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HIG2 promotes colorectal cancer progression via hypoxia-dependent and independent pathways

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Abstract

HIG2 (hypoxia-inducible gene 2) is a biomarker of hypoxia and elevated in several cancers. Here, we show that HIG2 also upregulated HIF-1 α expression under hypoxic conditions and enhanced AP-1 expression under normoxic conditions, which affects colorectal cancer cell survival. Importantly, over-expression of HIG2 promoted tumor growth by suppressing apoptosis in a mouse orthotopic model. These results are likely relevant to human disease since we found that the expression of HIG2 is gradually elevated as tumors progress. Collectively, these findings suggest that HIG2 plays an important role in promoting colorectal cancer growth in hypoxia-dependent and independent manners.

Keywords

hypoxia; normoxia; HIG2; HIF-1 α ; colorectal cancer; survival

1. Introduction

Hypoxia is one of the most common and critical factors identified in the regulation of cancer progression and metastasis. The cellular responses to hypoxia are mainly mediated through genes regulated by the family of hypoxia-inducible factor (HIF) transcription factors. HIF-1 α -regulated genes are involved in energy metabolism, angiogenesis, cell proliferation,

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Conflict of Interest

The authors have no conflict of interest

and survival [1-3]. HIF-1 α rapidly degrades during normoxia, but it becomes stabilized and activated under hypoxic conditions [4, 5]. The expression and activity of HIF-1 α are controlled by complex networks with positive regulators such as HIF1 and heat-shock proteins (HSPs) in facilitating tumor cells and tumor microenvironment to adapt to the hypoxic conditions [6-8].

Hypoxia-inducible gene 2 (HIG2) has been identified as one of the genes regulated by hypoxia [9]. Several studies have demonstrated HIG2 as a biomarker for a poor prognosis in a variety of cancers, including renal cell carcinoma (RCC) [10], uterine cancer [11] and ovarian cancer [12]. It has been reported that HIG2 is regulated by hypoxia-dependent and -independent mechanisms. For example, HIG2 is up-regulated by hypoxia in RCC and cervical epithelial cells [9, 13]. In RCC, HIG2 is a direct target of HIF-1 α and its promoter has functional hypoxia response elements (HREs) located in the proximal promoter region (307bp) [13]. In contrast, progesterone regulates HIG2 expression in T47D breast cancer cells via a hypoxia-independent manner [14]. In addition, LiCl, an activator of the β -catenin/Wnt pathway, increased HIG2 expression in RCC under normoxic conditions [10]. Although there are only two reports evaluating HIG2 function, the data presented in these reports are not consistent. Togashi *et al* reported that HIG2 enhanced oncogenic Wnt signaling and RCC cell proliferation [10]. In contrast, Gimm *et al* found that HIG2 overexpression neither stimulated proliferation nor activated Wnt signaling in HeLa cells. In contrast, their results suggested HIG2 as a novel lipid droplet protein that increases neutral lipid deposition and stimulates cytokine expression [13]. Therefore, the biological functions of HIG2 in cancer under both normoxic and hypoxic conditions remain poorly understood and are probably context specific. In this study, we investigated the role of HIG2 in promoting CRC progression under normoxic and hypoxic conditions.

2. Material and Methods

2.1. Cell culture and treatment

LS-174T, HT-29 and HCT-116 were purchased from the ATCC (Manassas, VA). All cells were routinely maintained in McCoy's 5A medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin in a 5% CO₂ humidified incubator at 37°C. Cells were exposed to hypoxia by placing them in a mixed-gas incubator that was infused with an atmosphere consisting of 94% N₂, 5% CO₂, and 1% O₂.

2.2. Real time-quantitative PCR (RT-qPCR)

Total RNA was isolated using TRIzol (Life Technology, Grand Island, NY). cDNA was synthesized from 2 μ g of total RNA by using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) and mixed with TaqMan® Gene Expression Assay Mix, sterile water and TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Real-time PCR was carried out using 7900 HT Fast system (Applied Biosystems, Foster City, CA) and expression of target genes mRNA relative to 18S rRNA was calculated.

2.3. Gene expression data of colon cancer patients

For the RT-qPCR analysis, thirty-six human colorectal carcinoma specimens, along with their normal counterparts, were obtained from the Tissue Procurement and Banking Facility (TPBF) at The University of Texas MD Anderson Cancer Center. Equal amounts of mRNA were analyzed by RT-qPCR for HIG2 and actin.

For the microarray analysis, we used a previously published expression data, which is comprised of 310 Affymetrix GeneChip Human Genome U133A Array containing 182

primary carcinoma, 46 polyps, 52 normal colon epithelium, 21 liver metastasis and 9 lung metastasis [15]. The data was subjected to Plier normalization, batch correction, modified Lowess correction [16] and Log2 transformation. Values below a threshold of $t=4$ were assigned with t .

2.4. DNA construction

HIG2 cDNA (Origene, Rockville, MD) was amplified with pfuUltra Hotstart DNA polymerase (Agilent Technologies, Santa Clara, CA). The PCR products were digested with BamHI and EcoRI and were cloned into pBMN-I-GFP (Addgen, Cambridge MA).

2.5. Establishment of stable cell line

pBMN-I-GFP and pBMN-I-GFP-HIG2 retroviral vectors were transfected into Phoenix cells in 60-mm dishes using Lipofectamine reagent (Life Technology, Grand Island, NY) according to the manufacturer's protocol. Culture medium containing virus particles was collected 48 h later and was added to LS-174T and HCT116 cells. Cells infected were sorted by green fluorescent protein (GFP) positivity to eliminate uninfected cells. shHIG2-1 (TRCN0000158518, Sigma-Aldrich, St. Louis, MO) and shHIG2-2 (TRCN0000158583, Sigma-Aldrich) were transfected into 293T cells. Culture medium containing virus particles was collected and was added to LS-174T and HCT116 cells. Cells infected were selected by puromycin and silencing of HIG2 was validated by RT-qPCR.

2.6. Western blotting

Whole cell lysates were prepared for Western blot analyses using lysis buffer containing 20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% TX-100, 1mmol/L EDTA, pH 8.0, and 1 mmol/L PMSF. Samples were denatured in a SDS sample buffer. Total proteins were separated by loading 20 μ g of total cell lysate on a denaturing 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.1% Triton X-100 and incubated with primary antibodies that recognize HIG2 (Santa Cruz Biotechnology, Dallas, Texas), FOS (BD Pharmingen, San Jose, CA), FOSB (Cell Singaling, Danvers, MA), JUN (Santa Cruz Biotechnology, Dallas, Texas) and Actin (Sigma-Aldrich, St. Louis, MO). Secondary antibody conjugated to horseradish peroxidase (Vector Laboratories Inc, Burlingame, CA) was used at 1:2,000 to detect primary antibodies and enzymatic signals were visualized by chemiluminescence. Western blots were quantitated with ImageJ.

2.7. Cell viability assay

Ninety-six-well plates were seeded with 5,000 cells per well and cells were incubated in serum-free medium for 3 d under normoxic or hypoxic conditions. Cell viability was determined using Cell Proliferation Reagent WST-1 (Roche Applied Science, Indianapolis, IN).

2.8. Immunofluorescent staining

Immunofluorescent staining was performed on paraffin-embedded sections using the Tyramide Signal Amplification system (Invitrogen, Carlsbad, CA). Paraffin-embedded specimens were treated with xylene and ethanol to remove the paraffin. The slides were immersed in Borg decloaker solution (Biocare Medical, Inc., Concord, CA) and boiled in a pressure cooker at 125°C for 5 min for antigen retrieval. Endogenous peroxidase activity was blocked by incubating in 3% H₂O₂ containing PBS solution for 10 min. The slides were blocked with 5% normal goat serum and incubated with anti-HIG2 (1:100, Santa Cruz Biotech, Dallas, Texas), and anti-HIF-1 α (1:100, Becton Dickinson, Franklin Lakes, NJ) at 4°C overnight. After washing with PBS, the slides were incubated with 1:200 biotinylated

goat anti-mouse IgG (Vector Laboratories, Burlingame, CA). Streptavidin-HRP (1:200) and was then applied to the slides. Thereafter, Alexa Flour® 488 labeled tyramide (1:100 in TSA amplification diluents) was used to detect the specific signals. The nuclei were stained with DAPI.

2.9. Luciferase assay

For dual luciferase reporter assays, cells were transfected with the firefly luciferase reporter constructs and the control renilla luciferase reporter pRL-CMV using Lipofectamine™ (Invitrogen, Carlsbad, CA). After treatment, cells were lysed with cell lysis buffer provided by the dual-luciferase reporter assay kit (Promega, Madison, WI). Luciferase activity was then measured according to the manufacture's instruction.

2.10. Xenograft studies

All mice were housed and treated in accordance with protocols approved by the Institutional Animal Care and Use Committee at The University of Texas M.D. Anderson Cancer Center. For the sub-Q mouse model, HIG2/LS-174T or GFP/LS-174T cells were injected into the flanks of nude mice. Tumor size was measured starting from 13 to 28 days after injection. For the orthotopic mouse model, HIG2/HCT116 or GFP/HCT116 cells were injected in to the cecal wall of nude mice. Tumor size was measured by bioluminescence imaging before euthanasia. After the mice were euthanized using CO₂ asphyxiation, necropsies were done to remove tumors and measure their weight and size.

2.11. In vivo apoptosis analysis

Apoptotic cells were detected with cleaved caspase-3 immunohistochemistry as well as the *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany) following the instructions of the manufacturer. Images were taken at 10X objective to quantify apoptotic cells. Fluorescein positive nuclei were counted and the percentages of positively stained nuclei to total nuclei were calculated.

2.12. Statistical analysis

Each experiment was completed at least three times, and data are presented as the mean $\pm$ SE. Statistical significance was determined using Student's *t* test, or two-factor ANOVA, where applicable. $P < 0.05$ was considered statistically significant.

3. Results

3.1. HIG2 expression is elevated in human colorectal carcinomas

To examine whether HIG2 is overexpressed in human CRC, RT-qPCR was performed. HIG2 mRNA levels were elevated in 28 of 36 (77%) colorectal carcinoma specimens as compared with the matched normal mucosa (Fig. 1A). To further test whether HIG2 is involved in CRC progression, we reanalyzed HIG2 mRNA levels from a published DNA microarray data set [15]. Indeed, HIG2 expression is up-regulated by ~1.9-fold in colorectal adenomas as compared to normal tissue, by ~1.4-fold in primary colorectal carcinomas as compared to adenomas, and by ~1.4-fold in liver and lung metastases as compared to primary colorectal carcinomas, suggesting an association of HIG2 with disease progression (Fig. 1B). Since HIG2 is upregulated during hypoxia [9, 13], we assessed whether HIG2 was expressed in hypoxic regions of human CRC specimens. As shown in Fig. 1C, immunofluorescent staining showed that HIG2 (green) was co-localized with HIF-1 α , a good indicator of hypoxic regions (red), but was also found in HIF-1 α negative regions, indicating that HIG2 is expressed in both normoxic and hypoxic regions of the tumor microenvironment.

3.2. HIG2 is a HIF-1 α target in regulating cell survival during hypoxia

As expected, HIG2 was up-regulated in several colorectal cancer cell lines exposed to hypoxia (Fig. 2A). In addition, we have confirmed our findings in experiments examining cobalt chloride-induced hypoxia (Supplementary Fig. 1A). The observation that HIF-1 α knockdown inhibited induction of HIG2 following hypoxia demonstrated that HIG2 is clearly one of HIF-1 α targets during hypoxia (Fig. 2B). To examine the role of HIG2 in CRC cell growth under hypoxic conditions, LS174T and HCT116 cells with knockdown of HIG2 were established (Fig. 2C). Silencing of HIG2 reduced cell viability during hypoxia (Fig. 2D). Similarly, knockdown of HIG2 resulted in decreased LS-174T cell viability following cobalt chloride-induced hypoxia (Supplementary Fig. 1B). These results demonstrate that HIG2 is one of HIF-1 α -regulated survival factors during hypoxia.

3.3. HIG2 regulates the HIF-1 α activity and the expression of its targets

We next determined that HIG2 downstream target genes are also hypoxia-regulated genes. We found that inhibition of HIG2 suppressed the expression of several hypoxia-inducible genes, including VEGF and BMP2 (Fig. 3A). More interestingly, HIG2 silencing reduced HIF-1 α transcriptional activity and HIF-1 α protein levels (Fig. 3B and C, *right panel*). We also confirmed that silencing of HIG2 led to reduction of HIF-1 α protein levels following cobalt chloride-induced hypoxia (Supplementary Fig. 1C). We also observed that there were two bands recognized by the antibody in HIF-1 α Western blots (Fig 3C, *right panel*). Our results are consistent with previous published results because hypoxia induces the multiple modifications of HIF-1 α such as phosphorylation and sumoylation [15]. These two bands represent HIF-1 α with different modifications. However, HIG2 silencing did not affect HIF-1 α mRNA levels (Fig. 3C, *left panel*). These results suggest that HIG2 induces HIF-1 α activity through enhancing HIF-1 α protein levels and indicate that a positive feedback regulation between HIG2 and HIF-1 α exists in the context of these experiments.

3.4. HIG2 promotes cell survival under normoxic conditions

Since HIG2 was expressed in normoxic regions of CRC specimens (Fig. 1C), we examined which factor regulates HIG2 expression under normoxia. We tested the effects of growth factors, which promote CRC progression, on HIG2 expression. Finally, we found that EGF increased HIG2 mRNA and protein levels (Fig. 4A). To determine the biological role of HIG2 under normoxic conditions, we established LS174T and HCT116 cells that over-express HIG2 (Fig. 4B). Over-expression of HIG2 enhanced LS174T and HCT116 cell viability (Fig. 4C). Since our results showed that HIG2 had no measurable effects on cell proliferation (data not shown), we postulated that HIG2 enhances cell viability via promoting cell survival. Little is known about HIG2 downstream targets that are involved in regulating programmed cell death. The microarray analysis revealed that the presence of elevated HIG2 resulted in increased expression of FOS, FOSB, and JUN, which are members of the AP-1 transcription factor family (data now shown). As shown in Fig. 4D, our results from RT-qPCR and Western blotting assays confirmed that overexpression of HIG2 up-regulated these genes at mRNA (*left panel*) and protein (*middle panel*) levels. Also, AP-1 transcriptional activity was increased by HIG2 over-expression (Fig. 4D, *right panel*). Taken together, these results suggest that HIG2 may enhance cancer cell survival through regulating the expression of AP-1 in normoxic conditions and possibly other immediate early genes.

3.5. HIG2 promotes tumor growth in vivo

To validate our *in vitro* results *in vivo*, HIG2-overexpressed LS-174T cells (HIG2/LS-174T) or their control cells (GFP/LS-174T) were injected into the flanks of nude mice. The tumor size was measured starting from 7 to 28 days after injection. Tumor weight was also

measured after mice were euthanized. Cells expressing HIG2 formed larger tumors than those found following injection of control cells (Fig. 5A). To further confirm the effect of HIG2 on tumor growth, an orthotopic mouse model of CRC was utilized. HIG2-overexpressing HCT-116 cells (HIG2/HCT-116) or control cells (GFP/HCT-116) were injected into the cecal wall of nude mice. Tumor growth was monitored by bioluminescence imaging and tumors were weighted following euthanization of the animals. Consistent with the above results, over-expression of HIG2 promoted tumor growth and inhibited tumor cell apoptosis as measured by caspase 3 activation and DNA fragmentation (Fig. 5B-D). These *in vivo* results demonstrate that HIG2 accelerates colorectal cancer progression via suppression of apoptosis.

4. Discussion

In this study, our results revealed that hypoxia-induced expression of HIG2 enhances CRC cell survival via up-regulation HIF-1 α expression, whereas overexpression of HIG2 also promotes CRC cell survival through activation of the AP-1 signaling pathway under normoxic conditions. These findings indicate that HIG2 utilizes different signaling pathways to promote CRC cell survival under different conditions depending on the context of the situation.

Several studies have shown the up-regulation of HIG2 in various cancers including renal cell carcinoma and ovarian cancer [10, 12]. HIG2 has been suggested as a possible biomarker for early detection of ovarian clear cell adenocarcinoma or for prediction of response to chemotherapy [12]. In this study, we showed for the first time that HIG2 is highly elevated in human CRC and its level is correlated with tumor stage, suggesting that HIG2 may be involved in promoting CRC progression as well.

HIF-1 α is an important transcription factor that targets genes related to cancer cell survival during hypoxia [1, 2]. High levels of HIF-1 α are present in colorectal carcinomas and its up-regulation and stabilization have been demonstrated previously [17]. Our study is the first to show that HIG2 regulates HIF-1 α protein levels, which leads to a positive feedback regulation between HIG2 and HIF-1 α . It has been reported that a group of positive regulators such as molecular chaperones, contributes to HIF-1 α stability [8, 18]. HSP90 can interact with HIF-1 α , which is crucial for rapid HIF-1 α accumulation [19] and Tid-1, a member of DnaJ chaperone protein family, interacts with VHL and enhances VHL binding to HIF-1 α , which leads to reduced HIF-1 α protein levels [20]. Our preliminary data showed that HIG2 over-expression affected several DnaJ members such as DnaJA1 and DnaJA4 as well as HSP70 family members such as HSPA1B and HSPA8 but did not affect VHL expression (data not shown). Therefore, future experiments would be needed to determine how HIG2 regulates HIF-1 α activity during hypoxia.

The AP-1 transcription factor is a heterodimeric protein composed of proteins mainly belonging to FOS and JUN in mammalian cells [21]. AP-1 plays a role in tumorigenesis by regulating genes engaged in cell cycle and apoptosis [22, 23] and is elevated in multiple human tumor types [24]. AP-1 activity and expression are stimulated by a complex network of signaling pathways that involves external signals such as growth factors and cytokines [21]. Since HIG2 has been reported to be a secreted protein and binds to the extracellular domain of Frizzled homology 10 (FZD), a G-protein coupled receptor [10], it is possible that HIG2 may regulate AP-1 expression via receptor-mediated signaling. In addition, HIG2 over-expression is sufficient to augment cytokine expression under normoxic conditions [13], suggesting that HIG2 induction of cytokines exerts a regulatory effect on AP-1. Therefore, further studies are required to investigate the precise mechanism by which HIG2 regulates AP-1 in promoting colorectal carcinoma growth.

In addition, our results demonstrated that HIG2 expression was also elevated in metastatic tumors as compared to primary tumor. Our preliminary data showed that several genes related to cell migration such as RAP1B, NES, SPHK1, and LRP8 were increased by HIG2 over-expression (data not shown), suggesting that HIG2 may be involved in CRC metastasis as well. Further investigation is needed to test this hypothesis.

Collectively, our results reveal a novel function of HIG2 in promoting tumor growth via enhancing tumor cell survival in both hypoxic and normoxic conditions. Our findings may offer another therapeutic option for CRC by targeting HIG2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Liu W, Shen SM, Zhao XY, Chen GQ. Targeted genes and interacting proteins of hypoxia inducible factor-1. *Int J Biochem Mol Biol.* 2012; 3:165–178. [PubMed: 22773957]
2. Giatromanolaki A, Harris AL. Tumour hypoxia, hypoxia signaling pathways and hypoxia inducible factor expression in human cancer. *Anticancer Res.* 2001; 21:4317–4324. [PubMed: 11908686]
3. Keith B, Johnson RS, Simon MC. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer.* 2011; 12:9–22. [PubMed: 22169972]
4. Baldewijns MM, van Vlodrop IJ, Vermeulen PB, Soetekouw PM, van Engeland M, de Bruine AP. VHL and HIF signalling in renal cell carcinogenesis. *J Pathol.* 2010; 221:125–138. [PubMed: 20225241]
5. Walmsley SR, McGovern NN, Whyte MK, Chilvers ER. The HIF/VHL pathway: from oxygen sensing to innate immunity. *Am J Respir Cell Mol Biol.* 2008; 38:251–255. [PubMed: 17932373]
6. Kim SH, Xia D, Kim SW, Holla V, Menter DG, Dubois RN. Human enhancer of filamentation 1 Is a mediator of hypoxia-inducible factor-1alpha-mediated migration in colorectal carcinoma cells. *Cancer Res.* 2010; 70:4054–4063. [PubMed: 20442290]
7. Yuan WC, Lee YR, Huang SF, Lin YM, Chen TY, Chung HC, Tsai CH, Chen HY, Chiang CT, Lai CK, Lu LT, Chen CH, Gu DL, Pu YS, Jou YS, Lu KP, Hsiao PW, Shih HM, Chen RH. A Cullin3-KLHL20 Ubiquitin ligase-dependent pathway targets PML to potentiate HIF-1 signaling and prostate cancer progression. *Cancer Cell.* 2011; 20:214–228. [PubMed: 21840486]
8. Henze AT, Acker T. Feedback regulators of hypoxia-inducible factors and their role in cancer biology. *Cell Cycle.* 2010; 9:2749–2763. [PubMed: 20603601]
9. Denko N, Schindler C, Koong A, Laderoute K, Green C, Giaccia A. Epigenetic regulation of gene expression in cervical cancer cells by the tumor microenvironment. *Clin Cancer Res.* 2000; 6:480–487. [PubMed: 10690527]
10. Togashi A, Katagiri T, Ashida S, Fujioka T, Maruyama O, Wakumoto Y, Sakamoto Y, Fujime M, Kawachi Y, Shuin T, Nakamura Y. Hypoxia-inducible protein 2 (HIG2), a novel diagnostic marker for renal cell carcinoma and potential target for molecular therapy. *Cancer Res.* 2005; 65:4817–4826. [PubMed: 15930302]

11. Nishimura S, Tsuda H, Nomura H, Kataoka F, Chiyoda T, Tanaka H, Tanaka K, Susumu N, Aoki D. Expression of hypoxia-inducible 2 (HIG2) protein in uterine cancer. *Eur J Gynaecol Oncol.* 2011; 32:146–149. [PubMed: 21614900]
12. Nishimura S, Tsuda H, Ito K, Takano M, Terai Y, Jobo T, Kigawa J, Sugiyama T, Yaegashi N, Aoki D. Differential expression of hypoxia-inducible protein 2 among different histological types of epithelial ovarian cancer and in clear cell adenocarcinomas. *Int J Gynecol Cancer.* 2010; 20:220–226. [PubMed: 20134266]
13. Gimm T, Wiese M, Teschemacher B, Deggerich A, Schodel J, Knaup KX, Hackenbeck T, Hellerbrand C, Amann K, Wiesener MS, Honing S, Eckardt KU, Warnecke C. Hypoxia-inducible protein 2 is a novel lipid droplet protein and a specific target gene of hypoxia-inducible factor-1. *FASEB J.* 2010; 24:4443–4458. [PubMed: 20624928]
14. Bray JD, Jelinsky S, Ghatge R, Bray JA, Tunkey C, Saraf K, Jacobsen BM, Richer JK, Brown EL, Winneker RC, Horwitz KB, Lyttle CR. Quantitative analysis of gene regulation by seven clinically relevant progestins suggests a highly similar mechanism of action through progesterone receptors in T47D breast cancer cells. *J Steroid Biochem Mol Biol.* 2005; 97:328–341. [PubMed: 16157482]
15. Brahimi-Horn C, Mazure N, Pouyssegur J. Signalling via the hypoxia-inducible factor-1alpha requires multiple posttranslational modifications. *Cellular signalling.* 2005; 17:1–9. [PubMed: 15451019]
16. Ballman KV, Grill DE, Oberg AL, Therneau TM. Faster cyclic loess: normalizing RNA arrays via linear models. *Bioinformatics.* 2004; 20:2778–2786. [PubMed: 15166021]
17. Furlan D, Sahnane N, Carnevali I, Cerutti R, Uccella S, Bertolini V, Chiaravalli AM, Capella C. Up-regulation and stabilization of HIF-1alpha in colorectal carcinomas. *Surg Oncol.* 2007; 16(Suppl 1):S25–27. [PubMed: 18023174]
18. Liu YV, Semenza GL. RACK1 vs. HSP90: competition for HIF-1 alpha degradation vs. stabilization. *Cell Cycle.* 2007; 6:656–659. [PubMed: 17361105]
19. Zhang D, Li J, Costa M, Gao J, Huang C. JNK1 mediates degradation HIF-1alpha by a VHL-independent mechanism that involves the chaperones Hsp90/Hsp70. *Cancer Res.* 2010; 70:813–823. [PubMed: 20068160]
20. Bae MK, Jeong JW, Kim SH, Kim SY, Kang HJ, Kim DM, Bae SK, Yun I, Trentin GA, Rozakis-Adcock M, Kim KW. Tid-1 interacts with the von Hippel-Lindau protein and modulates angiogenesis by destabilization of HIF-1alpha. *Cancer Res.* 2005; 65:2520–2525. [PubMed: 15805242]
21. Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer.* 2003; 3:859–868. [PubMed: 14668816]
22. Ashida R, Tominaga K, Sasaki E, Watanabe T, Fujiwara Y, Oshitani N, Higuchi K, Mitsuyama S, Iwao H, Arakawa T. AP-1 and colorectal cancer. *Inflammopharmacology.* 2005; 13:113–125. [PubMed: 16259733]
23. Vaiopoulos AG, Papachroni KK, Papavassiliou AG. Colon carcinogenesis: Learning from NF-kappaB and AP-1. *Int J Biochem Cell Biol.* 2010; 42:1061–1065. [PubMed: 20348011]
24. Matthews CP, Colburn NH, Young MR. AP-1 a target for cancer prevention. *Curr Cancer Drug Targets.* 2007; 7:317–324. [PubMed: 17979626]

Figure 1. HIG2 is up-regulated in human CRC specimens

A, Total RNA was isolated from 36 individual human colorectal cancer tissues and corresponding normal mucosa. Equal amounts of mRNA were analyzed by RT-qPCR for HIG2 expression. The dash means ratio of tumor/normal is equal to 1. B, HIG2 average expression in normal colon (n=52), polyp (n=46), CRC primary tumor (n=182) and CRC metastasis (n=30) human samples are shown according to Affymetrix DNA microarray analysis (probeset ID 218507_at). P-values for differences between the expression values of different pairs of groups were all below 0.0005 (ranksum test). C, Representative section shows immunofluorescent staining of HIG2 (green) and HIF-1 α (red) in human colorectal cancer specimens. Nuclei were stained with DAPI (blue).

Figure 2. Hypoxia increases HIG2 expression

A, LS174T, HCT116, and HT-29 cells were exposed to normoxia or hypoxia for 24 h and then HIF1 levels were determined using RT-qPCR analysis. *B*, Cells were transfected with non-targeting and HIF-1α siRNA, respectively. After twenty-four hours, transfecting cells were exposed to hypoxia for 24 h and subjected to analysis of RT-qPCR for HIG2 levels. *C*, LS174T and HCT116 cells were stably transfected with vectors containing nonsilencing control shRNA (shCTL) or HIG2 shRNA (shHIG2) and HIG2 levels were detected in these cell lines exposed to hypoxia for 24 h. *D*, Cells containing shCTL and shHIG2 were exposed to hypoxia for 3 d and then cell viability was measured.

Figure 3. HIG2 regulates HIF-1α protein level

A, shCTL/LS174T and shHIG2/LS174T cells were exposed to normoxia or hypoxia for 24 h and then analyzed by RT-qPCR for changes in hypoxia-inducible gene expression. B, shCTL/LS174T and shHIG2/LS174T cells were transiently cotransfected with HRE luciferase and pRL-CMV plasmids and cells were exposed to hypoxia for 25 h. The luciferase activity was determined. C, shCTL/LS174T and shHIG2/LS174T cells were exposed to hypoxia for 8 h (mRNA and protein) and 24 h (protein) and then analyzed by RT-qPCR (*left*) and Western blotting (*right*) for HIF-1α.

Figure 4. HIG2 expression increases cell proliferation under normoxia

A, LS174T cells were treated with 100 ng/ml EGF for 24 h (mRNA) and 48 h (protein) to determine the expression of HIG2 by RT-qPCR (*left*) and Western blotting (*right*). B, LS-174T and HCT116 cells were stably transfected with GFP or HIG2 constructs. HIG2 expression was determined by Western blotting. C, These cells were incubated in serum free medium for 3 days and then cell viability was tested. D, the expression levels of FOS, FOSB, JUN, and EGR1 were determined by RT-qPCR (*left*) and Western blotting (*middle*) in GFP/LS174T and HIG2/LS174T cells. These cell lines were cotransfected with AP-1 reporter plasmid and pRL-CMV plasmids, and the luciferase activity was determined (*right*).

Figure 5. Increased HIG2 promotes tumor growth *in vivo*

A, LS-174T cell stably transfected with GFP or HIG2 constructs were injected into the flanks of nude mice. The tumor size (*left*) was measured from 7 to 26 days after injection and tumor weight (*right*) was also measured after mice were euthanized. B, HCT116 cell stably transfected with GFP or HIG2 constructs were injected into the cecal wall of nude mice. The tumor size was imaged by bioluminescence (*left*) and tumor weight was also measured after mice were euthanized (*right*). C, Immunohistochemistry was performed to detect cleaved caspase 3 expression in tumor tissue formed by injection of either GFP/HCT116 or HIG2/HCT116 cells into the cecal wall of nude mice (*left*). Quantitation of cleaved caspase 3 staining. Columns present the number of positive cells per 10X field from 4 random fields (*right*). D, TUNEL assays were done to detect the DNA fragmentation. Apoptotic cells were stained bright green (*left*). Columns present the percentages of positively stained nuclei to total nuclei were calculated (*right*).