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Cancer cell-associated cytoplasmic B7–H4 is induced by hypoxia through hypoxia-inducible factor-1α and promotes cancer cell proliferation

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ABSTRACT

Aberrant B7–H4 expression in cancer tissues serves as a novel prognostic biomarker for poor survival in patients with cancer. However, the factor(s) that induce cancer cell-associated B7–H4 remain to be fully elucidated. We herein demonstrate that hypoxia upregulates B7–H4 transcription in primary $CD138^+$ multiple myeloma cells and cancer cell lines. In support of this finding, analysis of the Multiple Myeloma Genomics Portal (MMGP) data set revealed a positive correlation between the mRNA expression levels of B7–H4 and the endogenous hypoxia marker carbonic anhydrogenase 9. Hypoxia-induced B7–H4 expression was detected in the cytoplasm, but not in cancer cell membranes. Chromatin immunoprecipitation analysis demonstrated binding of hypoxia-inducible factor-1α (HIF-1α) to proximal hypoxia-response element (HRE) sites within the B7–H4 promoter. Knockdown of HIF-1α and pharmacological inhibition of HIF-1α diminished B7–H4 expression. Furthermore, knockdown of cytoplasmic B7–H4 in MCF-7 decreased the S-phase cell population under hypoxia. Finally, MMGP analysis revealed a positive correlation between the transcript levels of B7–H4 and proliferation-related genes including MKI67, CCNA1, and Myc in several patients with multiple myeloma. Our results provide insight into the mechanisms underlying B7–H4 upregulation and its role in cancer cell proliferation in a hypoxic tumor microenvironment.

1. Introduction

B7–H4, a co-inhibitory molecule that negatively regulates T cell immunity [1], is rarely observed in resting antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells [2], but is ectopically upregulated in a variety of cancer tissues including ovary, kidney, stomach, lung, pancreas, breast cancers, and melanoma [3–5]. Numerous immunohistochemical studies have demonstrated that B7–H4 is expressed not only in the membrane but also in the cytosol of cancer cells. Notably, an association appears to exist between cytoplasmic B7–H4 expression and higher cancer stages. For example, in endometrioid adenocarcinoma, high-risk tumors tend to preferentially express B7–H4 in the membrane and cytoplasm compared to low-risk tumors, which predominantly exhibit apical membrane B7–H4 expression [6]. This also applies to malignant breast cancer; membrane and cytoplasmic B7–H4 expression in invasive breast carcinomas is significantly greater compared to benign tumors exhibiting apical membrane B7–H4 [7]. These data suggest that cancer-associated B7–H4 is involved in cancer progression.

Unlike membrane B7–H4, which is directly involved in the suppression of T cell function [8], numerous gene knockdown studies have demonstrated that cancer cell-associated cytoplasmic B7–H4 is involved in basic cellular processes including cancer cell proliferation and apoptosis [9,10]. For example, knockdown of B7–H4 mRNA, and subsequent attenuation of cytoplasmic B7–H4 expression, inhibit cancer cell proliferation by

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downregulating ERK1/2 phosphorylation [9]. In a parallel study, B7–H4 knockdown in cancer cells enhanced apoptosis by increasing caspase activity, reducing Bcl-2 anti-apoptotic protein and increasing BAX pro-apoptotic protein, thereby adjusting the Bcl-2/BAX ratio [9].

Similarly, overexpression of B7–H4 in a human ovarian cancer cell line, in which B7–H4 expression was restricted to the cytoplasm, enhanced tumor formation in immunodeficient mice [10]. Collectively, these data indicate that ectopic expression of cytoplasmic B7–H4 in cancer cells may play an important role in the proliferation and survival of these cells. However, the factor(s) that induce upregulation of B7–H4 in cancer cells in a tumor microenvironment remain unclear. Regarding the mechanism regulating B7–H4 expression, several reports have suggested that immunosuppressive cytokines are an inducing factor for B7–H4 upregulation in immune cells. For example, peripheral blood monocytes, which do not express B7–H4, upregulate B7–H4 expression in response to immunosuppressive cytokines including IL-6 and IL-10, accounting for the observation that membrane B7–H4 is overexpressed in tumor macrophages derived from ovarian cancer ascites, in which these cytokines are abundant [11]. Notably, IL-6 and IL-10 failed to induce B7–H4 expression in these cancer cells, suggesting that B7–H4 expression is differentially regulated depending on cell type [11]. Another in vitro study revealed that a lung cancer cell line was able to induce B7–H4 by tumor macrophages [8]. However, the exact mechanism by which cancer cell-associated B7–H4 is induced in cancer cells is not fully understood.

In the present study, we demonstrated that hypoxia induced cytoplasmic, but not membrane B7–H4, in cancer cells, a process mediated by hypoxia-inducible factor (HIF)-1α binding to the first or second proximal hypoxia-response element (HRE) site in the B7–H4 promoter. We also assessed RNA expression using the Multiple Myeloma Genomics Portal (MMGP) data set [12] and observed a positive correlation between the expression of B7–H4 transcripts and carbonic anhydrogenase 9 (CA9), an endogenous hypoxia marker, in patients with multiple myeloma (MM). We also demonstrated that hypoxia-induced cytoplasmic B7–H4 expression increased the S-phase cell population, suggesting that it plays a role in cancer cell proliferation; this is supported by our analysis of the MMGP data set in which we observed a positive correlation between the transcript levels of B7–H4 and proliferation-related genes. Therefore, our results provide insight into the mechanisms underlying B7–H4 upregulation and its role in cancer cell proliferation in a hypoxic tumor microenvironment.

2. Materials and methods

2.1. Human samples

Bone marrow aspirates from patients were enriched for MM cells by negative selection using an MM-enrichment cocktail (StemCell Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's instructions. This study was approved by the institutional review board of Inje University College of Medicine, Busan, Korea.

2.2. Cell culture under hypoxia

Human MM cell lines including KMS-12-PE, KMS-20, and KMS-28-BM were kindly provided by Dr. T. Otsuki (Kawasaki Medical School, Okayama, Japan). ZR75-1, MCF-7, MDA-MB-453, and HeLa were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). Cell lines were cultured as described previously [13]. For hypoxic culture, cells were incubated in a hypoxic chamber with 1% oxygen, or grown in culture media containing desferrioxamine (DFO) or cobalt (II) chloride ($CoCl_2$) under normoxia.

2.3. Western blot and flow cytometry

Western blot and flow cytometry were conducted as previously described [13,14].

2.4. Knockdown with siRNA

Cancer cells (5×10^5 cell/well in 6 well plate) were transfected using TurboFect™ (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 300 pmol siRNA of human B7–H4, HIF-1α, and scrambled control siRNA. The sequences of each siRNA oligonucleotide were as follows:

B7–H4 #1: sense 5′-GGUGUUUUAGGCUUGGUCC(dTdT)-3′
B7–H4 #2: sense 5′-CAGUGUUUGCUGAUCAAGU(dTdT)-3′
HIF-1α: sense 5′-GUCUUCACGGCGGGCGGCCCCCAGG(dTdT)-3′
Control: sense 5′-GCAUCAAGGUGAACUUCAA(dTdT)-3′

2.5. Proliferation assay

Water Soluble Tetrazolium salts (WST) assay was used to evaluate cell proliferation using Cyto-X (LPS Solution, Seoul, Korea) according to the manufacturer's instructions. In some experiments, cells (1×10^5 cell/well in 24 well plate) were pulsed with 10 μM 5-bromo-2-deoxyuridine (BrdU) (Sigma–Aldrich, St. Louis, MO, USA) for 2 h, and then stained with anti-BrdU and 7-aminoactinomycin D (7-AAD). BrdU incorporation was detected by flow cytometry as described previously [14].

2.6. ChIP assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [14]. Briefly, MCF7 cells (5×10^6) were cross-linked, lysed, and sonicated according to the manufacturer's instructions (Exacta ChIP kit, R&D Systems, Minneapolis, MN, USA). Aanti–HIF–1α antibody (5 μg) was used to immunoprecipitate DNA fragments; precipitated fragments corresponding to B7–H4 promoter were amplified by polymerase chain reaction (PCR) using the following primers:

Forward: 5′-GCAGGGAATTCTGCACAGCCAGTT-3′
Reverse: 5′-TCCCGTGTATTGGCCTTAAATAGT-3′

2.7. Reporter gene assay

MCF-7 cells (1×10^5 cell/well in 24 well plate) were transfected with 0.5 μg of pGL-3 vector-containing the B7–H4 promoter using TurboFect™ and subjected to a luciferase assay using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.8. Site-directed mutagenesis

The putative HRE sites in B7–H4 promoter were mutated using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Two base pairs in each HRE site were changed from GC to AT. Mutations were confirmed by DNA sequence analysis.

2.9. Statistical analysis

Student's paired *t*-test was used to compare quantitative changes observed in a minimum of two independent experiments. A value of $p \leq 0.05$ was taken to indicate statistical significance.

3. Results

3.1. B7–H4 expression is positively associated with the expression of the endogenous hypoxia marker CA-9

Several immunohistochemical studies have demonstrated that upregulation of immune checkpoints such as B7–H1, B7–H3, and B7–H4 occurs in advanced cancers whose pathological activities are known to be associated with hypoxia [15]. Therefore, we examined the possible association between immune checkpoint gene expression and hypoxia by analyzing the MMGP RNA expression data set, which includes 414 patients with MM. Significant positive correlations were observed between the mRNA levels of B7–H4 and the endogenous hypoxia marker CA-9 ($p = 0.0001$, $r^2 = 0.0583$), and to a lesser extent, between B7–H1 and CA-9 expression levels ($p = 0.0002$, $r^2 = 0.0327$; Fig. 1A). We then analyzed the relationship between the expression levels of B7–H4 and CA-9 in subgroups of patients with MM exhibiting different molecular subtypes based on common gene signatures predominantly driven by recurrent translocations and hyperploidy [16]. A significant positive correlation was observed between B7–H4 levels and CA-9 expression in hyperploidy (Hy), PR, MF, and LB MM subgroups (Fig. 1B).

3.2. Hypoxia induces cytoplasmic B7–H4 in cancer cell lines

To determine whether B7–H4 expression is regulated by hypoxia, we isolated and incubated $CD138^+$ primary MM cells obtained from the bone marrow of patients under hypoxic conditions (0.5% oxygen) and measured B7–H4 transcripts using real-time PCR. We detected higher levels of B7–H4 mRNA in primary MM cells incubated during hypoxia vs. normoxia (Fig. 2A). In a KMS28BM culture (an MM cell line) obtained under hypoxic conditions, induction of B7–H4 mRNA occurred within 16 h of hypoxia exposure and peaked 24 h postexposure (Fig. 2B). Similar results were observed for MCF-7, a breast cancer cell line, when it was grown under hypoxia or treated with DFO, a chemical hypoxia inducer (Supplementary Fig. S1).

We then confirmed hypoxia-induced B7–H4 expression in protein levels. MM, cervical and breast cancer cell lines upregulated B7–H4 protein to a greater degree under hypoxia vs. normoxia (Fig. 2C). However, not all MM cell lines induced B7–H4 protein expression under hypoxia (data not shown). Notably, flow cytometry revealed that hypoxia did not upregulate surface B7–H4, but induced cytoplasmic B7–H4 in these cell lines (Fig. 2D). Cytoplasmic localization of hypoxia-induced B7–H4 was further indicated by biochemical analysis, which demonstrated that B7–H4 occurred in the cytoplasmic fraction, but not in the membrane or nuclear fractions of these cell lines (Supplementary Fig. S2). Notably, in endogenous membrane B7–H4-expressing breast cancer cell lines, B7–H4 was not induced by hypoxia (Supplementary Fig. S3).

3.3. HIF-1α is required for the regulation of B7–H4 transcription in response to hypoxia

Having demonstrated that hypoxia increases B7–H4 transcription, we then investigated the molecular mechanism underlying the hypoxic regulation of B7–H4 transcription. To achieve high transfection efficiency in promoter constructs, we used the adherent breast cancer cell line MCF-7 as the promoter activity assay. The B7–H4 promoter sequence contains five possible HREs with a core consensus RCGTG sequence that binds to the HIF family. The first and second HREs overlap in the region between −130

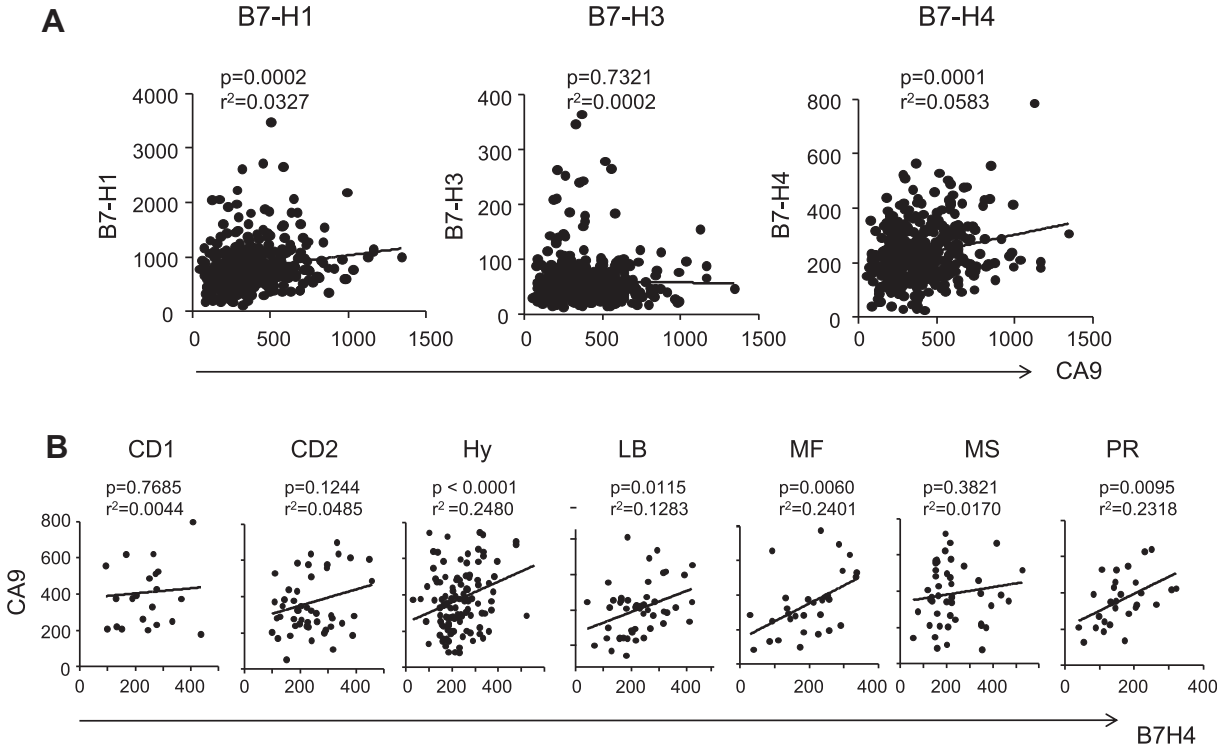

Fig. 1. Association between B7–H4 expression and an endogenous hypoxia marker. Data from the Multiple Myeloma Genomics Portal were analyzed. (A) Correlation between the expression levels of immune checkpoint genes and the hypoxia marker carbonic anhydrogenase 9 (CA9). (B) Correlation between the gene expression levels of B7–H4 and CA9 in subgroups of patients with MM having different molecular subtypes. Linear regression test was used to evaluate statistical significance.

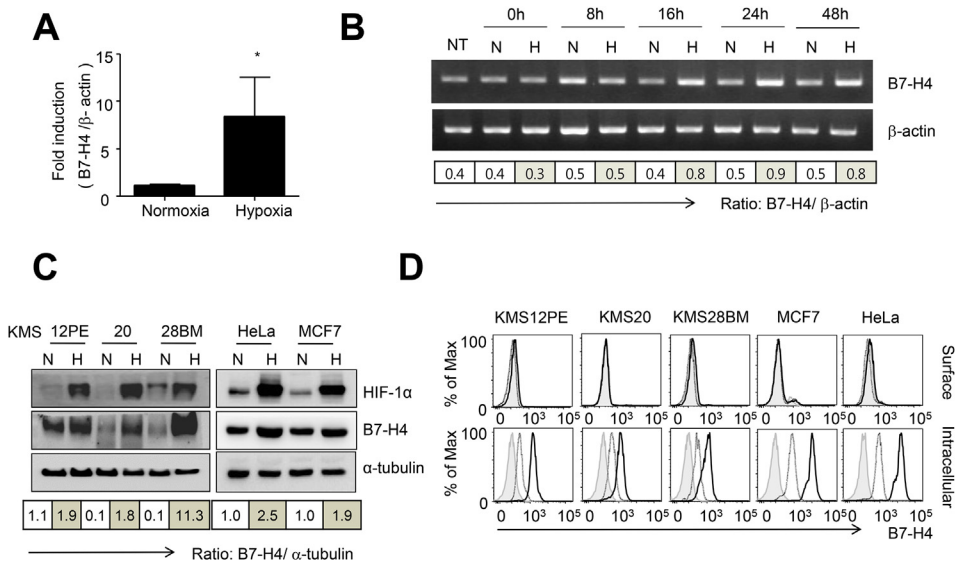

Fig. 2. Hypoxia induces cytoplasmic B7–H4 in cancer cells. (A) mRNA levels of B7–H4 were analyzed by real-rime PCR using the $CD138^+$ bone marrow cells of MM patients (n = 3) grown under normoxia or hypoxia. Data represent means ± SD. Student's *t* test was used to evaluate statistical significance (*, $p < 0.05$). (B) KMS-28BM cells were grown under hypoxia for the indicated durations. Reverse transcription (RT)-PCR analysis was performed for B7–H4. β-actin was used as an internal control. (C, D) The expression of B7–H4 in each cancer cell line grown under normoxia (N) or hypoxia (H) was examined via Western blotting (C) and flow cytometry (D). B7–H4 staining is indicated by filled histograms (isotype control), dotted lines (normoxia), and thick lines (hypoxia). Data are representative of three independent experiments.

and −121 (HRE-1/2), and all five HREs are located within 2 kb of the transcription start site (Supplementary Fig. S4).

We observed significantly greater induction of luciferase activity in cells transiently transfected with pGL3-luciferase constructs containing 1–2 kb of human B7–H4 promoter grown under chemical hypoxia compared to the vehicle (Fig. 3A). A similar degree of luciferase induction was observed in promoter construct-transfected cells grown under hypoxia vs. normoxia (data not shown). The HIF family of transcription factors are critical regulators of hypoxia-mediated transcription [17]. We then sought to determine if HIF-1α or HIF-2α plays regulatory roles in B7–H4 expression in response to hypoxia by simultaneously co-transfecting MCF-7 with a plasmid encoding HIF-1α or HIF-2α, and B7–H4 promoter luciferase constructs. We observed higher levels of luciferase activity in cells transfected with the HIF-1α vs. HIF-2α plasmid (Fig. 3B). Therefore, we focused on HIF-1α and its role in hypoxia-induced B7–H4 expression. First, HIF-1α knockdown was performed with siRNAs in MCF-7 cells grown under hypoxia; B7–H4 expression decreased in cells that otherwise induced B7–H4 (Fig. 3C). Furthermore, MCF-7 cells treated with PX-478, an HIF-1α inhibitor, reduced B7–H4 induction under hypoxia (Fig. 3D). Because the B7–H4 promoter contains five HREs, we sought to determine which site is responsible for hypoxic regulation of B7–H4 induction. We generated mutant reporter constructs using site-directed mutagenesis at each HRE site (mut-HRE-Luc). Twenty-four hour hypoxia failed to induce luciferase activity in mut-HRE-Luc, which contains mutations at HREs 1 and 2 (mut 1/2), but other mutants exhibited comparable luciferase activity to the parental promoter–reporter construct (Fig. 3E). We then performed a ChIP assay in MCF-7 cells following 24 h exposure to normoxia or hypoxia using an antibody against HIF-1α. The ChIP assay revealed that endogenous HIF-1α bound to HRE-1/2 of the B7–H4 promoter in hypoxic MCF-7 cells (Fig. 3F).

3.4. Hypoxia-induced B7–H4 facilitates cancer cell proliferation

Recently, hypoxia has emerged as a key regulator of tumor proliferation and malignant progression [18,19]. We sought to demonstrate whether B7–H4 is involved in the regulation of cancer cell proliferation under hypoxic conditions. Human MM cell lines exhibiting hypoxic induction of cytoplasmic B7–H4 were characterized by greater proliferation in response to 24 h hypoxia vs. normoxia (Fig. 4A), whose proliferation was dependent upon hypoxia exposure time (Fig. 4B). To directly examine the effect of B7–H4 on cancer cell proliferation, we assessed whether B7–H4 affects cell cycle progression in cancer cells under hypoxia. A BrdU incorporation assay revealed that in hypoxic MCF-7 cells transfected with B7–H4 siRNAs reduced the proportion of BrdU-positive S-phase cells to a significantly greater degree compared to those administered scrambled control siRNA (Fig. 4C, Supplementary Fig. S5A). In accordance with previous reports [9,10], we also observed increased apoptosis in hypoxic MCF-7 cells transfected with B7–H4 siRNA compared to control cells (Supplementary Fig. S5B). We then investigated whether B7–H4 expression was correlated with the expression of proliferation-associated genes such as MKI67, CCNA1, and Myc, in MM patient molecular subgroups by analyzing the MMGP data set. B7–H4 mRNA expression was positively correlated with MKI67 gene expression in CD2 and PR MM patient subgroups (Fig. 4D), with CCNA1 expression in the CD2, Hy, MF, MS, and PR subgroups, and with Myc in the CD2, Hy, MF, and PR subgroups (Supplementary Fig. S6). These data indicate that B7–H4 enhances cancer cell proliferation, which was further supported by an in vivo study demonstrating that B7–H4 knockdown HeLa cells grow markedly less rapidly in nude mice compared to control mock cells (Fig. 4E).

4. Discussion

In the present study, correlation analysis of the MMGP data set revealed that transcript levels of B7–H4 are positively correlated with those of hypoxic markers including CA9 in subgroups of patients with MM having different molecular signatures, suggesting that B7–H4 expression may be related to hypoxia. Western blot analysis confirmed hypoxic induction of B7–H4 in cancer cell lines, prompting us to investigate the molecular mechanism underlying the hypoxic induction of B7–H4. HIF transcription factors have been well established to mediate multiple transcriptional responses to hypoxic stress in both normal and cancerous cells, with

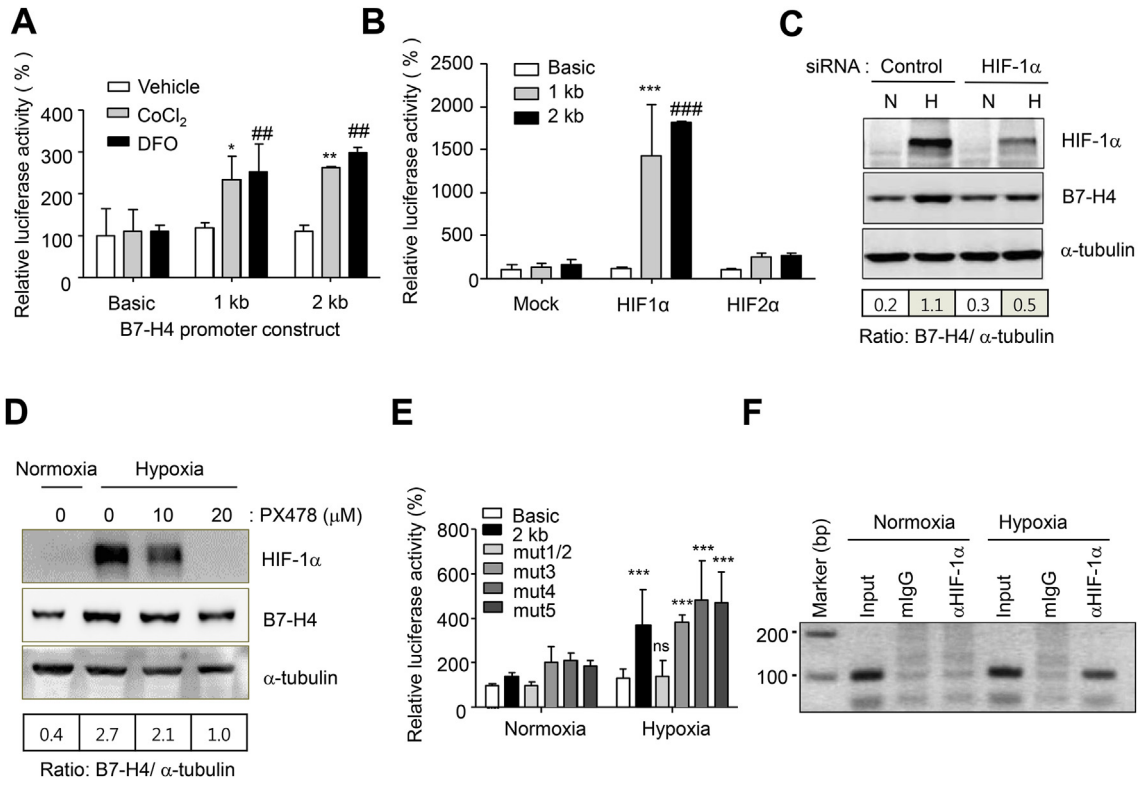

Fig. 3. HIF-1α is required for the hypoxic induction of B7–H4. MCF-7 cells were transfected with B7–H4-promoter luciferase constructs for 1 day and then subjected to luciferase assay. (A) Transfected cells were grown in media containing $CoCl_2$ and DFO. Data represent means ± SEM. (*, $p < 0.05$; **, $p < 0.01$; ##, $p < 0.01$; vs. basic). (B) MCF-7 cells were co-transfected with plasmid containing the HIF-1α or HIF-2α gene. Data represent means ± SEM. (***, $p < 0.001$; ###, $p < 0.001$; vs. mock). (C) MCF-7 cells were transfected with HIF-1α or control siRNA, or (D) treated with the HIF-1α inhibitor PX-478, grown under normoxia (N) or hypoxia (H) for 1 day, and subjected to Western blot analysis for the expression of B7–H4 or HIF-1α. (E) MCF-7 cells were transfected with luciferase constructs containing wild-type or mutated B7–H4 promoters for 1 day and subjected to luciferase assay after an additional 24 h of hypoxia exposure. Data represent means ± SEM. (***, $p < 0.001$; vs. 2 kb in hypoxia). (F) MCF-7 cells were grown under normoxia or hypoxia and subjected to a ChIP assay (see Materials and Methods). Anti–HIF–1α (αHIF-1α) or mouse IgG was used to precipitate sonicated chromatin. ANOVA was used to evaluate statistical significance (A, B, E). Each experiment was performed in triplicate in more than three separate experiments.

the majority of HIF transcriptional responses attributed to HIF-1α and HIF2α [20]. Our results using a promoter activity assay indicate that overexpression of HIF-1α under normoxic conditions is more strongly associated with increased transcriptional activity in the B7–H4 promoter than with HIF-2α overexpression, indicating that HIF-1α is more likely than HIF-2α to be involved in the hypoxic induction of B7–H4. The involvement of HIF-1α in the hypoxic induction of B7–H4 is further supported by our biochemical analysis; first, a mutation at the HRE sequence to which HIF-1α binds loses transcriptional activity of the B7–H4 promoter. Second, the ChIP assay revealed that HIF-1α directly binds to HRE sites of the B7–H4 promoter. Third, HIF-1α inhibitor reduces B7–H4 expression under hypoxic conditions. Expression of HIF-1α subunits is regulated not only by hypoxic stress, but also by oncogenic signaling including genetic lesions at the von Hippel–Lindau (VHL) locus [21]. Under normoxic conditions, HIF-1α subunits are modified by prolyl hydroxylase, which leads to VHL tumor suppressor gene-mediated proteosomal degradation. Several recent studies have indicated that VHL loss enhances HIF-1α expression, thereby promoting cancer cell proliferation and survival [22]. Therefore, further study of the relationship between transcriptional levels of VHL and B7–H4 would be instructive.

Flow cytometry and Western blot analysis revealed that hypoxic conditions induce cytoplasmic, but not membrane, B7–H4 expression in cancer cells that endogenously express cytoplasmic B7–H4 at very low levels such as HeLa and MCF-7 cells. Notably, in breast cancer cell lines that endogenously express membrane B7–H4, including SK-BR3, ZR75-1, and MDA-MB453, membrane B7–H4 is not induced by hypoxia. Further study of the molecular basis of differential regulation of membrane and cytoplasmic B7–H4 in response to hypoxia is warranted.

Recently, Zhang et al. suggested that cytoplasmic B7–H4 is mobilized to the nucleus under normoxic conditions, shuttling between the two compartments due to the presence of the nuclear localization sequence (NLS) in its C-terminus [23]. Consistent with this observation, we also observed hypoxia-induced B7–H4 in the nucleus in the presence of the nuclear export receptor inhibitor (LMB), which accumulates shuttling proteins in the nucleus (data not shown). However, the B7–H4 protein was not detected in the nuclear fraction in the absence of LMB even under hypoxic conditions. B7–H1 (PD-L1) is also induced under hypoxia through HIF-1α, but not HIF-2α, in immune-suppressing myeloid-derived suppressive cells (MDSCs) and cancer cells [24]. However, hypoxic stress does not always induce immune checkpoints. For example, hypoxia selectively diminishes the surface expression of CD80 [25], indicating that the upregulation of immune checkpoints is differentially controlled by hypoxia. At present, whether cytoplasmic localization of hypoxia-induced B7–H4 results from splicing variants with a deleted signal sequence or entrapment in cytoplasm by unknown mechanisms such as posttranslational modification including phosphorylation is not clear. Further study is required to elucidate the molecular mechanism underlying preferential localization of hypoxia-induced B7–H4.

Taken together, our results demonstrate that hypoxia induces B7–H4 transcription through HIF-1α, with subsequent B7–H4 expression localized in the cytoplasm of cancer cells. Hypoxia-

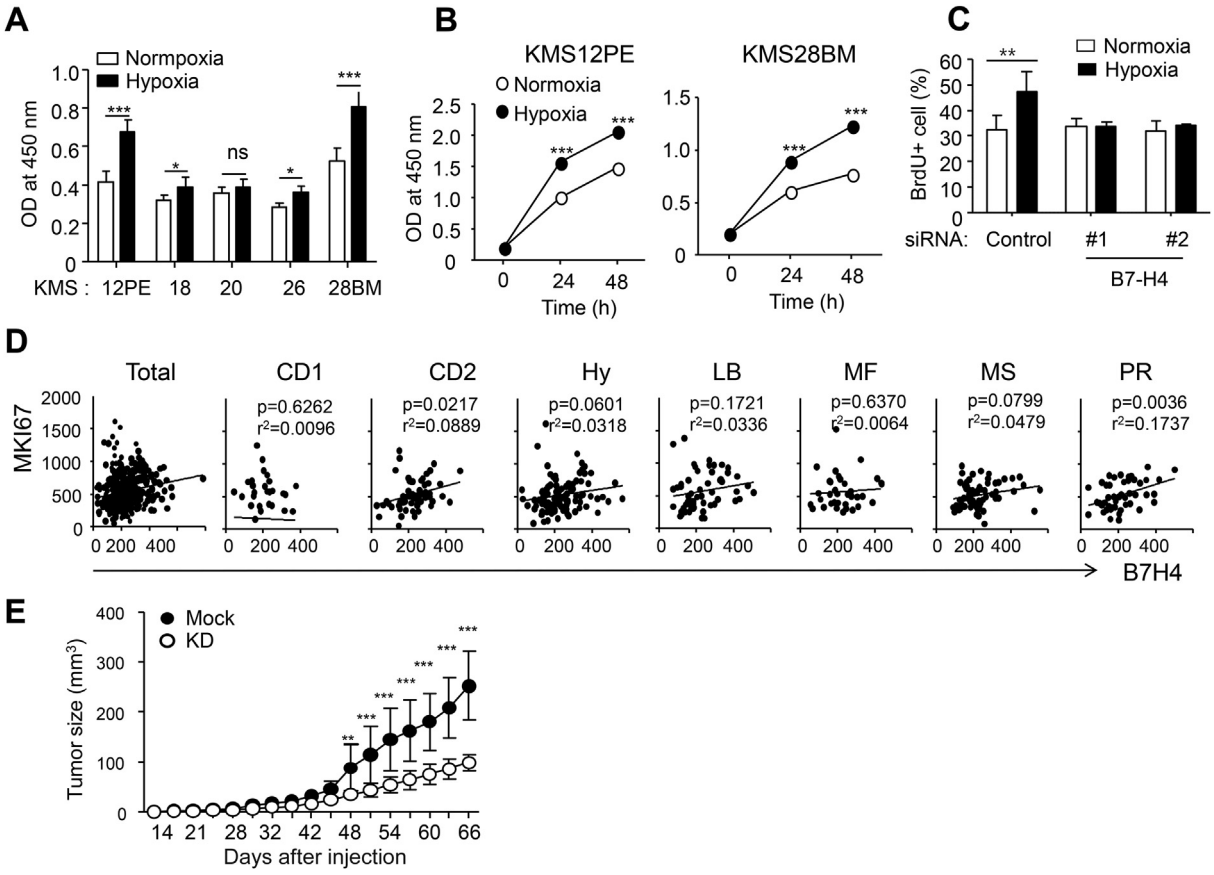

Fig. 4. Hypoxia-induced B7–H4 facilitates cancer cell proliferation. Cancer cells ((A) 1×10^4 or (B) 2×10^4 cells/well) were grown under normoxia or hypoxia for (A) 1 day or (B) the durations indicated, and cell proliferation was measured by a WST assay. Data represent means ± SEM. (*, $p < 0.05$; ***, $p < 0.001$). (C) MCF-7 cells were transfected with B7–H4 or control siRNA, and cultured under normoxia or hypoxia for 22 h. Cells were then pulsed with 10 μM BrdU for 2 h, stained with anti-BrdU-FITC antibody and 7-AAD, and analyzed by flow cytometry. Data represent means ± SEM. (**, $p < 0.05$). (D) Data from the Multiple Myeloma Genomics Portal was assessed for correlations between the gene expression levels of B7–H4 and MKI67. (E) Nude mice were implanted with HeLa cells transfected with mock or B7–H4 shRNA plasmid, and tumor size was measured at the indicated times. Data are provided as means ± SEM. (**, $p < 0.01$; ***, $p < 0.001$). Student's *t* test (A, B, C, E) and linear regression test (D) were used to evaluate statistical significance. Data are representative of three independent experiments.

induced cytoplasmic B7–H4 increases the proliferation of hypoxic cancer cells by enhancing the S-phase of cell cycle. In addition to its association with the negative regulation of antitumor immunity, cytoplasmic B7–H4 may play a critical role in tumor progression under hypoxic conditions in a tumor microenvironment; targeting cytoplasmic B7–H4 represents a possible therapeutic approach to cancer treatment.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.098.

Transparency document

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