Neuroglobin, a Novel Intracellular Hexa-Coordinated Globin, Functions as a Tumor Suppressor in Hepatocellular Carcinoma via Raf/MAPK/Erk

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ABSTRACT

Hypoxia and oxidative stress are critical factors in carcinogenesis and exist throughout cancer development; however, the underlying mechanisms are far from clear. Here, for the first time to our knowledge, we reported that neuroglobin (Ngb), an intracellular hexa-coordinated globin serving as an oxygen/reactive oxygen species (ROS) sensor, functions as a tumor suppressor in hepatocellular carcinoma (HCC). Ngb protein and mRNA expression were significantly down-regulated in tumor tissues, compared with its adjacent non-tumor tissues of human HCC samples and normal liver tissues. Knock-down of Ngb by RNA interference promoted human HCC cell line (HepG2) growth and proliferation, G0/G1-S transition in vitro, and tumor growth in vivo. On the contrary, overexpression of Ngb suppressed HepG2 cell growth and proliferation, G0/G1-S transition, colony formation in vitro, and tumorigenicity in vivo. These results established a tumor suppressor function of Ngb in HCC.

The underlying mechanisms were further investigated. Overexpression of Ngb suppressed Raf/MEK/extracellular signal-regulated kinase (Erk), whereas knockdown of Ngb enhanced Raf/MEK/Erk activation in HepG2 cells in vitro and in vivo. Glutathione S-transferase pull-down showed that Ngb interacted with c-Raf-1 in HepG2 cells. Overexpression of Ngb suppressed serum- and H2O2-stimulated Erk activation in HepG2 cells. Pharmacological inhibition of Erk activation abolished the proliferative effect of Ngb knockdown in HepG2 cells. Mutation of Ngb at its oxygen-binding site (H64L) abolished the inhibitory effects of Ngb on Erk activation and HepG2 cell proliferation. Therefore, we propose that Ngb controls HCC development by linking oxygen/ROS signals to oncogenic Raf/mitogen-activated protein kinase (MAPK)/Erk signaling. Our data suggest that neuroglobin could be a new target for cancer therapy.

Introduction

Liver cancer is the sixth most common malignant tumor and the third leading cause of cancer-related death in the world. Hepatocellular carcinoma (HCC) is the most common primary liver malignant tumor in adults. Hepatocarcinogenesis caused by virus infection, liver injury, or inflammation usually involves hepatocytic death and abnormal reconstruction (Severi et al., 2010; Pollicino et al., 2011), in which hypoxia or oxidative stress plays an important role. Hypoxia and oxidative stress might be independent initial factors in hepatocarcinogenesis by activating pro-oncogenic genes or causing genetic instability (El-Serag and Rudolph, 2007; Aravalli et al., 2008; Zender et al., 2010), which finally induces cellular transformation and promotes tumor cell growth. For example, diethylnitrosamine, a well-known liver carcinogen, induces hepatocyte injury and compensatory proliferation by accumulating reactive oxygen species (ROS) (Aravalli et al., 2008; Sakurai et al., 2008; Zender et al., 2010). The major signaling pathways involved in HCC genesis or development includes Raf/mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/AKT, and Wnt/β-catenin (El-Serag and Rudolph, 2007; Aravalli et al., 2008; Whittaker et al., 2010). Nevertheless, the exact mechanisms underlying hypoxia- or oxidative stress-induced HCC remain far from understood.

Neuroglobin (Ngb), a newly discovered intracellular respiratory globin with a hexa-coordinated heme (Burmester et al., 2000; Trent et al., 2001), plays an important role in cancer therapy.
His64 locks Ngb in a penta-coordinated state, increasing its
activity negatively. Mutation of His64 locks Ngb in a penta-coordinated state, increasing its catalytic nitrite binding and reductase activity. In addition, Ngb binds to G-protein subunit (Wakasugi et al., 2003), 14-3-3 (Jayaraman et al., 2011), or cytochrome C (Raychaudhuri et al., 2010), which is regulated by hypoxic or oxidative conditions. These evidences suggest that Ngb may regulate signal transduction by sensing changes of intracellular oxygen or ROS signals. On the other hand, the expression (Zhu et al., 2002) and phosphorylation (Raychaudhuri et al., 2010) of Ngb under ischemic or hypoxic conditions are also regulated by intracellular signaling pathways, such as Erk activation. These data suggest a complicated relationship between Ngb and Raf/MAPK/Erk cascades under physiologic or pathologic conditions.

There are emerging evidences showing that the expression levels of hexa-coordinated globins are associated with tumor genesis or development. Emara et al. (2010) reports that Ngb and cytoglobin (Cygb) are detected in most types of tumors by microarray analysis. Oleksiewicz et al. (2011) reports that Ngb is significantly up-regulated in lung cancer. However, Gorr et al. (2011) reports that neither Ngb nor Cygb is systematically up-regulated in tumors. In non–small cell lung cancer, the down-regulation of Ngb is found to be associated with Ngb promoter hypermethylation (Oleksiewicz et al., 2011). Hypermethylation of Cygb promoter has also been found in several tumors (Shivapurkar et al., 2008). These evidences indicate that Ngb expression in tumors may depend on specific cellular conditions, such as hypoxia or hypermethylation. Until now, the functional role of Ngb in tumor cells has not been directly studied, whereas the function of other globin members in tumor cells has already been reported. Because Ngb is a native oxygen or ROS sensor, clarifying the functional role of Ngb in tumor cells is important for understanding the role of hypoxia and ROS in tumor genesis and development.

Here, we reported that Ngb was down-regulated in HCC. Overexpression of Ngb suppressed HCC cell growth in vitro and tumor growth in vivo, and knockdown of Ngb showed the opposite effects in HCC cells. Furthermore, we found that Ngb controls HCC cell proliferation via regulating Raf/MAPK/Erk activities negatively.

Materials and Methods

Tissue Samples. Sixty male human HCC samples and lung cancer samples were used for immunohistochemical analysis. These samples were purchased from the Department of Pathology, Tongji Hospital, Huazhong University of Science and Technology (HUST). All samples were surgically resected during 2008–2009 in Tongji Hospital, HUST, and diagnoses were determined clinically and pathologically at Tongji Hospital, HUST. Among the 60 HCC samples, 48 samples had their corresponding adjacent non-tumor tissues (ANT). All human (male) normal liver samples were obtained from the Department of Forensic Medicine, HUST.

Seventeen fresh male HCC tissues with their corresponding adjacent non-tumor tissues were used for quantitative mRNA analysis. All samples were purchased from the Department of Hepatobiliary Surgery, Union Hospital, HUST, and diagnoses were confirmed clinically and pathologically at Union Hospital, HUST.

Immunohistochemical Analysis and Assessment. The expression of Ngb in HCC was examined by immunohistochemistry (IHC) using a rabbit polyclonal antibody against Ngb (Santa Cruz Biotechnology, Santa Cruz, CA). Four-micrometer sections were cut from paraffin-embedded HCC tissue blocks, deparaffinized, rehydrated, and put into endogenous peroxidase-blocking solution. The sections were then boiled for 10 minutes in 10 mmol/l citrate buffer (pH, 6.0) in a water bath. Thereafter, slides were incubated with Ngb antibody at a dilution of 1:100 at 4°C overnight. The expression of Ngb was detected using corresponding rabbit EnVision System-HRP (DAB) kit (DAKO Denmark) according to the manufacturer’s instructions. Slides were then washed in water, counterstained with Mayer’s hematoxylin (Merck, Darmstadt, Germany), and coverslipped. Negative controls without primary antibody were performed in parallel. Positive staining was envisioned as brown particles in the cytoplasm. Total immunostaining scores of each slide were calculated by multiplying the positive cell percentage scores and the intensity scores as previously described (Huynh, 2004). The positive cell percentage scores (0–4) were defined by the percentage of positively stained cells in tissues (0, none; 1, < 10%; 2, 10%–50%; 3, 50%–80%; 4, >80%). The intensity scores (0–3) represented the relative staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranged from 0 to 12. Ngb-positive staining was defined as a total score greater than or equal to 4.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and quantitative Real-Time PCR (qPCR). Total RNA was extracted using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Grand Island, NY). Conventional and qPCR was performed as previously described (Chen et al., 2005b; Ye et al., 2009). qPCR was performed using the Platinum SYBR Green qPCR superMix-UDG Detection Kit (Invitrogen) and the Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems, Inc., Foster City, CA). The Ngb primers were 5’-GAAGCACCCGGCAGTG-3’ and 5’-AGA-ACACCTTTCAGCAGTATGG-3’. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control, and its primers were 5’-AAGGATTGGTCGTAGTATG-3’ and 5’-GGGAATTGCTGATGA-TGGCATTC’. Triplicate PCR’s were analyzed using the comparative Ct (ΔΔCt) method according to the manufacturer’s instructions (Applied Biosystems Inc.). Relative Ngb expression level was expressed as the ratio of Ngb to GAPDH.

Cell Lines, Plasmids, and Transfection. Human HCC cell line (HepG2 cells) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA) at 37°C with 95% humidified atmosphere containing 5% carbon dioxide in an incubator (Heat Force Inc., Hong Kong, China). The Ngb overexpression plasmids (p-Ngb-EGFP-N1) were constructed previously (Chen et al., 2005b). Ngb mutant variants (Y44D, H64A, H64L, E53Q, E118Q, and K119N) were constructed using the Fast Mutagenesis System (TransGen Biotech Co., Beijing, China) with use of p-Ngb-EGFP-N1 as the template. The Ngb short hairpin RNA (shRNA)–expressing plasmids (p-Ngb-shRNA-Genesil-1, sh-Ngb) for Ngb knockdown were constructed by inserting the specific Ngb-shRNA DNA fragment into the p-Genesil-1 vector (Wuhan Genesil Biotechnology, Wuhan, China) as previously reported (Ye et al., 2009). The target sequence of Ngb-shRNA was 5’-GGTGGATGG-TTCGATGTACGTC-3’. A nonspecific DNA fragment was used as negative control. All plasmids were confirmed by sequencing. Transfection of HepG2 cells at 80%–90% confluence was performed using FuGENE HD reagent (Roche Diagnostics, Basel, Switzerland). Stably transfected HepG2 cell lines were established by neomycin (G418; Merck KGaA, Germany).
Western Blot Analysis. Total soluble proteins were extracted from tissues or cultured cells, and equal amounts of total proteins were used for Western blot analysis as previously reported (Chen et al., 2005a). After blocking with 5% of nonfat milk in phosphate-buffered saline (PBS), the blots were incubated with primary antibodies against Ngb, c-Raf-1, β-actin (Santa Cruz Biotechnology), p-Raf-1 Ser338, p-MEK1/2 Ser217/221, p-Erk1/2 Thr202/Tyr204, Erk1/2, p-OSK-3p Ser9, OSK-3p, p-PEN Ser380/Thr382/383, PTEN, p-β-catenin Ser33/37/Thr41, and β-catenin (Cell Signaling Technology, Danvers, MA). The blots were visualized with corresponding fluorescent secondary antibodies, and the bands were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Cell Viability Assay. Cell viability was measured using 3-(4,5-dimethylthiazole-2-y1)-2,5-bisphenyl tetrazolium bromide (MTT) assay as previously reported (Ye et al., 2009). Stably transfected HepG2 cells were seeded in 96-well plates (2000 cells/well), and MTT (1 mg/ml of final concentration; Amresco Inc., Solon, OH) was added to each well at a fixed time every day. The culture plates were incubated for 4 hours at 37°C, and 150 μl of dimethyl sulfoxide was applied to dissolve the blue formazan crystals. The optical density of solution was measured using absorbance spectrometry at 490 nm with use of a microtiter plate reader (Synergy 2; BioTek Instruments, Winooski, VT).

Colony Formation Assay in Soft Agar. Soft agar plates were prepared in 35-mm culture dishes with 1 ml of a bottom layer of 0.6% (VV) agar (Sigma) in DMEM containing 10% FBS. Stably transfected HepG2 cells were trypsinized into single-cell suspension, and 2000 cells were resuspended in 1 ml of top agarose layer containing 0.7% (V/V) agarose in DMEM with 10% FBS. The plates were then cultured under normal conditions for 3 weeks continuously (Huynh, 2004). The colonies larger than 50 μm were counted from the whole plates under the microscope after the cells in the plates were stained with 0.5 ml of 0.005% crystal violet.

Cell proliferation assay, stably or transiently transfected HepG2 cells were seeded in 96-well plates (1 × 10⁴ cells/well) for 10 hours and were incubated with 50 μM of 5-ethyl-2-deoxyuridine (EdU; RiboBio Co. LTD, Guangzhou, China) for another 3 hours according to the manufacturer’s instructions. The nuclei were stained with Hoechst 33342 (RiboBio Co. LTD). Micrographs were taken randomly from at least six different fields in each 96-well plate with a conventional inverted fluorescent microscope. The numbers of EdU-positive cells and total cells (determined by Hoechst staining) in all fields were counted, and the percentage of EdU-positive cells (representing proliferating cells) was calculated.

For cell cycle re-entry assay, stably transfected HepG2 cells were seeded in 6-well plates (2 × 10⁴ cells/well) and starved with DMEM without serum for 48 hours. The cultures were then replaced with fresh DMEM plus 10% FBS to allow cells re-entering into cell cycle. HepG2 cells were harvested at various time points (8, 12, or 16 hours), fixed in ice-cold 70% ethanol at 4°C overnight, washed with PBS, and resuspended with the binding buffer containing 0.2% (V/V) Triton X-100 and 1% (W/V) bovine serum albumin in PBS at a final density of 1 × 10⁶ cells/ml. Subsequently, RNase at a final concentration of 1 μg/ml was added, and cells were stained with propidium iodide (0.1μg/ml; Sigma) for 15 minutes. All cells were subjected to fluorescent-activated cell sorting (FACS) analysis in triplicate on a FACS Calibur system (Becton Dickinson, Franklin Lakes, NJ).

Tumor Xenograft in Nude Mice. All animal experiments were performed under the approval of the HUST Animal Welfare Committee. The protocol and procedures were approved by the Ethics Committee of HUST and performed with humane care. Female BALB/c nude mice (age, 4–5 weeks; body weight, 10–12 g) were purchased from Beijing HFK Bioscience Company, LTD (Beijing, China) and housed in specific pathogen-free barrier system of Animal Center, Union Hospital, HUST. Stably transfected parental HepG2 cells (2 × 10⁴ cells in 200 μl of PBS) were injected subcutaneously in both flanks of mice (Wang et al., 2010). Tumor volume was measured with calipers at 2- or 3-day intervals, and the tumor volume was calculated using the following formula: tumor volume = 0.5 × length × width² (Deng et al., 2010). Mice were sacrificed 1 month later, and tumors were removed completely, weighed, and photographed.

Glutathione S-transferase (GST) Pull-Down. Equal amount of p-GST-c-Raf-1 was cotransfected with p-Ngb-EGFP-N1 wild type or its mutants in HepG2 cells, and whole cell lysate was extracted using GST-lysis buffer (150 mM NaCl, 10 mM HEPES (pH, 7.45), 1% nonident P-40, 5 mM Na pyrophosphate, 5 mM NaF, 2 mM Na orthovanadate, 10 mg/l aprotinin, 10 mg/l leupeptin, and 1 mM PMSF) 2 days after transfection. After centrifugation, 400 total soluble proteins were incubated with 40 μl of GST aga-rose beads (Thermo Fisher Scientific Inc., Waltham, MA) for 4 hours at cold room temperature (4°C) with gentle rotation. After extensive washing with GST-lysis buffer, the immunoprecipitates were subjected to Western blot analysis with anti-GST or anti-green fluorescent protein (GFP) antibody (Santa Cruz Biotechnology).

Statistical Analysis. For animal experiments, 8 animals per group were randomly selected. Cell experiments were repeated at least three times independently. Categorical data were evaluated using χ² and Fisher’s exact tests. The weight and volume of xenografted tumors were evaluated using Mann-Whitney U test. Other values were expressed as mean ± S.E.M. and evaluated using 2-way analysis of variance followed by the Student-Newman-Keuls test. P value less than 0.05 was considered to be statistically significant.

Results

Ngb Is Down-Regulated in HCC. The expression of Ngb in human HCC tissues was examined using IHC. Figure 1, A and B, shows that the Ngb antibody that we used recognized Ngb protein specifically in Western blot analysis and fluorescent cytokinnoostaining assay. With use of lung cancer as positive control (Emara et al., 2010; Oleksiewicz et al., 2011), the up-regulation of Ngb in human lung cancer was verified by IHC (Fig. 1C). Figure 1D shows the representative micrographs of IHC from normal human liver and three different HCC samples with adjacent non-tumor tissues. Clearly, the intensity of Ngb staining in tumor tissues was much lower than that of their adjacent non-tumor tissues. The percentage of Ngb-positive tumor tissues (22 of 60 cases, 36.7%) was significantly decreased, compared with that of Ngb-positive adjacent non-tumor tissues (36 of 48 cases, 75.0%) and normal liver tissues (20 of 20, 100%) (Fig. 1E). Furthermore, the expression levels of Ngb mRNA in HCC (17 cases) were measured by conventional (Fig. 1F) and qPCR (Fig. 1F). Statistical analysis revealed that the relative level of Ngb mRNA in tumor tissue was significantly decreased, compared with that of corresponding adjacent non-tumor tissues (Fig. 1F). These data established a negative relationship between Ngb expression level and HCC.

Knockdown of Ngb Promotes HCC Cell Growth In Vitro and Tumor Growth In Vivo. The apparent low expression of Ngb in HCC prompted us to investigate the functional role of Ngb knockdown in HCC cell growth. Endogenous Ngb in HepG2 cells was knocked down by RNA interfering technique. Results of qPCR, conventional RT-PCR, and Western blot (Fig. 2A) confirmed that the expression level of Ngb was significantly decreased in human HepG2 cells stably transfected with the p-Ngb-shRNA-Genesil-1 plasmids (sh-Ngb), compared with that of negative

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control cells (transfected with the nonspecific plasmids). MTT assay revealed that knockdown of Ngb (sh-Ngb) significantly enhanced HepG2 cell viability (representing total cell number) in the culture, compared with that of corresponding negative controls (Fig. 2B), suggesting that the loss of Ngb promoted HCC cell growth. We further determined the effect of Ngb knockdown on tumor growth in vivo. Subcutaneous injection of parental HepG2/sh-Ngb cells (stably expressing sh-Ngb) developed much larger tumor xenograft in the nude mouse than did the tumor xenograft derived from same amounts of parental HepG2/N-con cells 30 days after cell injection (Fig. 2C). Statistical analysis revealed that the tumor volume derived from HepG2/sh-Ngb cells was significantly increased, compared with that derived from HepG2/negative control cells in the consecutive measurement for 30 days (Fig. 2D). The weight of tumor derived from HepG2/sh-Ngb cells was also significantly heavier than that derived from HepG2/ negative control cells (Fig. 2E). The reduction of Ngb in tumor xenograft derived from HepG2/sh-Ngb cells was confirmed by RT-PCR (Fig. 2E). These data revealed that Ngb down-regulation promoted HepG2 cell growth in vitro and in vivo.

**Overexpression of Ngb Suppresses HCC Cell Growth In Vitro and Tumor Growth In Vivo.** After establishing a causative relationship between the loss of Ngb and HCC cell growth, we asked whether increasing Ngb artificially might suppress HCC cell growth. HepG2 cells were stably transfected with p-Ngb-EGFP-N1 or p-EGFP-N1 plasmids, and the overexpression of Ngb in HepG2/Ngb cells (stably over-expressing Ngb) was verified by Western blot analysis (Fig. 3A) and RT-PCR (Fig. 3B). MTT assay revealed that over-expressing Ngb significantly reduced HepG2 cell viability (representing total cell number), compared with its corresponding vector control cells (GFP) along the 5 days of cell growth in the culture (Fig. 3C). We further investigated the effect of Ngb overexpression on anchorage-independent growth of HCC cells, a characteristic phenotype of malignant transformed cells in vitro. Colony-formation assay in soft agar showed that overexpressing Ngb evidently reduced HepG2 cell colonies, compared with its vector control (Fig. 3D). Statistical analysis revealed that the colony numbers derived from parental HepG2/Ngb cells (190.5 ± 40.3 colonies/dish) were significantly less than those derived from same amount of parental HepG2/vector cells (582.0 ± 113.8 colonies/dish) (Fig. 3E). The effect of Ngb overexpression on HepG2 tumor growth in vivo was further studied by subcutaneous injection of same amounts of HepG2/Ngb or HepG2/vector cells into either side of the same nude mouse. Four weeks later, the tumor derived from parental HepG2/Ngb cells was evidently smaller than that derived from parental HepG2/vector cells (Fig. 3F). The growth kinetic curve of HepG2/Ngb tumors was compared with that of HepG2/GFP tumors, which showed a significant reduced growth speed in HepG2/Ngb tumors (Fig. 3G). Finally, the weight of tumors derived from HepG2/Ngb cells was significantly decreased, compared with that derived from HepG2/GFP cells (Fig. 3H). The overexpression of Ngb in tumors derived from HepG2/Ngb cells was confirmed by Western blot analysis (Fig. 3I). These data revealed an inhibitory effect of Ngb on HCC cell growth in vitro and in vivo.

**Ngb Suppresses Proliferation and G1/G0-S Transition in HCC Cells.** Because Ngb controlled HCC cell growth, we further analyzed the functional role of Ngb in HepG2 cell

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Fig. 1. Ngb is down-regulated in human HCC. (A) Representative results of Western blot showing the specificity of Ngb antibody. 293T cells were transiently transfected with either pcDNA-Ngb or control vector for 2 days. Equal amount of total proteins extracted from transfected cells were subjected to Western blot analysis with anti-Ngb antibody, β-actin was used as a loading control. (B) Representative results of fluorescent cytoimmunostaining showed that the Ngb antibody recognized Ngb specifically. HepG2 cells were transiently transfected with either p-Ngb-EGFP or p-EGFP plasmids for 2 days. Cells in the cultures were fixed for fluorescent cytoimmunostaining with anti-Ngb antibody (red). Transfected cells were viewed by GFP fluorescence. (C) Results of immunohistochemical staining showed that Ngb was elevated in human lung cancer tissue but not in normal human lung tissue. (D) Representative results of immunohistochemical staining of Ngb in human HCC samples. The upper panels showed Ngb staining in normal human liver and HCC samples at lower magnifications (20×). The lower panels showed Ngb staining of another two HCC samples at higher magnifications (40×). (E) Statistical analysis of IHC results of Ngb staining in human HCC samples. The percentage of Ngb-positive stained tumor (T) was compared with that of Ngb-positive adjacent non-tumor tissues (ANT) and normal liver tissues (Nor). **p < 0.001, χ² test. (F) Conventional (inserted graphs) and quantitative PCR results of Ngb in human HCC samples. The expression levels of Ngb mRNA in HCC tumor tissues (T) and their adjacent non-tumor tissues (ANT) were measured quantitatively and normalized to that of GAPDH. The relative Ngb expression level (Ngb/GAPDH) in HCC tumor tissues (T) was significantly lower than that in adjacent non-tumor tissues (ANT). *p < 0.05, paired Student’s t test, n = 17.
division and cell cycle. EdU incorporation assay showed that overexpressing Ngb reduced EdU-positive stained cells (Fig. 4, A and B). The mean percentage of EdU-positive HepG2/Ngb cells was significantly reduced, compared with that of EdU-positive HepG2/GFP cells (Fig. 4B). On the contrary, knockdown of Ngb (sh-Ngb) significantly increased the percentage of EdU-positive HepG2 cells, compared with its control (Fig. 4, C and D). These data suggest that the inhibitory effect of Ngb on HCC cell growth was a direct inhibitory effect of Ngb on cell proliferation.

We then studied the effect of Ngb overexpression or knockdown on HepG2 cell cycle. Synchronized HepG2 cells were induced to re-enter the cell cycle by serum supplementation. FACS analysis showed that Ngb overexpression reduced the percentage of HepG2 cells in the S phase (38.4% versus 49.2% of control) and increased that of HepG2 cells in the G1/G phase (51% versus 41.1% of control) but did not alter the percentage of HepG2 cells in the G2/M phase after 12 hours of serum stimulation (Fig. 4E). Statistical analysis revealed that the percentage of HepG2/sh-Ngb cells in the S phase was significantly increased after 12 and 16 hours of serum stimulation, compared with that of corresponding negative controls (Fig. 4H). These data revealed that Ngb suppressed HepG2 cells entering into the S phase from the G1/G0 phase.

**Ngb Controls HCC Cell Proliferation via Regulating the Raf/MEK/Erk Signaling.** To understand the molecular mechanisms by which Ngb controlled HCC cell growth or proliferation, we investigated the effect of Ngb on several key signal pathways, which are closely related to the cellular proliferation of HCC cells (Huynh, 2004; Newell et al., 2009). Western blot analysis (Fig. 5A) showed that Ngb had a prominent effect on Raf/MEK/Erk signaling cascades but had no effect on GSK-3β or β-catenin signaling in HepG2 cells under normal culture conditions. Overexpression of Ngb evidently reduced the expression levels of p-Raf-1 (Ser338, p-MEK, and p-Erk Thr202/Tyr204 in HepG2 cells but did not alter total Erk and Raf-1 levels, compared with its GFP controls (Fig. 5A). On the contrary, knockdown of Ngb
(sh-Ngb) clearly increased the expression levels of p-Erk Thr202/Tyr204, p-MEK, and p-Raf-1 Ser338/Raf-1 but did not alter the expression levels of total Erk and Raf-1 in HepG2 cells, compared with its negative control (Fig. 5A). In HepG2 cell-derived tumor xenografts, activation of Erk was either enhanced by Ngb knockdown (Fig. 5B) or suppressed by Ngb overexpression (Fig. 5C), compared with their corresponding controls. To explore the possible mechanism by which Ngb regulates Raf/MEK/Erk cascade, the interaction of Ngb and c-Raf-1 was examined. GST pull-down assay showed that Ngb bound with c-Raf-1, and mutation of Ngb at its oxygen-bnding site (H64L) altered their interactions (Fig. 5D). Furthermore, we investigated the effects of Ngb on Erk signaling after serum stimulation (Fig. 6A) and H$_2$O$_2$ treatment (Fig. 6B), which mimic physiological and pathologic microenvironments. Western blot analysis showed that overexpression of Ngb suppressed Erk activation under both stimulants (Fig. 6, A and B). These in vitro and in vivo data together revealed that Ngb negatively regulated the oncogenic Raf-1/MEK/Erk signaling in HepG2 cells.

**Fig. 3.** Overexpression of Ngb suppressed HCC cell growth in vitro and in vivo. Representative Western blot (A) and RT-PCR (B) results showed the expression level of Ngb in HepG2 cells stably transfected with p-Ngb-GFP-N1 (Ngb) or p-EGFP-N1 (GFP). All experiments were repeated for least three times. The relative level of Ngb (Ngb/β-actin) in Ngb-stably transfected HepG2 cells (Ngb) was around 3-folds of that of GFP control cells. **P < 0.01 versus GFP control. (C) Results of MTT assay. Cell viability (representing total cell number) of HepG2/Ngb (Ngb) or HepG2/GFP (GFP) cells was determined by MTT assay at various time points (1, 2, 3, 4, and 5 days) after initial seeding (day 0) with same amount of cells in the cultures. *P < 0.05, **P < 0.01 versus corresponding GFP controls, n = 5. (D) Representative results of colony formation in soft agar derived from same amounts of parental HepG2/Ngb or HepG2/GFP cells. The experiments were repeated for three times. (E) Statistical analysis demonstrated that the mean number of HepG2/Ngb cell colonies (Ngb) was significantly reduced, compared with that of HepG2/GFP cell colonies (GFP). *P < 0.05, n = 3. (F) Representative tumor-bearing nude mouse and the xenografted tumor derived from either HepG2/Ngb cells (left side) or HepG2/GFP cells (right side). (G) Statistical analysis demonstrated that the tumor volume derived from parental HepG2/Ngb cells (Ngb) was significantly decreased, compared with that of parental HepG2/GFP cells (GFP). *P < 0.05; **P < 0.01 versus corresponding GFP controls, Mann-Whitney U test, n = 8. (H) Statistical analysis demonstrated that the tumor weight derived from HepG2/Ngb cells was significantly decreased, compared with that of HepG2/GFP controls. **P < 0.01, Mann-Whitney U test, n = 8. Inserted Western blot showed that Ngb was overexpressed in tumors derived from HepG2/Ngb cells.
Ngb Regulates HCC Cell Proliferation by Linking Oxygen/ROS Signals to Erk Signaling. Present evidence suggests that Ngb plays a major role in sensing oxygen or ROS signals (Trent et al., 2001; Herold et al., 2004; Burmester and Hankeln, 2009). Our data also showed that Ngb suppressed H₂O₂-induced Erk activation in HepG2 cells. To link the ability of Ngb in sensing oxygen/ROS signal directly to Erk signaling and HCC cell proliferation, the effect of Ngb mutants, including its oxygen/NO binding site (i.e., H64L) (Nienhaus et al., 2004; Vallone et al., 2004) and G-protein–binding site (i.e., E53Q and E118Q) (Wakasugi et al., 2003), on Erk activation and HCC cell proliferation were investigated. Western blot analysis showed that NgbH64L reversed the inhibitory effect of wild type Ngb on Erk activation, whereas mutation of NgbE53Q and NgbE118Q had no effect (Fig. 7, A and B). Consistently, mutation of Ngb at its oxygen/ROS-binding site but not G-protein–binding site abolished the inhibitory effect of Ngb WT on HepG2 cell proliferation (Fig. 7C). This evidence supported that Ngb controlled HCC proliferation by linking oxygen/ROS signals to Erk signaling cascades.

Discussion
In the present study, we reported that the loss of Ngb was associated to HCC development. We demonstrated a causative role of Ngb in HCC cell growth and proliferation in vitro and in vivo by the gain of function or knockdown of Ngb. Moreover, we identified a novel Ngb-regulated signaling pathway (i.e., Raf/MEK/Erk) that controls HCC cell proliferation. Furthermore,
our data suggest that Ngb is a molecular linker between oxygen/ROS signals and Raf/MEK/Erk cascade. Recently, Ngb was found to be up-regulated in many tumors (Emara et al., 2010); however, this up-regulation has been challenged by others (Gorr et al., 2011). Our finding that Ngb is expressed in normal liver tissues is consistent with the results of Gorr et al. (2011). However, we demonstrated that Ngb was significantly down-regulated in HCC tissues. The inconsistence of our data with those of Gorr et al. (2011) may be caused by the difference of human tissue samples and the amount of tissue samples used.

Hypoxia and oxidative stress are initial or stimulatory factors in the process of cancer genesis or development (Severi et al., 2010). However, the mechanisms underlying these processes are far from understood. At present, HIF1α is considered to be the major oxygen sensor that can certainly sense hypoxic signal and functions as a transcriptional factor in various tumor cells (Dewhirst et al., 2008). Ngb is the first hexa-coordinated oxygen-binding protein originally identified in mammalian brains and is considered to be a respiratory protein (Burmester et al., 2000). Although oxygen binds to Ngb with higher affinity, compared with hemoglobin, it is difficult for oxygen to be disassociated from Ngb (Trent et al., 2001). Therefore, it is unlikely for Ngb to function mainly as an oxygen supplier as hemoglobin or myoglobin does. Because Ngb has a native oxygen-binding property, it can serve as an intracellular oxygen sensor. Indeed, the expression of Ngb in brain cells is sensitive to severe ischemic or hypoxic conditions (Sun et al., 2001; Chen et al., 2005b). In tumor cells, the expression of Ngb could be either increased (Emara et al., 2010) or decreased (Oleksiewicz et al., 2011), suggesting a complicated regulation of Ngb in tumor cells. The increase of Ngb might represent the mechanism of hypoxic response of Ngb (Sun et al., 2001), and the decrease of Ngb might be caused by the hypermethylation of its promoter (Sun et al., 2001; Chen et al., 2005b). In tumor cells, the expression of Ngb could be either increased (Emara et al., 2010) or decreased (Oleksiewicz et al., 2011), suggesting a complicated regulation of Ngb in tumor cells. The increase of Ngb might represent the mechanism of hypoxic response of Ngb (Sun et al., 2001), and the decrease of Ngb might be caused by the hypermethylation of its promoter (Oleksiewicz et al., 2011). Hypermethylation of Cygb promoter, another hexa-coordinated member of hemoglobin families, is also found in several malignant tumors (Shivapurkar et al., 2008). The decrease of Ngb in HCC might be caused by the hypermethylation mechanism of its promoter during prolonged hypoxia. Further investigation of the status of Ngb promoter hypermethylation will elucidate the mechanism by which Ngb was down-regulated in HCC.

The altered expression of Ngb in tumor cells suggests a functional role of Ngb in cancer genesis or development; however, direct evidence demonstrating its function is

![Fig. 5. Ngb regulates Raf-I/MEK/Erk signaling and interacts with c-Raf-1. (A) Representative Western blot results showed that overexpression of Ngb decreased the expression levels of p-Erk 1/2, p-MEK, and p-Raf-1 Ser338, whereas knockdown of Ngb (sh-Ngb) increased their expression level, compared with corresponding controls. The expression levels of p-GSK-3β/Gsk-3β, p-β-catenin/β-catenin were not altered evidently. (B) Representative Western blot results showed that knocking down of Ngb (sh-Ngb) enhanced Erk activation in tumor xenografts derived from HepG2/sh-Ngb cells, compared with its control tumor xenografts (N-con) from different nude mice (No. 1 and 2). (C) Representative Western blot results showed that overexpression of Ngb (Ngb) suppressed Erk activation in tumor xenografts derived from HepG2/Ngb cells, compared with its control tumor xenografts (GFP) from different nude mice (No. 1 and 2). (D) Representative results of GST pull-down showed that Ngb bound to c-Raf-1. WCL, whole cell lysate. IB, immunobloting.](molpharm.aspetjournals.org)
missing. Here, we demonstrated that the decrease of Ngb promoted HCC cell growth (Fig. 2) and proliferation (Fig. 4, D and H), whereas the increase of Ngb inhibited HCC cell growth (Fig. 3) and proliferation (Fig. 4, B and F) in vitro and in vivo. These data clearly showed a negative causative relationship between the expression level of Ngb and HCC. Previous studies have shown a protective role of Ngb after ischemic death in primary cultures of neurons (Sun et al., 2001) and astrocytes (Chen et al., 2005b). However, administering Ngb directly to human neuronal SH-SY5Y cell line does not confer protection from oxygen and glucose deprivation (Peroni et al., 2007). These evidences indicate that the biologic functions of Ngb in distinct cellular contexts or tissues could be different. In malignant tumor cells, the energy metabolism pattern and key signaling transduction pathways are altered fundamentally as a result of chronic hypoxia or oxidative stress. It is likely that the pathologic functions of Ngb in malignant tumor cells are different form than in brain cells. Whether Ngb plays a specific inhibitory role in HCC cell growth or proliferation or has a similar function in other tumor cells deserves further investigations.

Ngb binds not only to oxygen but also to signaling proteins, such as the alpha subunit of heterotrimeric G protein (Gαi) (Wakasugi et al., 2003), 14-3-3 protein (Jayaraman et al., 2011), or cytochrome C (Raychaudhuri et al., 2010). The interaction of Ngb with Gαi/14-3-3/cytochrome C is controlled by hypoxic conditions (i.e., cellular oxygen signals), although the exact working model and function remain unclear. These evidences suggest that Ngb may function as a signaling molecule by coupling oxygen/ROS signals to intracellular signaling cascades. We found that Ngb interacted with c-Raf-1 and regulated the oncogenic Raf/MEK/Erk pathway in HCC in vitro and in vivo (Fig. 5). Blocking Erk signaling abolished the effect of Ngb knockdown on HCC cell proliferation (Fig. 6). Therefore, Raf/MEK/Erk was a downstream signaling pathway of Ngb in HCC cells. We have previously observed an early and robust activation of the Raf/MEK/Erk pathway in the primary cultures of cerebral cortical astrocytes but not in the primary cultures of cerebral cortical neurons after oxygen and glucose deprivation (Chen et al., 2005a). This suggests that the Raf/MEK/Erk signaling is not only sensitive to hypoxic signal but also specific to the cell type. Hypoxia is a common pathologic factor in tumor cells, and the Raf/MEK/Erk pathway is a well-known oncogenic proliferative signaling pathway in various tumors, including HCC (Huynh, 2004; Newell et al., 2009). The distal histidine of Ngb (i.e., His64) is the binding site of oxygen. In vitro studies reveal that Ngb mutation at this site alters its oxygen-binding affinity (Vallone et al., 2004), although its physiologic importance is unknown. We found that H64L mutation affected the binding of Ngb with c-Raf-1 (Fig. 5D) and abolished the inhibitory effects of NgbWT on Erk activation and HCC proliferation (Fig. 7), providing a direct link between oxygen sensing and Raf/MEK/Erk signaling. On the basis of these finding, we proposed a novel hypoxic signaling pathway in HCC cells (i.e., O2/Ngb/Raf/MEK/Erk) that links oxygen signal directly to the oncogenic signaling pathway. In addition, Ngb may regulate Raf/MEK/Erk activity via other proteins, such as 14-3-3 proteins (Zhao et al., 2011), suggesting a complicated regulatory machinery of Ngb on Raf/MEK/Erk cascades. It is known that p21-activated kinase can phosphorylate Raf-1 at Ser338 and regulates Raf-1 activity positively (King et al., 1998). It is
A recent study showed that Ngb overexpression could alter the spectrum of gene expression in neurons after ischemic conditions (Yu et al., 2009). We also reported previously that Ngb alters the expression level of 14-3-3-gamma protein in neuronal cells (Ye et al., 2009); 14-3-3 proteins are signaling hubs among intracellular signaling networks and are heavily involved in cell survival/death and growth/proliferation in tumor cells (Zhao et al., 2011). It is interesting to investigate whether Ngb may regulate gene expression in HCC cells as an oxidative stress sensor or scavenger to explore the role of Ngb in hepatocarcinogenesis. Further analyzing the relationships between Ngb expression and HCC clinical data (i.e., staging, grading, and survival time), together with other biomarkers, such as α-fetoprotein and HIF-1, is valuable to evaluate whether Ngb could be used as a prognosis-predicting biomarker. Because Ngb plays an important role in hypoxia/ROS-mediated signaling, which is essential for HCC development, manipulating Ngb expression or targeting to Ngb-mediated signaling might provide novel therapeutic approaches for advanced HCC.

In summary, we found that the loss of Ngb was associated with HCC development via suppression of Raf/MEK/Erk. Our data, for the first time to our knowledge, suggest that Ngb, an intracellular respiratory protein, might function as a tumor suppressor in HCC by coupling oxygen/ROS signals to the oncogenic Raf/MEK/Erk signaling.

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Neuroglobin Functions as a Tumor Suppressor


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