Carcinogenesis vol.31 no.8 pp.1367–1375, 2010
doi:10.1093/carcin/bgq116
Advance Access publication June 4, 2010

Hypoxia inducible factor-1 mediates expression of galectin-1: the potential role in migration/invasion of colorectal cancer cells

Xu-Yun Zhao[1,†], Ting-Ting Chen[1,†], Li Xia[1], Meng Guo[1], Ying Xu[2], Fei Yue[3], Yi Jiang[1], Guo-Qiang Chen[1,2] and Ke-Wen Zhao[1,*]

[1]Department of Pathophysiology, Key Laboratory of Cell Differentiation and Apoptosis of Ministry of Education of China and Chemical Biology Division of Shanghai Universities E-Institutes, Ruijin Hospital, Shanghai Jiao-Tong University School of Medicine, Shanghai 200025, China, [2]Institute of Health Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences/Shanghai Jiao-Tong University School of Medicine, Shanghai 200025, China and [3]Department of General Surgery, Ruijin Hospital, Shanghai Jiao-Tong University School of Medicine, Shanghai 200025, China

*To whom correspondence should be addressed. Department of Pathophysiology, Shanghai Jiao-Tong University School of Medicine, No.280, Chong-Qing South Road, Shanghai 200025, China.
Tel/Fax: +86 21 64154900;
Email: zkewen@shsmu.edu.cn
Correspondence may also be addressed to Guo-Qiang Chen.
Tel/Fax: +86 21 64154900;
Email: chengq@shsmu.edu.cn

The expression of galectin-1, one of the most important lectins participating in the malignant tumor development, has been shown to be regulated by hypoxia, but its exact mechanism remains elusive. Here, we find that ectopically expressed hypoxia-inducible factor (HIF) 1α protein, an oxygen-sensitive subunit of HIF-1 that is a master factor for cellular response to hypoxia, significantly increases galectin-1 expression in both messenger RNA and protein levels in all four colorectal cancer (CRC) cell lines tested. However, hypoxia-induced galectin-1 expression cannot be seen in sentrin/SUMO-specific protease 1 homozygous-null mouse embryonic fibroblasts that fail to accumulate HIF-1α protein. Furthermore, silence of HIF-1α or HIF-1β expression by specific short hairpin RNAs (shRNAs) antagonizes hypoxia-induced galectin-1 expression. All these results propose that galectin-1 is a direct target of transcriptional factor HIF-1. Applying luciferase reporter assay and chromatin immunoprecipitation, we identify that two hypoxia-responsive elements located at −441 to −423 bp upstream to transcriptional start site of *galectin-1* gene are essential for HIF-1-mediated galectin-1 expression. Finally, the knockdown of galectin-1 by its specific shRNA can significantly reduce hypoxia-induced invasion and migration of CRC cell line, and the ectopic expression of galectin-1 can remarkably restore invasion and migration abilities of HIF-1α-knocked SW620 cells, proposing that galectin-1 mediates the HIF-1-induced migration and invasion of CRC cells during hypoxia. Taken together, our results shed new light for understanding mechanism for hypoxia/HIF-1-mediated migration/invasion of CRC cells.

Introduction

Tumor hypoxia, mostly resulting from poor perfusion and anemia, is one of the key factors to induce the development of malignant cell clones with an aggressive and also treatment-resistant phenotype that leads to rapid progression and poor prognosis (1,2). It has been well known that the adaptive response of cell to hypoxia is mainly mediated by hypoxia-inducible factor (HIF)-1, a transcriptional heterodimer consisting of an oxygen-sensitive HIF-1α and constitutively expressed HIF-1β/aryl hydrocarbon nuclear translocator (3,4). In the normal air (normoxia), HIF-1α is rapidly degraded by E3 ligase von Hippel-Lindau tumor suppressor-mediated ubiquitin–proteasome pathway. However, the reduced oxygen availability (hypoxia) or treatment of hypoxia-mimetic agents such as cobalt chloride can stabilize HIF-1α protein, followed by its translocation into nucleus where it forms heterodimer with its partner HIF-1β (4–7). The HIF-1α/HIF-1β heterodimer binds to consensus sequence 5′-RCGTG-3′ named hypoxia-responsive elements (HREs) on promoters of its target genes such as vascular endothelial growth factor (VEGF) and glucose transporter 1, which participate in angiogenesis, erythropoiesis, energy metabolism, cell proliferation, survival and/or differentiation (4,8,9). HIF-1 and hypoxia was also shown to contribute to metastasis and invasion of cancers including colorectal cancer (CRC) (10–14). Some HIF-1-targeted genes promoting extracellular matrix remodeling and penetration of cancer cells through the basement membrane, such as autocrine motility factor, vimentin, fibronectin and matrix metalloproteinase 2, have been identified (4,15). But the mechanism about how HIF-1 mediates tumor progression and invasion remains to be further explored. In this work, we report that galectin-1, a 14 kDa ubiquitously expressed member of mammalian lectin family (16) and one of the most important lectins participating in the malignant tumor development (17), is a direct target gene of HIF-1, and the silence of galectin-1 expression by its specific short hairpin RNA (shRNA) can inhibit hypoxia-induced invasion and migration of CRC cells. Furthermore, enhancing the expression of galectin-1 can restore the invasion and migration abilities of CRC cells that is inhibited by shRNA against HIF-1α under hypoxia. These results would shed new light for understanding mechanism for hypoxia/HIF-1-mediated migration/invasion of CRC cells.

Abbreviations: CRC, colorectal cancer; FBS, fetal bovine serum; Glut-1, glucose transporter 1; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; mRNA, messenger RNA; MEF, mouse embryonic fibroblast; NC, negative control; SENP1, SUMO-specific protease 1; shRNA, short hairpin RNA; VEGF, vascular endothelial growth factor.

[†]These authors contributed equally to this work.

Materials and methods

Tissue samples and immunohistochemistry

Paraffin-embedded tumor tissues and normal adjacent tissues from 40 cases of CRC were collected from Ruijin hospital of Shanghai Jiao Tong University School of Medicine (SJTU-SM). The cancer stages were performed according to tumor, lymph node, metastasis classification. The immunohistochemical analysis was performed on the 4 μm thick fraction mounted on charged slides and sectioned from each clinical sample. Then, each slide was deparaffinized in 60°C, followed by treatment with xylene and graded alcohol. After the antigen retrieval and being blocked with 5% bovine serum albumin, tissue slides were immunohistochemically stained by antibodies against galectin-1 (Santa Cruz Biotechnology, Santa Cruz, CA), vascular endothelial growth factor (VEGF) and glucose transporter 1 (Glut-1) (Abcam, Cambridge, UK), respectively, then visualized by standard avidin-biotinylated peroxidase complex method. Then, hematoxylin was used for counterstaining and morphologic images were observed with Olympus BX51 microscope. All sections were evaluated systematically for galectin-1, VEGF and Glut-1 proteins according to a scale of three stages as described (18): grade 0, <10% immunoreactive cells; grade 1, 10–50% immunoreactive cells and grade 2, >50% immunoreactive cells. Samples in grade 0 were considered as negative expression and ones in grade 1 and 2 as positive expression.

Cell culture and treatment

CRC cell line LS174T, RKO, SW1116 and SW620 were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium (Sigma–Aldrich, St Louis, MI) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD). Mouse embryonic fibroblast (MEF) cells from sentrin/SUMO-specific protease 1 (SENP1) homozygous null (*SENP1*$^{-/-}$) and wild-type (*SENP1*$^{+/+}$) mice, which was provided by Dr J.K.Cheng in SJTU-SM, were cultured in Dulbecco's modified Eagle's medium with 10% FBS. All cell lines were cultured in 5% CO_2 and 95% air in a humidified atmosphere at 37°C. For hypoxic

© The Author 2010. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org

Downloaded from https://academic.oup.com/carcin/article/31/8/1367/2477336 by guest on 21 August 2022

exposure, cells were cultured in a specially designed hypoxia incubator (Thermo Electron, Forma, MA) in an atmosphere that consists of 94% N_2, 5% CO_2 and 1% O_2.

Quantitative real-time reverse transcription–polymerase chain reaction

Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Promega, Madison, WI). Reverse transcription was performed with TaKaRa RNA PCR kit (TaKaRa, Dalian, China). The double-stranded DNA dye SYBR Green PCR Master Mixture Reagents (Applied Biosystems, Warrington, UK) was used for quantitative real-time reverse transcription–polymerase chain reaction (PCR) analysis as described previously (9). The following specific primers used were 5′-CGCTAAGAGCTTCGTGCTGAAC-3′ (forward) and 5′-CACACCTCTGCAACACTTCCAG-3′(reverse) for galectin-1, 5′-TGATTGCATCTCCATCTCCTACC-3′ (forward) and 5′-GACTCAAAGCGACAGATAACACG-3′ (reverse) for HIF-1α, 5′-CAGTGAAAAAGGAAGGTCAGCA-3′ (forward) and 5′-CAAGTCCATTCCTGCATCTGTT-3′ (reverse) for HIF-1β and 5′-CATCCTCACCCTGAAGTACCC-3′ (forward) and 5′-AGCCTGGATAGCAACGTACATG-3′ (reverse) for β-actin as control. The folds of changes were shown as means ± SDs in three independent experiments with each triplicate.

shRNA design and transfection

Pairs of complementary oligonucleotides against galectin-1 were synthesized, annealed and ligated into pSIREN-RetroQ according to the manufacturer's instruction (Clontech, Mountain View, CA). The target sequence for galectin-1 was 5′-AACCTGTGCCTGCACTTCAAC-3′ and the sequence for HIF-1α and HIF-1β has been described previously (7). These target shRNAs and negative control (NC) shRNA-containing plasmids were transfected by retrovirus into SW620 cells. The viral supernatant was packaged in 293T cell by cotransfecting with pSIREN-RetroQ, pEQPAM (containing gag-pol, produced by Dr Lishan Su in University of North Carolina Chapel Hill, USA) and VSVG (Clontech). After transfection for 48 h, the viral supernatant was collected, filter-sterilized and added to SW620 cells (2×10^5 cells per well) in six-well plate containing polybrene with a final concentration of 4 μg/ml and puromycin (1.5 μg/ml) was added to select the stably transfected cells after another 48 h. SW620 cells with shRNA against galectin-1 and NC shRNA were named as SW620-sh-G8 and SW620-NC, respectively.

Plasmids and transfection

Human galectin-1 complementary DNA was amplified from total complementary DNA of SW620 cells with primers 5′-AGCGGATCCATGGCTTGTGGTCTGGTC-3′ and 5′-TATAAGCTTTCAGTCAAAGGCCACACA-3′. Its wobble mutant (called galectin-1^m), which bears quadruple-point mutation in the 19 bp target sequence of sh-G8 shRNA as described above, was produced by site-directed mutagenesis kit (Stratagene, La Jolla, CA) as the manufacturer's instructions. These sequences were inserted, respectively, into pcDNA3.1(−) expression vector. Transfections were performed by using FuGENE 6 Tranfection Reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. For stable transfection, the empty vector or pcDNA3.1(−)-galectin-1 plasmid were transfected into SW620 cells with stable expression of shRNA against HIF-1α (shR-α14 or shR-α16) or NC shRNA. Twenty-four hours after transfection, 1.8 mg/ml G418 (Calbiochem, San Diego, CA) was added to select the stable cell line, and the stable transformants with the expression of galectin-1 were confirmed by western blot.

Luciferase assay

The indicated sequences in promoter of *galectin-1* were obtained from National Center for Biotechnology Information, amplified by PCR from genomic DNA and subcloned into pGL3-Basic (Promega) to construct luciferase reporter plasmids. The site-directed mutagenesis kit (Stratagene) was used for the point mutations of the sequence of the reporter luciferase plasmids, as described by the manufacturer's instructions. For the luciferase assay, 293T cells were seeded in a 12-well plate (Becton Dickinson, Franklin lakes, NJ), and cotransfected with pEF-BOS-HIF-1β and pEF-BOS-HIF-1α (kind gifts from Dr K.Sogawa in Tohoku University, Japan), luciferase reporter plasmids driven by promoter fragments of *galectin-1* and pRLSV40-Renilla. After 36 h in normoxic or hypoxic conditions, cells were lysed and analyzed by the Dual-Luciferase Assay system according to the manufacturer's instructions (Promega).

Chromatin immunoprecipitation

After grown under normoxia and hypoxia for 24 h, SW620 cells were cross-linked with 1% formaldehyde at room temperature for 10 min, and cells were pelleted and resuspended in 400 μl lysis buffer (1% sodium dodecyl sulphate, 10 mM ethylenediaminetetraacetic acid, 50 mM Tris–HCl, pH 8.0). Then DNA of the cells was sonicated and sheared to small fragments of 500–1000 bp with Sonicator ultrasonic processor (Misonix, Farmingdale, NY). Subsequently, the supernatant of the sonicated cells was collected, diluted and precleared by protein A agarose (Santa Cruz Biotechnology). Furthermore, anti-human HIF-1α monoclonal antibody (BD Transduction Laboratories, Lexington, KY) was added to the supernatant for immunoprecipitation with normal pre-immuned mouse IgG (Santa Cruz Biotechnology) as a normal control. After overnight incubation, the protein A agarose were added and incubated for 3 h and then washed with low-salt, high-salt and LiCl buffers and the immunoprecipitated DNA was retrieved by 5 M NaCl at 65°C for 4 h and purified with a PCR purification kit (TaKaRa). PCR for the HREs in the promotor was performed with specific primers: 5′-GCATGCCCCTCATTTTGC-3′ (forward, P1) and 5′-CGGAGAAGTGCCTGGCTTT-3′ (reverse, P2) and 5′-CCCAGCCTTTCTTTAGCCTTCC-3′ (forward, P3) and 5′-GATGATGAGCTAGGCCCACAAG-3′ (reverse, P4).

Western blot

Cell extracts were prepared by using the following lysis buffer (4% sodium dodecyl sulphate, 20% glycerol, 100 mM dithiothreitol, Tris–HCl, pH 6.8). Twenty micrograms of cell lysates were loaded and separated by 10 or 15% sodium dodecyl sulphate–polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membrane (Bio-Rad, Richmond, CA). Then, 5% nonfat milk in Tris-buffered saline was used to block the membrane and immunoblotted with antibodies against HIF-1α, HIF-1β, E-cadherin (BD Transduction Laboratories), galectin-1 (Santa Cruz Biotechnology) and vimentin (Proteintech Group, Chicago, IL) together with β-tubulin as the internal control. Followed by horseradish peroxidase-linked second antibody (Cell signaling Technology, Beverly, MA) for 1 h at room temperature, detection was performed by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Scratch-wound assay

SW620 cells as indicated were seeded to form a monolayer on six-well plate surface; then, cells were rinsed and cultured in RPMI-1640 medium without FBS and a wound was made by scratching the cells in a line with a sterile pipette tip in the middle of the plate. After that, a photograph was taken immediately by Olympus BX51 fluorescence microscope, and the wound distance was calculated as a basic width. After 24 h of normoxic or hypoxic treatment, cells were washed three times by phosphate-buffered saline and another photograph was taken and the width of the wound distance was calculated. The wound closure (%) was determined as the width migrated after 24 h relative to the basic width.

Matrigel transwell invasion assay

Indicated SW620 cells were harvested and resuspended in RPMI-1640 medium without FBS and then, 5×10^5 cells were added in triplicates to the upper compartment of the prehydrated Matrigel-coated invasion chambers (Becton Dickinson Labware, Bedford, MA). The lower compartment of the invasion chamber was filled with RPMI-1640 medium containing 10% FBS as a chemoattractant. After 24 h of hypoxic or normoxic treatment, the upper surface of the membrane was scrubbed with moistened cotton swabs to remove Matrigel and non-invading cells. Then, the lower surface of the membrane were fixed by 100% methanol and stained with Wright–Giemsa staining. The numbers of cells invaded through the Matrigel layer was counted in five random fields with Olympus BX51 microscope and photographed with SPOT digital camera. The folds of invasive cells were calculated as the number of cells stained in lower surface treated in hypoxia against those in normoxia.

Statistical analysis

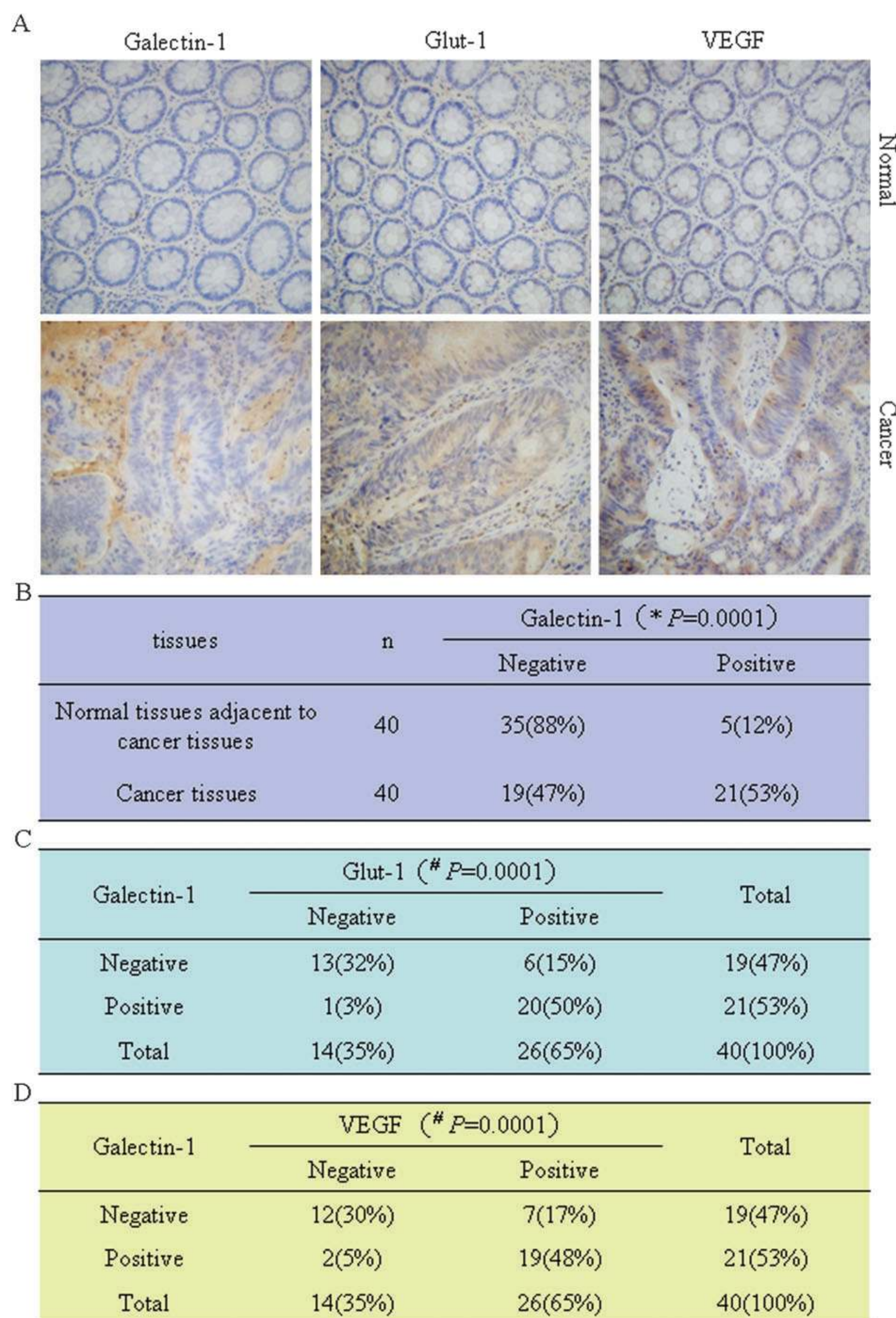
The results of immunohistochemistry were analyzed with Spearman's rank correlation and Pearson's chi-square test by SAS Enterprise software (SAS Institute, Cary, NC) as indicated. All experiments were repeated at least three times with the similar results. The values were expressed as mean ± SD. The paired *t*-test was used for statistical analysis between two groups. Significant level was set at $P < 0.05$.

Results

CRC tissues present higher expression of galectin-1 that can be induced by hypoxia

As well reviewed by Demydenko and Berest (19), galectin-1 expression is upregulated in tumors of different origin, such as prostate, lung, breast cancers, CRC and others. With the immunohistochemical analysis of tumor tissues and normal adjacent tissues from 40 cases of CRC, here we also showed that there was a significant increase of galectin-1 protein in CRC tissues compared with normal tissues adjacent to cancer tissues (Figure 1A and B). Furthermore, the potential correlations of galectin-1 expression with some clinicopathological

Downloaded from https://academic.oup.com/carcin/article/31/8/1367/2477336 by guest on 21 August 2022



B

tissues	n	Galectin-1 (* P=0.0001)	
		Negative	Positive
Normal tissues adjacent to cancer tissues	40	35(88%)	5(12%)
Cancer tissues	40	19(47%)	21(53%)

C

Galectin-1	Glut-1 (# P=0.0001)		Total
	Negative	Positive	
Negative	13(32%)	6(15%)	19(47%)
Positive	1(3%)	20(50%)	21(53%)
Total	14(35%)	26(65%)	40(100%)

D

Galectin-1	VEGF (# P=0.0001)		Total
	Negative	Positive	
Negative	12(30%)	7(17%)	19(47%)
Positive	2(5%)	19(48%)	21(53%)
Total	14(35%)	26(65%)	40(100%)

Fig. 1. Increased galectin-1 expression and its correlation with the expression of Glut-1/VEGF in CRC tissue. (**A**) Morphologic illustrations (×200) of representative immunohistochemical stainings of galectin-1, Glut-1 and VEGF in normal tissues adjacent to CRC and CRC tissues. (**B**–**D**) The expressions of galectin-1, Glut-1 and VEGF in normal and cancer tissues from CRC patients were assessed by the immunohistochemical staining described in Materials and Methods. The numbers in brackets represent the percentages of the cases related to the total number of patient samples. The symbol * indicates *P* value calculated by Pearson's chi-square test to evaluate the expression of galectin-1 between normal and tumor tissues (B). The symbol # indicates *P* value calculated by Spearman's rank correlation test to evaluate the correlation between the expression of galectin-1 and Glut-1 (C) or VEGF (D).

parameters were evaluated in these patients. The results showed that expression of galectin-1 was related to tumor invasion and lymph node involvement but not tumor location, tumor cell differentiation as well as sex and age of patients (supplementary Table I is available at *Carcinogenesis* Online), suggesting that galectin-1 expression is concerned with progression of CRC. Because hypoxia is regarded as a common environment for solid tumors, we asked whether the increased galectin-1 is related to hypoxia. Moreover, four representative CRC cell lines SW620, LS174T, SW1116 and RKO were incubated with 1% O_2 for 24 h, and the galectin-1 messenger RNA (mRNA) and protein were tested by quantitative real-time PCR and western blot, respectively. The results showed that both mRNA and protein of galectin-1 were significantly upregulated by hypoxia (1% O_2) in all four CRC cell lines (Figure 2A), which was time dependent (Figure 2B).

HIF-1 mediates hypoxia-induced galectin-1 expression

HIF-1 is a master factor for cellular adaptive response to hypoxia (20). Indeed, HIF-1α protein could be clearly detected under hypoxia in all four CRC cell lines (Figure 2). It deserved to point out that a protein

Downloaded from https://academic.oup.com/carcin/article/31/8/1367/2477336 by guest on 21 August 2022

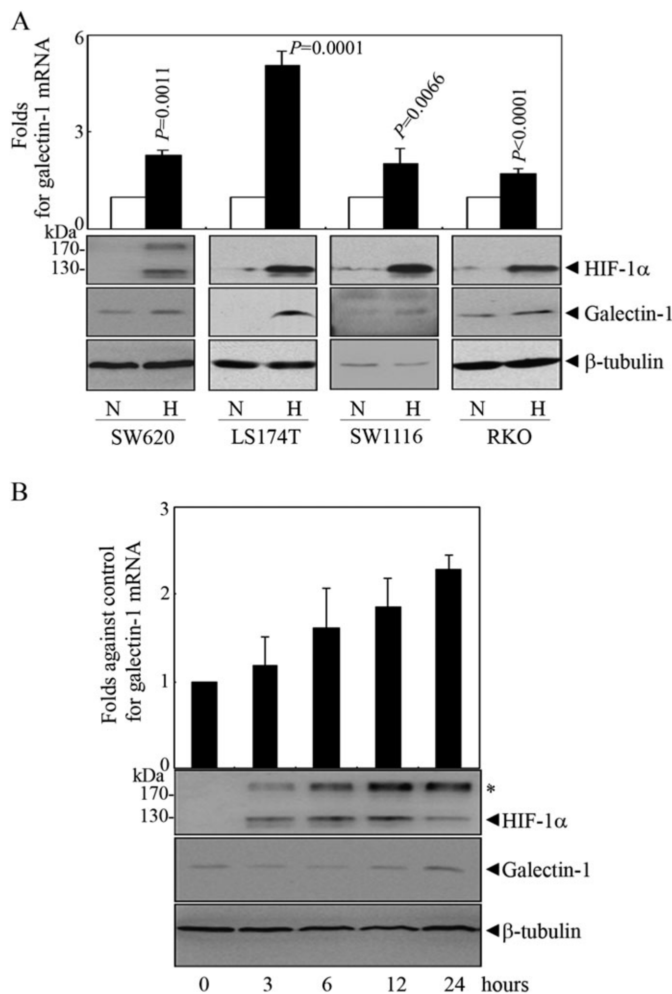


Fig. 2. Hypoxia induces mRNA and protein expression of galectin-1 in CRC cell lines. CRC cell lines as indicated were incubated in hypoxia (1% O_2) for 24 h (**A**) and SW620 cells were incubated in hypoxia for the indicated hours (**B**). Then, mRNA and protein levels of galectin-1 were detected, respectively, by quantitative real-time reverse transcription–PCR and western blot. N and H represent normoxia and hypoxia, respectively. All values were expressed as mean with bar as SD of three independent experiments. The *P* values were measured between cells under hypoxia and normoxia.

band with ~170 kDa was also induced by hypoxia in SW620 cell line. We extrapolated that the band might be a modified HIF-1α because the band could also be knocked down by shRNAs specifically against HIF-1α (Figure 3). In immunoprecipitation experiment, however, the antibody against HIF-1α that could effectively pull down HIF-1α failed to precipitate the 170 kDa protein (data not shown). Therefore, the band remains to be further investigated. According to the immunohistochemical analysis (Figure 1A, C and D), more intriguingly, the galectin-1 expression was highly correlated with the expression of VEGF and Glut-1, two known HIF-1α target proteins (8), in CRC tissue samples. To figure out whether HIF-1 also mediates hypoxia-related increase of galectin-1, HIF-1α-expressing plasmid was transiently transfected into SW620 and SW1116 cell lines. The transfection expressed detectable HIF-1α protein under normal air (Figure 3A). Notably, ectopic expression of HIF-1α could directly upregulate galectin-1 expression in these two cell lines (Figure 3A).

A recent report showed that SUMOylation can serve as a direct signal for ubiquitin-dependent degradation of HIF-1α protein, and SENP1 regulates HIF-1α stability through de-SUMOylation under hypoxia (21). Consistent with this, HIF-1α protein was undetectable even under hypoxia in *SENP1*$^{-/-}$ MEF cells (Figure 3B). Expectedly, hypoxia could induce galectin-1 expression in wild-type but not *SENP1*$^{-/-}$ MEF cells (Figure 3B). On the other hand, we stably transfected shRNAs specifically against HIF-1α (shR-α14 and shR-α16) with a scrambled NC shRNA into SW620 cell line. Consistent with our previous report (7), shR-α14/shR-α16 but not NC shRNA significantly suppressed HIF-1α but not HIF-1β mRNA and protein under hypoxia (Figure 3C). Accordingly, the knockdown of HIF-1α also suppressed hypoxia-induced galectin-1 expression in its mRNA and protein levels (Figure 3C). Although the expression of HIF-1α could be hardly detected under normoxia, notably, the basic expression level of galectin-1 could be attenuated by introducing shRNAs against HIF-1α (Figure 3C).

Considering that HIF-1α must form heterodimer with HIF-1β to exert its transcriptional activity (3), we also stably transfected shRNAs (shR-β2 and shR-β5) specifically against HIF-1β with an NC shRNA into SW620 cell line. As depicted in Figure 3D, these two shRNAs effectively suppressed HIF-1β but not HIF-1α expression, suggesting the specificity of these shRNAs for HIF-1β expression. The inhibition of HIF-1β expression significantly inhibited hypoxia-induced mRNA expression of VEGF, Glut-1 and PGK-1 (data not shown), three known HIF-1-targeted genes (22). Like that seen in HIF-1α-knockeddown cells, the suppression of HIF-1β also inhibited hypoxia-induced upregulation of galectin-1 mRNA and protein (Figure 3D). Totally, all these results strongly supported that HIF-1α mediates hypoxia-induced galectin-1 expression through its transcriptional activity.

Two HREs between −441 and −423 bp in the promoter of galectin-1 are required for HIF-1-driven galectin-1 expression

Bioinformatic analysis did show seven potential HREs within 2.2 kb regions upstream the transcriptional start site of *galectin-1* (Figure 4A). Then, we subcloned the DNA fragment carrying these seven potential HREs into a luciferase report vector pGL3-basic (upper panel, Figure 4A), which was transfected together with Renilla as the internal control into 293T cell line for the convenience of transfection. Twelve hours later, these cells were treated under normoxia or hypoxia for additional 24 h. The results revealed that hypoxia dramatically increased the luciferase activity (lower panel, Figure 4A). By the way, the ectopic expression of HIF-1α protein also increased the luciferase activity in a dose-dependent manner (Figure 4B). These data suggested that the fragment of *galectin-1* promoter does respond to HIF-1α protein. Thereafter, we tried to identify which HREs is indispensable for the HIF-1-mediated galectin-1 expression. For this purpose, luciferase reporter plasmids driven by fragments of *galectin-1* promoter containing HREs as indicated (Figure 4C) were transfected into 293T cells together with HIF-1α expressing plasmid. As shown in Figure 4C, only HRE1 at −427 to −423 and HRE2 at −441 to −437 could drive luciferase expression as strongly as that by the fragment containing all these seven potential HREs. Additionally, mutation (CG→AA) of HRE1 or/and HRE2 significantly damaged their activities (Figure 4C). These data proposed that two HREs between −441 and −423 (HRE1 and HRE2) in the promoter of *galectin-1* are required for HIF-1-driven galectin-1 expression. This conclusion could be consolidated by chromatin immunoprecipitation assay in hypoxia-incubated SW620 CRC cells. As shown in Figure 4D, anti-HIF-1α antibody but not normal IgG could precipitate HREs at −441 to −437 and −427 to −423 but not HRE7 at −2021 to −2017.

Galectin-1 mediates the HIF-1-induced migration and invasion of CRC cells during hypoxia

To understand the potential role of HIF-1-driven galectin-1 expression in CRC progression, SW620, a highly metastatic CRC line, was stably transfected with shRNA specifically against galectin-1 (sh-G8), which remarkably suppressed the expression of galectin-1 both in normal air and hypoxia (Figure 5A). Then, *in vitro* Matrigel invasion assay and scratch-wound assay were performed in

Downloaded from https://academic.oup.com/carcin/article/31/8/1367/2477336 by guest on 21 August 2022

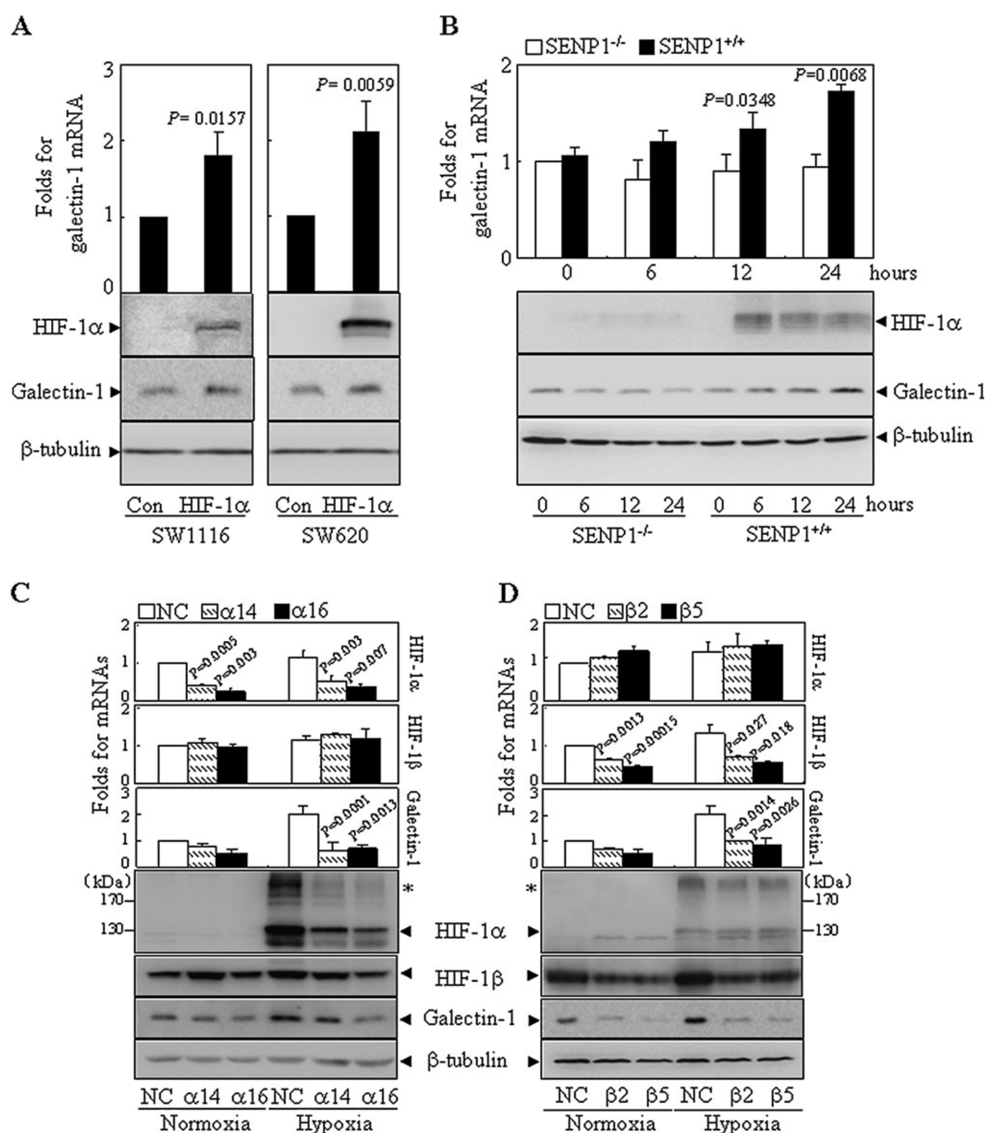


Fig. 3. Galectin-1 is a direct target of HIF-1 protein. (**A**) SW1116 and SW620 cell line were transiently transfected with either empty vector (Con) or pEF-BOS-HIF-1α (HIF-1α) for 24 h. (**B**) *SENP1*$^{-/-}$ and *SENP1*$^{+/+}$ MEF cells were treated under hypoxia for times as indicated. (**C** and **D**) SW620 cells that were stably transfected with shRNAs against HIF-1α (α14 and α16) (C) or against HIF-1β (β2 and β5) (D) or NC shRNA were exposed to normal air or hypoxia (1% O_2) for 24 h. The indicated mRNA and proteins were analyzed, respectively, by quantitative real-time reverse transcription–PCR and western blot. The column represents mean with bar as SD of three independent experiments with triplicate samples. The *P* values were calculated by compared with empty vector-transfected cells (A), untreated cells (B) and NC-transfected cells under hypoxia (C and D).

sh-G8-expressing SW620 cells with NC-transfected SW620 cells as control. The results revealed that suppression of galectin-1 expression by sh-G8 shRNA could significantly block hypoxia-induced invasion of SW620 cells through the Matrigel (Figure 5B and C). Similarly, the knockdown of gelactin-1 also inhibited hypoxia-induced migration of SW620 cells, as assessed by the scratch-wound assay (Figure 5D and E).

To further verify the effects of galectin-1 shRNA are specific, a wobble mutant galectin-1^{m} was transfected into SW620-sh-G8 cells. As depicted in supplementary Figure S1A (available at *Carcinogenesis* Online), galectin-1 could be clearly seen in the galectin-1^{m}-transfected cells in spite of galectin-1 shRNA expression, proposing the non-response of the galectin-1^{m} to sh-G8 and the specificity of sh-G8 to galectin-1. It was worth noting that hypoxia treatment also increased galectin-1^{m} protein. Re-expression of galectin-1 could remarkably restore the invasion (supplementary Figure S1B and C is available at *Carcinogenesis* Online) and migration (supplementary Figure S1D and E is available at *Carcinogenesis* Online) abilities of SW620-sh-G8 cells both under normoxia and hypoxia. Finally, SW620 cell line with stable expression of shR-α14, shR-α16 or NC shRNA as described above were stably transfected with galectin-1 expression plasmid and empty vector, respectively (Figure 6A). Consistent with previous reports (23–25), the inhibition of HIF-1α expression could modulate some downstream target genes that contribute to extracellular remodeling to mediate migration, such as downregulation of vimentin and up-regulation of E-cadherin (Figure 6). Notably, the silence of HIF-1α expression by shRNA significantly blocked invasion and migration abilities of SW620 cells under hypoxia as well as normoxia, which could be remarkably restored by the expression of galectin-1 (Figure 6B and C; supplementary Figure S2 is available at *Carcinogenesis* Online).

Downloaded from https://academic.oup.com/carcin/article/31/8/1367/2477336 by guest on 21 August 2022

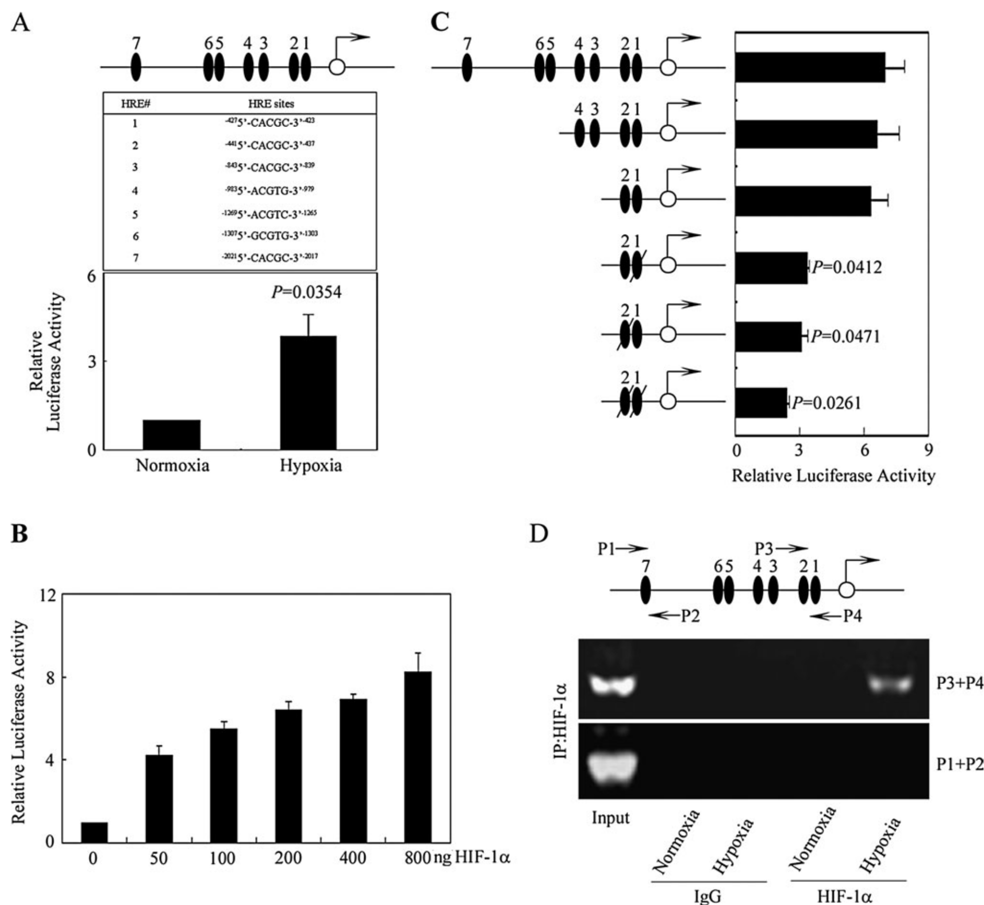


Fig. 4. Two HREs in promoter of *galectin-1* are essential for HIF-1α transactivity. (**A**) 293T cells were transfected with luciferase reporter plasmids driven by seven putative HREs in *galectin-1* promoter, which are shown on the top and middle panels, and were grown in the normal air or hypoxia for 24 h. In top diagram of putative HREs (black ovals), empty circles represent the transcriptional start point of *galectin-1*. (**B**) 293T cells were transfected with luciferase reporter plasmids driven by seven putative HREs in *galectin-1* promoter together with the indicated doses of HIF-1α and grown in the normal air for 36 h. (**C**) Luciferase reporter plasmids driven by the HREs or CG→AA mutated HRE sequences as indicated were transfected together with HIF-1α expressing vector or empty vector into 293T cells for 36 h in normal air. All the relative luciferase activities of *galectin-1* promoter were normalized by pSV40-Renilla and estimated as the relative folds against cells under normal air (A, lower panel) or empty vector-transfected cells (B and C). The *P* values were calculated between hypoxia- and normoxia-treated cells (A) or compared with full-length of *galectin-1* promoter driven luciferase plasmid transfected cells (C). All values represented the means with bar as SD of three independent experiments. (**D**) SW620 cells were grown under normoxia and hypoxia for 24 h. Chromatin immunoprecipitation assay was performed as described in Materials and Methods.

Discussion

Galectin-1 is a hypoxia-inducible β-galactoside-binding mammalian lectin produced by vascular, interstitial, epithelial and immune cells. Case *et al.* (26) reported that galectin-1 mRNA was regulated in lung mesenchyme by acute hypoxia at the transcriptional level *in vitro* and in protein level *in vivo*. The hypoxic regulation of galectin-1 at mRNA and protein levels has also been demonstrated in tumor biology and galectin-1 expression has been used as a prognostic marker of malignancy (19,27–29). For instance, galectin-1 presented higher expression in mild dysplasia, severe dysplasia and invasive carcinomas of the colon than in normal cases (30). Moreover, patients with galectin-1-positive colon tumors had shorter survival periods than those with galectin-1-negative colon tumors on the same stages (31). Here, we also showed that CRC tissues presented higher galectin-1 expression compared with the normal tissues adjacent to cancer. However, it is unclear how hypoxia increases galectin-1 expression. In this work, we provided evidence that galectin-1 is a direct target gene of HIF-1: Firstly, hypoxia-increased and ectopically expressed HIF-1α protein significantly increased galectin-1 expression in both mRNA and protein levels in all four CRC cell lines tested. Secondly, hypoxia-induced galectin-1 expression could not be seen in *SENP1*$^{-/-}$ MEF cells, in which HIF-1α protein could not be detected because of the failure of de-SUMOylation of HIF-1α protein (21). Thirdly, silence of HIF-1α expression by specific shRNA antagonized hypoxia-induced galectin-1 expression. Recent works showed that besides its transcriptional activity in the heterodimeric form with HIF-1β subunit, HIF-1α can also exert its biological roles through its interaction with some transcriptional factors such as Myc, CCAAT/enhancer binding protein-alpha, Runx1 and others (32–35). Therefore, we also tested whether HIF-1α-mediated galectin-1 expression is its transcriptional activity dependent. Our results showed that the suppression of HIF-1β by shRNA could also blocked hypoxia-induced expressions of galectin-1, indicating the dependence of transcriptional activity of HIF-1 for galectin-1 expression. Of great importance, hypoxia and HIF-1α overexpression significantly enhanced luciferase expression driven by the promoter sequence of *galectin-1* gene. Chromatin immunoprecipitation assay did show the binding of HIF-1α with two HREs in the promoter of *galectin-1*, which were essential for HIF-1-mediated galectin-1 expression, as evidenced by mutagenesis-based luciferase reporter assay.

Galectins are a family of animal lectins with a conserved carbohydrate recognition domain responsible for β-galactoside binding.

Downloaded from https://academic.oup.com/carcin/article/31/8/1367/2477336 by guest on 21 August 2022

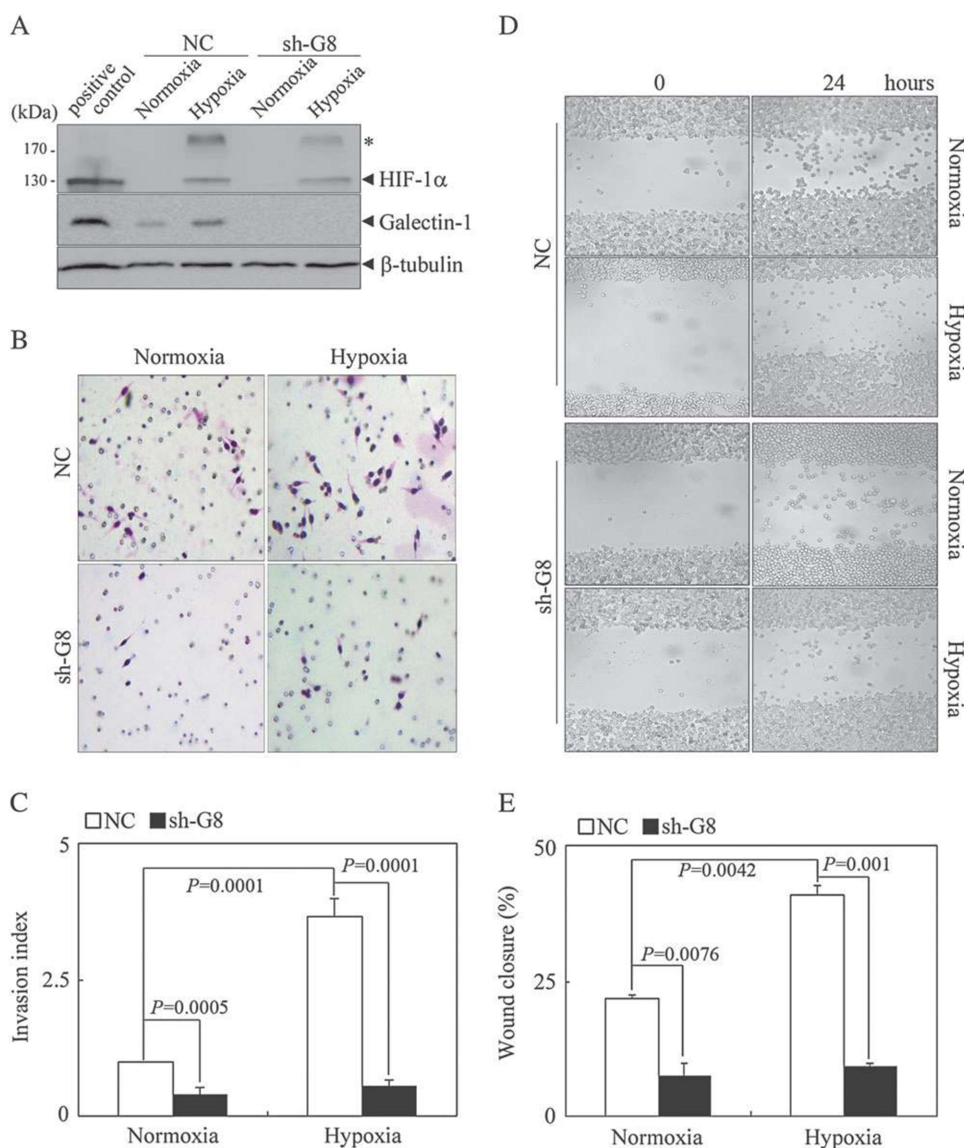


Fig. 5. The migration and invasion of CRC during hypoxia is blocked by the suppression of galectin-1 expression. (**A**) SW620-NC/sh-G8 cells were selected as described in Materials and Methods and treated under hypoxia for 24 h. The protein level of HIF-1α and galectin-1 were analyzed by western blot with β-tubulin as a loading control. SW620 cells transfected with ectopic HIF-1α was used as positive control. Matrigel invasion assay (**B**) and scratch-wound assay (**D**) were performed as illustrated in Materials and Methods. The invasion index (**C**) and wound closure (%, **E**) were calculated and all values were expressed as mean with bar as SD of three independent experiments, the *P* values were shown between two groups linked by lines.

To date, at least fifteen galectins have been identified. They affect a variety of cellular processes extracellularly by binding to cell surface and extracellular matrix glycans and intracellularly through protein–protein interactions with other cytoplasmic and nuclear proteins (36). Galectin-1 has been shown to participate in various physiological and pathophysiological processes such as immune and inflammatory responses, neural degeneration, atherosclerosis, diabetes and wound repair (36,37). Increasing lines of evidence also support that galectin-1 is one of the most important lectins participating in the malignant tumor development to various levels, including cell adhesion, protecting cancer cells from immune response, invasion, angiogenesis and metastasis (17,38–40). For example, galectin-1 is regarded to be essential in tumor angiogenesis because tumor growth is markedly impaired due to insufficient tumor angiogenesis in *galectin-1*-null mice (38). Expression of galectin-1 in tumor or stromal cell influences tumor migration and invasion (39,41). Overexpression of galectin-1 protein in oral squamous cell carcinoma cell lines results in increased level of tumor cell migration and invasion possibly through upregulation of matrix metalloproteinase 2/matrix metalloproteinase 9, metalloproteases involving in tumor cell invasion (42). Galectin-1 could also be observed in extracellular milieu in some colon tumor cell line and could be involved in the regulation of cell migration or invasion (43). Considering that HIF-1 is a critical factor in tumor migration and invasion and HIF-1α is more crucial than HIF-2α during CRC cancer progression (44), we investigated, besides regulation of known HIF-1 target genes, such as vimentin and E-cadherin, which implicated their roles in extracellular migration, whether galectin-1 overexpression in CRC cells contribute to hypoxia-induced migration/invasion of CRC cells. Our results showed that knockdown galectin-1 by shRNA significantly blocked hypoxia-induced cell migration/invasion and re-expression of galectin-1 could be sufficient to rescue HIF-1α-knockdown-caused inhibition effect, which was estimated by the scratch-wound and Matrigel invasion assay. Taken altogether, galectin-1 is identified as a novel target of HIF-1α that

Downloaded from https://academic.oup.com/carcin/article/31/8/1367/2477336 by guest on 21 August 2022

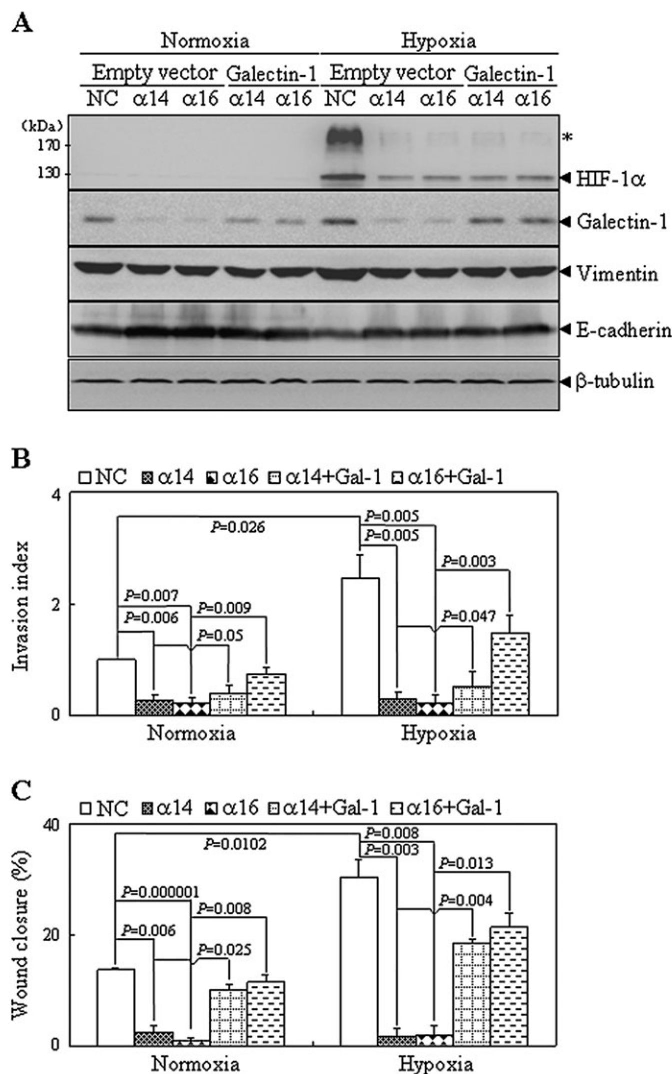


Fig. 6. The ectopic expression of galectin-1 restores invasion and migration abilities of HIF-1α-knocked SW620 cells. Empty vector or pcDNA3.1(−)-galectin-1 were stably transfected into SW620 cells expressing shR-α14, -α16 or NC. These cells were treated under hypoxia for 24 h. The protein level of HIF-1α, galectin-1, vimentin and E-cadherin were analyzed by western blot with β-tubulin as a loading control (**A**). Matrigel invasion assay and scratch-wound assay were performed as illustrated in Materials and Methods. The invasion index (**B**) and wound closure (%, **C**) were calculated and all values were expressed as mean with bar as SD of three independent experiments. The *P* values were shown between two groups linked by lines.

mediates the hypoxia-induced migration and progression of CRC. These findings would shed new light on the diagnosis and treatment of CRC to improve survival and reduce the risk for CRC-associated mortality.

Supplementary material

Supplementary Figures S1 and S2 and Table I can be found at http: //carcin.oxfordjournals.org/

Funding

National Key Program (973) for Basic Research of China (NO2009CB918404); National Natural Science Foundation (90813034, 30800452, 30971274); Science and Technology Committee of Shanghai (08JC1413700, 08ZR1412200); Shanghai Municipal Education Commission (09YZ94); Shanghai Ling-Jun Talent Program to Dr G.Q.C.

Acknowledgements

We appreciate Dr.K.Sogawa for generously providing pEF-BOS-HIF-1 plasmids, and we thank Dr J.K.Cheng of SJTU-SM for MEFs derived from *SENP1*$^{-/-}$ and *SENP1*$^{+/+}$ mice and Dr Yong Zuo for his help on immunohistochemical experiment.

Conflict of Interest Statement: None declared.

References

1. Harris,A.L. (2002) Hypoxia—a key regulatory factor in tumour growth. *Nat. Rev. Cancer*, **2**, 38–47.
2. Vaupel,P. (2008) Hypoxia and aggressive tumor phenotype: implications for therapy and prognosis. *Oncologist*, **13** (suppl. 3), 21–26.
3. Wang,G.L. *et al*. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl Acad. Sci. USA*, **92**, 5510–5514.
4. Semenza,G.L. (2007) Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci. STKE*, **2007**, cm8.
5. Jaakkola,P. *et al*. (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*, **292**, 468–472.
6. Huang,Y. *et al*. (2003) Cobalt chloride and low oxygen tension trigger differentiation of acute myeloid leukemic cells: possible mediation of hypoxia-inducible factor-1alpha. *Leukemia*, **17**, 2065–2073.
7. Song,L.P. *et al*. (2008) Hypoxia-inducible factor-1alpha-induced differentiation of myeloid leukemic cells is its transcriptional activity independent. *Oncogene*, **27**, 519–527.
8. Wenger,R.H. *et al*. (2005) Integration of oxygen signaling at the consensus HRE. *Sci. STKE*, **2005**, re12.
9. Liao,S.H. *et al*. (2009) Proteomics-based identification of two novel direct targets of hypoxia-inducible factor-1 and their potential roles in migration/invasion of cancer cells. *Proteomics*, **9**, 3901–3912.
10. Isaacs,J.S. *et al*. (2005) HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell*, **8**, 143–153.
11. Kim,W.Y. *et al*. (2004) Role of VHL gene mutation in human cancer. *J. Clin. Oncol.*, **22**, 4991–5004.
12. Khandrika,L. *et al*. (2009) Hypoxia-associated p38 mitogen-activated protein kinase-mediated androgen receptor activation and increased HIF-1alpha levels contribute to emergence of an aggressive phenotype in prostate cancer. *Oncogene*, **28**, 1248–1260.
13. Fan,L.F. *et al*. (2008) Role of Hypoxia-inducible factor-1 alpha and Survivin in colorectal carcinoma progression. *Int. J. Colorectal Dis.*, **23**, 1057–1064.
14. Hsieh,S.H. *et al*. (2008) Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. *Oncogene*, **27**, 3746–3753.
15. Funasaka,T. *et al*. (2005) Regulation of phosphoglucose isomerase/autocrine motility factor expression by hypoxia. *Faseb J.*, **19**, 1422–1430.
16. Barondes,S.H. *et al*. (1994) Galectins: a family of animal beta-galactoside-binding lectins. *Cell*, **76**, 597–598.
17. Liu,F.T. *et al*. (2005) Galectins as modulators of tumour progression. *Nat. Rev. Cancer*, **5**, 29–41.
18. Wincewicz,A. *et al*. (2007) Significant coexpression of GLUT-1, Bcl-xL, and Bax in colorectal cancer. *Ann. N. Y. Acad. Sci.*, **1095**, 53–61.
19. Demydenko,D. *et al*. (2009) Expression of galectin-1 in malignant tumors. *Exp. Oncol.*, **31**, 74–79.
20. Semenza,G.L. (2003) Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer*, **3**, 721–732.
21. Cheng,J. *et al*. (2007) SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. *Cell*, **131**, 584–595.
22. Elson,D.A. *et al*. (2000) Coordinate up-regulation of hypoxia inducible factor (HIF)-1alpha and HIF-1 target genes during multi-stage epidermal carcinogenesis and wound healing. *Cancer Res.*, **60**, 6189–6195.
23. Rankin,E.B. *et al*. (2008) The role of hypoxia-inducible factors in tumorigenesis. *Cell Death Differ.*, **15**, 678–685.
24. Krishnamachary,B. *et al*. (2003) Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res.*, **63**, 1138–1143.
25. Krishnamachary,B. *et al*. (2006) Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal

Downloaded from https://academic.oup.com/carcin/article/31/8/1367/2477336 by guest on 21 August 2022

cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Res.*, **66**, 2725–2731.
26. Case,D. *et al*. (2007) Mice deficient in galectin-1 exhibit attenuated physiological responses to chronic hypoxia-induced pulmonary hypertension. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **292**, L154–L164.
27. Le,Q.T. *et al*. (2005) Galectin-1: a link between tumor hypoxia and tumor immune privilege. *J. Clin. Oncol.*, **23**, 8932–8941.
28. Le Mercier,M. *et al*. (2008) Evidence of galectin-1 involvement in glioma chemoresistance. *Toxicol. Appl. Pharmacol.*, **229**, 172–183.
29. Le,Q.T. *et al*. (2007) Expression and prognostic significance of a panel of tissue hypoxia markers in head-and-neck squamous cell carcinomas. *Int. J. Radiat. Oncol. Biol. Phys.*, **69**, 167–175.
30. Hittelet,A. *et al*. (2003) Upregulation of galectins-1 and -3 in human colon cancer and their role in regulating cell migration. *Int. J. Cancer*, **103**, 370–379.
31. Nagy,N. *et al*. (2003) Refined prognostic evaluation in colon carcinoma using immunohistochemical galectin fingerprinting. *Cancer*, **97**, 1849–1858.
32. Dang,C.V. *et al*. (2008) The interplay between MYC and HIF in cancer. *Nat. Rev. Cancer*, **8**, 51–56.
33. Yang,L. *et al*. (2008) CCAAT/enhancer-binding protein alpha antagonizes transcriptional activity of hypoxia-inducible factor 1 alpha with direct protein-protein interaction. *Carcinogenesis*, **29**, 291–298.
34. Yoo,Y.G. *et al*. (2009) An essential role of the HIF-1alpha-c-Myc axis in malignant progression. *Ann. N. Y. Acad. Sci.*, **1177**, 198–204.
35. Peng,Z.G. *et al*. (2008) Physical and functional interaction of Runt-related protein 1 with hypoxia-inducible factor-1alpha. *Oncogene*, **vol. 27**, 839–847.
36. Yang,R.Y. *et al*. (2008) Galectins: structure, function and therapeutic potential. *Expert Rev. Mol. Med.*, **10**, e17.
37. Camby,I. *et al*. (2006) Galectin-1: a small protein with major functions. *Glycobiology*, **16**, 137R–157R.
38. Thijssen,V.L. *et al*. (2006) Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. *Proc. Natl Acad. Sci. USA*, **103**, 15975–15980.
39. Elola,M.T. *et al*. (2007) Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. *Cell. Mol. Life Sci.*, **64**, 1679–1700.
40. Georgiadis,V. *et al*. (2007) Lack of galectin-1 results in defects in myoblast fusion and muscle regeneration. *Dev. Dyn.*, **236**, 1014–1024.
41. Jung,E.J. *et al*. (2007) Galectin-1 expression in cancer-associated stromal cells correlates tumor invasiveness and tumor progression in breast cancer. *Int. J. Cancer*, **120**, 2331–2338.
42. Wu,M.H. *et al*. (2009) Galectin-1-mediated tumor invasion and metastasis, up-regulated matrix metalloproteinase expression, and reorganized actin cytoskeletons. *Mol. Cancer Res.*, **7**, 311–318.
43. Ohannesian,D.W. *et al*. (1994) Concomitant increases in galectin-1 and its glycoconjugate ligands (carcinoembryonic antigen, lamp-1, and lamp-2) in cultured human colon carcinoma cells by sodium butyrate. *Cancer Res.*, **54**, 5992–6000.
44. Imamura,T. *et al*. (2009) HIF-1alpha and HIF-2alpha have divergent roles in colon cancer. *Int. J. Cancer*, **124**, 763–771.

Received December 23, 2009; revised May 19, 2010; accepted May 29, 2010

Downloaded from https://academic.oup.com/carcin/article/31/8/1367/2477336 by guest on 21 August 2022