Suppression of FBI-1 by hypoxia induces MCT4-mediated lactate efflux in colon cancer cells

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Suppression of FBI-1 by hypoxia induces MCT4-mediated lactate efflux in colon cancer cells

Directed by Professor Man-Wook Hur

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Min-Young Kim

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Suppression of FBI-1 by hypoxia induces MCT4-mediated lactate efflux in colon cancer cells

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Tumor hypoxia is the situation where tumor cells have been deprived of oxygen. In order to support continuous growth and proliferation in hypoxic condition, cancer cells are found to alter their metabolism by modulating expression of many relevant genes. Investigation of the metabolic reprogramming and underlying mechanism of cancer cells under hypoxic condition is critical in our understanding of cancer cell physiology and development of anti-cancer therapeutics.

Unexpectedly, I found that FBI-1, initially characterized as

proto-oncogene, represses *MCT4* gene transcription under both normoxic and more potently, under hypoxic conditions by binding to the FRE (FBI-1 responsive element) of the promoter. FBI-1 also binds to the HRE (HIF-1α responsive element) of the MCT4 promoter and inhibits HIF-1α binding. FBI-1 enhances HIF-1α protein degradation.

Interestingly, FBI-1 expression can be negatively regulated at transcription level by RelA/p65 binding to the FBI-1 promoter under hypoxic condition, which increases MCT4 expression. Considering that NF-kB is activated in the cells under hypoxic condition, repression of FBI-1 expression might facilitate cancer cell growth by exporting glycolysis derived lactate through induction of MCT4.

Overall, in hypoxic cancer cells, FBI-1 is repressed to induce MCT4-mediated efflux of lactate and H^+, which makes extracellular pH acidic and support cancer cell proliferation.

Key words: FBI-1, ZBTB7A, MCT4, Hypoxia, RelA/p65, Transcription factor, Cancer metabolism, Lactate efflux, HIF-1α

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I. INTRODUCTION

Otto Warburg proposed a hypothesis that rapidly proliferating cells such as cancer cells consume glucose at a surprisingly high rate compared to normal cells and produce excess lactate rather than oxidizing glucose completely through TCA cycle, a phenomenon known as the ‘‘Warburg effect’’ in the 1920s.[1] After Warburg effect hypothesis was reported, many of researchers became interested in cancer specific metabolic reprogramming.

Two major features of cancer cells are the ability to grow under hypoxic conditions and unique metabolic regulation.[2, 3] Tumor hypoxia is the situation

where tumor cells have been deprived of oxygen. As tumor grows, parts of the tissue are in limited blood supply and expose to hypoxic condition. Vast majority of oxygen being consumed within 70 to 150 μm of tumor vasculature, the cells beyond this area have limited supply of oxygen available and have to reprogram metabolism to support continuous growth and proliferation in hypoxic environments.[4]

To survive and proliferate under hypoxic condition, cancer cells have unique metabolic program and alteration in gene expression. HIF-1 is a transcription factor that plays a central role in hypoxic condition.[5, 6] HIF-1 is a heterodimer composed of a hypoxia-inducible α subunit (HIF-1α or HIF-2α) and a constitutively expressed β subunit (HIF-1β). HIF-1α expression is regulated by cellular oxygen level, but HIF-1β is constitutively expressed. HIF-1α protein is regulated by proteasomal degradation. HIF-1α is hydroxylated by specific prolyl hydroxylases (PHD1, PHD2 and PHD3) at two conserved proline residues (Pro402 and Pro564) located within oxygen-dependent degradation (ODD) domain. Hydroxylated HIF-1α is recognized by von Hippel-Lindau (pVHL) protein, and ubiquitinated by pVHL/Elongin B/C/Cul2 ubiquitin E3 ligase, and degraded by the 26S proteasome.[7] Low oxygen under hypoxic conditions results in inhibition of prolyl hydroxylase and consequently stabilization of HIF-1α protein. HIF-1α

translocates into nucleus and forms a heterodimeric complex with HIF-1β. The HIF-1 complex binds to HIF-1α responsive element (HRE) and activates HIF-1α target genes such as *VEGF*, *EPO*, *HK2*, *MCT4* and *MMP*.[8] Immuno-histochemical analysis of human tumor biopsy specimens showed dramatic overexpression of HIF-1α.[9, 10]

Cancer cells under hypoxic condition rely on aerobic glycolysis to support their proliferation. In cancer cells, glucose is preferentially catabolized to lactate via glycolysis, rather than metabolized to carbon dioxide via mitochondrial TCA cycle/oxidative phosphorylation (OXPHOS).[11] Accordingly, cancer cells produce massive amount of lactate. Cancer-derived lactate is used by stromal cells, oxidative cancer cells and vascular endothelial cells to support their proliferation.[12, 13] Low intracellular pH is detrimental for cell survival. Cancer cell pump out lactate and H^+ by monocarboxylate transporters (MCTs) and maintain intracellular pH neutral. Acidic extracellular pH is beneficial for cancer cells adaptation and survival under hypoxic condition.[14-16]

The MCT protein families are composed of 14 members and expressed in various tissues. The MCT proteins are predicted to have 12 transmembrane domains, with the N- and C-termini facing the intracellular side of the membrane and a large cytosolic loop between domains 6 and 7.

Transmembrane domains are well conserved and MCTs differ mainly in their N- and C-termini and intermediary loop sequences.[17] Only 4 isoforms (MCT1 ~ MCT4) have been reported to transport monocarboxylates in an electroneutral transport mode of 1 H : 1 monocarboxylate with different substrate affinities. MCT1, which is found in nearly all tissues studied, has a Km value of 3–5 mM for L-lactate, and plays an active role in the uptake of lactate in the heart, skeletal muscle and red blood cells, as well as in liver. MCT4 has a low-affinity for lactate (Km value for lactate of 17–35 mM) and is found prominently in glycolytic tissues such as white skeletal muscle and hypoxic cancer.[18, 19] MCT4 is overexpressed in most of cancer cells, and supports cancer proliferation.[20-23] MCT4 is more important in hypoxic cancer cells. In cancer cells under hypoxic condition, to avoid toxic intracellular acidosis, HIF-1α increases MCT4 expression by binding HRE of the MCT4 promoter, and up-regulated MCT4 contributes to the acidification of the extracellular space and increases intracellular pH by pumping out glycolysis-derived lactate and H^+.[24, 25] Cell surface expression of MCT1 and MCT4 transporters requires the formation of complexes with their chaperone protein, CD147, which is a transmembrane protein of the immunoglobulin superfamily and is also known as basigin (BSG).[26-28]

The BTB domain (also known as POZ domain) was first discovered in

1993 and named after the Drosophila zinc finger transcription factor Bric-a-brac, Tramtrack, and Broad Complex. This domain is an evolutionary conserved protein-protein interaction motif found throughout the eukaryotes. Among the members of this group, more than 45 members have one or more of C-terminal C_2H_2 Krüppel-type zinc finger DNA binding domain.). Many of the POZ-ZF proteins are characterized as important transcription factors implicated in various cellular pathways including cancer.[29-31] FBI-1 (ZBTB7A) is a proto-oncoprotein of the POZ-domain Krüppel-like (POK) family of transcription factors. It plays important roles in the cell cycle, cell differentiation, proliferation, fatty acid synthesis, immune responses and oncogenesis. FBI-1 promotes cellular transformation by repressing alternative reading frame (ARF), p21 and Rb expression, and has been shown to promote cell proliferation and oncogenesis in the thymus, liver and spleen in transgenic mice.[32-35] In addition, expression of the fatty acid synthase (FASN), which is important in palmitate synthesis and cell proliferation in cancer cells, is potently activated by FBI-1 in the presence of sterol regulatory element binding protein-1 (SREBP-1).[36] FBI-1 also binds both non-methylated and methylated DNA and that MBD3 is recruited to the CDKN1A promoter through its interaction with FBI-1, where it enhances transcriptional repression by FBI-1.[37]

However, the function of FBI-1 as a tumor suppressor has been published recently. Pandolfi et al. showed that LRF (mouse FBI-1) inactivation promotes Pten loss−driven prostate tumorigenesis by activating Sox9-dependent oncogenic pathways, suggesting FBI-1 might act as a tumor suppressor gene.[38] FBI-1 expression is lost in a subset of human advanced prostate cancers. Furthermore, FBI-1 inhibits glycolysis metabolism by suppressing the expression of multiple glycolysis genes such as glucose transporter 3 (GLUT3), phosphofructokinase (PFKP) and pyruvate kinase (PKM). Recent report showed that chromosome19p13.3 containing *FBI-1* gene is frequently lost in various human cancers using the Cancer Genome Atlas (TCGA) databases. Intriguingly, FBI-1 is located in chromosome19p13.3 and a significant decrease of FBI-1 copy number variation (CNV) was seen in many types of human carcinoma, especially at the late stage of tumors. A close association of reduced mRNA level and CNV of FBI-1 was discovered in colon carcinoma. FBI-1 transcription was significantly lower in the late stage than the early stage of tumors, and low FBI-1 expression was associated with poorer patient survival.[39] In addition, FBI-1 represses transcription of *Melanoma Cell Adhesion Molecule (MCAM)* gene, which is widely known to play a pivotal role in metastatic progression of melanoma. FBI-1 was proposed to suppress melanoma metastasis.[40]

I investigated the function of FBI-1 in colon cancer cells under hypoxic condition. FBI-1 represses colon cancer cell growth in hypoxic condition through repression of *MCT4* gene by binding to the FRE and blocking HIF-1α binding at the HRE of MCT4 promoter. I also showed that FBI-1 de-stabilized HIF-1α protein by increasing ubiquitination in hypoxic condition. My results indicate that FBI-1 expression is repressed to increase efflux of glycolysis-derived lactate and H^+ and thereby support to effective colon cancer cell proliferation in hypoxic condition.

II. MATERIALS AND METHODS

1. Plasmids

To express FBI-1 and RelA/p65 proteins, full-length human FBI-1 and RelA/p65 cDNA fragment were PCR amplified from a cDNA library and cloned into pcDNA3 vector (Invitrogen). To prepare the pGL2-MCT4-Luc and pGL3-FBI-1-Luc plasmids, the human MCT4 (bp -873 to +53) or FBI-1 promoter region (bp -796 to +96) was PCR-amplified from human genomic DNA and cloned into pGL2- or pGL3-Basic vector. Other pGL2-MCT4-Luc constructs with shorter promoter fragments were cloned similarly. The pGL3-$(HRE)_{3X}$-Luc construct contains 3 copies of HIF-1 responsive element(5'-AGGCTGTGTGAGACAGCACGTAGGGCTTCGAACGGCCG GTAGGTCGA-3') of the erythropoietin gene promoter. The pNF-κB-Luc plasmid was purchased from Clontech (Palo Alto, CA, USA). All plasmid constructs were verified by sequencing.

2. Antibodies and reagents

Antibodies against FBI-1 (13E9: sc-33683), MCT4 (H-90: sc-50329), GAPDH (FL-335: sc-25778), HDAC1 (H-51: sc-7872) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). A purified Mouse anti-human

HIF-1α (610959) was purchased from BD Biosciences. Specific antibody against RelA/p65 (ab7970) was purchased from Abcam (Calbiochem). Cobalt chloride ($CoCl_2$) (C-8661) were purchased from Sigma (SIGMA-Aldrich, St-Louis, MO, USA). Proteasome inhibitor MG132 and cycloheximide (CHX) were purchased from Calbiochem (San Diego, CA, USA). Most of the chemical reagents were purchased from Sigma.

3. Site-directed mutagenesis

The HIF-1α point mutants were constructed by site-directed mutagenesis of the HIF-1α expression vector. The mutagenesis primers were used to convert proline residues at positions 402 and 564 to arginine (HIF-1α P402A and P564A). Conditions of PCR were as follows: denaturation step 5 min at 95 ℃, followed by 18 cycles 30 sec at 95 ℃, 1 min at 55 ℃ and 10 min at 68 ℃ for and final elongation step 10 min at 68 ℃. The primers for site-directed mutagenesis were used as listed in Table 1.

4. Cell culture

HCT116, RKO, DLD-1, SW480, HT29, Caki-1, A548 and U343 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, NY, USA) and 1% of

penicillin-streptomycin (Gibco-BRL, NY, USA). The cells were maintained at 37 ℃ in a humidified incubator with 5% CO_2.

5. Hypoxia and $CoCl_2$ incubation.

Hypoxic conditions were achieved with a hypoxia chamber with a gas mixture of 1% O_2, 5% CO_2 and 94% N_2. To prepare cells under hypoxia-mimicking condition, the cells were treated with Cobalt chloride ($CoCl_2$) at a 200 μM concentration.

6. Transcription analysis

pGL2-MCT4-Luc, pGL3-FBI-1-Luc and pGL3-$(HRE)_{3X}$-Luc reporter fusion plasmids with FBI-1, RelA/p65 or HIF-1α PA expression vector in various combinations were transiently transfected into HCT116 cells using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA). After 24 hr of incubation, cells were exposed to hypoxia (O_2, 1%) or treated with 200 μM $CoCl_2$. The cells were harvested and analyzed for luciferase activity. Reporter activity was normalized with co-transfected ß-galactosidase activity for transfection efficiency. Luciferase activities were measured using a SpectraMAX 250 ELISA reader (Molecular Device Co., Sunnyvale, CA) at 420 nm and is shown as the average of three independent assays.

7. Knock-down of FBI-1 and RelA/p65 expression by siRNA

siRNA against FBI-1 and RelA/p65 mRNA were purchased from Bioneer (Bioneer, Daejeon, South Korea). siRNA (60 pmoles) were transfected into HCT116 cells using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA). After transfection, the cells were harvested and the total RNA was extracted, Semi-quantitative PCR and RT-qPCR analysis of mRNA were performed described as Material & Method 9. The siRNA were used as listed in Table 2.

8. Western blot analysis

Cells were harvested and lysed in 1X IP buffer (0.05 M Tris (pH 7.4), 0.15 M NaCl, 0.1% NP-40). Cell lysates (30 μg) were separated using 10% SDS-PAGE gel electrophoresis, transferred onto Immun-Blot™ PVDF membranes (Bio-Rad, Philadelphia, PA, USA), and blocked with 5% skim milk (BD Biosciences, San Jose, CA, USA). Blotted membranes were incubated with indicated antibodies and further incubated with anti-mouse or rabbit or hamster secondary antibody conjugated with HRP (Vector Laboratory, Burlingame, CA, USA / (Santa Cruz, CA, USA). Protein bands were visualized with ECL solution (PerkinElmer, Waltham, MA, USA).

9. RT-PCR and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from the cells using QIAzol reagent (QIAGEN, CA, USA). cDNAs were synthesized using 3 μg of total RNA, random hexamer (10 pmol) and reverse transcriptase II (200 units) in a 20 μl reaction volume, using a reverse transcription kit (Promega, WI, USA). RT-qPCR was performed using SYBR Green Master Mix (Applied Biosystems) in a StepOnePlus real-time PCR instrument (Applied Biosystems). Based on DNA sequence information of cDNA, I designed quantitative real time PCR primers. The oligonucleotide primers sets of RT-qPCR were used as listed in Table 3. All reactions were performed in triplicate.

10. Oligonucleotide pull-down assays

HCT116 cells were transfected with indicated plasmid, cultured for 48 hr, and lysed in HKMG buffer (10 mM HEPES pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0.5% NP-40). Cell lysates were then incubated with 1 μg of biotinylated double-stranded oligonucleotide (FRE and HRE of the MCT4 promoter / NF-κB RE#1 and #2 of the FBI-1 promoter) at 4 ℃ overnight. The oligonucleotide primers sets of oligo pull down assay were used as listed in Table 4. Both forward and reverse oligonucleotides were incubated with 10X reaction buffer (3) (500 mM

Tris-HCl pH 8.0, 100 mM $MgCl_2$, 1 M NaCl) at 95℃ for 5 min. After incubation, the temperature was gradually reduced to room temperature. To collect DNA-bound proteins, the mixtures were incubated overnight with streptavidin agarose beads (Thermo Scientific), washed with PBS buffer, and precipitated by centrifugation. The precipitates were then analyzed by western blot as described above, using anti-FBI-1 or anti-HIF-1α or anti-RelA/p65 antibody.

11. Chromatin immunoprecipitation and quantitative PCR analysis (ChIP-qPCR)

HCT116 cells were transfected with transcription factor expression plasmids, grown for additional 24 hr. The cells were exposed to hypoxia (O_2, 1%) and treated with formaldehyde (final concentration 1%) to cross-link proteins to DNA, followed by cell lysis in RIPA buffer. Chromatin was sheared 3 times (amplitude: 50%, cycle: 0.5, Sec: 30 sec) by sonication (Bioruptor, Diagenode) and the supernatant then incubated with specific antibodies at 4 ℃ overnight. The next day, the mixture was incubated with salmon sperm DNA/protein-A agarose beads at 4 ℃ for 4 hr. The beads were then collected, washed with PBS, and eluted with elution buffer (1 % SDS, 0.1 M $NaHCO_3$). Input and immunoprecipitated chromatin were then incubated with 5 M NaCl at 65 ℃ for 4 hr to reverse crosslinks. After

incubation, the temperature was gradually reduced to room temperature. To precipitate the DNA, phenol/chloroform/isoamyl alcohol (Calbiochem) was added and the DNA pellets were obtained by centrifugation. As a negative control for the qChIP assays, IgG was used. To amplify the promoter regions, PCR reactions of the immunoprecipitated DNA were carried out. The oligonucleotide primers sets used for ChIP assay were listed in Table 5. PCR was performed using the following cycling conditions: denaturation 5 min at 94 °C, followed by 40 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 35 sec at 72 °C, and a final extension reaction 5 min at 72 °C, using a GeneAmp PCR system 9700 (Applied Biosystems).

12. Flow cytometry cell-cycle analysis

HCT116 and RKO cells were transfected with the pcDNA3-FBI-1 plasmid, and exposed to hypoxia (O_2, 1%). The cells were then washed, fixed using methanol, and stained with a solution containing propidium iodide (50 μg/mL) and ribonuclease A (100 μg/mL) for 30 min at 37℃ in the dark. DNA contents, cell cycle profiles, and forward scatter were analyzed using a FACS Calibur flow cytometer (BD Biosciences) at detection wavelengths 488 nm (excitation) and 575 nm (peak emission). The obtained data were analyzed using ModFit LT 2.0 (Verity Software House, Inc).

13. Cell growth assay

HCT116 and RKO colon cancer cells were grown in 35 mm dishes to 50% confluency after transfection with pcDNA3-FBI-1 plasmid, and cultured for 0–3 days. The cell growth was then measured by directly counting the number of cells with a hematocytometer.

14. Crystal violet staining

HCT116 and RKO cells were plated in 60 mm dishes. The cells were transfected with pcDNA3-FBI-1 plasmid and exposed to hypoxia (O_2, 1%) for 0 and 2 days. After removing culture medium, the cells were washed with PBS twice, fixed using 10% formalin, and then stained with 2% crystal violet (C3886, SIGMA-Aldrich, St. Louis, MO, USA) for 5 min, followed by three rinses with water. The dishes were air-dried and photographed. Crystal violet dye was extracted using 2ml of 1% SDS and absorbance was read at 570 nm with a microplate reader (VERSA max).

15. Cycloheximide (CHX) chase assay

HCT116 cells were transfected with pcDNA3-FBI-1 plasmid and exposed to hypoxia (O_2, 1%). After 9 hr incubation, cycloheximide (CHX) was used at a final concentration of 50 μg/ml for 0, 15, 30, 60, 120 min, and analyzed by

western blot analysis. Relative amounts of HIF-1α were calculated by normalizing to GAPDH.

16. Ubiquitination Assays

HCT116 cells grown in 60 mm dishes were co-transfected with pcDNA3-HIF-1α PA and His-ubiquitin expression vector in the presence or absence of pcDNA3-FBI-1 plasmid. After 24 hr, the cells were treated with 50 μM MG132 for 6 hr and harvested. The cells were re-suspended in 1X lysis buffer and 500 μg of the cell lysates was incubated with MagneHis^{TM} nickel particles for 1 hr at 4 ℃ and precipitated. The precipitated pellets were washed three times with buffer (0.5 % NP-40, 20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA), and analyzed by western blot using an anti-HIF-1α antibody.

17. Cytosolic and nuclear protein fractionation

HCT116 cells were exposed to hypoxia (O_2, 1%) or treated with 200 μM $CoCl_2$. The cells were lysed by buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.2 mM PMSF, 0.1% NP-40) and incubated on ice for 15 min. After centrifugation at 3,000 rpm for 10 min, supernatants of cell lysates were collected (cytoplasmic fractions) and pellet was

re-suspended in buffer A' (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.2 mM PMSF). After centrifugation at 4,000 rpm for 10 min, the pellets were re-suspended in buffer B (20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF) and incubated on ice for 30 min. Nuclear fractions were collected by centrifugation at 13,000 rpm for 10 min. Nuclear of cytosol expression of RelA/p65 under hypoxia or $CoCl_2$ treatment was analyzed by western blot using an anti-RelA/p65 antibody. HDAC1 was used as a cytoplasmic protein marker and GAPDH was used as nuclear protein marker.

18. Analysis of lactate concentration

HCT116 cells were transfected with pcDNA3-FBI-1 plasmid and exposed to hypoxia (O_2, 1%). Extracellular lactate concentration of cell culture medium was determined by measuring absorbance at 570 nm using a colorimetric assay kit (Sigma, cat. MAK064). Lactate efflux was calculated using formula " extracellular lactate concentration / total lactate concentration (Lactate $_{intracellular}$ + Lactate $_{extracellular}$ concentration)".

19. Extracellular pH measurement

HCT116 and RKO cells were transfected with pcDNA3-FBI-1 plasmid

and the cells were exposed to hypoxia (O_2, 1%) for indicated time period. Extracellular pH of the cell culture medium was measured using pH-indicator paper strips or was determined using pH meter.

20. DNA-protein binding assay using Episeeker assay kit

To measuring FBI-1 and HIF-1α of DNA binding activity, we used an EpiSeeker DNA-protein Binding Assay kit (ab117139). In this assay, a biotin-labeled double stranded oligonucleotide, containing a DNA-binding consensus sequence for the target transcription factor, is incubated with a protein mixture in the binding assay buffer. The active form of transcription factor in the protein mixture then binds to its consensus sequence. The oligo-protein complex is then captured onto the assay microwell. The target protein can be recognized with a high affinity antibody and colorimetrically measured using a detection antibody color-development reagent reaction system.

21. Statistical analysis

Student's t-test was used for the statistical analyses. P-values of < 0.05 were considered statistically significant.

Table 1. Sequence of oligonucleotide primers used for site-directed mutagenesis

Oligo	Primer	Sequence (5'→ 3')
HIF-1α	Forward	TTTGCTGGCCGCAGCCGCTGGAG (23mer)
-P402A	Reverse	CTCCAGCGGCTGCGGCCAGCAAA (23mer)
HIF-1α	Forward	GATGTTAGCTGCATATATCCCA (23 mer)
-P564A	Reverse	TTGGGATATATGCAGCTAACATC (23 mer)

Table 2. Sequence of siRNA of FBI-1 and RelA/p65

siRNA	Sequence (5'→ 3')
siFBI-1	CCUCACAAUAAAACCAACU (dTdT)
	AGUUGGUUUUAUUGCGAGG(dTdT)
siRelA/p65	CCUGAGCACCAUCAACUAU(dTdT)
	AUAGUUGAUGGUGCUCAGG(dTdT)

Table 3. List of forward primer and reverse primer used for amplification using RT-qPCR

Gene	Primer	Sequence (5'→ 3')
18S rRNA	Forward	AGTCCCTGCCCTTTGTACACA (21 mer)
	Reverse	GATCCGAGGGCCTCACTAAAC (21 mer)
FBI-1	Forward	TATGTCGCCAGATGCCAGGA (20 mer)
	Reverse	GTCTCAGTGCAGCAGAGCGTCTA (23 mer)
MCT4 (SLC16A3)	Forward	GAGTTTGGGATCGGCTACAG (20 mer)
	Reverse	CGGTTCACGCACACACTG (18 mer)
CD147	Forward	TGCTGGTCTGCAAGTCAGAG (20 mer)
	Reverse	GCGAGGAACTCACGAAGAAC (20 mer)
HIF-1α	Forward	ACAGTATTCCAGCAGACTCAA (21 mer)
	Reverse	CCTACTGCTTGAAAAAGTGAA (21 mer)
RelA/p65	Forward	CCCCACGAGCTTGTAGGAAAG (21 mer)
	Reverse	CCAGGTTCTGGAAACTGTGGAT (22 mer)

Table 4. List of primers used for Oligo pull down assay

Gene	Oligo name	Sequence (5'→ 3')
FBI-1	NF-κB	CCTTGCGGGGGGATCCCCACCGCCTCAC (28 mer)
	RE #1	GTGAGGCGGTGGGGATCCCCCCGCAAGG (28 mer)
FBI-1	NF-κB	GGCAAGAAGGGACCTGCCGAGCCTGTTC (28 mer)
	RE #2	GAACAGGCTCGGCAGGTCCCCTCTTGCC (28 mer)
MCT4 (SLC16A3)	FRE	GCACCACCTGCCCTGGGCGGGA (22 mer)
		TCCCGCCCAGGGCAGGTGGTGC (22 mer)
MCT4 (SLC16A3)	HRE	GCTCACAGGCACGTGCCCTCATCTT (25 mer)
		AAGATGAGGGCACGTGCCTGTGAGC (25mer)

Table 5. List of primers used for Chromatin Immunoprecipitation (ChIP)

Gene	Oligo name	Position	Sequence (5'→ 3')
FBI-1	NF-κB RE #1	-676/-570	GCAAGTCCCACTGAGGCCCG (20 mer)
			TGTGGGTCGCGCGGCCTGGG (20 mer)
FBI-1	NF-κB RE #2	-261/-107	AATAACAGCCCGGTCTCCCC (20 mer)
			TTAAAGGTGCCGCAGCCGCC (20 mer)
MCT4 (SLC16A3)	FRE	-865/-681	AACTGATGAATCGCCTGGTG (20 mer)
			AACTTCACAGATGAAGACTC (20 mer)
MCT4 (SLC16A3)	HRE	-671/-557	TCACCACTTGCCACAGGCCT (20 mer)
			ACAGAGGCTGGGTCTGGCCC (20 mer)

III. RESULTS

1. FBI-1 represses MCT4 expression and FBI-1 expression is repressed in colon cancer cells under hypoxic condition

FBI-1, a protein previously known as a proto-oncoprotein, represses various gene expression including p21, RB, and ARF. Recently, Liu et al. reported that FBI-1 inhibits glycolysis by repressing critical glycolytic genes, including GLUT3, PFKP and PKM, suggesting that FBI-1 might function as a tumor suppressor in metabolic perspective. MCT4, a monocarboxylate transporter 4, can transport glycolysis-derived lactate outside of the plasma membrane. MCT4 maintains intracellular pH neutral and makes cancer cells microenvironment acidic, which is important for cancer cells under hypoxic condition. Interestingly, differential mRNA expression analyses of doxycycline-inducible HEK293-Trex-FBI-1 cells showed that the MCT4 mRNA was decreased by doxycycline-induced FBI-1 overexpression (unpublished data), implying that *MCT4* might be the target gene of FBI-1.

In the cells under hypoxic condition, MCT4 expression is increased by hypoxia inducible transcription factor (HIF-1α) binding to the HRE of the MCT4 promoter. I investigated the regulatory relationship between *FBI-1*, *MCT4* and *HIF-1α* gene expression in the human colon cancer HCT116 cells

under hypoxic condition (O_2, 1% ; 0, 1, 3, 6, 9, 24 and 48 hr). Under hypoxic condition, MCT4 and HIF-1α expression is increased as reported, and FBI-1 expression is gradually decreased in time dependent manner at both mRNA and protein level (Figure 1A and B). I investigated whether the regulatory relationship between FBI-1 and MCT4 or HIF-1α is applicable to other cancer cell types; colon cancer cells, DLD-1, HT29, RKO, SW480; renal cancer cell, Caki-1; lung carcinoma, A549; glioblastoma, U343. As in HCT116 cells, 4 colon cancer cells showed that FBI-1 expression was decreased under hypoxic condition in time-dependent manner and decrease of FBI-1 expression varies depending on the cell types (Figure 1C). Interestingly, FBI-1 expression in Caki-1 and U343 was not affected by hypoxia and in A549 cell, FBI-1 expression was increased up to 6 hr and decreased by 9 hr of cultured under hypoxic condition (Supplementary Figure 1).

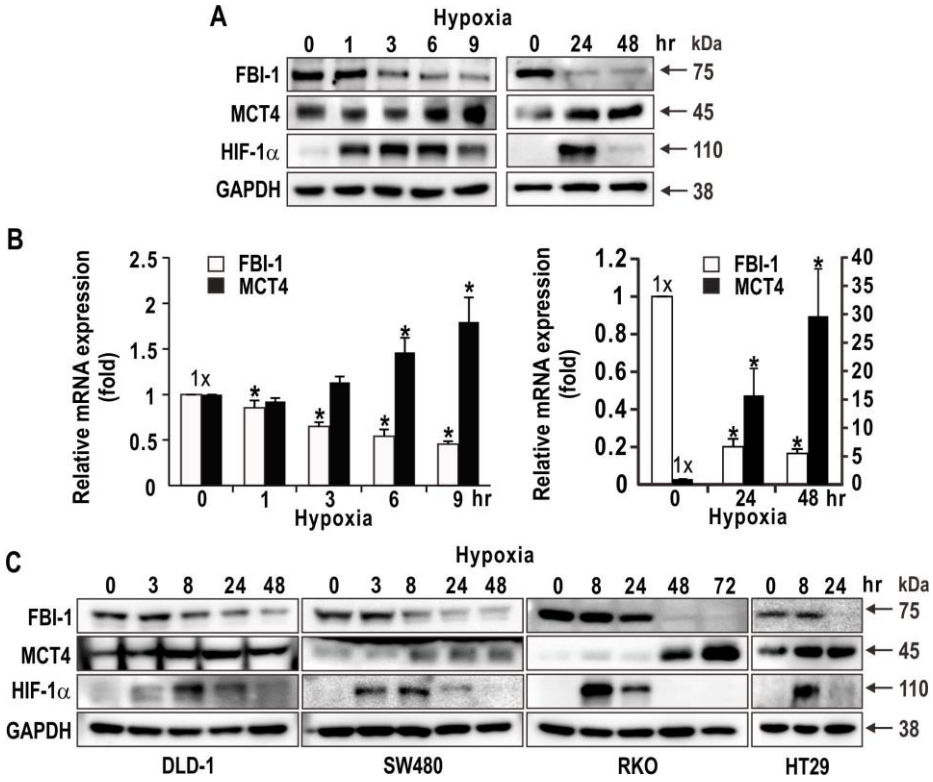


Figure 1. Under hypoxic condition, FBI-1 expression is decreased and MCT4 expression is increased in colon cancer cells. (A) Western blot analysis. HCT116 cells were exposed to hypoxia (O_2, 1%) for indicated time. FBI-1, MCT4, HIF-1α, GAPDH protein in whole-cell extracts of HCT116 cells were analyzed using indicated antibodies. HIF-1α, a marker of hypoxic cancer cells. GAPDH, loading control. (B) RT-qPCR analysis of FBI-1 and MCT4 mRNA expression under hypoxic condition. HCT116 cells were exposed to hypoxia (O_2, 1%). RT-qPCR data were normalized with 18S ribosomal RNA.

The data presented are the average of three independent assays. Error bars represent standard deviation. *, $p < 0.05$, statistically significant. (C) Western blot analyses. DLD-1, SW480, RKO and HT29 cells were exposed to hypoxia (O_2, 1%) for indicated time. Western blot analyses were performed as described above.

2. FBI-1 represses transcription of *MCT4* gene and transcription activation of *MCT4* gene by hypoxia was decreased by FBI-1 in HCT116 colon cancer cells

Using RT-qPCR and western blot analysis, I investigated whether FBI-1 regulates transcription of *MCT4* gene. In HCT116 human colon cancer cells, ectopic FBI-1 repressed MCT4 transcription by 0.65- fold, and knock-down of FBI-1 increased transcription of *MCT4* gene by 1.8-fold. In contrast, CD147, a chaperon glycoprotein important in MCT4 expression, was not changed by FBI-1 (Figure 2A and B). At the protein level, ectopic FBI-1 significantly decreased MCT4 expression, while FBI-1 knock-down increased MCT4 expression (Figure 2C and D). These data further suggested that FBI-1 represses *MCT4* gene expression.

I also investigated that whether FBI-1 can repress MCT4 transcription under hypoxic condition. In HCT116 cells, when the cells were exposed to hypoxic condition, endogenous FBI-1 expression was reduced and MCT4 expression was induced at both mRNA and protein levels as in Figure 1. FBI-1 also significantly decreased transcription activation of MCT4 gene at mRNA and protein levels by hypoxia (Figure 2E and F). Additionally, I also investigated whether FBI-1 can repress *MCT4* gene expression in the HCT116 cells treated with cobalt chloride ($CoCl_2$, 200 μM for 4 hr), a condition

mimicking hypoxia. MCT4 transcriptional activation by $CoCl_2$ treatment was decreased by FBI-1 (Figure 2G and H). These results suggested that FBI-1 can repress transcription activation of the *MCT4* gene in normoxic and hypoxic condition.

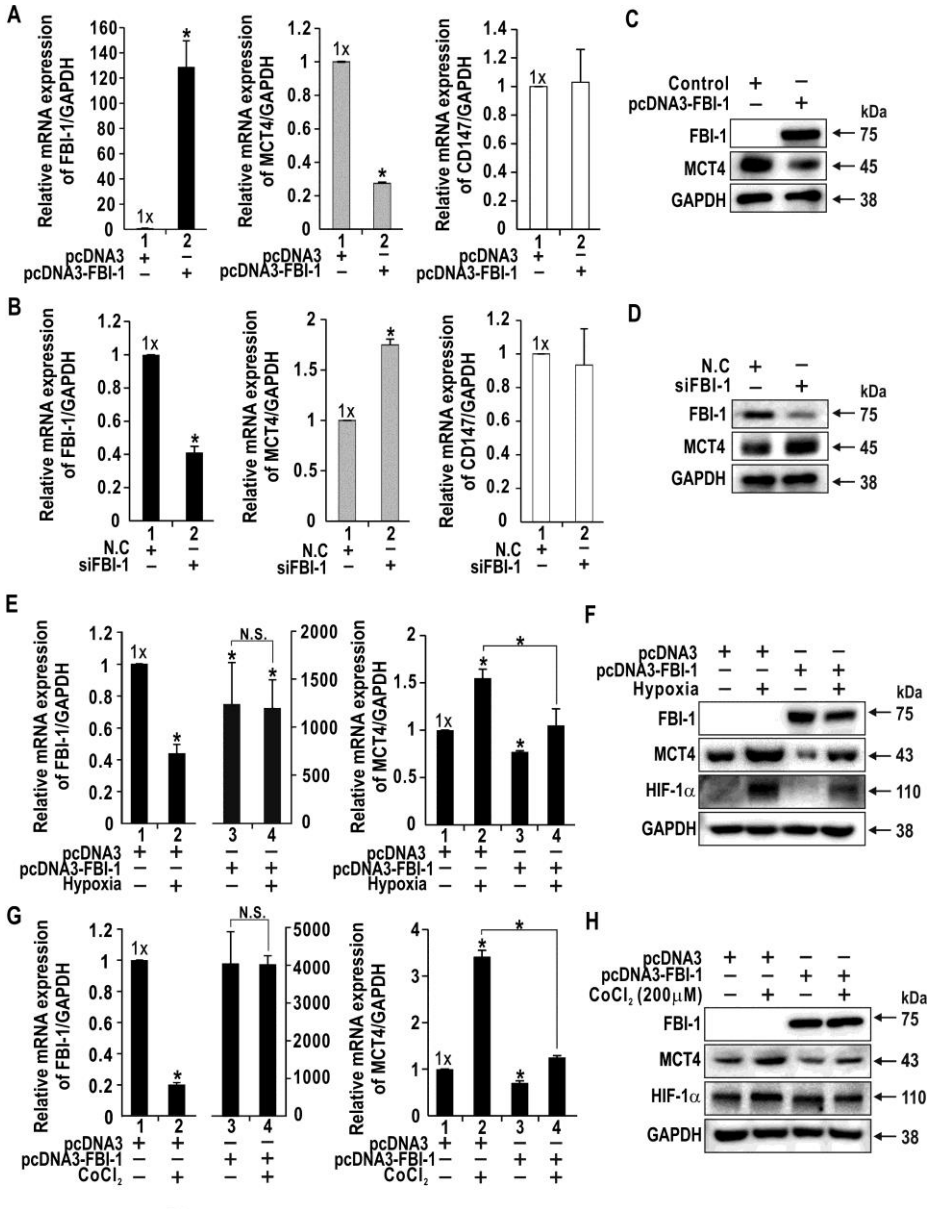


Figure 2. FBI-1 represses MCT4 expression in both normoxic and hypoxic condition. (A, B) RT-qPCR analysis of mRNA expression of FBI-1, MCT4

and CD147 in the HCT116 cells transfected with pcDNA3-FBI-1 or FBI-1 siRNA. After 48 hr, total mRNA was isolated from cells and the levels of FBI-1, MCT4 and CD147 mRNA were determined by RT-qPCR. The data were normalized with 18S ribosomal RNA. (C, D) Western blot analysis. HCT116 cells were transfected with pcDNA3-FBI-1 or FBI-1 siRNA and analyzed for FBI-1, MCT4, and GAPDH protein expression. GAPDH, loading control. (E, F) RT-qPCR and western blot analysis of FBI-1 and MCT4 expression in the HCT116 cells transfected with pcDNA3-FBI-1under hypoxic condition (O_2, 1%). After 24 hr, mRNA and protein were isolated from the cells and were used for analysis. RT-qPCR and western blot analysis were performed as described above. (G, H) RT-qPCR and western blot analysis of FBI-1 and MCT4 expression with cobalt chloride ($CoCl_2$). HCT116 cells with ectopic FBI-1 or not were stimulated with cobalt chloride ($CoCl_2$) 200 μM. After 8 hr, mRNA and protein were isolated from the cells and were used for analysis. RT-qPCR and western blot analysis were performed as described above. Error bars represent standard deviation. *, $p < 0.05$, statistically significant.

3. FBI-1 represses MCT4 transcription via the distal promoter element containing novel 5 copies of FBI-1 and HIF-1α responsive elements

In the above, I found that FBI-1 is a transcriptional repressor of *MCT4* gene. I analyzed the promoter sequence of human *MCT4* gene to find potential FBI-1 responsive elements. Ullah MS et al. showed that MCT4 promoter contains a HRE (5'-GCTCACAGGCACGTGCCCTCATCTT-3') and HIF-1α activates transcription of *MCT4* gene potently. Interestingly, I found the FRE (5'-GCACCACCTGCCCTGGGCGGGA-3') of the MCT4 promoter which is GC-rich region based on previous studies. Human MCT4 promoter has 5 copies of potential FRE in a head to tail orientation (Figure 3A).

To test how the transcription of MCT4 promoter is regulated by FBI-1, I prepared pGL2-human MCT4-Luc constructs and two other MCT4 promoter and luciferase gene fusion constructs differing in the 5' upstream regulatory sequence. And, pGL2-MCT4-Luc (1) contains both FRE and HRE, and pGL2-MCT4-Luc (2) has no FRE, and pGL2-MCT4-Luc (2) has no FRE and HRE. HCT116 cells were co-transfected with reporter plasmid and control or FBI-1 expression vector and exposed to hypoxia (O_2, 1%) for 6 hr. Interestingly, luciferase activity assays showed that the reporter expression of pGL2-MCT4-Luc (1) was decreased by 60~70% in the presence of FBI-1 under normoxic condition. Also transcriptional activation induced by hypoxia

was decreased by FBI-1. Reporter expression of pGL2-MCT4-Luc (2) lacking 5 copies of FRE was modestly decreased by 30~40% in the presence of ectopic FBI-1 and transcriptional activation induced by hypoxic condition was decreased by 30%. In contrast, pGL2-MCT4-Luc (3) which does not contain the FRE and HRE, was neither activated by hypoxia nor repressed by FBI-1, indicating that FBI-1 represses transcription of MCT4 by acting on the both FRE and HRE, preferentially by acting to the FRE (Figure 3B and C).

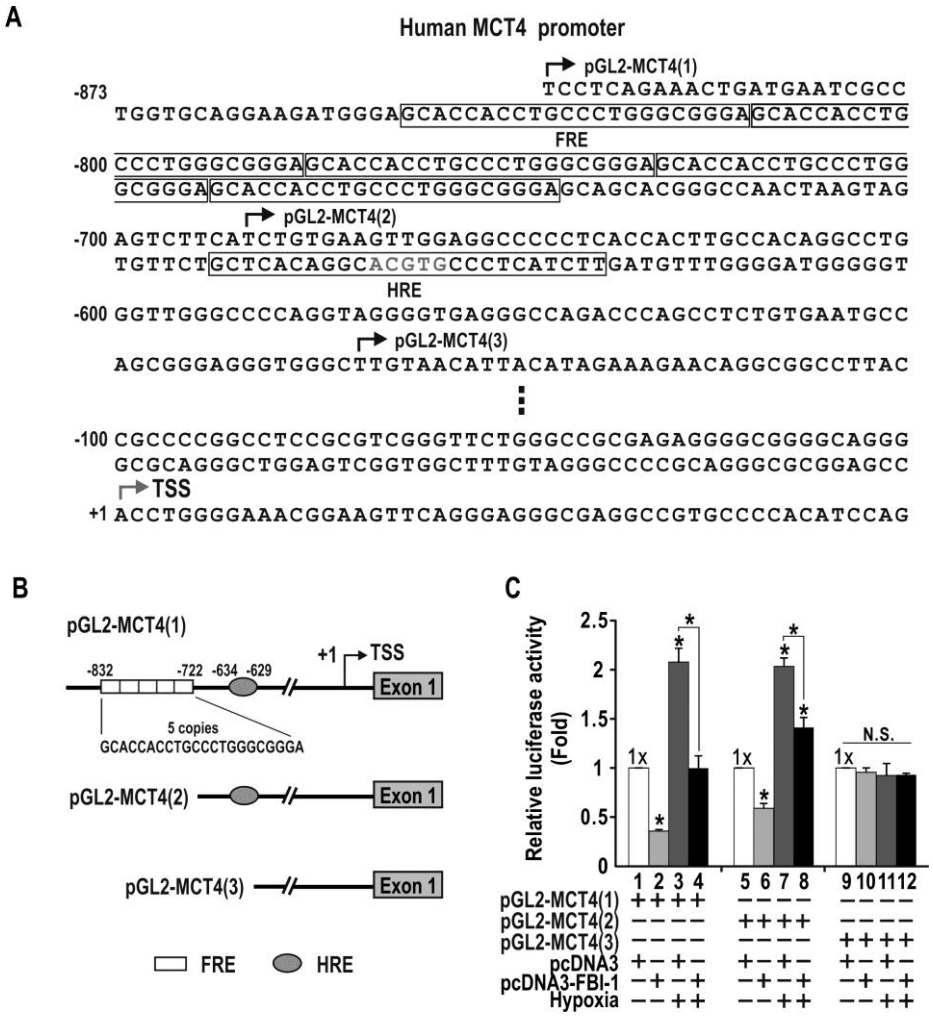


Figure 3. FBI-1 represses MCT4 promoter and transcription activation of MCT4 promoter by hypoxic condition. (A, B) The sequence of human MCT4 promoter and the structure of pGL2-MCT4-Luc reporter construct. +1, transcription start point. FBI-1 responsive element is in box. Core sequences of HRE are in gray. (C) Transient transfection and transcription assays in HCT116

cells. HCT116 cells were transfected with expression vector of FBI-1 and pGL2-MCT4-Luc construct, and exposed to hypoxia (O_2, 1%) for 6 hr. Luciferase activities were normalized with co-expressed β-galactosidase activity, and data presented are the average of three independent assays. Error bars represent standard deviation. *, $p < 0.05$, statistically significant.

4. FBI-1 binds to the FRE and the HRE of MCT4 promoter, and FBI-1 blocks HIF-1α binding to the HRE

Transcription factors often regulate the target gene transcription by blocking DNA binding of other transcription factors by physical interaction and/or binding competition.

To determine how the transcription of *MCT4* gene is regulated, I analyzed molecular events at the MCT4 promoter. First, I tested whether FBI-1 can affect the transcription activation of pGL3-$(HRE)_{3X}$-Luc with the well-established HRE of erythropoietin gene promoter at the proximal promoter. Transient transcription assays showed that reporter expression was increased by ectopic HIF-1α PA (stable form of HIF-1α, proline 402 and 564 replaced with alanine) or hypoxic condition, and which were decreased by FBI-1 (Figure 4B). The result, along with the data shown in Figure 3, suggested that FBI-1 may repress transcription activation of MCT4 by HIF-1α. Accordingly, I investigated whether FBI-1 can bind two critical elements FRE and HRE of MCT4 by oligonucleotide pull-down assays and ChIP assays. Oligonucleotide pull-down assays of the whole cell lysates transfected with FBI-1 or HIF-1α expression vector showed that FBI-1 binds to both FRE and HRE. FBI-1 binds to the FRE much stronger than the HRE. HIF-1α binds to the HRE only (Figure 4A and C). Chromatin-immunoprecipitation (ChIP) of

HCT116 cells transfected with FBI-1 expression vector also showed that FBI-1 binds the FRE and the HRE. Interestingly, FBI-1 binding was significantly decreased probably due to repression of FBI-1 by hypoxic condition. HIF-1α binding was increased under hypoxic condition and ectopic FBI-1 decreased HIF-1α binding at the HRE (Figure 4D). Oligonucleotide pull-down assays of whole cell lysates (prepared from the HCT116 cells transfected with FBI-1 and/or HIF-1α PA expression vector) showed that HIF-1α binding to the representative HRE oligo was gradually decreased by the presence of FBI-1 in the dose-dependent manner (Figure 4E). I also showed that recombinant HIF-1α binding to the HRE was gradually decreased by recombinant FBI-1 using in vitro binding assay (Figure 4F). These data indicate that FBI-1 not only represses transcription of *MCT4* gene by binding at the distal FRE, but also represses transcription by blocking HIF-1α binding at the HRE.

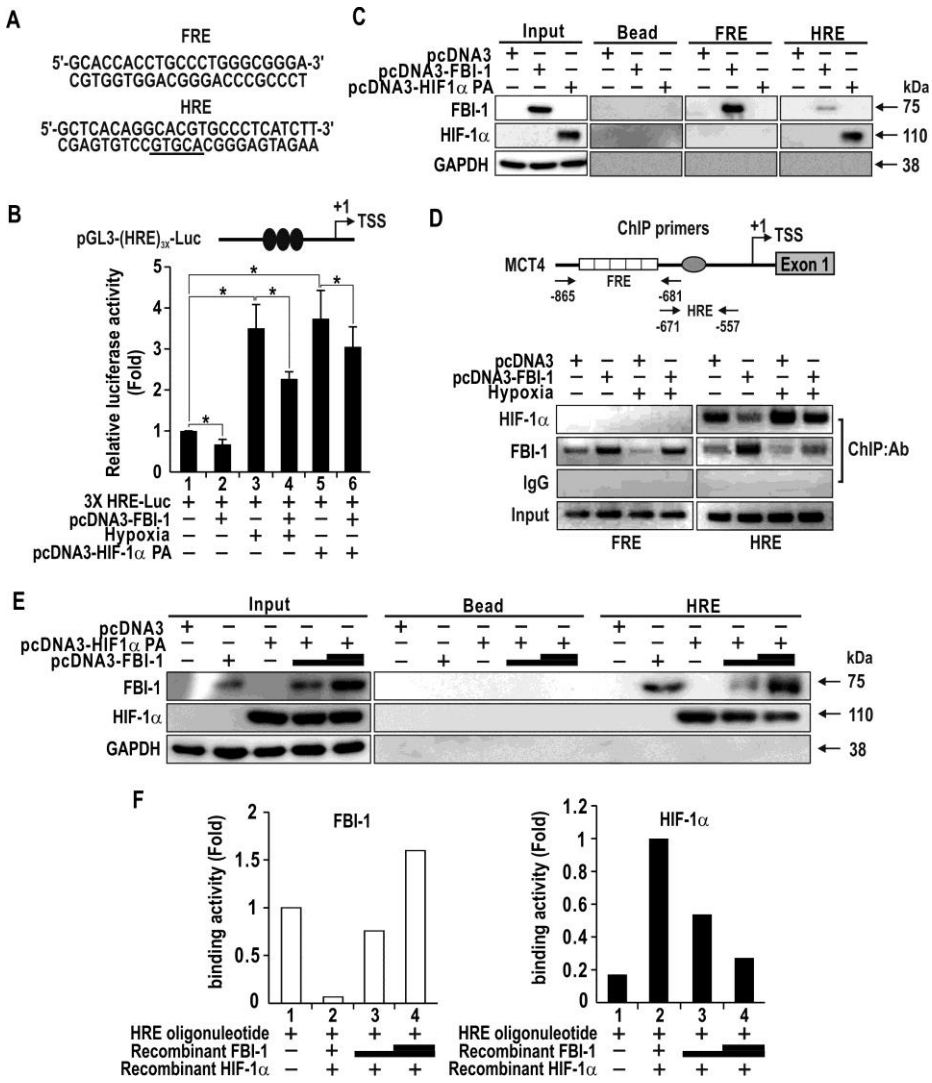


Figure 4. FBI-1 binds the FRE and HRE of the MCT4 promoter, and FBI-1 inhibits HIF-1α binding at the HRE. (A) Nucleotide sequences of the FRE and HRE of the MCT4 promoter used for oligonucleotide pull-down assay. HRE core sequence is underlined. (B) Transient transfection and

transcription assays in HCT116 cells. HCT116 cells were transfected with pcDNA3-FBI-1 or pcDNA3-HIF-1α PA (P402A, P564A) expression vector and pGL3-$(HRE)_{3X}$-Luc construct. The cells were exposed to hypoxia (O_2, 1%) for 6 hr. Luciferase activities were normalized with co-expressed β-galactosidase activity, and data presented are the average of three independent assays. Error bars represent standard deviation. *, $p < 0.05$, statistically significant. (C) Oligonucleotide pull-down assays of FBI-1 and HIF-1α binding to the MCT4 promoter. HCT116 cells were transfected with pcDNA3-FBI-1 or pcDNA3-HIF-1α PA (P402A, P564A) expression vector and harvested after 48 hr. Cells were lysed using protein extraction buffer. Biotinylated oligonucleotide probes including FRE or HRE were incubated with the cell lysates and were incubated with neutravidin agarose beads. The precipitates were analyzed by western blot analysis using anti-FBI-1, anti-HIF-1α and anti-GAPDH antibodies. GAPDH, loading control. (D) ChIP assays of FBI-1 and HIF-1α binding at the FRE and HRE. HCT116 cells were transfected with pcDNA3-FBI-1 and the cells were exposed to hypoxia (O_2, 1%) for 8 hr. Chromatins were immunoprecipitated with the anti-FBI-1 antibody, anti-HIF-1α antibody and anti-IgG antibody and analyzed by qRT-PCR. IgG, negative control. (E) Oligonucleotide pull-down assays of FBI-1 and HIF-1α binding to the HRE of MCT4 promoter. HCT116 cells were

transfected with increasing amount of pcDNA3-FBI-1 or pcDNA3-HIF-1α PA expression vector and harvested after 48 hr. (F) In vitro DNA binding assay. DNA binding of recombinant FBI-1 or recombinant HIF-1α to the HRE of the MCT4 promoter.

5. FBI-1 decreases lactate efflux across the cell membrane under hypoxic condition

Having shown that FBI-1 represses *MCT4* gene transcription directly and/or by binding competition with HIF-1α, I analyzed lactate efflux and extracellular pH of the HCT116 cells transfected with pcDNA3 or pcDNA3-FBI-1 expression vector and cultured under hypoxic condition (O_2, 1%) for 24 hr. Extracellular lactate concentration was significantly increased under hypoxic condition, and it was decreased by FBI-1. FBI-1 does not affect extracellular lactate concentration under normoxic condition (Figure 5A). This result reflects that not only *MCT4* gene is repressed by FBI-1 is not induced but also there is no excess lactate to transport under normoxic condition. Also, FBI-1 decreased the lactate efflux induced by hypoxia (Figure 5B).

I also measured extracellular pH using pH-indicator paper strip and pH meter. Ectopic FBI-1 in RKO and HCT116 cells decreased acidification of culture medium and increased extracellular pH under hypoxic condition, but FBI-1 did not affect extracellular pH under normoxic condition (Figure 5C, D and E). These results indicated that FBI-1 inhibits lactate efflux and increases extracellular pH by repressing *MCT4* gene expression under hypoxic condition.

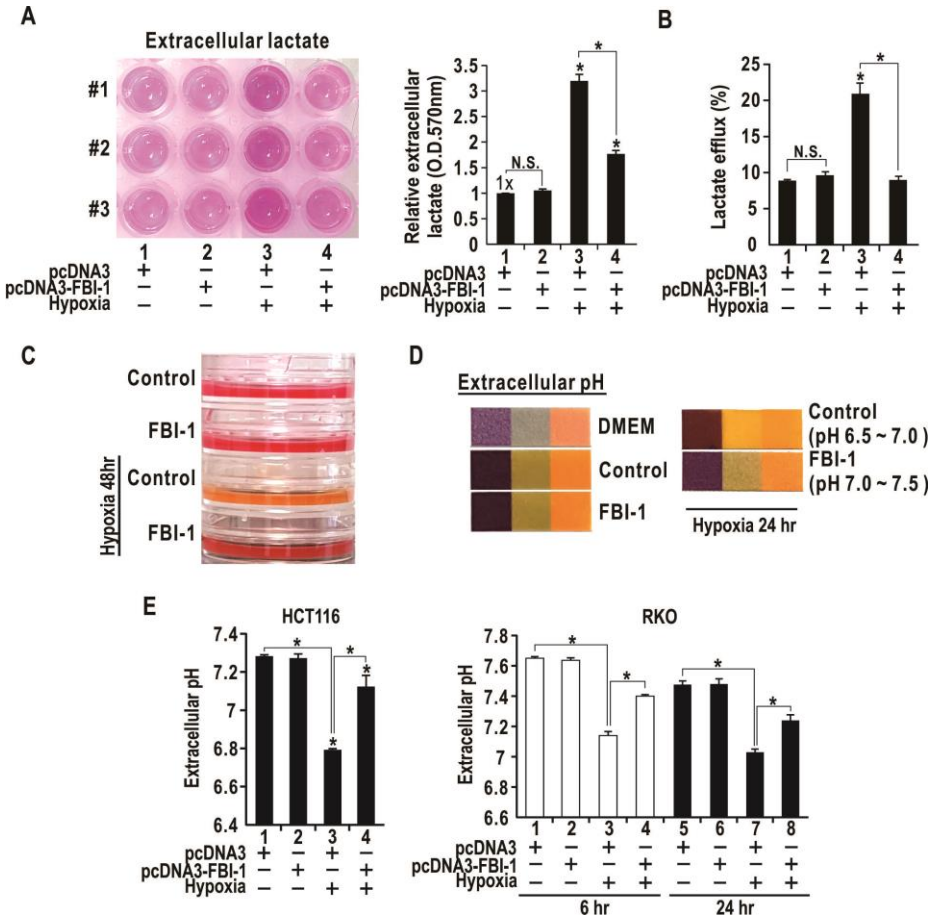


Figure 5. FBI-1 decreases extracellular lactate concentration by inhibiting lactate efflux, and increases extracellular pH under hypoxic condition. (A, B) Lactate concentration measurement. HCT116 cells were transfected with pcDNA3-FBI-1 expression vector and the cells were exposed to hypoxia (O_2, 1%) for 24 hr. Extra-and intracellular lactate concentration was determined by measuring absorbance at 570 nm using colorimetric lactate assay kit. Error bars

represent standard deviation. *, $p < 0.05$, statistically significant. (C) Acidification of the culture medium. RKO cells were transfected with pcDNA3-FBI-1 and the cells were exposed to hypoxia (O_2, 1%). After 48 hr, the photo of the culture dish was taken to compare acidification of culture medium. (D) Extracellular pH measurement. RKO cells were transfected with pcDNA3-FBI-1 and the cells were exposed to hypoxia (O_2, 1%) for 24 hr. Extracellular pH was visible as a change in color of the pH-indicator strips dipped into the cell culture medium. (E) Extracellular pH measurement. HCT116 and RKO cells were transfected with pcDNA3-FBI-1 and were exposed to hypoxia (O_2, 1%) for 6 hr or 24 hr. pH of culture medium was determined using pH meter. Error bars represent standard deviation. *, $p < 0.05$, statistically significant.

6. FBI-1 decreases HIF-1α expression by ubiquitin-mediated proteasomal degradation

Transcriptional activation of *MCT4* gene by hypoxic condition was attenuated by FBI-1 in Figure 2. The data also showed that hypoxia-induced HIF-1α expression might be decreased by FBI-1. I investigated whether FBI-1 can decrease HIF-1α expression induced by hypoxia or $CoCl_2$ treatment. FBI-1 decreased HIF-1α expression induced by hypoxia or $CoCl_2$ treatment (Figure 6A), without affecting mRNA expression of HIF-1α (Figure 6B).

HIF-1α is rapidly degraded at normoxic condition by the VHL-meditated ubiquitin-proteasome pathway. HIF-1α hydroxylation at prolines 402 and 564 increases interaction with VHL, and HIF-1α is degraded. Hypoxic condition blocks hydroxylation of HIF-1α at the proline residues and stabilizes HIF-1α. The HIF-1α mutant (P402A and P564A) (stable form of HIF-1α, named as HIF-1α-PA) can't be hydroxylated and can't interact with VHL under normoxic condition. Using the stable form of HIF-1α (HIF-1α-PA), I investigated whether HIF-1α PA expression is also affected by FBI-1. While mRNA expression of HIF-1α was not changed, HIF-1α-PA protein was decreased by FBI-1 under normoxic condition (Figure 6C and D), suggesting that FBI-1 might decrease HIF-1α expression at protein level.

I further investigated how FBI-1 decreased HIF-1α expression in the

HCT116 cells exposed to hypoxia (O_2, 1%) for 9 hr. First, cycloheximide (CHX) chase assays showed that FBI-1 decreased HIF-1α protein stability more rapidly up to 64.5%, while HIF-1α protein expression decreased only by 38.2% in absence of FBI-1 at 120 min (Figure 6E). As the stability of HIF-1α was mainly reported to be regulated by ubiquitin mediated proteasomal degradation, I tested whether FBI-1 affects HIF-1α protein stability via modulation of poly-ubiquitination and proteasomal degradation. FBI-1 enhanced degradation of HIF-1α, which was reversed by proteasome inhibitor MG132 under hypoxic condition (Figure 6F). FBI-1 increased ubiquitination of HIF-1α protein (Figure 6G). Furthermore, the microarray data showed that a variety of HIF-1α target genes were down-regulated in the doxycycline-inducible HEK293-Trex cells (Supplementary Figure 2). The results suggested that FBI-1 may have potential role in increasing ubiquitin-mediated proteasomal degradation of HIF-1α.

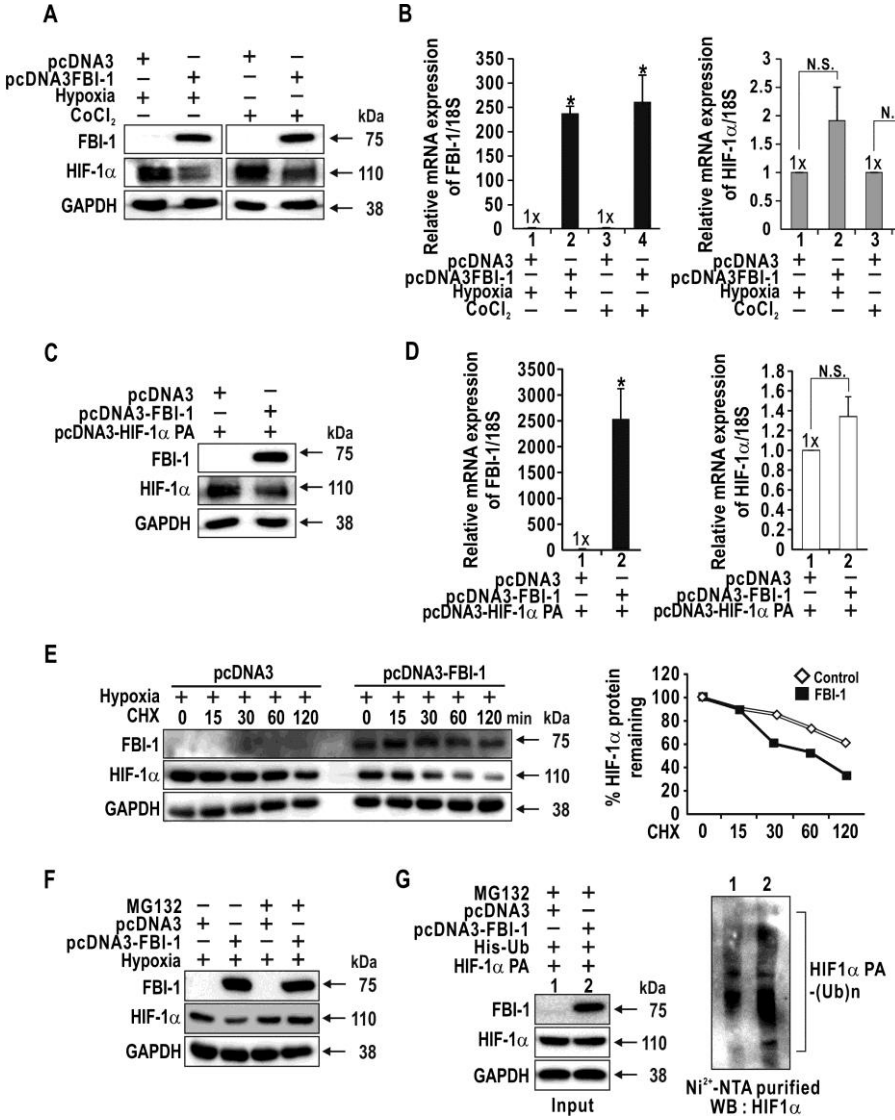


Figure 6. FBI-1 inhibits HIF-1α expression by increasing ubiquitination of HIF-1α under hypoxic condition. (A, B) RT-qPCR and western blot analysis of FBI-1 and HIF-1α expression under hypoxic condition or with cobalt

chloride ($CoCl_2$) treatment. HCT116 cells were exposed to hypoxia (O_2, 1%) for 9 hr or were stimulated with cobalt chloride ($CoCl_2$) for 9 hr. RT-qPCR data were normalized with 18S ribosomal RNA. Western blot analysis of FBI-1, HIF-1α, GAPDH expression in the HCT116 cells were analyzed using indicated antibodies. GAPDH, loading control. Error bars represent standard deviation. *, $p < 0.05$, statistically significant. (C, D) RT-qPCR and western blot analysis of FBI-1 and HIF-1α expression. HCT116 cells were transfected with pcDNA3-HIF-1α PA and/or pcDNA3-FBI-1 expression vector. RT-qPCR and western blot analysis were performed as described above. *, $p < 0.05$, statistically significant. (E) A protein stability assay. HCT116 cells were transfected with pcDNA3-FBI-1 and exposed to hypoxia (O_2, 1%) for 9 hr. Cycloheximide (CHX) was used at a final concentration of 10 μg/ml for the indicated time, and analyzed by western blot analysis. Relative amounts of HIF-1α were calculated after normalizing to GAPDH and are shown in the right panel. (F) Proteasomal dependent degradation of HIF-1α by FBI-1. HCT116 cells were transfected with pcDNA3-FBI-1 and exposed to hypoxia (O_2, 1%) for 9 hr. The cells were treated with 50 mM MG132 for 9 hr, followed by western blot analysis. (G) Ubiquitination of HIF-1α. HCT116 cells were transfected with His-ubiquitin and pcDNA3-HIF-1α PA and/or pcDNA3-FBI-1 expression vector. The cells were exposed to hypoxia (O_2, 1%) for and treated

with 50 mM MG132 for 9 hr. After purification of ubiquitinated proteins using Ni^{2+}-NTA beads, total extracts (Input) and His-purified proteins were detected by western blot analysis using anti-FBI-1, anti- HIF-1α and anti-GAPDH antibodies. GAPDH, loading control.

7. FBI-1 inhibits colon cancer cell proliferation by inducing G1 phase arrest under hypoxic condition

Hypoxic condition is detrimental to proliferation of both cancer cells and normal cells, but cancer cells acquired ability to proliferation by genetic and metabolic reprogramming. Cancer cells produce excess lactate by metabolic shift to glycolysis and acquired ability to export lactate outside of membrane via MCT4 expression, which is critical in cancer cells survival and proliferation. Because FBI-1 inhibits MCT4 expression by repressing transcription activation of MCT4 by HIF-1α, and also by decreasing HIF-1α stability, I investigated whether FBI-1 could inhibit colon cancer cell proliferation under hypoxic condition. HCT116 and RKO colon cancer cells transfected with FBI-1 expression vector were exposed to hypoxia (O_2, 1%) for 0, 1, 2, and 3 day. As reported by others, cell proliferation of two cell types was inhibited by hypoxia, and ectopic FBI-1 significantly inhibited cell proliferation over the 3 days period of cell culture under hypoxia (Figure 7A, B and Supplementary Figure 3A, B). As shown in Figure 2F and H, FBI-1 repressed MCT4 induction by hypoxia (Figure 7C and Supplementary Figure 3C).

Alternatively, I also investigated whether FBI-1 can inhibit cancer cell proliferation under hypoxic condition using crystal violet staining assay.

HCT116 and RKO colon cancer cells were transfected with FBI-1 expression vector and exposed to hypoxia (O_2, 1 %) for 0 ~ 2 day. Crystal violet staining assay showed that the two cells grew similarly regardless of FBI-1 presence in normoxic condition. However, under hypoxic condition, two cells with ectopic FBI-1 grow more slowly (Figure 7D and Supplementary Figure 3D).

Furthermore, I examined whether FBI-1 affect cell cycle progression using flow cytometry analysis (FACS) of HCT116 cells transfected with FBI-1 expression vector. FBI-1 increased the cell population of G1 phase from 53.8% to 63.1% and decreased the cell population of S phase from 22.9% to 15.0% under hypoxic condition, but the cell population of G2-M phase was not changed under both normoxic and hypoxic conditions (Figure 7E). These results suggested that FBI-1 inhibits colon cancer cell proliferation by inducing G1 phase arrest under hypoxic condition.

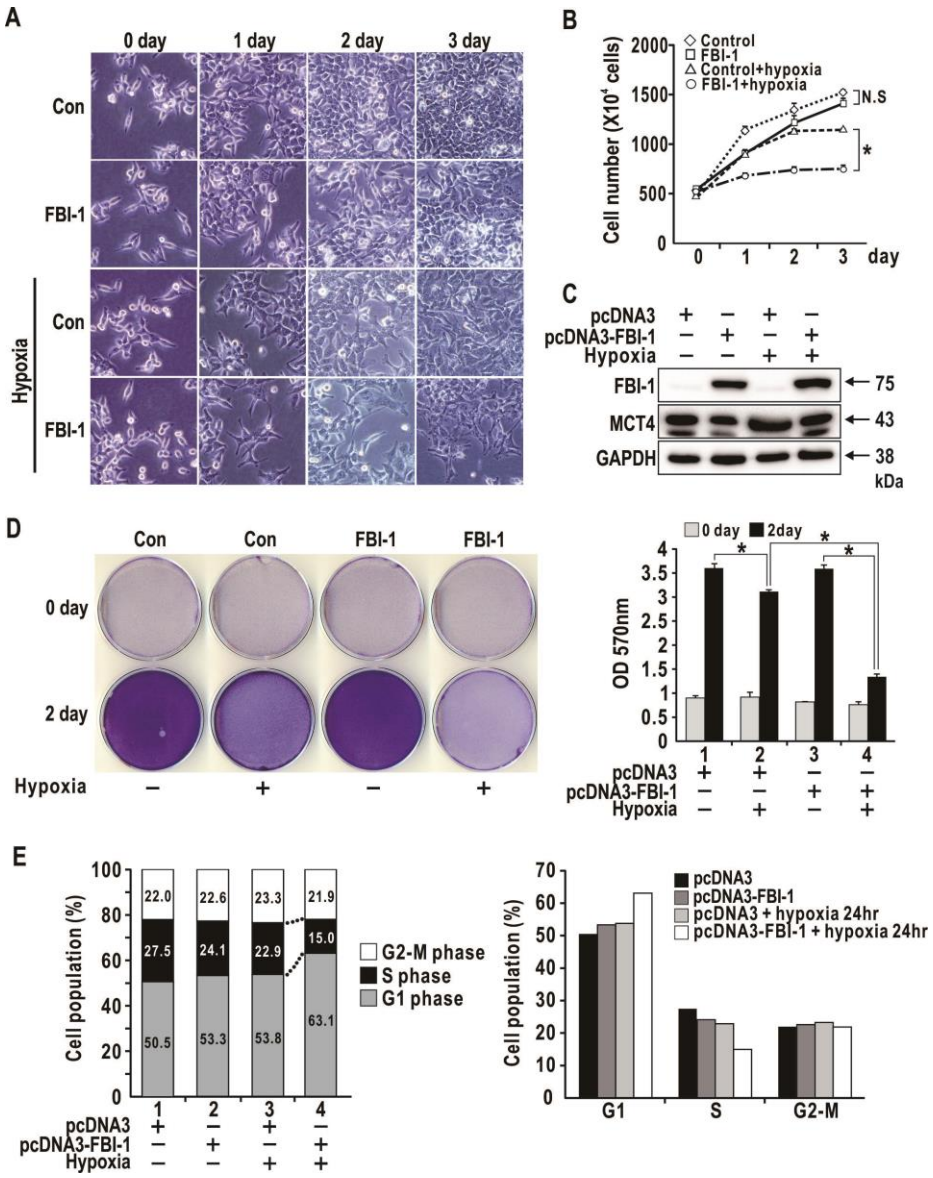


Figure 7. FBI-1 inhibits colon cancer cell growth by inducing G1 phase arrest under hypoxic condition. (A) The morphology of cells. HCT116 cells

were transfected with pcDNA3-FBI-1 and exposed to hypoxia (O_2, 1%) for 0 ~ 3 days. Morphology of cells was photographed daily. (B) Cell growth curve analysis. HCT116 cells were transfected with pcDNA3-FBI-1 and exposed to hypoxia (O_2, 1%) for 0 ~ 3 day. Cell number was counted daily using hematocytometer. *, $p < 0.05$, statistically significant. (C) Western blot analysis. The cells used for growth curve analysis were lysed and analyzed by western blot using antibodies indicated. GAPDH, loading control. (D) Crystal violet staining assay. HCT116 cells were cultured in 35 mm cell culture dish and transfected with pcDNA3-FBI-1 and/or exposed to hypoxia (O_2, 1%). After 0 and 2 day, the cells were fixed and stained with crystal violet. Crystal violet stain was dissolved using 1% SDS buffer and measured absorbance at 570 nm. *, $p < 0.05$, statistically significant. (E) Flow cytometry analysis. HCT116 cells were transfected with FBI-1 and exposed to hypoxia (O_2, 1%) for 24 hr. Cells were collected, fixed, stained with propidium iodide, and analyzed by FACS.

8. RelA/p65 induced by hypoxia represses FBI-1 transcription

FBI-1 represses *MCT4* gene expression at transcription level. Interestingly, under hypoxic condition, FBI-1 transcription was gradually decreased while that of MCT4 increased (Figures 1 and 2). Intrigued by this observation, I investigated how FBI-1 gene transcription is repressed under hypoxic condition. I searched for potential transcription binding sites of human FBI-1 promoter using QIAGEN Epitect ChIP qPCR primer design program (http://www.sabiosciences.com/chipqpcrsearch.php?app=TFBS). Interestingly, I was able to locate two potential NF-κB responsive elements and also two potential HIF-1α responsive elements on the human FBI-1 promoter (Figure 8A).

Previous studies showed that, under hypoxic condition, IκB kinase (IKK) is activated through a calcium/calmodulin-dependent kinase 2 (CaMK2)-dependent pathway, resulting in increasing RelA/p65 binding to the target genes promoters. HCT116 cells were transfected with pNF-κB-Luc reporter construct and exposed to hypoxia for 6 hr. The transient transcription assay showed increase in reporter expression from pNF-κB-Luc, suggesting NF-κB induced by hypoxia activates transcription from the pNF-κB-Luc (Figure 8B).

Accordingly, to investigate *FBI-1* gene expression is negatively regulated

by NF-κB induced by hypoxia, I prepared pGL3-FBI-1-Luc reporter construct containing two HRE and two NF-κB RE elements (Figure 8C). In HCT116 colon cancer cells, FBI-1 promoter activity was repressed by ectopic RelA/p65 or hypoxic condition. However, HIF-1α didn't show significant effect on the FBI-1 promoter although two potential HRE are present at the FBI-1 promoter (Figure 8D). These data suggested that activated RelA/p65 by hypoxic condition might be a major repressor of FBI-1 gene transcription.

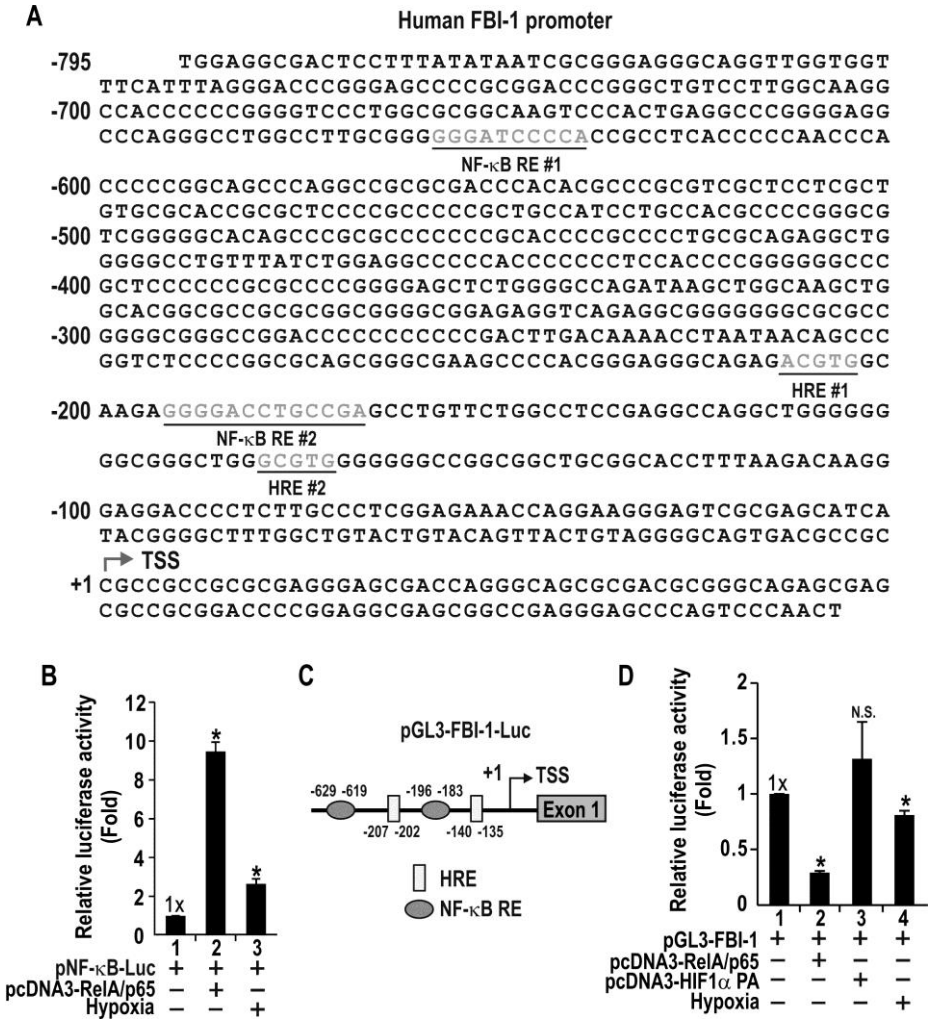


Figure 8. The nucleotide sequence and NF-κB and HIF-1α RE of human FBI-1 promoter and the FBI-1 is repressed by RelA/p65 and hypoxic condition. (A) The sequence of human FBI-1 promoter. +1, transcription start point. NF-κB RE are indicated by underlines. Core sequences of HIF-1α RE

are in gray. (B) Transient transfection and transcription assays. HCT116 cells were transfected with indicated plasmid and were exposed to hypoxia (O_2, 1%) for 6 hr. Luciferase activities were normalized with co-expressed β-galactosidase activity, and data presented are the average of three independent assays. Error bars represent standard deviation. *, $p < 0.05$, statistically significant. (C) The structure of pGL3-FBI-1-Luc construct. +1, transcription start point. NF-κB and HIF-1α responsive elements are indicated. (D) Transient transfection and transcription assays. HCT116 cells were transfected with the indicated plasmid and were exposed to hypoxia (O_2, 1%) for 6 hr. Luciferase activities were measured as described in (B).

9. FBI-1 transcription is decreased by RelA/p65 binding to the FBI-1 promoter under hypoxic condition

Transient transcription assays suggested that RelA/p65 represses transcription of FBI-1. I tested whether RelA/p65 decreases FBI-1 gene expression at mRNA and protein level under normoxic and hypoxic conditions. Ectopic RelA/p65 decreased FBI-1 expression under normoxic condition and knock-down of RelA/p65 de-repressed FBI-1 expression under hypoxic condition. The results suggest that *FBI-1* gene is target of RelA/p65 and RelA/p65 induced by hypoxia can repress FBI-1 expression (Figure 9A and B).

I investigated whether RelA/p65 could bind the two NF-κB responsive elements of FBI-1 promoter using the cell lysates prepared from the HCT116 cells transfected with the pcDNA3-RelA/p65 expression vector. Oligonucleotide pull down assays showed that both endogenous and ectopic RelA/p65 binds to the NF-κB RE#1 and -#2 in vitro (Figure 9C). Also, ChIP assay showed that RelA/p65 binds to the NF-κB RE#1 and -#2 in vivo and binding was increased when RelA/p65 is overexpressed (Figure 9D).

Reports by others showed that activated form of RelA/p65 translocate from cytoplasm into nucleus and activates transcription of target genes under hypoxic condition. I also observed that nuclear RelA/p65 is increased by hypoxia or $CoCl_2$ treatment in HCT116 cells (Figure 9E). ChIP assay showed

that RelA/p65 binding to the NF-κB RE#1 and #2 of FBI-1 promoter was increased by hypoxia, reflecting increased nuclear translocation of RelA/p65 (Figure 9F). The results suggested that RelA/p65 may functions as a transcriptional repressor of *FBI-1* gene in the cells under hypoxic condition.

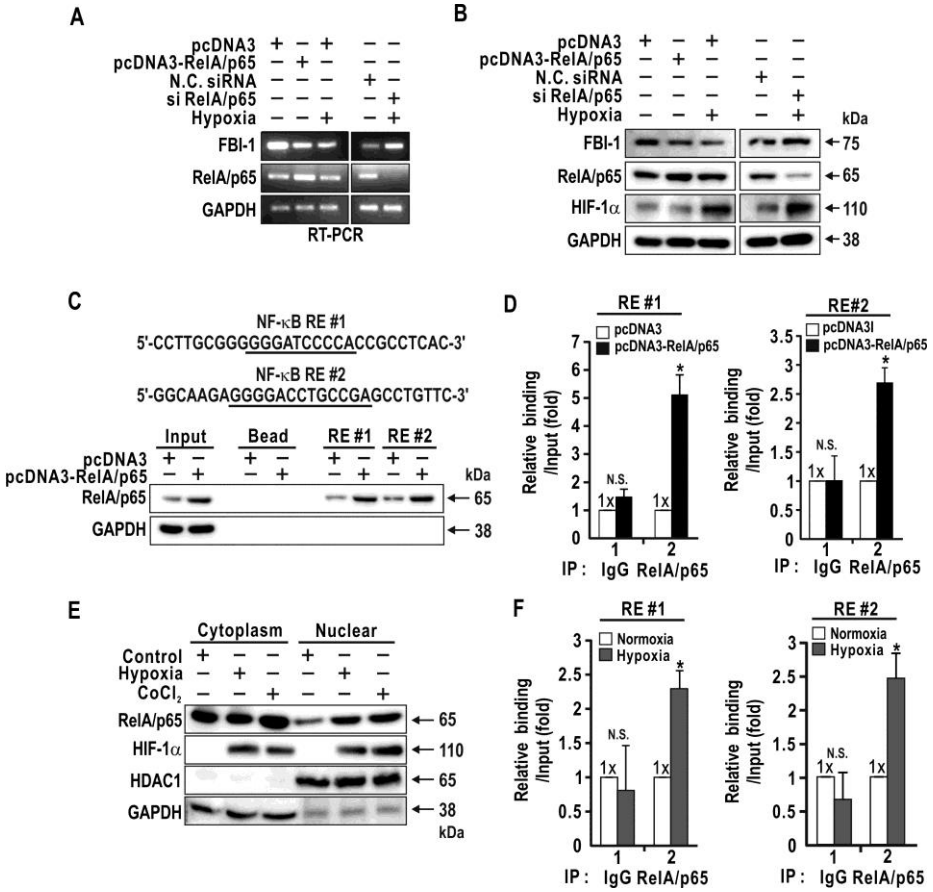
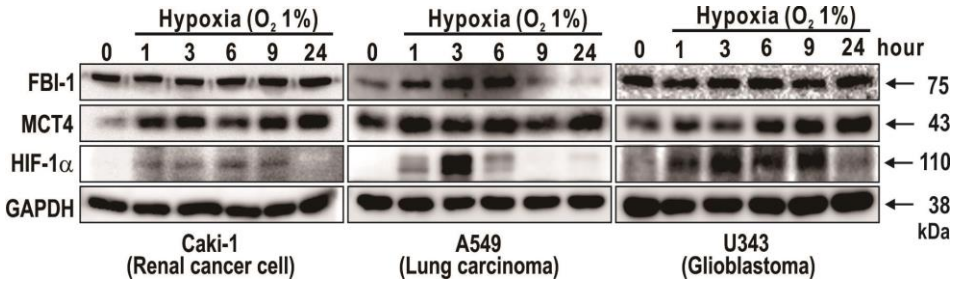


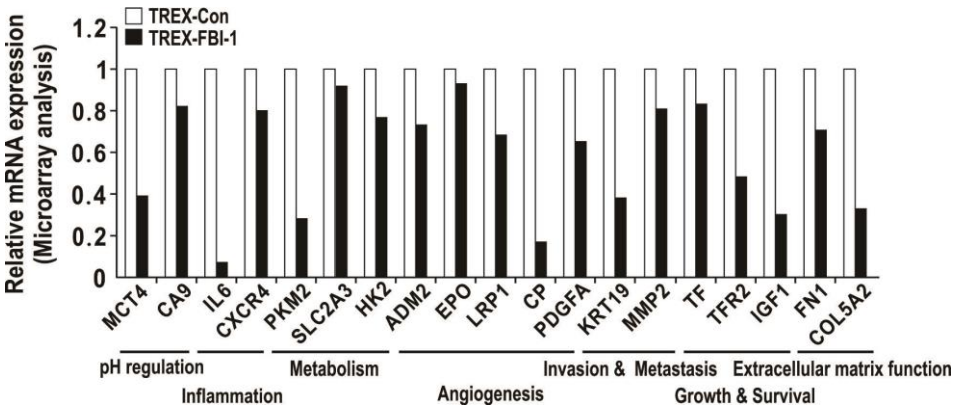
Figure 9. Hypoxia-induced RelA/p65 nuclear translocation represses FBI-1 gene transcription by binding to the NF-κB responsive elements of the FBI-1 promoter. (A) PCR analysis. HCT116 cells transfected with pcDNA3-RelA/p65 or RelA/p65 siRNA, and were exposed to hypoxia (O_2, 1%) for 24 hr. GAPDH, loading control. (B) Western blot analysis of FBI-1 expression. HCT116 cells with pcDNA3-RelA/p65 or RelA/p65 siRNA were

exposed to hypoxia (O_2, 1%). After 24 hr, protein was isolated from the cells and used for analysis. FBI-1, RelA/p65, HIF-1α, GAPDH protein in whole-cell lysates of cells were analyzed using indicated antibodies. HIF-1α, hypoxia marker. GAPDH, loading control. (C) Oligonucleotide pull-down assays of RelA/p65 binding to the FBI-1 promoter. HCT116 cells were transfected with pcDNA3-RelA/p65 expression vector and harvested after 48 hr. Cells were lysed using protein extraction buffer. Biotinylated oligonucleotide probes NF-κB RE#1 and -#2 were incubated with the cell lysates and were incubated with neutravidin agarose beads. The precipitates were analyzed by western blot analysis using anti-RelA/p65 and anti-GAPDH antibody. GAPDH, loading control. (D) ChIP assays of RelA/p65 binding at the NF-κB RE#1 and #2. HCT116 cells were transfected with pcDNA3-RelA/p65 expression vector and chromatins were immunoprecipitated with the anti-RelA/p65 antibody, and analyzed by qRT-PCR. IgG, negative control. (E) Cell fractionation assay. HCT116 cells were exposed to normoxia or hypoxia or treated with 200μM of $CoCl_2$ for 4 hr. Cell lysates were fractionated in cytoplasmic and nuclear proteins. RelA/p65, HIF-1α, HDAC1 and GAPDH protein in total cell lysates were analyzed by western blot assays using the antibodies indicated. HDAC1, nuclear protein marker. GAPDH, cytoplasmic protein marker. (F) ChIP assays of RelA/p65 binding at the NF-κB RE#1 and -#2. HCT116 cells were exposed

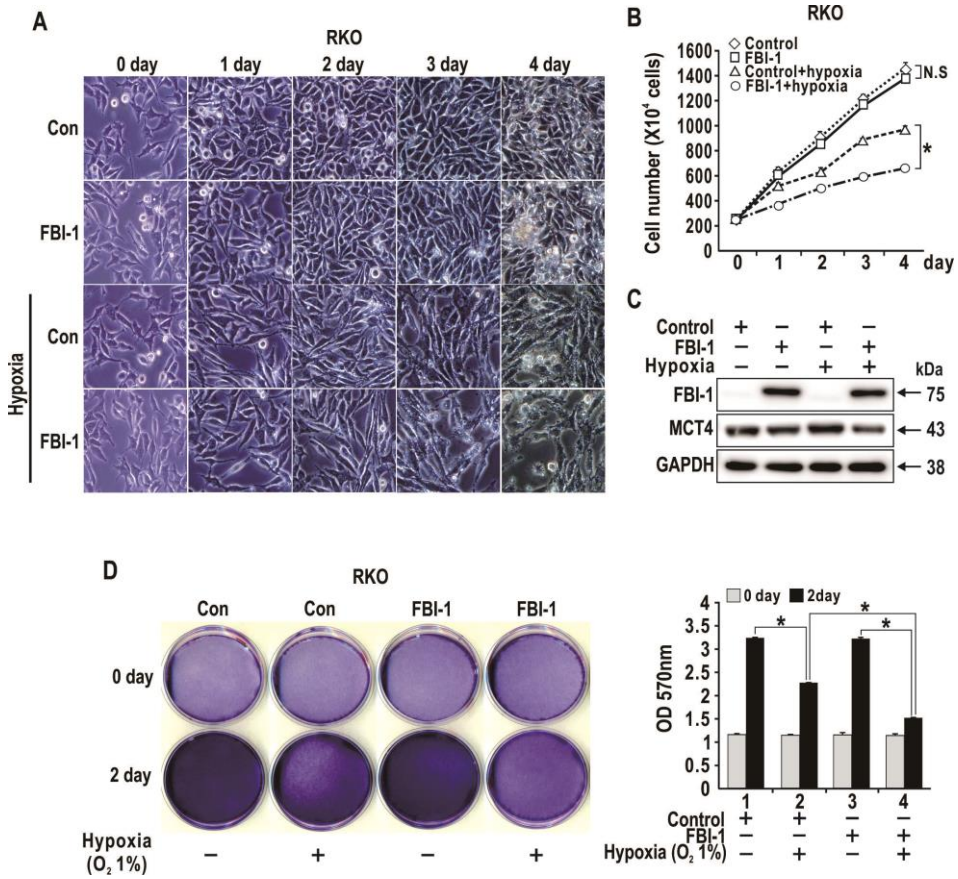
to normoxia or hypoxia (O_2, 1%) for 8 hr. ChIP assay was performed as described in (D).



Supplementary figure 1. FBI-1 and MCT4 expression in various cancer cells under hypoxic condition. Western blot analysis of FBI-1, MCT4, HIF-1α, and GAPDH. Caki-1(Renal cancer cell), A549(Lung carcinoma) and U343(Glioblastoma) cells were exposed to hypoxia (O_2, 1%). FBI-1, MCT4, HIF-1α, GAPDH protein in whole-cell lysates of the cells were analyzed using indicated antibodies. HIF-1α, hypoxia marker. GAPDH, loading control.



Supplementary figure 2. FBI-1 decreases expression of the various HIF-1α target genes. Microarray analysis of HIF-1α target genes mRNA expression in the doxycycline-inducible HEK293-Trex-FBI-1 cells. HIF-1α target genes were grouped based on their biological function.



Supplementary figure 3. FBI-1 represses RKO colon cancer cell proliferation under hypoxic condition. (A) The morphology of cells. RKO cells were transfected with pcDNA3-FBI-1 and exposed to hypoxia (O_2, 1%) for 0 ~ 3 days. The cells were photographed daily. (B) Cell growth curve analysis. RKO cells were transfected with pcDNA3-FBI-1 and exposed to hypoxia (O_2, 1%) for 0 ~ 3 days. Cells number was analyzed daily for three

days using hematocytometer. *, $p < 0.05$, statistically significant. (C) Western blot analysis. The cells used growth curve analysis were lysed and detected by western blot analysis using indicated antibodies. GAPDH, loading control. (D) Crystal violet staining assay. RKO cells were cultured in 35 mm cell culture dish and transfected with pcDNA3-FBI-1 expression vector and exposed to hypoxia (O_2, 1%). After 0 and 2 day, the cells were fixed and stained with crystal violet. Crystal violet stain was dissolved using 1% SDS buffer and measured absorbance at 570 nm. *, $p < 0.05$, statistically significant.

IV. DISCUSSION

Two major features of cancer cells are ability to grow under hypoxic conditions and unique metabolic regulation. Hypoxia is a condition in which tissue is deprived of adequate oxygen supply. Unlike normal tissues, hypoxia develops in most solid tumors as a result of inefficient vascular development and/or abnormal vascular architecture, especially in late stages of cancers.[1, 2] Tumor growth is restricted by oxygen and nutrients when tumor cells are located distant from blood vessels. Cancer hypoxia is associated with cancer survival, malignant progression, and resistance to therapy. Cancer progression under hypoxic condition has become a central issue in cancer physiology and treatment. Investigation on the factors that are required for cancer cell survival and proliferation under hypoxic condition is important to understand cancer biology.[3, 4] In this study, I found that FBI-1 is a transcription repressor of *monocarboxylate transporters 4 (MCT4)* gene in colon cancer cells under hypoxic condition.

Differential mRNA expression analyses of doxycycline-inducible HEK293-Trex-FBI-1 stable cells showed that FBI-1 induction represses MCT4 mRNA. In hypoxic cancer cells, hypoxia-induced HIF-1α binds HRE of human MCT4 promoter and up-regulated MCT4 transcription. I

investigated whether the expression patterns of FBI-1, MCT4 and HIF-1α in the various colon cancer cells are altered under hypoxic condition. Interestingly, MCT4 and HIF-1α expression was induced as reported,[24] but FBI-1 expression was decreased in colon cancer cells under hypoxic condition at mRNA and protein levels. I also analyzed FBI-1, MCT4 and HIF-1α expression in other cancer cells including Caki-1, A549 and U343 cells. FBI-1 expression was increased up to 6 hr and decreased by 9 hr in A549 cells. However, in Caki-1 and U343 cells, FBI-1 expression was not changed by hypoxic condition as shown in supplementary figure 1. Liu et. al showed that chromosome 19p13.3 including *FBI-1* gene is frequently deleted in some human cancers (bladder urothelial carcinoma, lung carcinoma, colon adenocarcinoma, etc.), especially at the late stage of cancers (Cancer Genome Atlas (TCGA) databases). However, there was no change in FBI-1 expression between early cancer stage and late tumor stage in kidney renal clear cell carcinoma and head & neck carcinoma.[39] Decreasing of FBI-1 expression under hypoxic condition appears to be unique to colon cancer cells.

Cellular responses to reduced oxygen level are mediated by the HIF-1α. In the hypoxic cancer cells, HIF-1α is induced by protein stabilization in the von Hippel-Lindau tumor suppressor protein (pVHL) dependent manner. Induced HIF-1α binds to hypoxia responsive elements (HREs) in the promoters of

target genes at early time of hypoxia.[5, 6, 10] However, HIF-1α expression was decreased at late time of hypoxia. If hypoxic condition lasts too long or is too severe, p53 protein expression is induced.[41-43] The p53 reduces the expression of HIF-1α by ubiquitin-dependent proteasomal degradation. One of the hypothesis proposed p53 as a scaffold protein, recruits MDM2 into the complex with HIF-1α and p53 could lead to a MDM2-dependent ubiquitination and subsequent proteasomal degradation of HIF-1α.[44-46] However, later studies have questioned the involvement of MDM2 in p53 mediated HIF-1α degradation. Since HIF-1α is acutely induced and degraded during early time of hypoxia, HIF-2α, which was reported to be induced during later time of hypoxia and last prolonged periods of time, may play a role replacing HIF-1α during severe hypoxic condition to survive.[47, 48] FBI-1 may play a role in MCT4 and HIF-1α expression at the early stage of cellular response to hypoxia.

Next, I investigated whether FBI-1 can repress transcription of MCT4 under normoxic and hypoxic condition. FBI-1 repressed endogenous MCT4 expression under normoxic condition and also significantly decreased transcription of MCT4 activated by hypoxic condition or cobalt chloride treatment. I am also able to show that FBI-1 repress *MCT4* gene transcription by binding to the 5 copies putative FBI-1 responsive element

(5'-GCACCACCTGCCCTGGGCGGGA-3') of the MCT4 promoter. Interestingly, the reporter expression of pGL2-MCT4-Luc promoter construct without FRE was significantly increased by 4-fold comparing to the construct with FRE, implying that FRE plays a critical role in repressing *MCT4* gene transcription. FBI-1 also interacts with a methyl-CpG binding protein, MBD3. FBI-1 recruits BCoR and the Mi-2/NuRD-HDAC complex to the CDKN1A promoter through interaction with MBD3, which is followed by histone modifications, DNA methylation, HP1 binding and epigenetically silencing CDKN1A.[37] Fisel et. al showed that MCT4 promoter can be epigenetically repressed by DNA methylation.[49] FBI-1 may act on the FRE of MCT4 promoter by similar mechanism that was proposed for CDKN1A.

FBI-1 also binds to the HRE of the MCT4 promoter, and represses reporter gene transcription activation of pGL3-$(HRE)_{3X}$-Luc construct by hypoxic or ectopic HIF-1α PA. Moreover, HIF-1α protein induced by hypoxic condition or cobalt chloride was decreased by FBI-1, while mRNA expression of HIF-1α was not changed, suggesting that FBI-1 might affect various HIF-1α target gene expression by decreasing HIF-1α expression. Differential mRNA expression analyses of doxycycline-inducible HEK293-Trex-FBI-1 cells showed that FBI-1 down-regulated a variety of HIF-1α target gene mRNA.

Interestingly, FBI-1 affected HIF-1α-PA, a stable form of HIF-1α which

can't interact with VHL. This implies that FBI-1 may regulate HIF-1α stability in VHL-independent manner. HIF-1α is mainly regulated by VHL E3 ubiquitin ligase and VHL-dependent regulation depends on the oxygen level. HIF-1α expression can be regulated independent of oxygen level. HIF-1α interacts with heat shock protein 90 (HSP90) which is a molecular chaperone that protects hsp90 interacting proteins from mis-folding and degradation.[50-53] HSP90 inhibits RACK1 (E3 ligase) binding to the PAS-A domain by interacting with HIF-1α, result in stabilization of HIF-1α protein. Inhibitors of HSP90, such as 17-allylamino-17-demethoxygeldanamycin (17-AAG), block the binding of HSP90 to the PAS-A domain of HIF-1α, allowing RACK1 to bind at this site and recruit the ElonginC ubiquitin ligase complex.[54-56] I tested whether FBI-1 can interact with HSP90 protein. FBI-1 interacts with HSP90 (data not shown), suggesting that FBI-1 might be decreased HIF-1α stability via HSP90-RACK1 pathway.

One of the physiological consequences of the metabolic switch to glycolysis in hypoxic cancer cells is excessive lactate production, which needs to be exported outside of the cells in order to maintain neutral intracellular pH to avoid cellular damage. Excessive lactate exported out makes cancer microenvironment acidic, which contributes to tumor cell proliferation, invasion and metastasis.[12-15] The lactate efflux is mediated through the MCT4.

Because cancer cells produce massive lactate under hypoxic condition, MCT4 is important in survival and proliferation of hypoxic cancer cells.[16, 18]

In this study, I found that FBI-1 inhibits lactate efflux and increases extracellular pH of hypoxic cancer by repressing *MCT4* gene expression, and FBI-1 also increases cell population of G1 phase and inhibits colon cancer cell proliferation under hypoxic condition. The inhibition of lactate efflux by FBI-1 affects colon cancer cell proliferation. However, the possibility of other mechanisms on the hypoxic cancer cell proliferation by FBI-1 can't be overlooked, because FBI-1 still has the possibility of regulating various other genes of hypoxic cancer cells.

I also found that FBI-1 expression was gradually decreased by hypoxia. I first analyzed human FBI-1 promoter sequences for potential transcription factors binding sites, and I found the putative two NF-κB RE and two HRE at the human FBI-1 promoter. Previous studies by others showed that hypoxic condition activates NF-κB pathway by inducing IκB kinase (IKK) activity, resulting in increasing RelA/p65 binding to the target genes promoter.[57-59] I was curious as to whether NF-κB can affect FBI-1 expression in response to hypoxic condition. Intriguingly, RelA/p65, NF-κB subunit, repressed the transcription of FBI-1 by increasing RelA/p65 binding to the NF-κB RE #1 and -#2 of FBI-1 promoter under hypoxic condition. RelA/p65 activates

various target genes by binding target gene promoter, and can also functions as transcription repressor. RelA/p65 negatively regulates transcription of cyclin E by binding NF-κB RE of cyclin E promoter.[60] Also, RelA/p65 interacts with histone deacetylase (HDAC) corepressor proteins such as HDAC1 and HDAC2, implying that the association of RelA/p65 with the HDAC1 and HDAC2 corepressor proteins might be function to repress expression of NF-κB target genes.[61] The relationship between RelA/p65 and HDAC corepressor in down-regulating FBI-1 expression of hypoxic cancer cells need to be determined in detail. Furthermore, since FBI-1 expression is decreased by hypoxia-induced RelA/p65, the investigation of NF-κB inhibitor effect on cancer cells exposed to hypoxic condition might be also interesting. If NF-κB inhibitor treatment blocks down-regulation of FBI-1 expression in hypoxic cancer cells, it may have a negative influence on survival of hypoxic cancer cells.

In summary, my study has uncovered novel FBI-1 function in lactate efflux of cancer cells under hypoxic condition. FBI-1 may inhibit cancer cell proliferation by repressing *MCT4* gene expression under hypoxic condition.

V. CONCLUSION

Two major features of cancer cells are ability to grow under hypoxic conditions and unique metabolic regulation. Hypoxia is a condition in which tissue is deprived of adequate oxygen supply. Unlike in normal tissues, hypoxia develops in most solid tumors as a result of inefficient vascular development and/or abnormal vascular architecture, especially in late stages of cancers. Investigating cancer cell specific metabolic regulatory program under hypoxic condition is important for the understanding of cancer cell survival and proliferation.

My study demonstrated that FBI-1 plays a critical role in survival and proliferation of colon cancer cells under hypoxic condition. FBI-1 is expressed in normal level in colon cancer cells under normoxic condition. FBI-1 represses MCT4 transcription by binding to the FRE and HRE of the MCT4 promoter. When the cancer cells are in hypoxic condition, RelA/p65 translocate into the nucleus. RelA/p65 represses FBI-1 transcription by binding to the NF-κB RE#1 and -#2 of the FBI-1 promoter. As a result, *MCT4* gene is induced by decreasing FBI-1 protein expression and by binding hypoxia-induced HIF-1α protein to the HRE of the MCT4 promoter. Consequently, cancer cells pump out lactate and H^+ by the MCT4 and

maintain neutral intracellular pH and make extracellular pH acidic (Figure 10). However, once FBI-1 is overexpressed under hypoxic condition, MCT4 expression is potently repressed by FBI-1 binding to the FRE and HRE of the MCT4 promoter and proteasomal degradation of HIF-1α. FBI-1 overexpression in colon cancer cells under hypoxic condition is detrimental for cancer cell survival by deregulation of lactate efflux. These findings are important addition to the various functions of the FBI-1.

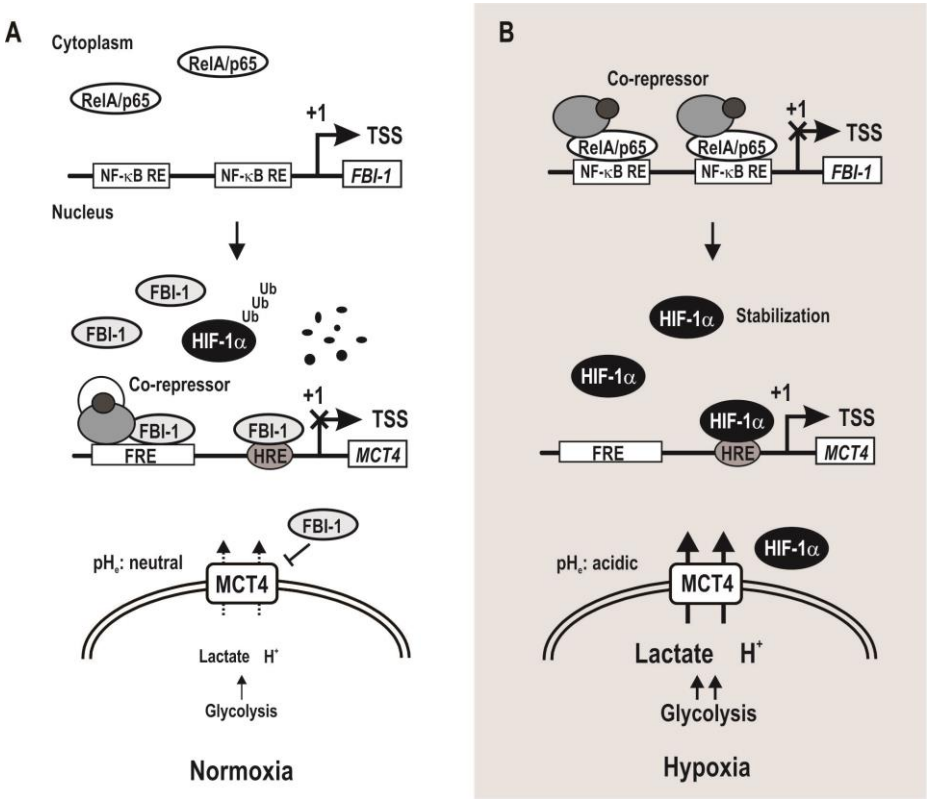


Figure 10. Hypothetical model of *FBI-1* and *MCT4* gene regulation under hypoxic condition. (A) FBI-1 exists in normal level in colon cancer cells under normoxic condition. FBI-1 represses MCT4 transcription by binding to the FRE and HRE of the MCT4 promoter. (B) Hypoxia induced RelA/p65 represses FBI-1 transcription by binding to the NF-κB RE#1 and -#2 of the FBI-1 promoter. As a result, *MCT4* gene is induced by decreasing FBI-1 protein expression and by hypoxia-induced HIF-1α protein binding to the HRE. Consequently, cancer cells pump out lactate and H^+ to cytoplasm by the MCT4.

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ABSTRACT (in Korean)

저산소 상태의 대장암 세포에서 FBI-1의 억제는 MCT4를 통한 젖산 배출을 유도한다

<지도교수 허 만 욱>

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김 민 영

암 세포는 저산소 상태에서 세포의 생존과 성장을 유지하기 위하여 다양한 유전자들을 조절하여 세포 내 대사를 변화시킨다. 저산소 상태의 암 세포의 대사과정의 변화와 메커니즘을 연구하는 것은 암 세포의 생리학적 과정의 이해와 항암제 개발에 중요하다.

본 연구자는 발암인자로 알려진 FBI-1가 정상 산소와 저산소 상황에서 MCT4 유전자 프로모터에 존재하는 FBI-1 결합 부위에 작용하여 *MCT4* 유전자를 전사적으로 억제함을 발견하였다. 또한, FBI-1은 MCT4 프로모터 내 HIF-1α 결합 부위에 작용하여 HIF-1α 단백질의 결합을 저해하고, 또한 HIF-1α 단백질 분해를

촉진시켜 *MCT4* 유전자의 발현을 억제할 수 있다.

흥미롭게도, 저산소 상태에서 *MCT4* 유전자의 발현은 증가하는 반면에, RelA/p65가 FBI-1 프로모터 내 결합부위에 작용함으로써 FBI-1 유전자의 발현은 전사수준에서 억제된다. 저산소 상태의 암 세포에서 NF-kB가 활성화됨에 따른 FBI-1의 발현 감소는 *MCT4* 유전자의 발현을 유도하여 과도하게 생성된 젖산을 세포 밖으로 배출시킴으로써 암 세포의 생존 및 성장을 촉진한다.

요약하면, 저산소 상태의 암 세포는 세포의 성장을 유지하기 위해 FBI-1의 발현을 감소시켜 *MCT4* 유전자 발현을 증가시키고 암 세포에서 해당과정에 의해 생성된 과도한 젖산과 수소 이온을 세포 밖으로 배출시켜 세포 내 pH를 중성으로 유지하고, 세포 밖의 pH를 산성화 시킨다.

핵심되는 말: FBI-1, ZBTB7A, MCT4, 저산소증, RelA/p65, 전사인자, 암 대사, 젖산 배출, HIF-1α

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