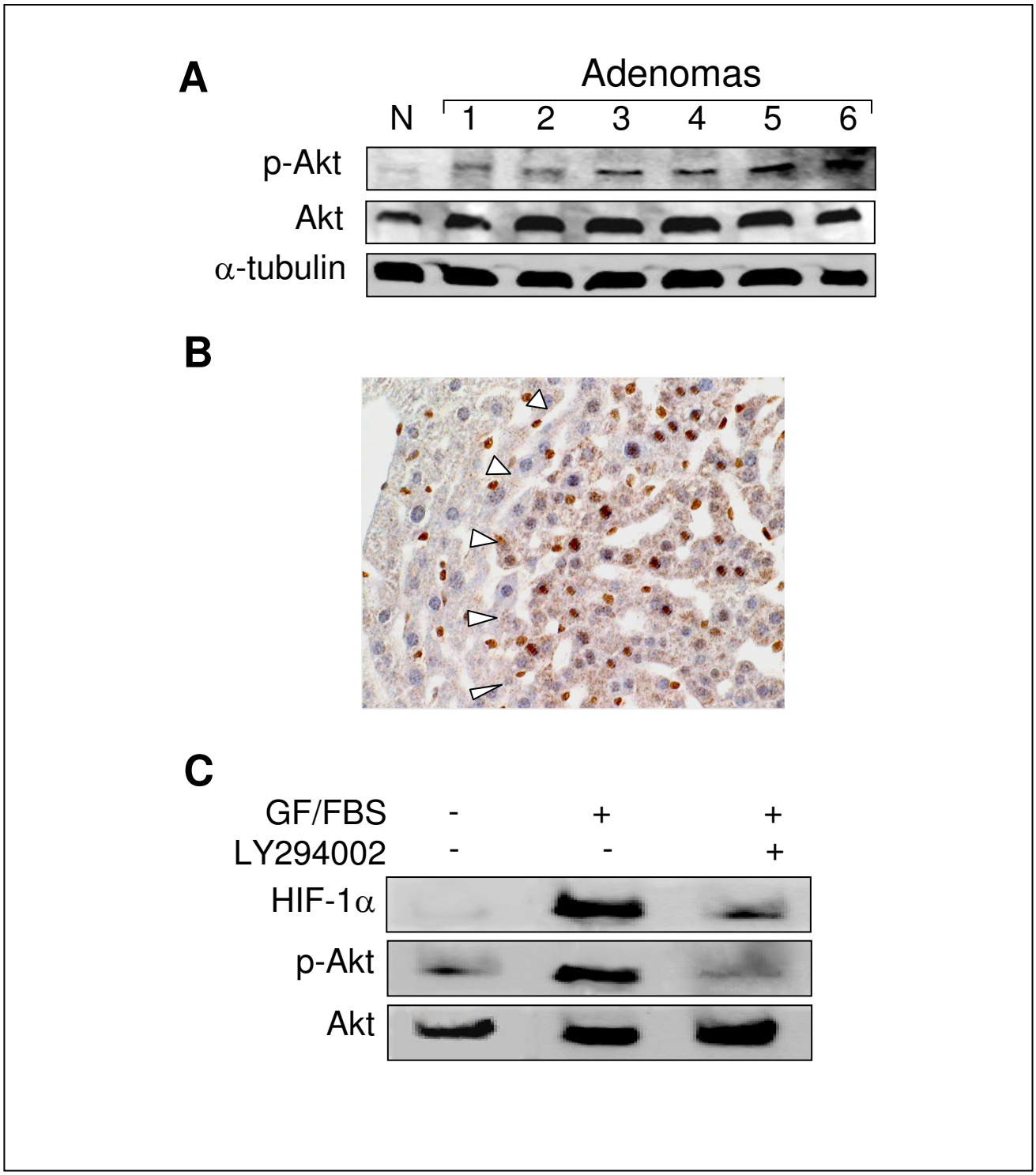

Figure 4 Hiroki Tanaka

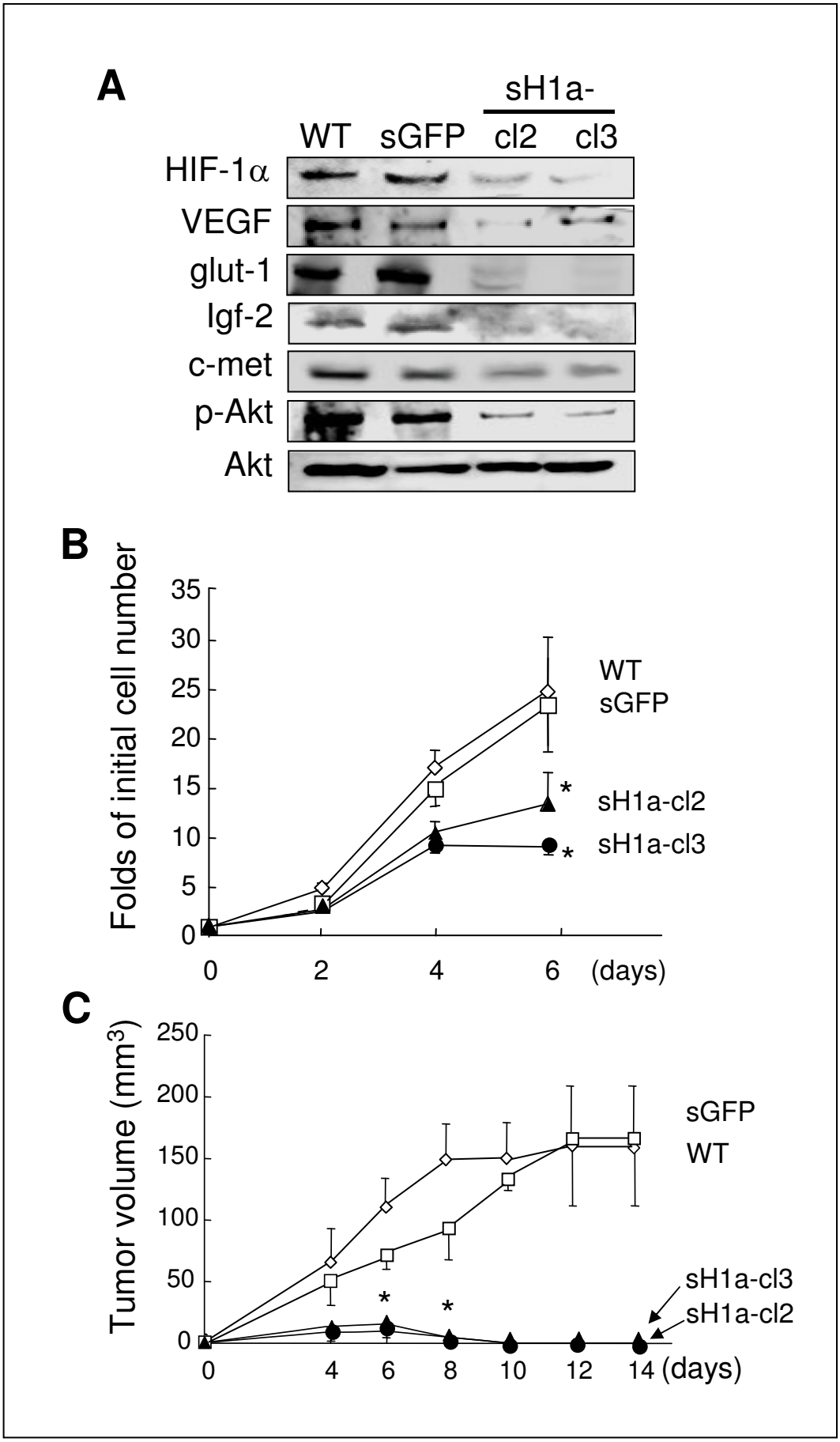

Figure 5

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