Cancer Science
The official journal of the Japanese Cancer Association

Hypoxia-induced β-catenin downregulation involves p53-dependent activation of Siah-1

Dapeng Wang,[1,5] Yongxing Wang,[2,5] Tao Kong,[3] Feiyue Fan[1] and Yongguang Jiang[2,4]

[1]Institute of Radiation Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin; [2]Department of Urology, Beijing Anzhen Hospital Affiliated with Capital Medical University, Beijing Institute of Heart Lung and Blood Diseases, Beijing; [3]Department of Urology, Beijing University of Traditional Chinese Medicine Subsidiary Dongfang Hospital, Beijing, China

(Received February 16, 2011/Revised March 29, 2011/Accepted March 30, 2011/Accepted manuscript online April 5, 2011/Article first published online May 12, 2011)

Solid tumors contain extensive hypoxic areas and it is of considerable importance to decipher the potential role of hypoxia in signaling pathway regulation. In the present study, we examined the impact of hypoxia on β-catenin status and the mechanisms involved. Hypoxia significantly decreased β-catenin protein, but had no effect on glycogen synthase kinase (GSK)-3β or adenomatous polyposis coli (APC) levels. However, hypoxia-induced β-catenin downregulation seemed to require APC but not GSK-3β. Further investigation revealed that hypoxia significantly upregulated Siah-1, the human homolog of *Drosophila* seven in absentia. In addition, hypoxia augmented the interaction between β-catenin and SIP and Skp1. Silencing of Siah-1, as well as the use of a dominant negative Siah-1 mutant, attenuated these responses to hypoxia and rescued β-catenin transactivation. The Siah-1-mediated degradation of β-catenin during hypoxia may involve p53, but not hypoxia-inducible factor-1, activation. Together, the results suggest that hypoxia downregulates β-catenin by increasing Siah-1 expression in a p53-dependent manner. (*Cancer Sci* 2011; 102: 1322–1328)

Solid tumors frequently outgrow their blood supply during the course of progression to advanced stages. This deficiency in blood supply can deprive tumor cells of oxygen, resulting in hypoxia.[1] The key regulator of cellular and systemic responses to hypoxia is the hypoxia-inducible factor (HIF) system, which consists of a constitutively expressed HIF-1β subunit and one of either hypoxia-inducible α-subunits, HIF-1α or HIF-2α.[2]

The Wnt signaling pathway plays a critical role in cell fate determination, tissue homeostasis, and tumorigenesis.[3] The central player in this signaling pathway is the cytoplasmic protein β-catenin. The stability of β-catenin is regulated by a destruction complex consisting of glycogen synthase kinase (GSK)-3β,[4] the adenomatous polyposis coli (APC) tumor suppressor protein,[5] and axin.[6] Inappropriate activation of the Wnt pathway leads to accumulation of nuclear β-catenin, which, in complex with T-cell factor (TCF),[7] drives tumor progression via the upregulation of target genes such as c-myc[8,9] and cyclin D1.[10]

There are reports that hypoxia induces β-catenin overexpression and/or intracellular accumulation in macrophages and hepatic cancer cells by downregulating the endogenous degradation machinery.[11,12] In addition, both hypoxia and the hypoxia mimetic desferrioxamine (DFO) enhance β-catenin transcriptional activity in osteoblasts and C3H10T1/2 cells by suppressing sclerostin and enhanced phosphorylation of GSK-3β, respectively.[13,14] However, in the colon adenocarcinoma cell line SW480, neither β-catenin nor TCF-4 levels were changed in response to hypoxia and immunocytochemistry revealed no obvious changes in their subcellular localization.[15] In contrast, downregulation of β-catenin under hypoxic conditions was observed in the human colorectal cancer cell line RKO, which may have resulted from hypoxia-induced endoplasmic reticulum stress.[16] Another *in vitro* study found reduced β-catenin and increased GSK-3β activity under hypoxic conditions in retinal endothelial cells, thus impairing the integrity of cell–cell junctions.[17]

It is not clear why there are so many apparently discrepant reports regarding β-catenin regulation under hypoxic conditions. In the present study, we investigated β-catenin status under hypoxic conditions in multiple cancer cell lines and explored potential underlying mechanisms.

Materials and Methods

Cell culture and hypoxia treatment. LNCaP, 786-0, and NCI-H460 cells were cultured in RPMI 1640 plus 10% FBS, whereas the MCF7, HeLa, SW480, HCT-116p53$^{+/+}$ and HCT-116 p53$^{-/-}$ cell lines were cultured in DMEM plus 10% FBS. To create hypoxic conditions, cells were grown under 1% oxygen.

Plasmids and siRNA. The plasmid encoding wild-type APC used in the present study, namely pCMV-APC, was purchased from Addgene (Cambridge, MA, USA). The dominant negative Siah-1 mutant plasmid (designated pcDNA3.1-Siah-1-ΔDN) was constructed as described previously.[18] Scrambled, GSK-3β-specific, Siah-1-specific, and p53-specific siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Western blot analysis. Total proteins were extracted using modified RIPA buffer and quantified using the Bradford assay (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were semi-dry transferred to nitrocellulose membranes. Membranes were blocked and incubated with appropriately diluted primary antibodies (all from Abcam, Cambridge, MA, USA) overnight at 4°C. After incubation with HRP-conjugated secondary antibody, the HRP signal was detected using Super ECL Plus Detection Reagent (Applygen, Beijing, China) and exposed to X-ray film. β-Actin served as a loading control. For quantification of band intensity, appropriate films were scanned and band densities were determined using Quantity One software (Bio-Rad), normalized against β-actin, and presented as a ratio of control (arbitrarily designated as ''1'').

Co-immunoprecipitation. Cellular extracts were precleared with protein A sepharose beads for 1 h at 4°C and incubated with β-catenin antibody (Abcam) overnight at 4°C. Then, protein A sepharose beads were added and samples were incubated for a further 1 h. The beads were then washed four times with ice-cold lysis buffer and suspended in SDS sample loading buffer. Western blotting was then performed using anti-Skp1 and anti-SIP antibodies (Abcam).

Real-time quantitative PCR. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). After DNase I treatment, 5 μg total RNA was reverse-transcribed into cDNA using

[4]To whom correspondence should be addressed.
E-mail: yongguang.jiang@yahoo.com.cn
[5]These authors contributed equally to this work.

doi: 10.1111/j.1349-7006.2011.01950.x

the Superscript First-Strand Synthesis System (Invitrogen). Real-time PCR was performed with an ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Ex Taq II kit (TaKaRa, Dalian, China). The specific primer pairs used for quantitative (q) PCR were as follows: β-catenin, 5′-GATCCTCGCAGGGACTACAG-3′ (sense) and 5′-TACCCGGGTCTTCTACATGC-3′ (antisense); Siah-1, 5′-GACTGGCACAACTGCATCCA-3′ (sense) and 5′-AGCCAAGTGCGAATGGATC-3′ (antisense); β-actin, 5′-CTGGAACGGTGAAGGTGACA-3′ (sense) and 5′-AAGGGACTTCCTGTAACAATGCA-3′ (antisense); 28S RNA, 5′-GGTAAACGGCGGGAGTAACTATG-3′ (sense) and 5′-TAGGTAGGGACAGTGGGAATCTCG-3′ (antisense); and RNA pol II, 5′-GCACCACGTCCAATGACAT-3′ (sense) and 5′-GTGCGGCTGCTTCCATAA-3′ (antisense). The expression levels of the target genes were calculated using the $\Delta\Delta C_t$ method and were normalized using the geometric mean of three housekeeping genes (β-actin, 28S rRNA, and RNA pol II), as described previously.[19] Changes in gene expression are expressed as percent changes compared with the corresponding control (defined as 100%). All experiments were performed in triplicate and repeated three times.

Transfection. One day before transfection, cells were seeded in appropriate culture disks to reach 80–90% confluency at transfection. Cell transfection was performed using Lipofectamine 2000 (Invitrogen). After 24 h transfection, cells were exposed to hypoxia for specific times.

Luciferase reporter assays. Cells were transfected with TCF/leukemia inhibitory factor (LEF)-responsive luciferase reporter TOPflash (Millipore/Upstate Labs, Temecula, CA, USA), FOPflash (mutant TCF binding sites, Millipore/Upstate Labs), and Renilla luciferase reporter plasmids together with test plasmids. Renilla luciferase reporter plasmids were used to normalize transfection efficiencies. After exposure to hypoxia, cellular luciferase activities were determined using the Dual Luciferase Assay System kit (Promega, Madison, WI, USA).

Statistical analysis. All real-time PCR results and quantified western blot results are presented as the mean ± SD. Student's *t*-test was used to identify significant differences between groups. Statistical significance was set at $P < 0.05$.

Results

Significant downregulation of β-catenin, but not obvious changes in GSK-3β and APC, during hypoxia in various cancer cell lines. To more precisely examine β-catenin status during hypoxia and exclude the possibility of cell type specificity, we performed experiments in six different tissue-derived cell lines. All cell lines carried wild-type APC and β-catenin, except for HCT-116 cells, which carry a gain-of-function mutation (deletion of amino acid Ser[45]). First, the cellular response to hypoxia was verified by observing the levels of HIF-1α protein. As shown in Figure 1(a), hypoxia significantly increased HIF-1α protein expression in all cell lines except in HIF-1α-deficient 786-0 cells, suggesting that the cells were experiencing and responding to hypoxia. Then, β-catenin protein levels were determined under hypoxic conditions. In all cells lines, hypoxia significantly downregulated β-catenin levels. This downregulation was time dependent, with a marked downregulation seen at 6 h in most cell lines (LNCaP, MCF7, HCT-116 and 786-0) or at 12 h or later in the NCI-H446 and HeLa cells. Of note, the HIF-1α-deficient cell line 786-0 showed a marked loss of β-catenin after exposure to hypoxia, indicating that hypoxic β-catenin downregulation may not involve HIF-1α activation.

To determine the reason for β-catenin protein downregulation in hypoxia, we examined β-catenin mRNA levels using quantitative RT-PCR. As shown in Figure 1(b), there were no significant changes in β-catenin mRNA levels in any of the cell lines tested after exposure to 6 or 12 h hypoxia. After 24 h hypoxia, moderate β-catenin mRNA downregulation was observed in MCF-7, 786-0, and HeLa cells, whereas β-catenin mRNA levels in the other cell lines were maintained at normal levels. This suggests that the transcriptional status of β-catenin is unaffected by hypoxia, at least not by early hypoxia (6 or 12 h). Therefore, the downregulation of β-catenin protein levels may be due to a post-transcriptional mechanism induced by hypoxia.

Because GSK-3β and APC are two important regulators of β-catenin degradation, we examined GSK-3β and APC levels under hypoxic conditions. Hypoxia had no significant effect on GSK-3β phosphorylation, the total amount of GSK-3β protein (Fig. 1a) or APC levels. We further examined levels of phosphorylated (p-) β-catenin (S33/S37/T41), which is phosphorylated by GSK-3β, under hypoxic conditions. As shown in Figure 1(a), hypoxia did not induce a significant increase in p-β-catenin (S33/S37/T41). Together, these findings indicate that the hypoxia-induced degradation of β-catenin may not be due to changes in GSK-3β or APC.

Hypoxia-induced β-catenin degradation requires APC, but not GSK-3β. Although hypoxia did not induce significant changes in APC or GSK-3β, this does not exclude their roles in β-catenin degradation under hypoxic conditions. Therefore, we investigated

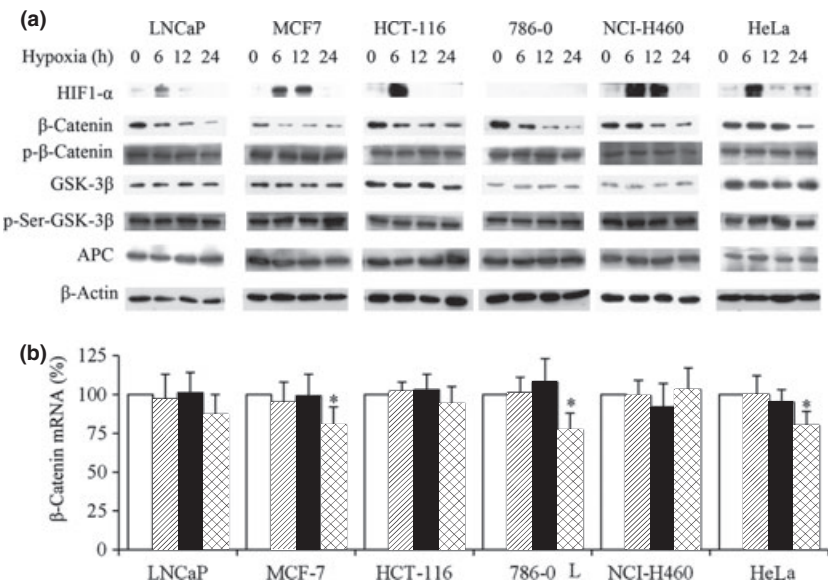

Fig. 1. Expression of β-catenin, glycogen synthase kinase (GSK)-3β, and adenomatous polyposis coli (APC) in different cell lines under hypoxic conditions. (a) Cells were exposed to hypoxia for the time indicated and western blot analysis was used to determine levels of hypoxia-inducible factor (HIF)-1α, β-catenin, phosphorylated (p-) β-catenin (S33, S37, and T41), GSK-3β, p-Ser-GSK-3β and APC. (b) Real-time PCR was performed to determine β-catenin mRNA levels following hypoxia for 0 (□), 6 (▨), 12 (■), or 24 h (▩). Changes in β-catenin mRNA levels, normalized against β-actin, are expressed as percentage changes compared with untreated (0 h) cells (in which β-catenin expression was set at 100%). Data are the mean ± SD of three experimental replicates. *$P < 0.05$ compared with untreated (0 h) cells.

whether hypoxia-induced β-catenin degradation requires GSK-3β activity. To this end, we examined β-catenin levels under hypoxic conditions in the presence of LiCl, a potent GSK-3β inhibitor, in MCF-7 cells, which carry wild-type APC and β-catenin. As shown in Figure 2(a), LiCl treatment significantly increased β-catenin levels under normoxic conditions. However, in the presence of hypoxia, the LiCl-induced increase in β-catenin levels were attenuated. As a negative control, KCl treatment did not induce any obvious changes in β-catenin levels under normoxic or hypoxic conditions (data not shown). In parallel, we also examined the effects of siRNA-mediated GSK-3β silencing on hypoxic β-catenin status. As shown in Figure 2(b), β-catenin levels were increased in MCF-7 cells transfected with GSK-3β siRNA, but not scrambled siRNA. After exposure to hypoxia, both GSK-3β siRNA- and scrambled siRNA-transfected cells showed a marked loss of β-catenin ($P < 0.05$). These results suggest that hypoxia-mediated degradation of β-catenin may be independent of GSK-3β.

Next, we sought to investigate whether APC is required for β-catenin degradation under hypoxic conditions by exposing SW480 cells, a colon cancer cell line containing truncated APC, to hypoxia for specific times (0, 6, 12 and 24 h). As shown in Figure 2(c), there was no apparent β-catenin loss after hypoxia. Then, cells were transfected with a plasmid encoding full-length wild-type APC and β-catenin levels under hypoxic conditions were reassessed. In SW480 cells transfected with the wild-type APC plasmids, marked β-catenin degradation was observed compared with cells transfected with an empty vector or untransfected cells (Fig. 2d). When exposed to hypoxia, cells transfected with wild-type APC exhibited further degradation of β-catenin, whereas those cells transfected with empty vector or untransfected cells did not exhibit β-catenin loss ($P < 0.05$). Together, these data provide evidence of the requirement for APC, but not GSK-3β, for β-catenin degradation in hypoxia.

Increased Siah-1 expression and enhanced interaction between β-catenin and SIP and Skp1 during hypoxia. The results reported above indicate that other, GSK-3β-independent and APC-dependent, pathways may be involved in hypoxia-induced β-catenin degradation. Previous studies have reported another important regulator of β-catenin degradation, namely Siah-1, which serves as an E3 ubiquitin ligase that promotes β-catenin degradation in a phosphorylation-independent manner.[20,21] Accordingly, we investigated whether Siah-1 levels were affected by hypoxia. As seen in Figure 3(a), varying degrees of Siah-1 mRNA upregulation were seen in all cell lines except for HeLa cells. The upregulation of Siah-1 was time dependent, starting 6 h after the initiation of hypoxia and persisting for 12 or 24 h. Moreover, Siah-1 protein levels were also increased in most cell lines, especially MCF-7 and HCT-116 cells, which corresponds with the mRNA results. However, Siah-1 levels were not increased in HeLa cells under hypoxic conditions. Importantly, obvious Siah-1 upregulation was observed in 786-0 cells under hypoxic conditions, suggesting that HIF-1α may not be involved in the hypoxia-induced upregulation of Siah-1.

Based on previous reports that Siah-1 regulates β-catenin degradation by recruiting it to SIP and Skp1, we evaluated the interaction between β-catenin and SIP and Skp1 under hypoxic conditions. As shown in Figure 3(b), under normoxic conditions there was little or no SIP and Skp1 co-immunoprecipitated with β-catenin in MCF-7 and HCT-116 cells. In contrast, under hypoxic conditions more SIP and Skp1 were detected in the β-catenin immunoprecipitates compared with normoxia (Fig. 3b). These immunoprecipitates also contained β-transducin repeat-containing protein (TrCP), an E3 ligase participating in phosphorylation-dependent β-catenin degradation, but there were no significant differences in β-TrCP levels between normoxic and hypoxic conditions. Together, these results indicate that the Siah-1 pathway may be involved in hypoxia-induced β-catenin degradation.

Hypoxia-induced β-catenin degradation requires Siah-1. To verify the role of Siah-1 in β-catenin downregulation during hypoxia, we examined β-catenin levels and the interaction between β-catenin and SIP and Skp1 during hypoxia after Siah-1 silencing. To this end, HCT-116 cells, which have a relatively high basal expression of Siah-1, were transfected with either

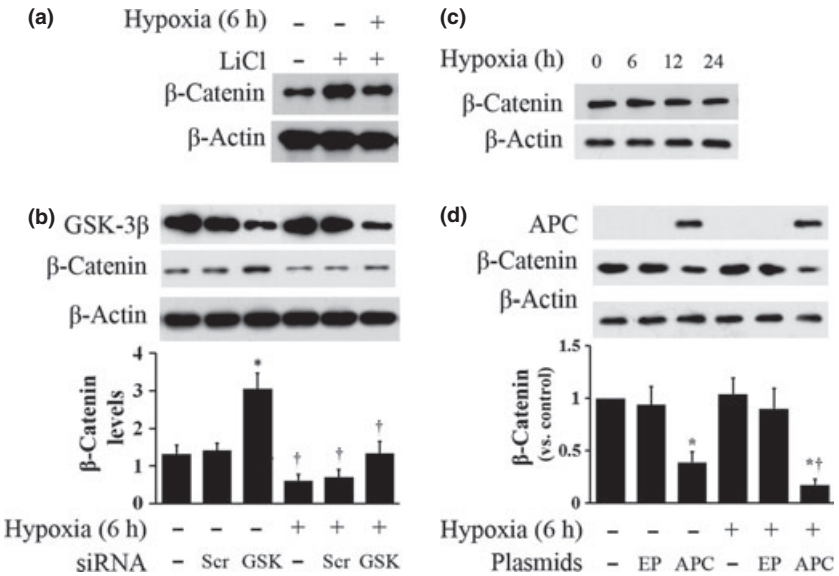

Fig. 2. Role of glycogen synthase kinase (GSK)-3β and adenomatous polyposis coli (APC) in β-catenin degradation under hypoxic conditions. (a) MCF-7 cells were exposed to hypoxia or normoxia in the presence of 20 mM LiCl or KCl (control) for 6 h and β-catenin levels were then determined. (b) MCF-7 cells were transfected with scrambled (Scr) or GSK-3β specific (GSK) siRNA or not transfected (–). Then, 48 h after transfection, cells were exposed to hypoxia and β-catenin levels were determined quantitatively using western blot analysis. Data are the mean ± SD. *$P < 0.05$ compared with untransfected or Scr siRNA-transfected cells.; †$P < 0.05$ compared with normoxic conditions. (c) SW480 cells were exposed to hypoxia for times indicated and β-catenin levels were determined. (d) SW480 cells were transfected with wild-type APC plasmids or empty plasmids (EP) for 36 h before being exposed to hypoxia for 6 h. β-Catenin levels were then determined quantitatively by western blot analysis. Data are the mean ± SD. *$P < 0.05$ compared with untransfected or EP-transfected cells; †$P < 0.05$ compared with normoxic conditions.

doi: 10.1111/j.1349-7006.2011.01950.x

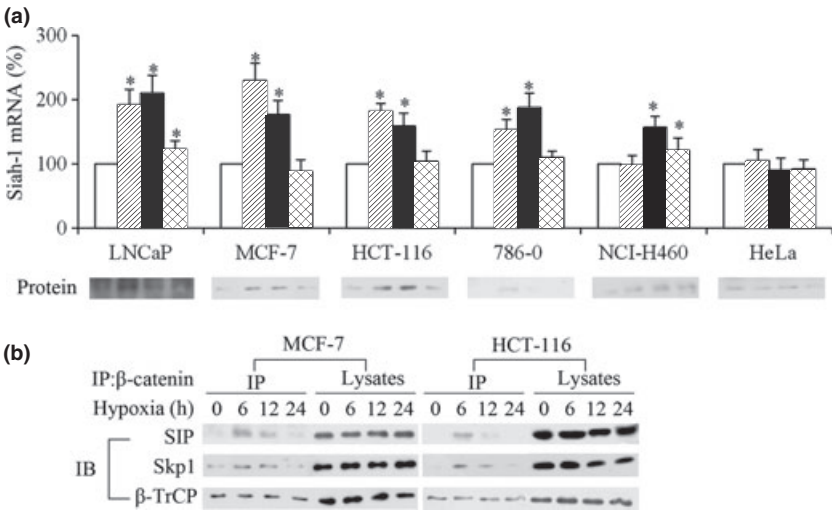

Fig. 3. Expression of Siah-1 and the interaction between β-catenin with SIP and Skp1 under hypoxic conditions in different cell lines. (a) Cells were exposed to hypoxia for 0 (□), 6 (▨), 12 (■), or 24 h (▩) before determination of Siah-1 mRNA and protein levels using real-time PCR and western blot analysis, respectively. Changes in Siah-1 mRNA levels, normalized against β-actin, are expressed as percentage changes compared with untreated (0 h) cells (in which β-catenin expression was set at 100%). Data are the mean ± SD. $*P < 0.05$ compared with normoxic control (0 h). (b) A co-immunoprecipitation (IP) assay was performed to determine the interaction between β-catenin and SIP and Skp1, or β-transducin repeat-containing protein (TrCP), under hypoxic conditions in MCF-7 and HCT-116 cells. IB, immunoblotting.

Siah-1-specific siRNA or control scrambled siRNA. In cells transfected with the scrambled siRNA, significant β-catenin loss was observed under hypoxic conditions (Fig. 4a). In contrast, significantly less loss of β-catenin was observed in Siah-1-silenced cells ($P < 0.05$). In addition, although hypoxia induced significant increases in SIP and Skp1 in the β-catenin immunoprecipitates from untransfected and scrambled siRNA-transfected cells, this induction was potently attenuated by transfection of Siah-1 siRNA (Fig. 4b).

To further confirm the role of Siah-1 in hypoxia-induced β-catenin degradation, we analyzed the effect of dominant negative interference of Siah-1 on hypoxia-induced β-catenin degradation by transfecting HCT-116 cells with pcDNA3.1-Siah-1-ΔDN, a plasmid expressing an N-terminal really interesting new gene (RING) domain-lacking mutant of Siah-1 that encodes a dominant negative form of Siah-1.[18,22] As anticipated, transfection of cells with the dominant negative Siah-1 construct significantly suppressed β-catenin degradation under hypoxic conditions compared with control (empty plasmid; $P < 0.05$), whereas hypoxia-induced upregulation of Siah-1 was not affected (Fig. 4c).

To gain further insight into the role of Siah-1 in hypoxia-induced degradation of β-catenin, the impact of dominant negative interference of Siah-1 on the transactivating ability of β-catenin under hypoxic conditions was assessed using luciferase reporter assay. In this assay, HCT-116 cells were transfected with the β-catenin/TOPflash or its mutant derivative (FOPflash) together with pcDNA3.1-Siah-1-ΔDN or empty plasmids before being exposed to hypoxia. As shown in Figure 4(d), although the dominant negative Siah-1-transfected cells exhibited decreased luciferase activity after exposure to hypoxia, the decrease was much less than that in empty plasmid-transfected cells ($P < 0.05$). The luciferase activity of FOPflash was not affected by hypoxia or transfection. Together, these data provide evidence supporting a role for Siah-1 in hypoxia-induced β-catenin degradation.

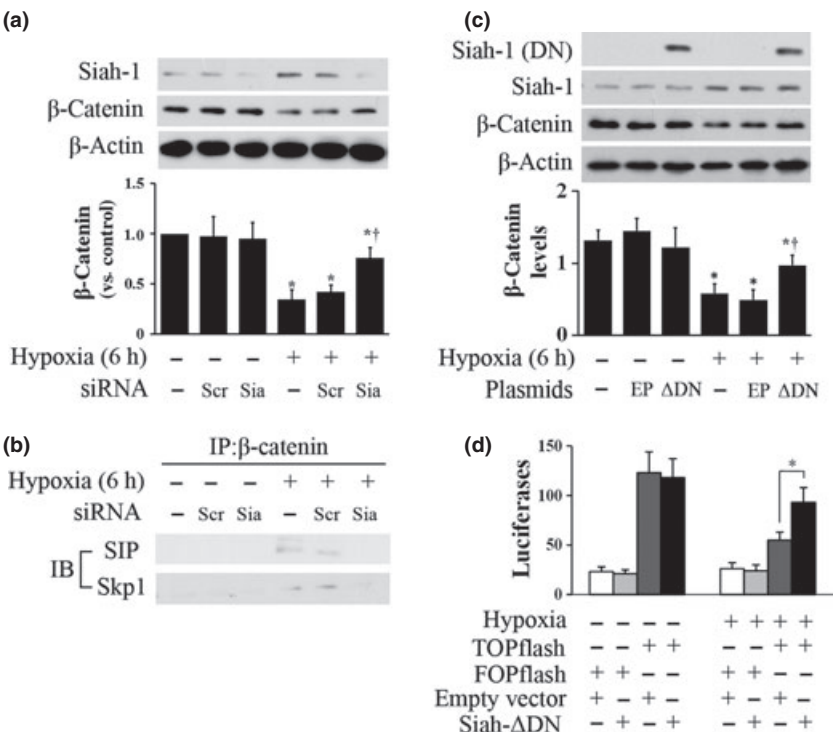

Fig. 4. Effect of silencing or dominant negative (DN) interference of Siah-1 on β-catenin levels under hypoxic conditions. HCT-116 cells were transfected with scrambled (Scr) or Siah-1-specific siRNA (Sia) or not transfected (–). Then, 24 h after transfection, cells were exposed to hypoxia and (a) β-catenin levels and (b) the interaction between β-catenin and SIP and Skp1 were determined. For quantitative analysis of β-catenin levels, data are the mean ± SD. $*P < 0.05$ compared with normoxia; $\dagger P < 0.05$ compared with untransfected or Scr-transfected hypoxic cells. (c) HCT-116 cells were transfected with pcDNA3.1-Siah-1-ΔDN (ΔDN) or empty plasmids (EP) or not transfected (–). Then, 24 h after transfection, cells were exposed to hypoxia and β-catenin levels were determined quantitatively by western blot analysis. Data are the mean ± SD. $*P < 0.05$ compared with normoxia; $\dagger P < 0.05$ compared with untransfected or EP-transfected hypoxic cells. (d) HCT-116 cells were transfected with TOPflash or FOPflash reporter plasmids together with pcDNA3.1-Siah-ΔDN (ΔDN) or empty plasmids (EP) for 48 h. Then, cells were exposed to hypoxia and luciferase activities were determined. Data are the mean ± SD of three experimental replicates. $*P < 0.05$.

Hypoxia induces Siah-1 upregulation and β-catenin degradation in a p53-dependent manner. Because p53 serves as an upstream regulator of Siah-1,[20,21] we investigated whether p53 plays a role in hypoxia-induced Siah-1 upregulation and β-catenin degradation. To this end, we examined β-catenin and Siah-1 levels in p53 wild-type and knockout HCT-116 cells under hypoxic conditions. In accordance with the results described above, hypoxia produced a marked decrease in β-catenin expression in HCT-116 $p53^{+/+}$ cells (Fig. 5a). In contrast, no significant changes were found in β-catenin expression in $p53^{-/-}$ cells after exposure to hypoxia. In addition, hypoxia induced a less profound upregulation of Siah-1 in $p53^{-/-}$ cells than observed in $p53^{+/+}$ cells ($P < 0.05$). However, p53 levels were not affected by hypoxia in $p53^{+/+}$ HCT-116 cells.

We further tested the above hypothesis by evaluating hypoxic β-catenin levels in HCT-116 p53-silenced cells. As expected, the p53 siRNA, but not scrambled siRNA, potently decreased p53 protein levels (Fig. 5b). After exposure of cells to hypoxia, both untransfected and scrambled siRNA-transfected cells exhibited a significant loss of β-catenin. However, the loss of β-catenin was significantly less in p53-silenced cells ($P < 0.05$). Moreover, hypoxic Siah-1 upregulation was markedly suppressed by p53 silencing ($P < 0.05$).

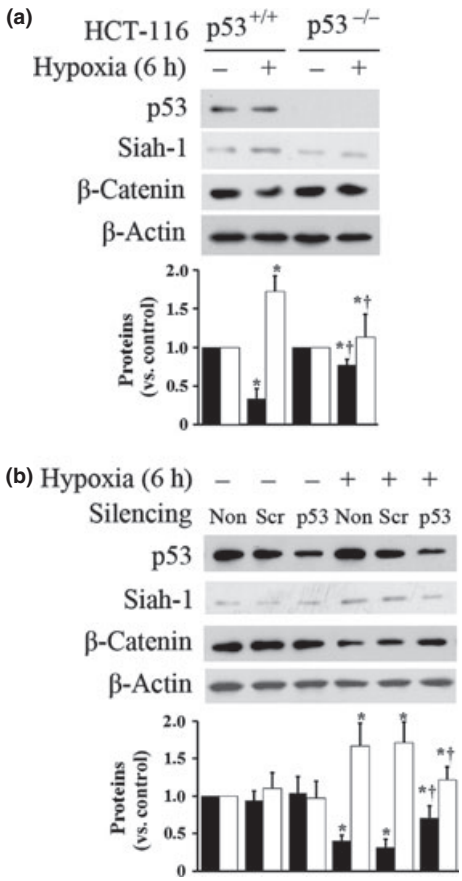

Fig. 5. Effects of p53 knockout or knockdown on β-catenin and Siah-1 levels under hypoxic conditions. (a) Both HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells were exposed to hypoxia and the expression of β-catenin (■) and Siah-1 (□) was determined quantitatively using western blot analysis. Data are the mean ± SD. *$P < 0.05$ compared with normoxia; †$P < 0.05$ compared with $p53^{+/+}$ hypoxic cells. (b) HCT-116 $p53^{+/+}$ cells were transfected with scrambled (Scr) or p53-specific siRNA (p53) or untransfected (Non). Then, 24 h after transfection, cells were exposed to hypoxia and the expression of β-catenin (■) and Siah-1 (□) was determined quantitatively by western blot analysis. Data are the mean ± SD. *$P < 0.05$ compared with normoxia; †$P < 0.05$ compared with untransfected or Scr-transfected hypoxic cells.

Therefore, p53 may be involved in hypoxia-induced β-catenin degradation.

Discussion

In the present study, we investigated β-catenin levels under hypoxic conditions. Hypoxia enhanced β-catenin degradation in all cell lines examined. These results are in agreement with a previous study that also reported marked β-catenin degradation during severe hypoxia (<0.01%) in Wnt3a-expressing cells.[16] Another study also reported β-catenin loss under hypoxic conditions in normal human retinal microvascular endothelial cells.[17] However, there are some reports that have reported conflicting results. For example, a study in macrophages reported that chronic hypoxia induced β-catenin accumulation through AKT activation and GSK-3β inactivation.[12] This apparent discrepancy may stem from the fact that the authors of that study used chronic moderate hypoxia,[12] whereas we used acute severe hypoxia. In addition, tumor cells may build up better tolerance to hypoxia than normal cells because solid tumors experience more severe and persistent hypoxia than do normal cells.[23]

In searching for the fundamental mechanism underlying hypoxia-induced β-catenin degradation, we excluded the possibility of decreased β-catenin gene transcription, which has also been reported in RKO cells.[16] Then, we examined canonical β-catenin degradation machinery under hypoxic conditions. Unfortunately, we did not detect any significant fluctuations in GSK-3β and APC levels. However, hypoxic β-catenin degradation still requires APC, but not GSK-3β. These findings led us to examine other possible GSK-3β-independent mechanisms involved in β-catenin degradation under hypoxic conditions. Recent research has revealed a novel β-catenin degradation pathway, namely p53–Siah-1. The critical protein in this pathway, Siah-1, mediates β-catenin degradation in a phosphorylation-independent manner, thereby downregulating transcription of Wnt target genes. This pathway is initiated by increased expression of proteins in the Siah family, namely Siah-1 and Siah-2, which function as E3 ubiquitin ligases by recruiting E2 ligase Ubc-H5 through its RING domain and interact sequentially with SIP, Skp1, and Ebi proteins.[20,21] The Ebi protein, a member of the F-box protein family, binds and recruits β-catenin to the Siah-1–SIP–Skp1 complex for polyubiquitination and subsequent proteosome-mediated degradation. Cellular Siah-1 protein levels are kept low under normal physiological conditions.[18] However, under conditions of cellular stress, Siah-1 expression can be induced.[24] Clinicopathological studies have demonstrated that Siah-1 expression appears to be modified in human tumor tissues compared with normal controls.[25] In most cases, Siah-1 mRNA is decreased in tumor tissues compared with that in normal tissues.[26] The results of the present study clearly demonstrate a significant upregulation of Siah-1 and increased interaction between β-catenin and SIP and Skp1under hypoxic conditions, implying a potential role for Siah-1 in hypoxia-induced β-catenin degradation. Further experiments using siRNA-mediated knockdown and dominant negative interference confirmed the role of Siah-1 in hypoxia-induced β-catenin degradation. A previous study has shown that Siah-2, another member of the Siah family, is upregulated in both severe and mild hypoxia, suggesting a role in hypoxia signaling.[27] In addition, a recent study has reported a significant increase in Siah-1 in preterm placental explants during hypoxia.[28] These findings are consistent with those of the present study, suggesting Siah-1 as a putative hypoxia-sensing molecule.

It is widely considered that p53 is a key regulator in tumor hypoxia. In the present study, we provide evidence supporting the involvement of p53 in hypoxia-induced upregulation of Siah-1. This finding is in agreement with previous reports that

doi: 10.1111/j.1349-7006.2011.01950.x

Siah-1 is a p53-inducible regulator in mammalian cells.[20,21] However, we are puzzled by the result that there was no significant increase in p53 expression under hypoxic conditions, which seems to undermine our presumption of p53 acting as an upstream modulator of Siah-1 in hypoxia. Indeed, other studies have demonstrated that p53 cannot be induced by hypoxia alone, but that it does respond to the hypoxic microenvironment.[29,30] A previous Siah-1 promoter study isolated a genomic fragment of the Siah-1 promoter region and found an Sp1 recognition site that is required for basal Siah-1 transcription.[31] However, p53 recognition sites are absent in this region. Perhaps, p53 may act as a coactivator of Siah-1 transcription or regulate its enhancer.

It is worth noting that hypoxia-induced Siah-1 upregulation occurs in a time-dependent manner and only persists for 12–24 h. After 24 h hypoxia, cellular Siah-1 levels return to normal. Thus, we hypothesized that Siah-1 may act as a hypoxia-inducible immediate early gene. There are several lines of evidence supporting this assumption. An earlier study has reported that Siah-1 expression is increased significantly under high glucose conditions in Müller Cells and that this is sustained for up to 24 h.[24] Similarly, hypoxia-induced Siah-2 expression starts as early as 2 h after hypoxia and reaches a maximum after approximately 5 h.[27] However, hypoxia induces gradual β-catenin downregulation, with the most significant β-catenin loss at 24 h after hypoxia. This discordance suggests other regulators may be involved in β-catenin downregulation. Moreover, Siah-1 silencing or dominant negative interference of Siah-1 cannot completely rescue β-catenin loss under hypoxic conditions, which also indicates the presence of other mechanisms contributing to hypoxia-induced β-catenin degradation. Given the observation that HIF-1α-deficient 786-0 cells exhibited decreased β-catenin and increased Siah-1 under hypoxic conditions, we suggest that HIF-1α may not be one of the factors contributing to hypoxia-induced β-catenin degradation.

It is a widely accepted that hypoxia can block tumor cell survival, eventually leading to apoptosis or cell death.[32] However, tumor hypoxia can also induce a more aggressive clinical outcome. These two drastically different outcomes can be explained by the assumption that hypoxia kills most of the sensitive tumor cells and leaves only the most resistant, eventually enhancing the malignancy of the surviving cells.[16] Therefore, tumor hypoxia has been considered as a selective pressure for tumor cell populations. The attenuated β-catenin signaling observed in the present hypoxia study may be one of the selective pressures, because β-catenin signaling is an important driving force for tumor progression. An earlier report suggested that hypoxia selects for tumor variants that have lost their apoptotic potential and, in particular, for cells acquiring p53 mutations.[33] Given the present finding that p53 is required for hypoxia-induced Siah-1 upregulation and β-catenin downregulation, we argue that p53 deficiency could allow tumor cells to circumvent the ''β-catenin loss pressure'', thus maintaining the malignancy-promoting ability of β-catenin signaling. In addition, we also hypothesize that β-catenin degradation in hypoxia may be critical for the prevention of constitutive cell proliferation, because decreasing energy consumption is an urgent need for the survival of hypoxic cells.

In conclusion, the present study has revealed a substantial decrease in β-catenin during hypoxia. Although other mechanisms contributing to β-catenin degradation in hypoxia cannot be ruled out, our findings provide a model in which hypoxia-induced Siah-1 upregulation leads to β-catenin degradation in a p53-, but not HIF1α-dependent manner.

Acknowledgments

The authors are grateful to Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, Maryland, USA) for providing the HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells. This work was supported by the National Natural Science Foundation of China (grant no. 30872934).

Disclosure Statement

The authors declare no competing financial interests.

References

1 Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* 2001; **93**: 266–76.
2 Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; **3**: 721–32.
3 Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature* 2005; **434**: 843–50.
4 Blagosklonny MV, An WG, Romanova LY, Trepel J, Fojo T, Neckers L. p53 inhibits hypoxia-inducible factor-stimulated transcription. *J Biol Chem* 1998; **273**: 11 995–8.
5 Kinzler KW, Nilbert MC, Su LK *et al.* Identification of FAP locus genes from chromosome 5q21. *Science* 1991; **253**: 661–5.
6 Kikuchi A. Regulation of beta-catenin signaling in the Wnt pathway. *Biochem Biophys Res Commun* 2000; **268**: 243–8.
7 Korinek V, Barker N, Morin PJ *et al.* Constitutive transcriptional activation by a beta-catenin-Tcf complex in $APC^{-/-}$ colon carcinoma. *Science* 1997; **275**: 1784–7.
8 van de Wetering M, Sancho E, Verweij C *et al.* The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002; **111**: 241–50.
9 An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature* 1998; **392**: 405–8.
10 Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 1999; **398**: 422–6.
11 Liu L, Zhu XD, Wang WQ *et al.* Activation of beta-catenin by hypoxia in hepatocellular carcinoma contributes to enhanced metastatic potential and poor prognosis. *Clin Cancer Res* 2010; **16**: 2740–50.
12 Deguchi JO, Yamazaki H, Aikawa E, Aikawa M. Chronic hypoxia activates the Akt and beta-catenin pathways in human macrophages. *Arterioscler Thromb Vasc Biol* 2009; **29**: 1664–70.
13 Genetos DC, Toupadakis CA, Raheja LF *et al.* Hypoxia decreases sclerostin expression and increases Wnt signaling in osteoblasts. *J Cell Biochem* 2010; **110**: 457–67.
14 Kimura S, Kitadai Y, Kuwai T *et al.* Expression of p53 protein in esophageal squamous cell carcinoma: relation to hypoxia-inducible factor-1alpha, angiogenesis and apoptosis. *Pathobiology* 2005; **72**: 179–85.
15 Kaidi A, Williams AC, Paraskeva C. Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nat Cell Biol* 2007; **9**: 210–7.
16 Verras M, Papandreou I, Lim AL, Denko NC. Tumor hypoxia blocks Wnt processing and secretion through the induction of endoplasmic reticulum stress. *Mol Cell Biol* 2008; **28**: 7212–24.
17 Hoang MV, Smith LE, Senger DR. Moderate GSK-3beta inhibition improves neovascular architecture, reduces vascular leakage, and reduces retinal hypoxia in a model of ischemic retinopathy. *Angiogenesis* 2010; **13**: 269–77.
18 Hu G, Fearon ER. Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. *Mol Cell Biol* 1999; **19**: 724–32.
19 Vandesompele J, De Preter K, Pattyn F *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; **3**: doi:10.1186/gb-2002-3-7-research0034.
20 Liu J, Stevens J, Rote CA *et al.* Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. *Mol Cell* 2001; **7**: 927–36.
21 Matsuzawa SI, Reed JC. Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. *Mol Cell* 2001; **7**: 915–26.
22 Jang KL, Shackelford J, Seo SY, Pagano JS. Up-regulation of beta-catenin by a viral oncogene correlates with inhibition of the seven in absentia homolog 1 in B lymphoma cells. *Proc Natl Acad Sci U S A* 2005; **102**: 18431–6.
23 Semenza GL. Hypoxia and cancer. *Cancer Metastasis Rev* 2007; **26**: 223–4.
24 Yego EC, Mohr S. Siah-1 protein is necessary for high glucose-induced glyceraldehyde-3-phosphate dehydrogenase nuclear accumulation and cell death in Müller cells. *J Biol Chem* 2010; **285**: 3181–90.

25 Bruzzoni-Giovanelli H, Fernandez P, Veiga L *et al.* Distinct expression patterns of the E3 ligase SIAH-1 and its partner Kid/KIF22 in normal tissues and in the breast tumoral processes. *J Exp Clin Cancer Res* 2010; **29**: 10.
26 Brauckhoff A, Ehemann V, Schirmacher P, Breuhahn K. [Reduced expression of the E3-ubiquitin ligase seven in absentia homologue (SIAH)-1 in human hepatocellular carcinoma]. *Verh Dtsch Ges Pathol* 2007; **91**: 269–77 (In German).
27 Nakayama K, Ronai Z. Siah: new players in the cellular response to hypoxia. *Cell Cycle* 2004; **3**: 1345–7.
28 Rolfo A, Many A, Racano A *et al.* Abnormalities in oxygen sensing define early and late onset preeclampsia as distinct pathologies. *PLoS ONE* 2010; **5**: e13288.
29 Pan Y, Oprysko PR, Asham AM, Koch CJ, Simon MC. p53 cannot be induced by hypoxia alone but responds to the hypoxic microenvironment. *Oncogene* 2004; **23**: 4975–83.
30 Achison M, Hupp TR. Hypoxia attenuates the p53 response to cellular damage. *Oncogene* 2003; **22**: 3431–40.
31 Maeda A, Yoshida T, Kusuzaki K, Sakai T. The characterization of the human Siah-1 promoter(1). *FEBS Lett* 2002; **512**: 223–6.
32 Papandreou I, Krishna C, Kaper F, Cai D, Giaccia AJ, Denko NC. Anoxia is necessary for tumor cell toxicity caused by a low-oxygen environment. *Cancer Res* 2005; **65**: 3171–8.
33 Graeber TG, Osmanian C, Jacks T *et al.* Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996; **379**: 88–91.

doi: 10.1111/j.1349-7006.2011.01950.x