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## **Enhancement of *in vitro* cell motility and invasiveness of human malignant pleural mesothelioma cells through the HIF-1 $\alpha$ -MUC1 pathway**

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**Abstract**

In this study, we examined the effects of hypoxia on the malignancy of human malignant pleural mesothelioma (MPM) cell lines, and found 1) hypoxia enhanced motility and invasiveness of human malignant pleural mesothelioma (MPM) cells; 2) this phenomenon resulted from increased expression of sialylated MUC1 through the activation of HIF-1 pathway; 3) two HIF-binding sites located in the promoter region of MUC1 were important for MUC1 transactivation under hypoxia. These findings are useful for better understanding molecular mechanisms of aggressive behavior of MPM cells and for targeting them in the clinical therapies for MPM patients.

**Keywords:** mesothelioma, hypoxia, HIF, MUC1, motility, invasiveness

## **1. Introduction**

Malignant pleural mesothelioma (MPM) is an intractable tumor of mesothelial cells lining the visceral and parietal pleura. Often MPM progresses to advanced stages without clinical signs or symptoms and has involved other organs by the time of diagnosis; its prognosis is very poor even after multi-modality treatments [1]. Most well-known etiology of MPM is exposure to asbestos and MPM may develop long after exposure to asbestos (usually after 30-40 years) [2]. Unfortunately MPM incidence is increasing and the peak of MPM mortality is expected to be in the near future [3].

In solid tumors, hypoxia, a condition that the oxygen supply to tissues decreases, often occurs during the expansion of a tumor mass. Namely, in contrast to normal cells, tumor cells have uncontrolled cell proliferation, and because of an increased cell number, oxygen supply becomes insufficient; this hypoxic condition stimulates angiogenesis, but the tumor vessels have functional and structural abnormalities, and the blood flow is irregular and sluggish, which can not deliver enough oxygen to tumor cells [4]. Another cause for hypoxia in a solid tumor is the distance of cells to a vessel; if they are away from a vessel by more than 70  $\mu\text{m}$ , they do not receive enough oxygen [5]. Hypoxic regions exist in mesothelioma tissues as well as in other tumor tissues.

It is well known that tumor hypoxia has the Janus face. Namely, in some cases, hypoxia suppresses cell proliferation and may induce differentiation, cell cycle arrest, apoptosis or even necrosis [6]. On the other hand, some populations of tumor cells in a tumor tissue can adapt themselves to hypoxia for their survival. Further hypoxia occasionally promotes malignant progression such as invasion, metastasis and acquiring

resistance to chemo- and radio-therapies [4, 6].

Adaptive cellular responses to hypoxia are mainly mediated by the activation of heterodimeric transcription factors belonging to hypoxia-inducible factor (HIF) family [7]. HIF heterodimer consists of  $\alpha$  subunit and  $\beta$  subunit:  $\alpha$  subunit (mainly HIF-1 $\alpha$  or HIF-2 $\alpha$ ) is an oxygen-regulated subunit and  $\beta$  subunit (HIF-1 $\beta$ , also called ARNT) is a constitutively expressed subunit. Once HIF complex has been activated, it stimulates transcription of a series of genes for adaptation to hypoxia including angiogenic factors, glycolytic enzymes and glucose transporters. It is also known that HIF activates genes related to invasion and metastasis [4, 8]. These reports have prompted us to hypothesize that hypoxia may enhance malignant behavior such as motility and invasiveness of MPM cells.

In this study, we aimed to determine (1) whether hypoxia enhances motility and invasiveness of MPM cells and (2) and if it does, (b) by which mechanism(s) hypoxia involves the malignancy.

## **2. Materials and methods**

### *2.1. Antibodies*

Mouse monoclonal antibodies to HIF-1 $\alpha$  (54/HIF-1 $\alpha$ , BD Biosciences San Jose, CA), HIF-2 $\alpha$  (190b, Santa Cruz Biotechnology Santa Cruz, CA) and  $\beta$ -actin (C4, Millipore Billerica, MA) were used for Western blot analyses. Two kinds of mouse monoclonal antibodies to MUC1, VU-4H5 (Cell Signaling Technology, Danvers, MA) and MY.1E12 were used for Western blot and flow cytometric analyses: VU-4H5 and

MY.1E12 recognizes hypoglycosylated PDTRPAP and sialylated VTS of MUC1 tandem repeat, respectively [9, 10]. Horseradish peroxidase-conjugated sheep anti-mouse IgG (GE Health Care, Buckinghamshire, UK) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel, Aurora, OH, USA) were used as secondary antibodies for Western blot and flow cytometry, respectively.

### *2.2. Cells and cell culture*

Six human malignant pleural mesothelioma (MPM) cell lines were used in this study: Of those, HMM-1 and HMM-3 were established from pleural effusions of patients with MPM [11]; TCC-MESO-1 and TCC-MESO-2 were established respectively from the primary and metastatic tumors of a patient with MPM [12]; TCC-MESO-3 was established from the primary tumor (left pleura) of a patient with MPM [12]; NCI-H226 was obtained from National Institute of Health, National Cancer Institute, Rockville, MD. All cell lines were grown on tissue culture dishes in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (DME/F12) supplemented with 10% of fetal bovine serum (FBS). The cells were maintained at 37°C under normoxia in a CO<sub>2</sub> incubator (21% O<sub>2</sub>, 5% CO<sub>2</sub>). The cells in the hypoxic group were incubated at 37°C in a hypoxic chamber (Wakenyaku, Tokyo, Japan) gassed with 1% O<sub>2</sub> (balanced with N<sub>2</sub>) and 5% CO<sub>2</sub>.

### *2.3. Luciferase reporter assay*

Promoter activity of HIF was evaluated by using the following reporter vectors. pGL3-5xHRE had five tandem repeats of the sequence 5'-CGCCCTACGTGCTGTCTCACACAGCCTGTCTGA-3', including a

hypoxia-response element (HRE) from the erythropoietin gene upstream of firefly luciferase gene [13]. An empty pGL3-promoter vector was used as a negative control against pGL3-5xHRE reporter vector (Promega, Madison, WI). For MUC1 promoter assay, different sizes of human MUC1 promoter regions (-1468/+28 (1.4 kb), -758/+28 (0.7 kb) and -187/+28 (0.2 kb)) were cloned by PCR of genomic DNA extracted from TCC-MESO-1 cells. The following PCR primers used for cloning the MUC1 promoter regions were designed based on human genomic DNA sequence (NT\_004487.19):

Forward primer for -1468Luc (1.4 kb)  
5-TGCGCTAGCTGCTCCCCAAAGGATAGTTCTGTGTCCG-3, for -758Luc (0.7 kb)  
5-TGCGCTAGCCGCAAGGCTCCCGGTGACCACTAGA-3, and for -187Luc (0.2 kb)  
5-TGCGCTAGCACGGGTAGTCAGGGGGTTGAGCGATTAG-3. Reverse primer for all of cloned fragments was 5-GCAAAGCTTAGGCGCTGGCTGCTTGAGAGGTG-3.

The PCR products were digested with *Hind*III and *Nhe*I, and ligated to pGL3-basic vector (Promega). Substitution of 5'-TTAAT-3' for 5'-(A/G)CGTG-3', which was a consensus sequence of HIF-binding site (HBS), at -1130/-1126 and/or -1072/-1068 of pGL3-basic-MUC1-1.4, was performed by site-directed mutagenesis. Sequences of all the reporter vectors were checked, and designated as pGL3-basic-MUC1-1.4, pGL3-basic-MUC1-0.7, pGL3-basic-MUC1-0.2, pGL3-basic-MUC1-1.4-mut (-1130/-1126), pGL3-basic-MUC1-1.4-mut (-1072/-1068) and pGL3-basic-MUC1-1.4-mut (-1130/-1126 and -1072/-1068). An empty pGL3-basic vector was used as a negative control against the MUC1 promoter reporter vectors. In both HIF and MUC1 promoter assays, pRL-CMV (Promega) which had *Renilla*

luciferase gene constitutively driven by CMV promoter was used as an internal transfection control. MPM cells suspended in Opti-MEM I (Invitrogen, Carlsbad, CA) were transfected with 10 µg of the reporter vectors along with 20 ng of pRL-CMV by using an electroporator (CUY21 Pro-Vitro-SM, NEPA GENE, Ichikawa, Japan). Immediately after the transfection, the cells were seeded on wells (24-well plates) and incubated all under normoxia for 24 h. And then the cells were divided into two groups and incubated for another 24 h, one under normoxia and the other under hypoxia. The cells were lysed and subjected to measuring luciferase activities by using a dual-luciferase assay system (Promega) and Berthold Mini Lumat LB950L (Berthold, Tokyo, Japan). Relative luciferase activity was expressed as the firefly luciferase activity normalized by the *Renilla* luciferase activity.

#### *2.4. Flow cytometry*

Cell preparation for flow cytometric analysis was performed by the same method described in our previous report [14]. Fluorescence intensity was analyzed with the use of FACSCanto (Becton Dickinson, San Jose, CA).

#### *2.5. Phagokinetic track assay*

Phagokinetic track assay was performed to assess the cell motility by the same method described in our previous report [14]. The phagokinetic tracks were observed with dark-field illumination under an inverted microscope at a magnification of x200. The distribution of track area was summarized by using box and whisker plots.

#### *2.6. Assay for invasion of type I collagen gel by MPM cells*

Assay for invasion of type I collagen gel were performed by using Transwell chambers



with 8  $\mu\text{m}$  pore-membrane (Corning, Corning, NY) by the same method described in our previous report [15]. with some modification. Type I collagen solution (Cellmatrix type I-A, Nitta Gelatin, Yao, Japan), 10-fold concentrated D-MEM,  $\text{NaHCO}_3$ /HEPES and FBS were mixed in the volume at 8: 1: 1: 0.5, respectively. The mixed collagen solution (0.6 ml) was placed in the wells, and a Transwell was placed on each well. The Transwells were pressed down to make sure of their tight contact with the collagen solution. After gelation in a normoxic  $\text{CO}_2$  incubator, 0.1 ml of cell suspension ( $2 \times 10^5/\text{ml}$ , DME/F12-10% FBS) was placed into the upper compartment of each Transwell. After 24 h-incubation in the normoxic or hypoxic condition, the Transwells were removed and the cells in the collagen gel were observed under an inverted phase-contrast microscope. Invasiveness of the cells was evaluated from the number of cells per field at  $\times 100$  magnification (mean  $\pm$  SD,  $n = 45$ ).

#### *2.8. Protein extraction and Western blot analysis*

The cells on culture dishes were quickly washed twice with cold PBS(-) and whole cell lysates were prepared by using cold RIPA buffer [25 mM Tris.HCl (pH 7.6), 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN)]. After incubation of the lysates on ice for 5 min, the lysates were centrifuged at 20,000  $\times g$  for 15 minutes, and the supernatants were collected as whole cellular proteins. The protein concentration was evaluated by using a DC protein assay kit (BioRad), with bovine serum albumin (Pierce, Rockford, IL) as a standard protein. The aliquots were separated on SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA) in a semi-dry trans-blot

apparatus. The membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% skim milk for 1 h at room temperature, and then were probed with primary antibodies. The membranes were next incubated with horseradish peroxidase-conjugated antibodies for 1 hr at room temperature, and developed by using the Enhanced Chemiluminescent Detection System (Millipore).

### 2.9. Transfection of small interfering RNA (siRNA)

TCC-MESO-1 and NCI-H226 cells ( $1 \times 10^6$  cells) were transfected with 2.5  $\mu\text{g}$  of appropriate siRNA duplex by using an electroporator and seeded in 6-well plates and incubated for 24 h in normoxic condition. After the incubation under normoxia or hypoxia for another 24 h, the cells were used for further analysis. The nucleotide sequences of siRNAs used in this study were siHIF-1 $\alpha$ :  
5'-CUGAUGACCAGCAACUUGAdTdT-3' (sense) and  
5'-UCAAGUUGCUGGUCAUCAGdTdT-3' (antisense); siHIF-2 $\alpha$ :  
5'-CAGCAUCUUUGAUAGCAGUdTdT-3' (sense) and  
5'-ACUGCUAUCAAAGAUGCUGdTdT-3' (antisense); siMUC1:  
5'-GAUCGUAGCCCCUAUGAGAdTdT-3' (sense) and  
5'-UCUCAUAGGGGCUACGAUCdTdT-3' (antisense). AllStar negative control conjugated with AlexaFlour647 (Qiagen) was used as a negative control siRNA.

### 2.10. RNA extraction and RT-PCR

Total RNA was extracted from monolayer cultures of MPM cells with TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. For preparation of cDNA, 1  $\mu\text{g}$  of total RNAs was subjected to reverse transcription with Oligo(dT)

primers and Superscript III reverse transcriptase (Invitrogen). PCR amplification of cDNA was performed in 25  $\mu$ l reaction mixture containing 1  $\mu$ l cDNA, 5  $\mu$ l of 5x Green GoTaq Buffer (Promega), 3 $\mu$ l of 2 mM dNTPs mix, 1  $\mu$ l of 5  $\mu$ M of each specific primers and 0.125  $\mu$ l GoTaq DNA polymerase (Promega). PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide and intensity of the bands was observed under an ultraviolet transilluminator. Nucleotide sequences of primers used were listed in Table I.

#### *2.11. Quantitative real-time RT-PCR (qRT-PCR)*

qRT-PCR was performed by using ABI Prism 7900HT (Applied Biosystems, Grand Island, NY) with 2x Quantifast SYBR Green PCR Master mix (Qiagen) with 1 mM primers. Specific primer sets for HIF1 $\alpha$ , HIF2 $\alpha$  and ACBT ( $\beta$ -actin, as internal control) were listed in Table 1. Each target gene was analyzed in three independent qRT-PCR assays. Thermal cycling was started with an initial activation step for 5 min at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Relative expression of the target gene was analyzed by the  $\Delta\Delta$ Ct method (the reference gene:  $\beta$ -actin). Results were normalized relative to the non-treatment control.

#### *2.12. Statistical analysis*

Statistical significance was determined by one-way analysis of variance followed by Fisher's probable least-squares difference analysis as a *post hoc* test for data from phagokinetic track assay, and by two-sided Student's *t*-test for data from invasion assays. In all statistical comparisons,  $P < 0.01$  was used to indicate statistically significant difference.

### **3. Results**

#### *3.1. Hypoxia activates HIF pathways in the MPM cell lines*

We firstly examined whether hypoxia activated HIF signaling pathway in human MPM cell lines. The activation of HIF pathways is one of the earliest and most important responses to hypoxia, and usually the activation is evaluated from the increases in HIF-1 $\alpha$  and/or HIF-2 $\alpha$  proteins, transactivation of downstream genes of HIF transcription complexes or an increase in promoter activity to hypoxia response element (HRE) [4, 16]. As shown in Fig. 1A, the expressions of both HIF-1 $\alpha$  and HIF-2 $\alpha$  at protein levels were increased in almost all the MPM cell lines used while they were cultured for 24 h under hypoxia. Next, we analyzed the expression levels of GLUT-1, VEGFA and HK2 genes by RT-PCR; they are known to be target genes of HIF transcriptional complexes. The expression levels of these 3 genes in all the cell lines were higher in hypoxia than in normoxia (Fig. 1B). Further, to confirm the activation of HRE-dependent transcription in MPM cells under hypoxia, we analyzed HRE-dependent luciferase activity. As a result, it was revealed that hypoxia activated HRE-dependent transcription in all the 6 cell lines (Fig. 1C). These data suggested that the hypoxic condition activated HIF pathways in all the MPM cell lines used.

#### *3.2. Enhancement of in vitro cell motility and invasiveness of MPM cells by hypoxia*

We next examined influences of hypoxia on cell biological behaviors such as motility and invasiveness, which were especially related to aggressiveness of MPM. Phagokinetic track assay showed that hypoxia significantly enhanced cell motility in all

the cell lines except TCC-MESO-3 (Fig. 2A). Invasiveness was evaluated from their invasion of type I collagen gel. Hypoxia significantly enhanced invasion of type I collagen gel by all of MPM cell lines (Fig. 2B).

### *3.3. Hypoxia-enhanced motility and invasiveness of MPM cells are dependent on HIF-1 $\alpha$ activation*

In many kinds of human cells, HIF-pathways are known to be activated by HIF-1 $\alpha$ - and/or HIF-2 $\alpha$ -mediated mechanisms for oxygen sensing [17, 18]. We therefore investigated which HIF $\alpha$  isoform was involved in hypoxia-enhanced motility and invasiveness. siRNAs targeting HIF-1 $\alpha$  (siHIF-1 $\alpha$ ) or HIF-2 $\alpha$  (siHIF-2 $\alpha$ ) were transfected into two of the cell lines (TCC-MESO-1 and NCI-H226) to knock down the expressions of each HIF isoform. Twenty-four hours after the siRNA transfection, the expressions of HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-1 $\beta$  were analyzed by conventional RT-PCR, quantitative RT-PCR and Western blot. As shown in Fig. 3A, B and C, siHIF-1 $\alpha$ -treatment blocked specifically the expression of HIF-1 $\alpha$  but not HIF-2 $\alpha$ , whereas siHIF-2 $\alpha$  blocked the expression of HIF-2 $\alpha$  but not HIF-1 $\alpha$ , in both cell lines. The treatment with the two siRNAs together blocked the expressions of both genes at an equal level to the blocking with either one of the siRNAs. These results indicated that siHIF-1 $\alpha$  and siHIF-2 $\alpha$  could effectively and specifically knock down the expressions of HIF-1 $\alpha$  and HIF-2 $\alpha$ , respectively. Using this system, we examined whether hypoxia-enhanced motility and invasiveness were via HIF-1 $\alpha$ - or HIF-2 $\alpha$ -pathway. As a result, knockdown of HIF-1 $\alpha$  abolished hypoxia-enhanced motility in TCC-MESO-1 and NCI-H226 cells whereas knockdown of HIF-2 $\alpha$  did not affect it in either cells (Fig.

3C and D). Knockdown of both HIF-1 $\alpha$  and HIF-2 $\alpha$  abolished hypoxia-enhanced motility likewise. Invasiveness enhanced by hypoxia was also reduced when siHIF-1 $\alpha$ , but not siHIF-2 $\alpha$ , was transfected into TCC-MESO-1 and NCI-H226 cells (Fig. 3E and F). However, in normoxia, transfection of any of the siRNAs did not affect either motility or invasiveness.

#### *3.4. MUC1, possibly a HIF-1 target gene involved in hypoxia-enhanced motility and invasion*

The complex of HIF-1 $\alpha$  and HIF-1 $\beta$  is well known to regulate the transcription of a variety of genes, some of which are closely related to motility and invasion [19]. We examined by RT-PCR whether the expressions of the motility- and invasion-related genes, which are reported to be upregulated in HIF-1 $\alpha$ -mediated manner, and other genes relevant to them underwent changes in hypoxia [4, 19-24]. As shown in Fig.4, the expression of MUC1 gene increased in all the MPM cell lines when they were under hypoxia, but no common change was observed among the cell lines in the expressions of other genes whether they were under hypoxia or normoxia.

Besides matrix metalloproteinase (MMP)-2 and MMP-9, we further analyzed the expressions of the following: extracellular matrix-degradative enzymes and their inhibitors including MMP-1, -3, 7, -14, and -15; tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 and heparanase since they play important roles in tumor invasion. We did not find any gene of which expression was changed in hypoxia commonly in the 6 cell lines (data not shown).

#### *3.5. HIF-1 $\alpha$ -dependent MUC1 up-regulation in MPM cells under hypoxia*

We speculated from RT-PCR analysis that MUC1 was one of the genes which possibly enhanced motility and invasion via HIF-1 pathway under hypoxia. As shown in Fig. 5A and 6A, RT-PCR analysis of MUC1 expressions in MPM cells with knockdown of HIF-1 $\alpha$  and/or HIF-2 $\alpha$  revealed that the increase in MUC1 expression under hypoxia depended on HIF-1 $\alpha$  expression levels but not on those of HIF-2 $\alpha$ . Then, we explored the region responsible for transactivation of MUC1 promoter in NCI-H226 and HMM-3 cells under hypoxia by luciferase reporter assay. As shown in Fig. 5B, promoter analysis by using reporter plasmids with serial deletions of MUC1 promoter revealed that the region from -1468 to -759 upstream from the transcription starting site was necessary for hypoxia-induced MUC1 transactivation. We found 6 consensus sequences of HIF-binding sites (HBSs: 5'-(A/G)CGTG-3'), two on sense strands and the other four on antisense strands in the region -1468 to -759. This finding coincided with the indication by Kimura et al. that an HBS and its downstream HIF-1 ancillary sequence (HAS) within the HRE are required as *cis*-elements for the transcriptional activation of VEGF by hypoxia [25]. We recognized the third (-1114/-1110) and fifth (-1052/-1048) HBSs from the distal site of promoter region of MUC1 were similar to HAS of HRE in the promoter region of HO-1 since the sequence of HAS in HO-1 was identical to the antisense sequence of 5'-(A/G)CGTG-3' [25]. From these we speculated that the second and the third (likewise the fourth and the fifth) ones formed HRE in the promoter region of MUC1. To determine whether the second and fourth HBS were essential for transactivation of MUC1 under hypoxia, we made mutant reporters of those HBSs by site-directed mutagenesis, and found that the both sites were essential

for hypoxia-induced transactivation of MUC1 (Fig. 5C). We next confirmed that hypoxia up-regulated MUC1 expressions not only at mRNA level but also at protein level. As shown in Fig. 5D, analysis by flow cytometry revealed that the expressions of sialylated MUC1 on cell surfaces increased under hypoxia in both TCC-MESO-1 and NCI-H226 cells. Immunoblot analysis also showed increased expressions of sialylated and hypoglycosylated MUC1 under hypoxia in both cell lines (Fig. 6B).

### *3.6. Inhibition of motility and invasiveness by MUC1 knockdown*

We finally examined whether increased expression of MUC1 played a crucial role in hypoxia-enhanced motility and invasiveness. MUC1 was knocked down in TCC-MESO-1 and NCI-H226 cell lines by transfection with siRNA targeting MUC1 (siMUC1). The transfection decreased MUC1 expression in both cell lines at mRNA and protein levels but did not affect HIF-1 $\alpha$  expression (Fig. 6A and B). The decrease in MUC1 expression was detected not only in hypoxia but also in normoxia. After treating TCC-MESO-1 and NCI-H226 cells, respectively with siHIF-1 $\alpha$  and siMUC1, their motility and invasiveness were analyzed by phagokinetic track assay and invasion assay by using type I collagen gel. As a result, there was no difference in motility of both cell lines, whether treated with siMUC1 or siHIF-1 $\alpha$ , between normoxia and hypoxia (Fig. 6C). Motility of siMUC1-treated cells was significantly low, compared to that of siHIF-1 $\alpha$ -treated cells, in both normoxia and hypoxia. Invasiveness of siMUC1-treated cells was also significantly lower than that of siHIF-1 $\alpha$ -treated cells under hypoxia in both cell lines (Fig. 6D). Moreover, MUC1 knockdown significantly decreased invasiveness under normoxia although HIF-1 $\alpha$  knockdown did not.



#### 4. Discussion

Hypoxia is a common feature in a variety of solid tumors [26]. Hypoxia-induced cellular responses are often linked to malignant progression such as invasion, metastasis, and resistance to chemo- and radio-therapy [8]. In this study, we demonstrated that hypoxia enhanced cell motility and invasiveness of MPM cells.

Adaptive cellular responses to hypoxia are mainly mediated by the activation of heterodimeric transcription factors belonging to HIF family [7]. In normoxia, HIF $\alpha$  subunit is subjected to proline hydroxylation and degraded by proteasome, but in hypoxia it forms a complex with HIF-1 $\beta$  and the complex transactivates target genes for adaptation to hypoxia. It has come to be known that HIF-1 $\alpha$  and HIF-2 $\alpha$  function differently; for example, some target genes are transcribed in a HIF-1 $\alpha$ -dependent manner whereas others are in a HIF-2 $\alpha$ -dependent manner [27-30]. Sowter et al. demonstrated that a target gene is transcribed by a particular transcription factor, for example, in breast cancer cells, by HIF-1 $\alpha$ , and in renal carcinoma cells, by HIF-2 $\alpha$  [17]. Hence we examined which HIF $\alpha$  protein was involved in hypoxia-enhanced motility and invasiveness of MPM cells. Our knockdown experiments using siRNA targeting HIF-1 $\alpha$  and/or HIF-2 $\alpha$  showed that HIF-1 $\alpha$ -knockdown reduced hypoxia-enhanced motility and invasiveness whereas HIF-2 $\alpha$ -knockdown did not. This indicated that HIF-1 $\alpha$  signaling played a major role in the hypoxia-driven malignancy of MPM cells.

Next we explored genes transactivated by HIF-1 complex which were involved

in the enhancement of motility and invasiveness under hypoxia. Of the genes we analyzed by RT-PCR, the expression of only MUC1 was upregulated under hypoxia in all the 6 cell lines. And the upregulated MUC1 expression under hypoxia was subdued by HIF-1 $\alpha$  knockdown, but not by HIF-2 $\alpha$  knockdown (Fig. 5A). The hypoxia-induced upregulation of MUC1 expression coincided with the phenomenon that hypoxia-enhanced motility and invasiveness were dependent on activation of HIF-1 $\alpha$  but not of HIF-2 $\alpha$ . Our reporter assay with serial deletions of MUC1 reporter plasmids suggested that a region from -1468 to -759 in MUC1 promoter was essential for the transactivation under hypoxia. Two other groups have reported different views from ours concerning the region related to responses to hypoxia. Aubert *et al.* demonstrated that two HIF-1 binding sites (HBSs) located further away from -1468 were important for responses to hypoxia and able to mediate activation of the MUC1 promoter in HEK293 cells and human renal cell carcinoma Caki-2 cells they used [31]. Mikami *et al.* described that three HBSs within 2.8 kb of MUC1 promoter, which were predicted in the Web site of Genomatrix, did not work as cis-elements for regulating the promoter activity of MUC1 in human lung cancer A549 cells in hypoxia [32]. Discrepancies among the three study groups may imply that mechanisms of transcriptional activation of MUC1 could differ depending on the origins of the cell lines used. And our site-directed mutagenesis studies further revealed that two HBSs (-1130/-1126 and -1072/-1068) in the region from -1468 to -759 were essential for transcriptional activation of MUC1 gene under hypoxia. Interestingly, there was an HBS (-1114/-1110 and -1052/-1048) on antisense strands downstream of each HBS (-1130/-1126 and

-1072/-1068). It is known that HRE of some hypoxia-inducible genes consists of HBS and its downstream HIF-1 ancillary sequence (HAS) [25]. The positional relation between HBS (-1130/-1126 or -1072/-1068) and antisense HBS (-1114/-1110 or -1052/-1048) is equal to that between HBS and HAS within HRE of HO-1, although the number of nucleotides between HBS and HAS is larger in MUC1 than in HO-1 [25]. These findings suggest that two putative HREs exist in the region from -1468 to -759 and that they can function as *cis*-elements for MUC1 promoter.

In renal cell carcinoma (RCC), it has already been shown that overexpression of MUC1 through HIF-1 signaling pathway enhanced motility and invasiveness under hypoxia [31]. Our next question was whether MUC1 was actually implicated in hypoxia-enhanced motility and invasiveness of MPM cells as well as RCC cells. As expected, MUC1-knockdown reduced the motility and invasiveness enhanced by hypoxia. And the motility and invasiveness of MUC1-knocked-down cells were lower than those of HIF-1 $\alpha$ -knocked-down cells under hypoxia. This discrepancy between MUC1-knocked-down and HIF-1 $\alpha$ -knocked-down cells may be caused by a difference in the levels of MUC1 expression between the cells since MUC1 expression levels of HIF-1 $\alpha$ -knocked-down cells were relatively high compared to those in MUC1-knocked-down cells. And MUC1 knockdown slightly reduced cell motility and invasiveness even in normoxic conditions whereas HIF-1 $\alpha$  knockdown did not. From these findings, we speculated that MUC1 contributed to motile and invasive activities more under hypoxia than under normoxia.

How does the increase in expression of MUC1 lead to the enhancement of

motility and invasiveness of MPM cells under hypoxia? MUC1 gene encodes a type I transmembrane protein [33]. And MUC1 protein has an N-terminal extracellular domain consisting of variable number of tandem repeats (VNTR) of 20 amino acids and a C-terminal domain, which consists of an extracellular region, a transmembrane domain and a cytoplasmic tail (MUC1CT). Serine and threonine residues in VNTR are *O*-glycosylated, and the *O*-linked glycans are fully sialylated during the maturation of MUC1 mucin through a *trans*-Golgi network [34, 35].

We noted, first, that extracellular domain of MUC1 functions as anti-adhesion molecules. Sialylated MUC1 mucin expressed on tumor cells suppresses homotypic cellular aggregation through masking E-cadherin, homophilic cell adhesion molecules known as an invasion-suppressor [36, 37], and promotes invasion and metastasis [38, 39]. Since MUC1 knockdown reduced the hypoxia-enhanced motility and invasiveness of MPM cells, even those of TCC-MESO-1 cells whose E-cadherin expression was extremely low, it is unlikely, from our results, that disturbance in E-cadherin function by MUC-1 overexpression was involved in their responses to hypoxia.

Recently it has come to be known that cytoplasmic domain (CD) of MUC1CT (MUC1CD) works as an intracellular signaling subunit. Namely, MUC1CD of MUC1 bound to ligands such as intercellular adhesion molecule-1 (ICAM-1) behaves like a scaffold protein: MUC1CD recruits Src and CT10 regulator of kinase like (CrkL), and this complex triggers Rac1- and Cdc42-dependent actin cytoskeletal protrusive activity, which stimulates motility and invasion [40]. Or MUC1CD makes a complex with

$\beta$ -catenin and the complex is translocated to the nucleus, which initiates epithelial-to-mesenchymal transition (EMT) [41]. Further, MUC1 is known to interact with receptor tyrosine kinases including epidermal growth factor receptor (EGFR), platelet derived growth factor receptor  $\beta$  (PDGFR $\beta$ ) and hepatocyte growth factor receptor (c-Met), and to participate in their downstream signaling pathways [42-44]. Taken together, it is likely that MUC1 facilitates motility and invasiveness of MPM cells by accompanying other molecules. Interestingly a recent report has shown that hypoxia activates the signal transduction pathway of EGFR without EGF-stimulation [45]. EGFR is expressed in most of MPM [46, 47] and we observed that c-Met was also expressed in MPM cells as shown in Fig. 4B. Therefore, it is very likely that the interaction of MUC1 with receptor tyrosine kinases, such as EGFR and c-Met, activated by exposure to hypoxia, may play a pivotal role in hypoxia-enhanced motility and invasiveness of MPM cells.

Our data presented here showed that HIF-1 signaling upregulated MUC1 expression. On the other hand, Chaika *et al.* reported that MUC1 functioned as a modulator of the hypoxic response in pancreatic cancer cells by regulating the expression/stability and activity of HIF-1 $\alpha$  [48]. Thus, it is possible that MUC1 and HIF-1 co-regulate under hypoxia, which may enhance malignant properties of cancer cells.

In summary, we report that hypoxia enhances motility and invasiveness of MPM cells through activation of the HIF-1 $\alpha$ -MUC1 pathway. Considering the extremely aggressive phenotype of mesothelioma, we suggest that

HIF-1 $\alpha$ -MUC1-initiated signaling pathway in response to hypoxia can be a clue to elucidate the molecular mechanisms of MPM malignancy, and could be used as a target in the clinical therapies for MPM patients.

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### **Conflict of Interest Statement**

The authors declare no conflict of interest.

### **References**

- [1] P.A. Zucali, G. Giaccone, Biology and management of malignant pleural mesothelioma, *Eur. J. Cancer* 42 (2006) 2706-2714.
- [2] J.C. McDonald, Health implications of environmental exposure to asbestos, *Environ. Health Perspect.* 62 (1985) 319-328.
- [3] T. Aziz, A. Jilaihawi, D. Prakash, The management of malignant pleural mesothelioma; single centre experience in 10 years, *Eur. J. Cardiothorac. Surg.* 22 (2002) 298-305.
- [4] G.L.Semenza, Molecular mechanisms mediating metastasis of hypoxic breast

cancer cells, *Trends Mol. Med.* 18 (2012) 534-543.

[5] P. Vaupel, Tumor microenvironmental physiology and its implications for radiation oncology, *Sem. Rad. Oncol.* 14 (2004) 198-206.

[6] P. Vaupel, A. Mayer, Hypoxia in cancer: significance and impact on clinical outcome, *Cancer Metastasis Rev.* 26 (2007) 225-239.

[7] C.J. Schofield, P.J. Ratcliffe, Oxygen sensing by HIF hydroxylases, *Nat. Rev. Mol. Cell Biol.* 5 (2004) 343-354.

[8] A.L. Harris, Hypoxia--a key regulatory factor in tumour growth, *Nat. Rev. Cancer* 2 (2002) 38-47.

[9] P. Ciborowski, O.J. Finn, Non-glycosylated tandem repeats of MUC1 facilitate attachment of breast tumor cells to normal human lung tissue and immobilized extracellular matrix proteins (ECM) in vitro: potential role in metastasis, *Clin. Exp. Metastasis* 19 (2002) 339-345.

[10] H. Takeuchi, K. Kato, K. Denda-Nagai, F.G. Hanisch, H. Clausen, T. Irimura, The epitope recognized by the unique anti-MUC1 monoclonal antibody MY.1E12 involves sialyl alpha 2-3galactosyl beta 1-3N-acetylgalactosaminide linked to a distinct threonine residue in the MUC1 tandem repeat, *J. Immunol. Meth.* 270

(2002) 199-209.

- [11] H. Goudarzi, Y. Hida, H. Takano, H. Teramae, H. Iizasa, J. Hamada, Hypoxia affects in vitro growth of newly established cell lines from patients with malignant pleural mesothelioma, *Biomed. Res.* 34 (2013) 13-21.
- [12] K. Yanagihara, M. Tsumuraya, M. Takigahira, K. Mihara, T. Kubo, K. Ohuchi, T. Seyama, An orthotopic implantation mouse model of human malignant pleural mesothelioma for in vivo photon counting analysis and evaluation of the effect of S-1 therapy, *Int. J. Cancer* 126 (2010) 2835-2846.
- [13] M. Natsuzaka, M. Ozasa, S. Darmanin, M. Miyamoto, S. Kondo, S. Kamada, M. Shindoh, F. Higashino, W. Suhara, H. Koide, K. Aita, K. Nakagawa, T. Kondo, M. Asaka, F. Okada, M. Kobayashi, Synergistic up-regulation of Hexokinase-2, glucose transporters and angiogenic factors in pancreatic cancer cells by glucose deprivation and hypoxia, *Exp. Cell Res.* 313 (2007) 3337-3348.
- [14] J. Hamada, T. Omatsu, F. Okada, K. Furuuchi, Y. Okubo, Y. Takahashi, M. Tada, Y.J. Miyazaki, Y. Taniguchi, H. Shirato, K. Miyasaka, T. Moriuchi, Overexpression of homeobox gene HOXD3 induces coordinate expression of metastasis-related genes in human lung cancer cells, *Int. J. Cancer* 93 (2001) 516-525.



- [15] Y.J. Miyazaki, J. Hamada, M. Tada, K. Furuuchi, Y. Takahashi, S. Kondo, H. Katoh, T. Moriuchi, HOXD3 enhances motility and invasiveness through the TGF- $\beta$ -dependent and -independent pathways in A549 cells, *Oncogene* 21 (2002) 798-808.
- [16] G.L. Wang, G.L. Semenza, Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia, *J. Biol. Chem.* 68 (1993) 21513-21518.
- [17] H.M. Sowter, R.R. Raval, J.W. Moore, P.J. Ratcliffe, A.L.Harris, Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia, *Cancer Res.* 63 (2003) 6130-6134.
- [18] V.A. Carroll, M. Ashcroft, Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway, *Cancer Res*, 66 (2006) 6264-6270.
- [19] G.L.Semenza, Targeting HIF-1 for cancer therapy, *Nat. Rev. Cancer* 3 (2003) 721-732.

- [20] M.H. Yang, M.Z. Wu, S.H. Chiou, P.M. Chen, S.Y. Chang, C.J. Liu, S.C. Teng, K.J.Wu, Direct regulation of TWIST by HIF-1alpha promotes metastasis, *Nat. Cell Biol.* 10 (2008) 295-305.
- [21] C. Sahlgren, M.V. Gustafsson, S. Jin, L. Poellinger, U. Lendahl, Notch signaling mediates hypoxia-induced tumor cell migration and invasion, *Proc. Natl. Acad. Sci. USA* 105 (2008) 6392-6397.
- [22] X. Lu, Y. Kang, Hypoxia and hypoxia-inducible factors: master regulators of metastasis, *Clin. Cancer Res.* 16 (2010) 5928-5935.
- [23] H. Niizeki, M. Kobayashi, I. Horiuchi, N. Akakura, J. Chen, J. Wang, J-I. Hamada, P. Seth, H. Katoh, H. Watanabe, A. Raz, M. Hosokawa, Hypoxia enhances the expression of autocrine motility factor and the motility of human pancreatic cancer cells, *Br. J. Cancer* 86 (2002) 1914-1919.
- [24] D. Eguchi, N. Ikenaga, K. Ohuchida, S. Kozono, L. Cui, K. Fujiwara, M. Fujino, T. Ohtsuka, K. Mizumoto, M. Tanaka, Hypoxia enhances the interaction between pancreatic stellate cells and cancer cells via increased secretion of connective tissue growth factor, *J. Surg. Res.* (in press).
- [25] H. Kimura, A. Weisz, T. Ogura, Y. Hitomi, Y. Kurashima, K. Hashimoto, F.

D'Acquisto, M. Makuuchi, H. Esumi, Identification of Hypoxia-inducible Factor 1 Ancillary Sequence and Its Function in Vascular Endothelial Growth Factor Gene Induction by Hypoxia and Nitric Oxide, *J. Biol. Chem.* 276 (2001) 2292–2298.

[26] L. Harrison, K. Blackwell, Hypoxia and anemia: factors in decreased sensitivity to radiation therapy and chemotherapy? *Oncologist* 9 (2004) 31-40.

[27] R.R. Raval, K.W. Lau, M.G. Tran, H.M. Sowter, S.J. Mandriota, J.L. Li, C.W. Pugh, P.H. Maxwell, A.L. Harris, P.J. Ratcliffe, Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma, *Mol. Cell. Biol.* 25 (2005) 5675-5686.

[28] B. Keith, R.S. Johnson, M.C. Simon, HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression, *Nat. Res. Cancer* 12 (2012) 9-22.

[29] M.Y. Koh, R.Jr. Lemos, X. Liu, G. Powis, The hypoxia-associated factor switches cells from HIF-1alpha- to HIF-2alpha-dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion, *Cancer Res* 71 (2011) 4015-4027.

[30] C.J. Hu, L.Y. Wang, L.A. Chodosh, B. Keith, M.C. Simon, Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene

regulation, *Mol. Cell. Biol.* 23 (2003) 9361-9374.

- [31] S. Aubert, V. Fauquette, B. Hémon, R. Lepoivre, N. Briez, D. Bernard, I. Van Seuninghen, X. Leroy, M. Perrais M, MUC1, a new hypoxia inducible factor target gene, is an actor in clear renal cell carcinoma tumor progression, *Cancer Res.* 69 (2009) 5707-5715.
- [32] Y. Mikami, A. Hisatsune, T. Tashiro, Y. Isohama, H. Katsuki, Hypoxia enhances MUC1 expression in a lung adenocarcinoma cell line, *Biochem. Biophys. Res. Commun.* 379 (2009) 1060-1065.
- [33] S.J. Gendler, C.A. Lancaster, J. Taylor-Papadimitriou, T. Duhig, N. Peat, J. Burchell, L. Pemberton, E.N. Lalani, D. Wilson, Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin, *J. Biol. Chem.* 265 (1990) 15286-15293.
- [34] S.V. Litvinov, J. Hilkens, The epithelial sialomucin, episialin, is sialylated during recycling, *J. Biol. Chem.* 268 (1993) 21364-21371.
- [35] T. Irimura, K. Denda, S. Iida, H. Takeuchi, K. Kato, Diverse glycosylation of MUC1 and MUC2: potential significance in tumor immunity, *J. Biochem.* 126 (1999) 975-985.
- [36] M.J. Ligtenberg, F. Buijs, H.L. Vos, J. Hilkens, Suppression of cellular aggregation by high levels of episialin, *Cancer Res.* 52 (1992) 2318-2324.

- [37] K. Kondo, N. Kohno, A. Yokoyama, K. Hiwada, Decreased MUC1 expression induces E-cadherin-mediated cell adhesion of breast cancer cell lines, *Cancer Res.* 58 (1998) 2014-2019.
- [38] T. Suwa, Y. Hinoda, Y. Makiguchi, T. Takahashi, F. Itoh, M. Adachi, M. Hareyama, K. Imai, Increased invasiveness of MUC1 and cDNA-transfected human gastric cancer MKN74 cells, *Int. J. Cancer* 76 (1998) 377-382.
- [39] S. Satoh, Y. Hinoda, T. Hayashi, M.D. Burdick, K. Imai, M.A. Hollingsworth, Enhancement of metastatic properties of pancreatic cancer cells by MUC1 gene encoding an anti-adhesion molecule, *Int. J. Cancer* 88 (2000) 507-518.
- [40] Q. Shen, J.J. Rahn, J. Zhang, N. Gunasekera, X. Sun, A.R. Shaw, M.J. Hendzel, P. Hoffman, A. Bernier, J.C. Hugh, MUC1 initiates Src-CrkL-Rac1/Cdc42-mediated actin cytoskeletal protrusive motility after ligating intercellular adhesion molecule-1, *Mol. Cancer Res.* 6 (2008) 555-567.
- [41] L.D. Roy, M. Sahraei, D.B. Subramani, D. Besmer, S. Nath, T.L. Tinder, E. Bajaj, K. Shanmugam, Y.Y. Lee, S.I. Hwang, S.J. Gendler, P. Mukherjee, MUC1 enhances invasiveness of pancreatic cancer cells by inducing epithelial to mesenchymal transition, *Oncogene* 30 (2011) 1449-1459.

- [42] B.G. Bitler, A. Goverdhan, J.A. Schroeder, MUC1 regulates nuclear localization and function of the epidermal growth factor receptor, *J. Cell Sci.* 123 (2010) 1716-1723.
- [43] P.K. Singh, Y. Wen, B.J. Swanson, K. Shanmugam, A. Kazlauskas, R.L. Cerny, S.J. Gendler, M.A. Hollingsworth, Platelet-derived growth factor receptor beta-mediated phosphorylation of MUC1 enhances invasiveness in pancreatic adenocarcinoma cells, *Cancer Res.* 67 (2007) 5201-5210.
- [44] P.K. Singh, M.E. Behrens, J.P. Eggers, R.L. Cerny, J.M. Bailey, K. Shanmugam, S.J. Gendler, E.P. Bennett, M.A. Hollingsworth, Phosphorylation of MUC1 by Met modulates interaction with p53 and MMP1 expression, *J. Biol. Chem.* 283 (2008) 26985-26995.
- [45] Y. Wang, O. Roche, C. Xu, E.H. Moriyama, P. Heir, J. Chung, F.C. Roos, Y. Chen, G. Finak, M. Milosevic, B.C. Wilson, B.T. Teh, M. Park, M.S. Irwin, M. Ohh, Hypoxia promotes ligand-independent EGF receptor signaling via hypoxia-inducible factor-mediated upregulation of caveolin-1, *Proc. Natl. Acad. Sci. USA* 109 (2012) 4892-4897.
- [46] H. Dazzi, P.S. Hasleton, N. Thatcher, S. Wilkes, R. Swindell, A.K. Chatterjee,

Malignant pleural mesothelioma and epidermal growth factor receptor (EGF-R). Relationship of EGF-R with histology and survival using fixed paraffin embedded tissue and the F4, monoclonal antibody, Br. J. Cancer 61 (1990) 924-926.

[47] A. Destro, G.L. Ceresoli, M. Falleni, P.A. Zucali, E. Morengi, P. Bianchi, C. Pellegrini, N. Cordani, V. Vaira, M. Alloisio, A. Rizzi, S. Bosari, M. Roncalli, EGFR overexpression in malignant pleural mesothelioma. An immunohistochemical and molecular study with clinico-pathological correlations, Lung Cancer 51 (2006) 207-215.

[48] N.V. Chaika, T. Gebregiworgis, M.E. Lewallen, Purohit V, P. Radhakrishnan, X. Liu, B. Zhang, K. Mehla, R.B. Brown, T. Caffrey, F. Yu, K.R. Johnson, R. Powers, M.A. Hollingsworth, P.K. Singh, MUC1 mucin stabilizes and activates hypoxia-inducible factor 1 alpha to regulate metabolism in pancreatic cancer, Proc. Natl. Acad. Sci. USA. 109 (2012) 13787-13792.

### **Figure legends**

Figure 1. HIF pathway activation in MPM cells under hypoxia. The cell were cultured for 24 h under normoxia or hypoxia. (A) Equal amounts of whole cell lysates (20  $\mu$ g) of 6 MPM cell lines were separated by SDS-PAGE and immunoblotted by anti-HIF-1 $\alpha$  and -HIF-2 $\alpha$  antibodies.  $\beta$ -actin was used as internal control. N, normoxia; H, hypoxia. (B) Elevation of mRNA levels of HIF-inducible genes in MPM cells exposed

to hypoxia.  $\beta$ -actin was used as internal control. N, normoxia; H, hypoxia. (C) HRE-dependent transcriptional activity of MPM cells under hypoxia. MPM cells were transiently co-transfected with pGL3-5xHRE reporter vector (or control reporter vector, pGL3-promoter) and internal control vector (pRL-CMV). Values from reporter luciferase were normalized to internal control *Renilla* luciferase. Each column and bar represents the mean  $\pm$  SD of quadruplicate samples. Open column, normoxia; solid column, hypoxia. \*  $P < 0.01$  compared to control reporter.

Figure 2. Enhancement of *in vitro* cell motility and invasiveness of MPM cells under hypoxia. (A) Motility of MPM cells under hypoxia or normoxia, evaluated by phagokinetic track assay. The phagokinetic tracks (areas cleared of gold particles) by randomly selected 20 cells were measured after 24 h incubation. The distribution of the track areas is summarized by using boxplots. The central box in each plot shows the interquartile (25th to 75th percentile) range. The line in the box shows the median. The whiskers (vertical bars) were drawn to the 90th and 10th percentiles. Extreme values higher than the 90th percentile or lower than the 10th percentile were marked with circles individually. Open box, normoxia; solid box, hypoxia. \*  $P < 0.01$  compared to values under normoxia.

(B) Invasiveness of MPM cells under hypoxia or normoxia. Invasion assay was performed by using Transwell chambers with membranes embedded in type I collagen gel. Invasiveness was evaluated from the number of cells invaded per Transwell chamber 24 h after the cell seeding into the chamber. Each column and bar represents



the mean  $\pm$  SD of quadruplicate samples. Open column, normoxia; solid column, hypoxia. \* $P$ <0.01 compared to value under normoxia.

Figure 3. Subsiding of hypoxia-enhanced motility and invasiveness by knockdown of HIF-1 $\alpha$ . The cells were cultured for 24 h under normoxia or hypoxia. (A) RT-PCR analysis of expressions of HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-1 $\beta$  mRNA in TCC-MESO-1 and NCI-H226 cells treated with siRNAs (siHIF-1 $\alpha$  and/or siHIF-2 $\alpha$ ). Neg. Cont., siRNA as negative control; N, normoxia; H, hypoxia. (B) Quantitative RT-PCR analysis of expressions of HIF-1 $\alpha$  and HIF-2 $\alpha$  mRNA in TCC-MESO-1 and NCI-H226 cells treated with siRNAs (siHIF-1 $\alpha$  and/or siHIF-2 $\alpha$ ). Results were normalized relative to the non-treatment control. Neg. Cont., siRNA as negative control; Open column, normoxia; solid column, hypoxia. (C) Western blot analysis of expressions of HIF-1 $\alpha$  and HIF-2 $\alpha$  protein in TCC-MESO-1 and NCI-H226 cells treated with siRNAs (siHIF-1 $\alpha$  and/or siHIF-2 $\alpha$ ). Neg. Cont., siRNA as negative control; N, normoxia; H, hypoxia. (D) Motility of MPM cells under normoxia or hypoxia when treated with siRNAs. Data represents in the same manner as in Figure 2A. Open box, normoxia; solid box, hypoxia. \* $P$ <0.01 compared to value under normoxia. (E) Invasiveness of MPM cells under normoxia or hypoxia when treated with siRNAs. Open column, normoxia; solid column, hypoxia. \* $P$ <0.01 compared to value under normoxia.

Figure 4. Expression patterns of the hypoxia-inducible genes which are involved in cell motility or invasion, and their related genes. Twenty four hours after the cell culture under normoxia or hypoxia, RNA extraction was performed. Expression levels were

analyzed by RT-PCR using  $\beta$ -actin as internal control. N, normoxia; H, hypoxia.

Figure 5. MUC1 expression of MPM cells under hypoxia or normoxia. The cells were cultured for 24 h under normoxia or hypoxia. (A) Expression of MUC1 mRNA in TCC-MESO-1 and NCI-H226 cells treated with siHIF-1 $\alpha$  and/or siHIF-2 $\alpha$ . (B, C) Luciferase reporter assay. Deletion analysis to identify HRE in the promoter of MUC1 gene under hypoxia (B). Mutagenesis of the putative HBSs located at positions -1130/-1126 and -1072/-1068 on MUC1 promoter activity (C). Left panel: Constructs of different lengths of the 5' flanking parts of MUC1 promoter. Black and gray arrows indicate the putative HBSs (5'-(A/G)CGTG-3') on sense and antisense strand, respectively. X on the arrow indicates mutated HBS ((A/G)CGTG-to-TTAAT substitution). Right panel: NCI-H226 and HMM-3 cells were transiently co-transfected with each reporter vector (or control reporter vector, pGL3basic) and internal control vector (pRL-CMV). Values from reporter luciferase were normalized to internal control *Renilla* luciferase. Each column with bar represents the mean  $\pm$  SD of quadruplicate samples. Open column, normoxia; solid column, hypoxia. \* $P$ <0.01 compared to values under normoxia. # $P$ <0.01 compared promoter activity of reporter with or without one mutated HBS. (D) Flow cytometric analysis of MUC1 expression on cell surfaces of TCC-MESO-1 and NCI-H226 cells. The cells were incubated with monoclonal antibodies to sialylated MUC1 (MY.1E12) and then reacted with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG. Fluorescence intensity was analyzed by using FACSCanto. Abscissa and ordinate indicate fluorescence

intensity and cell number, respectively. N, normoxia; H, hypoxia; Control, the cells treated with FITC-conjugated anti-mouse IgG alone.

Figure 6. Subsiding of hypoxia-enhanced motility and invasiveness by knockdown of MUC1. The cells were cultured for 24 h under normoxia or hypoxia. (A) Expression of HIF-1 $\alpha$  and MUC1 mRNA in TCC-MESO-1 and NCI-H226 cells treated with siRNAs (siHIF-1 $\alpha$  or siMUC1). Neg. Cont., siRNA as negative control; N, normoxia; H, hypoxia. (B) Expression of MUC1 protein in TCC-MESO-1 and NCI-H226 cells treated with siRNAs (siHIF-1 $\alpha$  or siMUC1). Sialylated and hypoglycosylated MUC1 was detected with MY.1E12 and VU4H5 monoclonal antibody, respectively. Neg. Cont., siRNA as negative control; N, normoxia; H, hypoxia. Arrows indicate protein markers (180 kDa). (C) Motility of MPM cells under normoxia or hypoxia when treated with siRNAs. Data represents in the same manner as in Figure 2A. Open box, normoxia; solid box, hypoxia. \* $P$ <0.01 compared to value under normoxia. # $P$ <0.01 compared to value in siHIF-1 $\alpha$ -treated cells. (D) Invasiveness of MPM cells under normoxia or hypoxia when treated with siRNAs. Open column, normoxia; solid column, hypoxia. \* $P$ <0.01 compared to value under normoxia. # $P$ <0.01 compared to value in siHIF-1 $\alpha$ -treated cells.

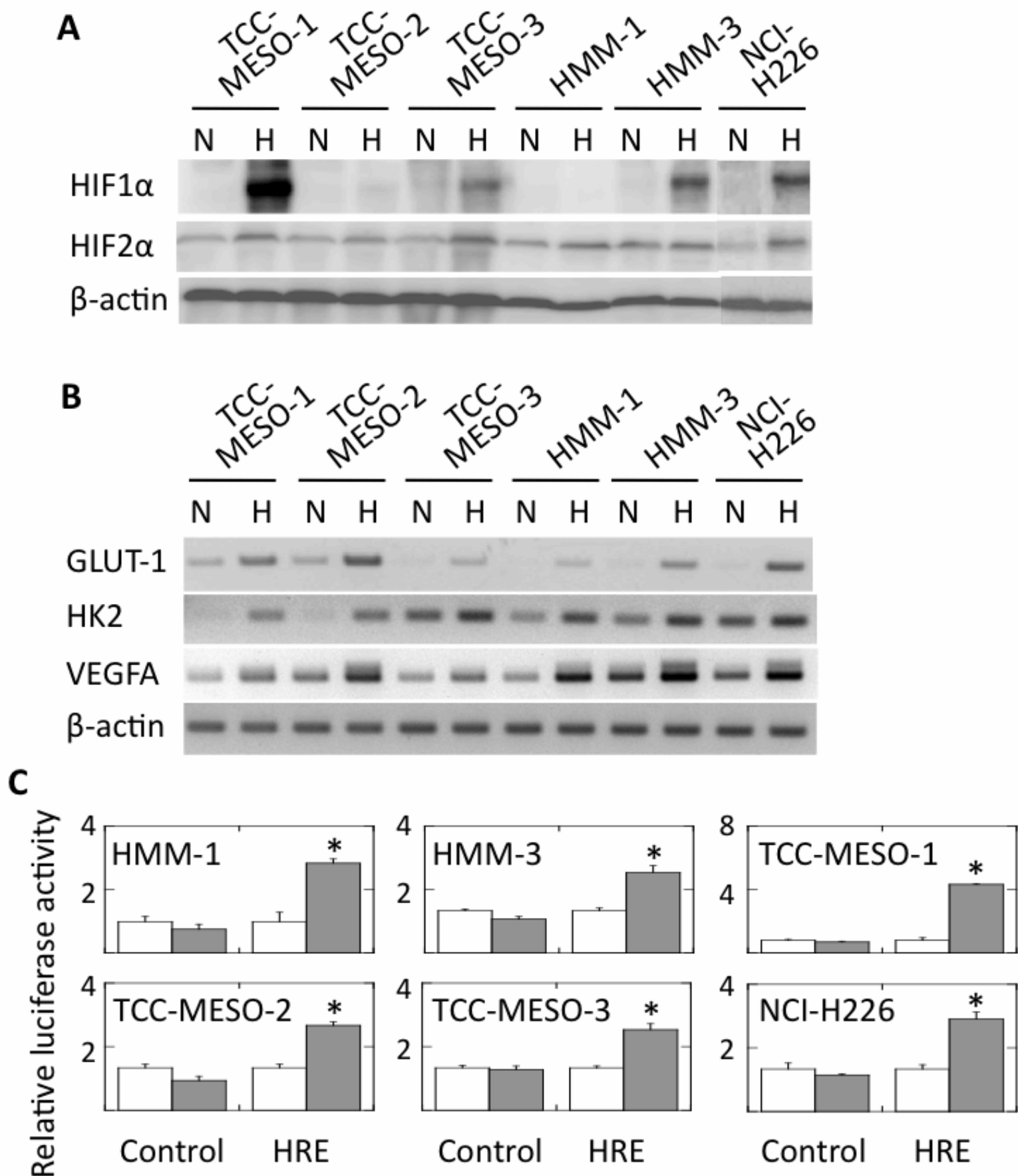


Figure 1 Goudarzi et al.

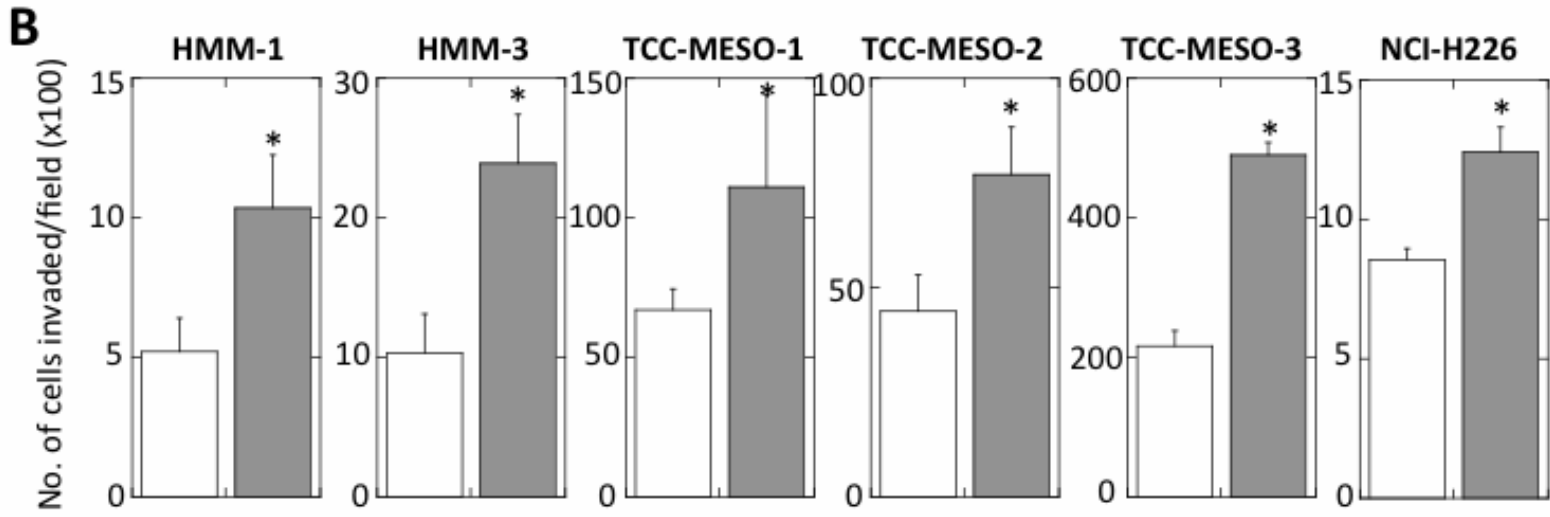
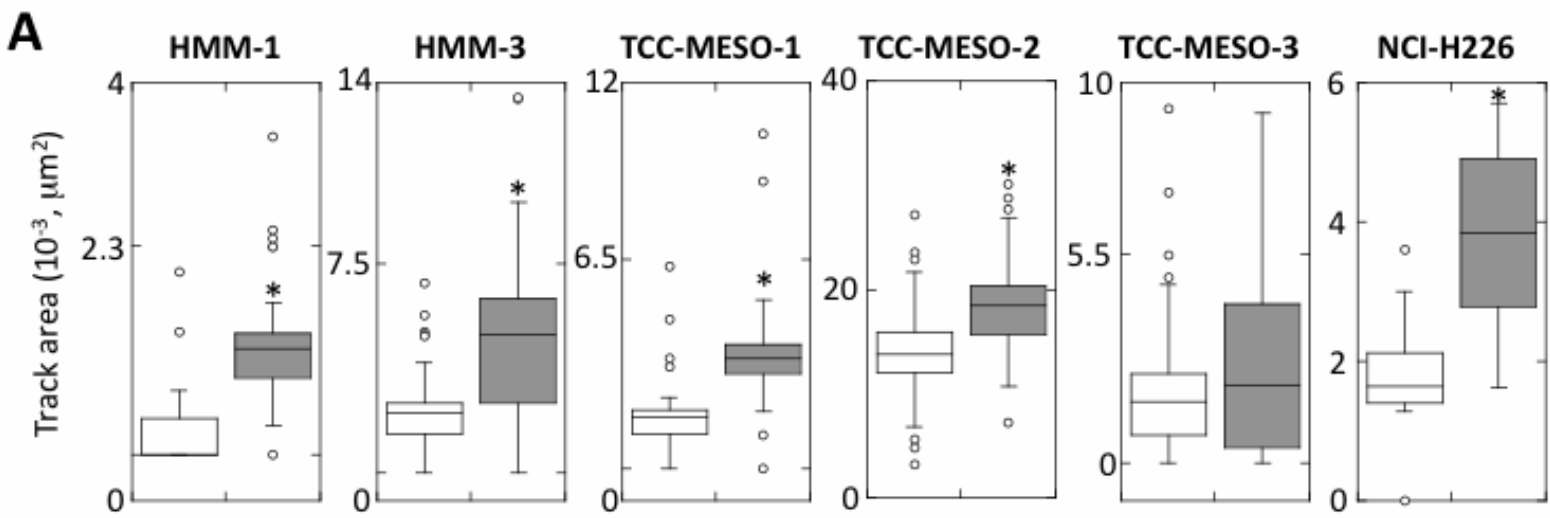


Figure 2 Goudarzi et al.

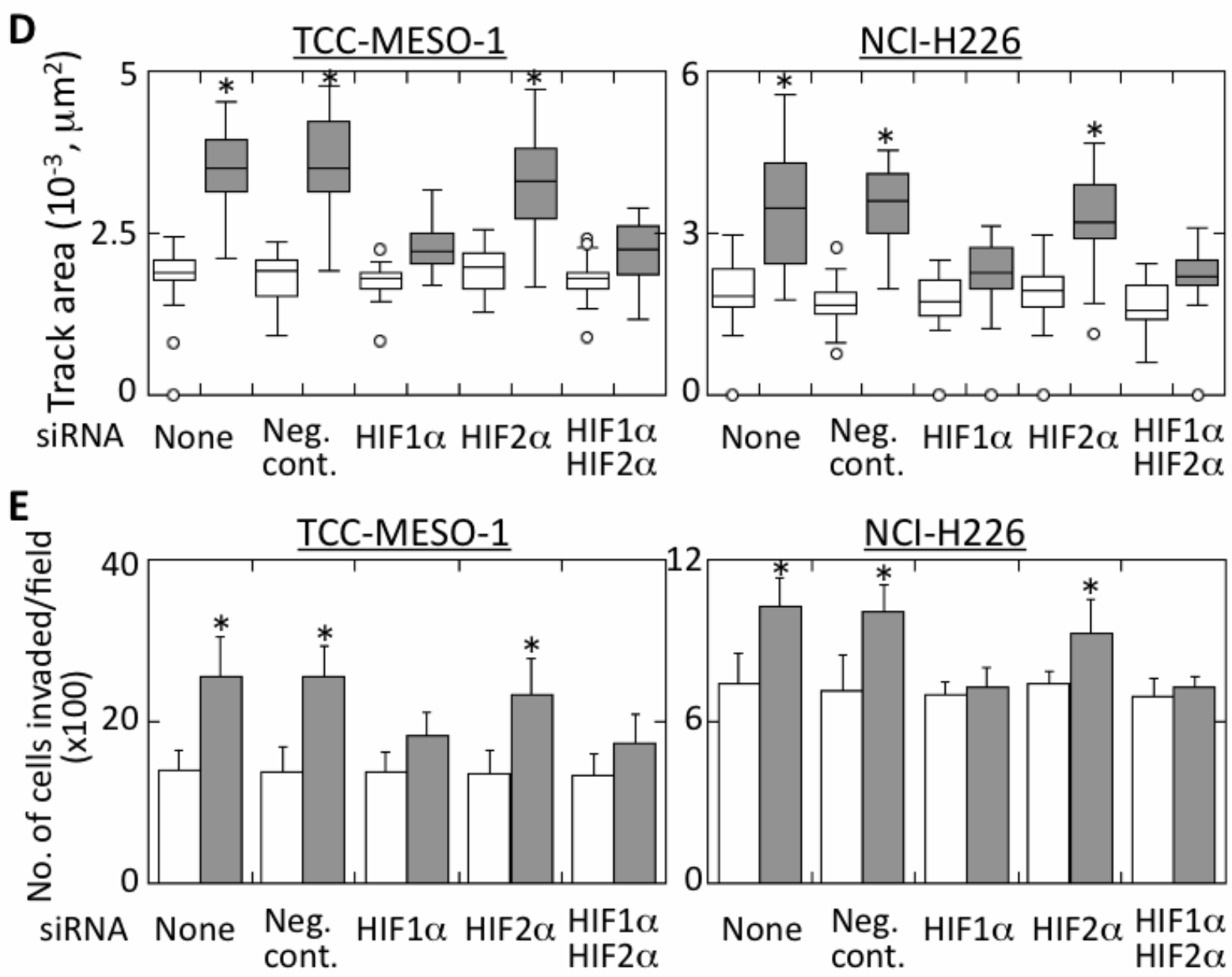


Figure 3 Goudarzi et al.

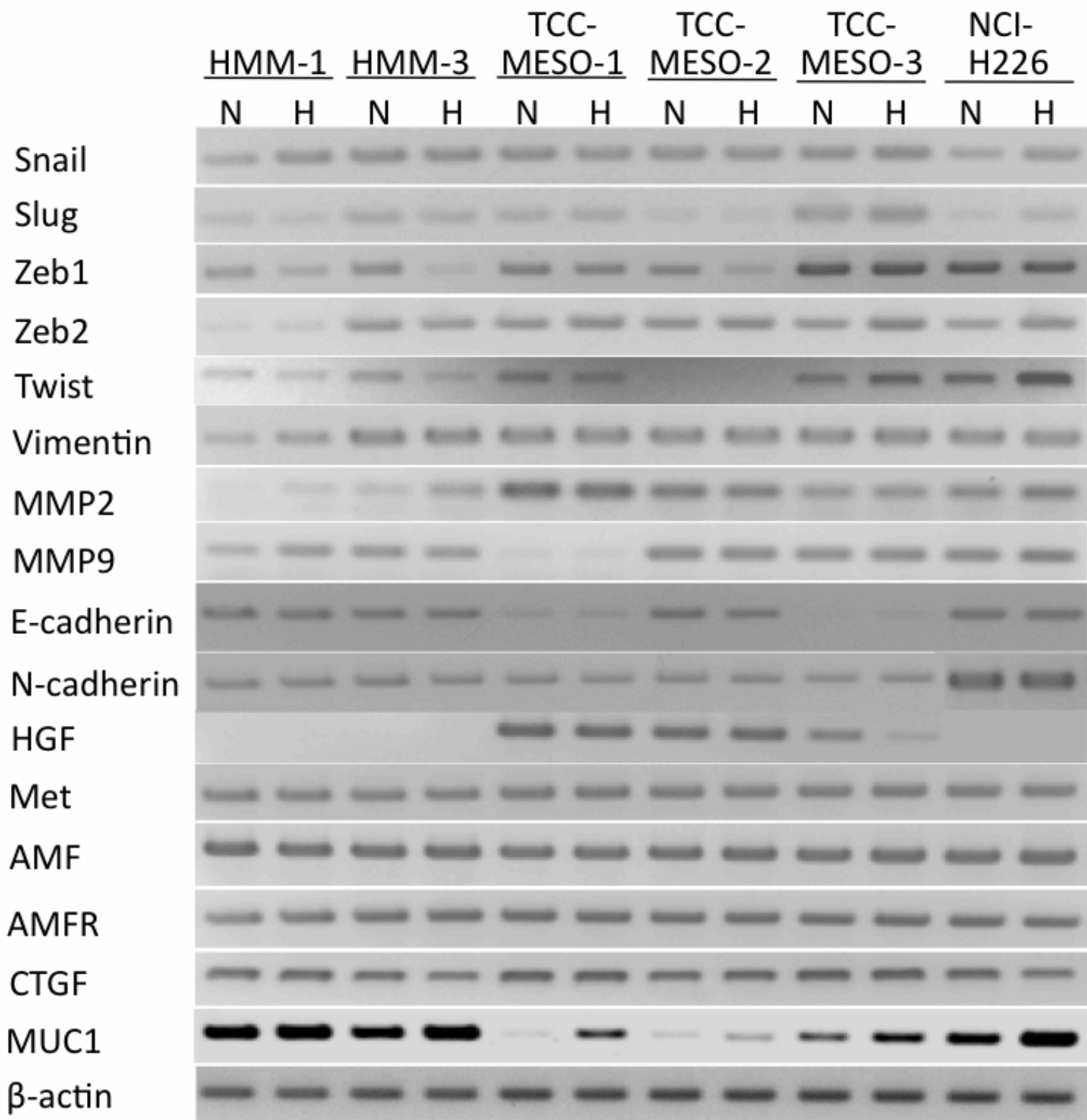


Figure 4 Goudarzi et al.

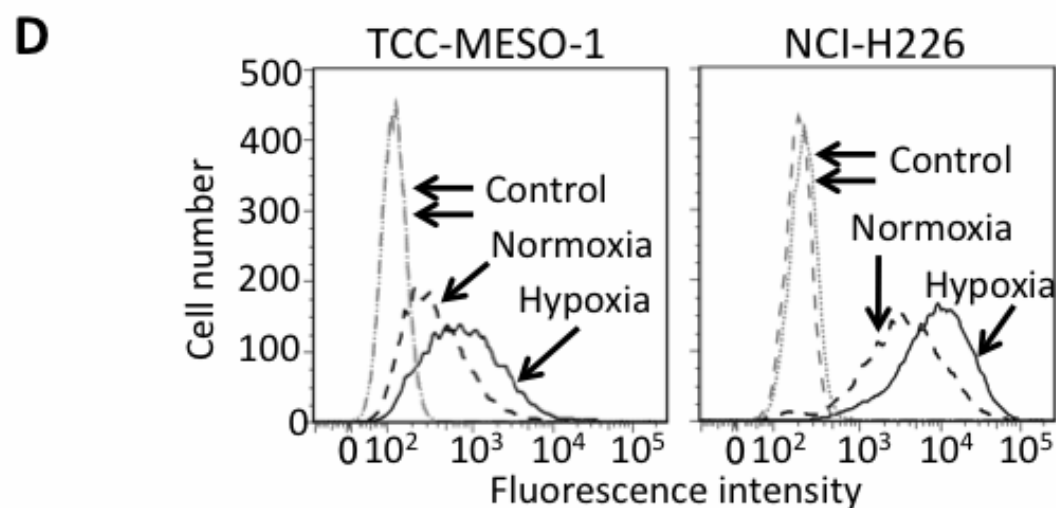
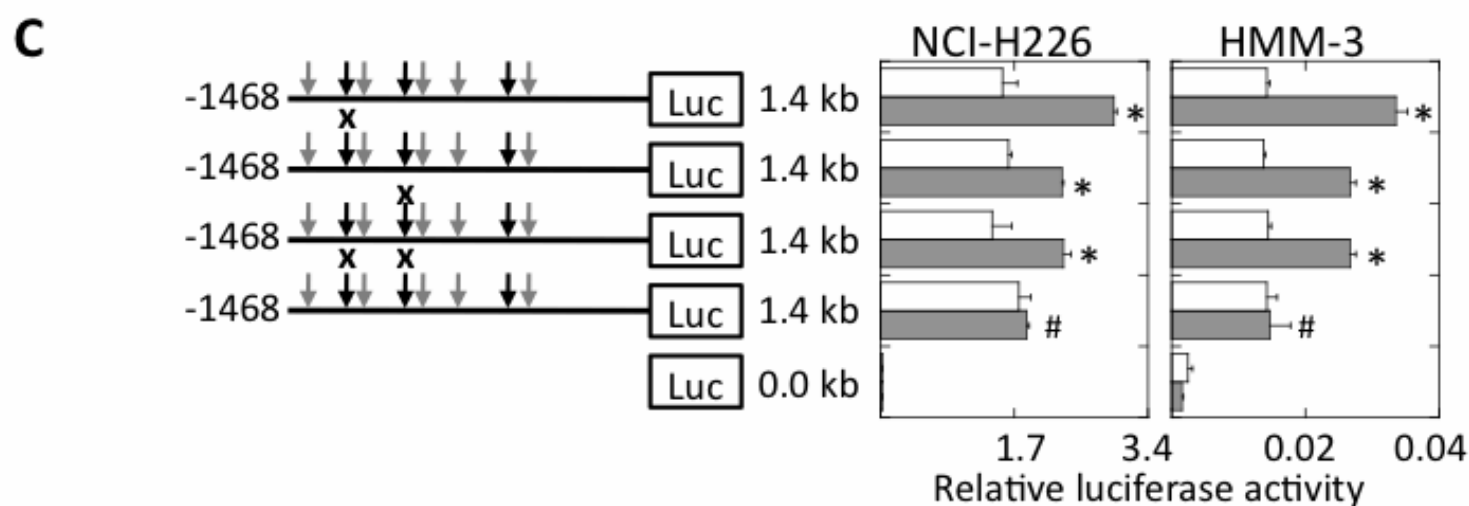
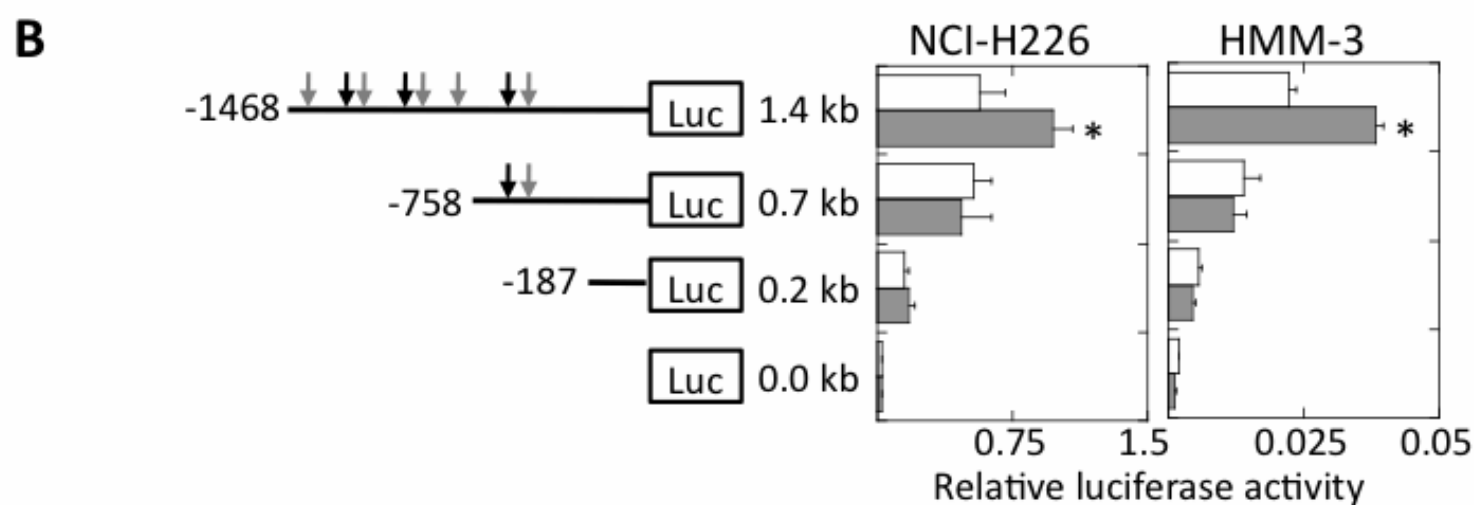
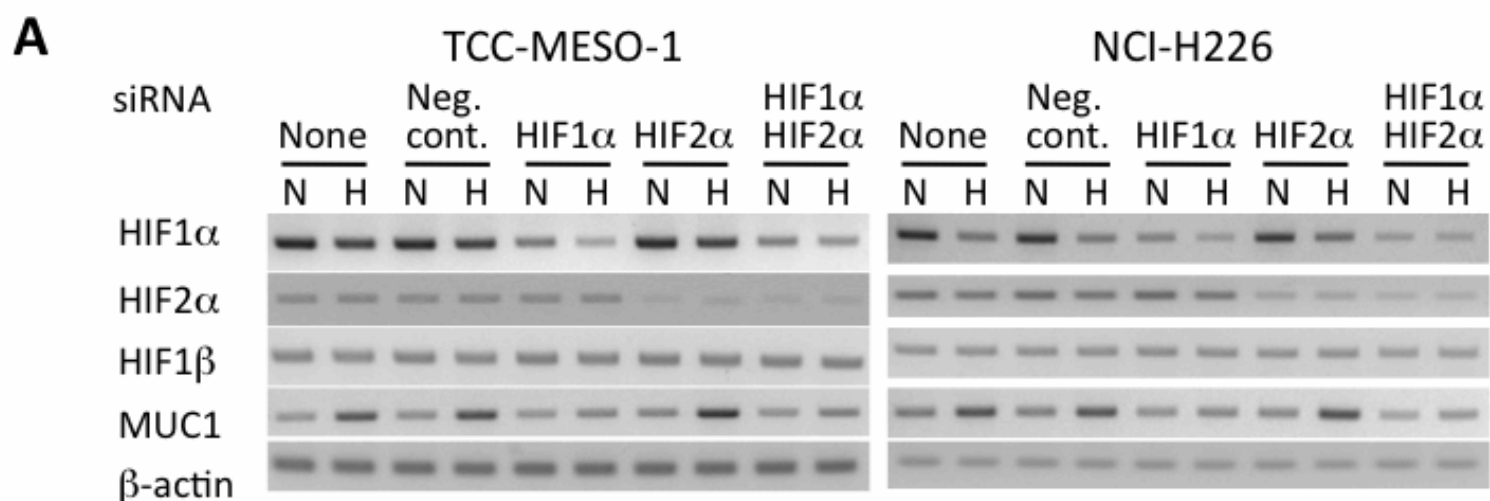


Figure 5 Goudarzi et al.



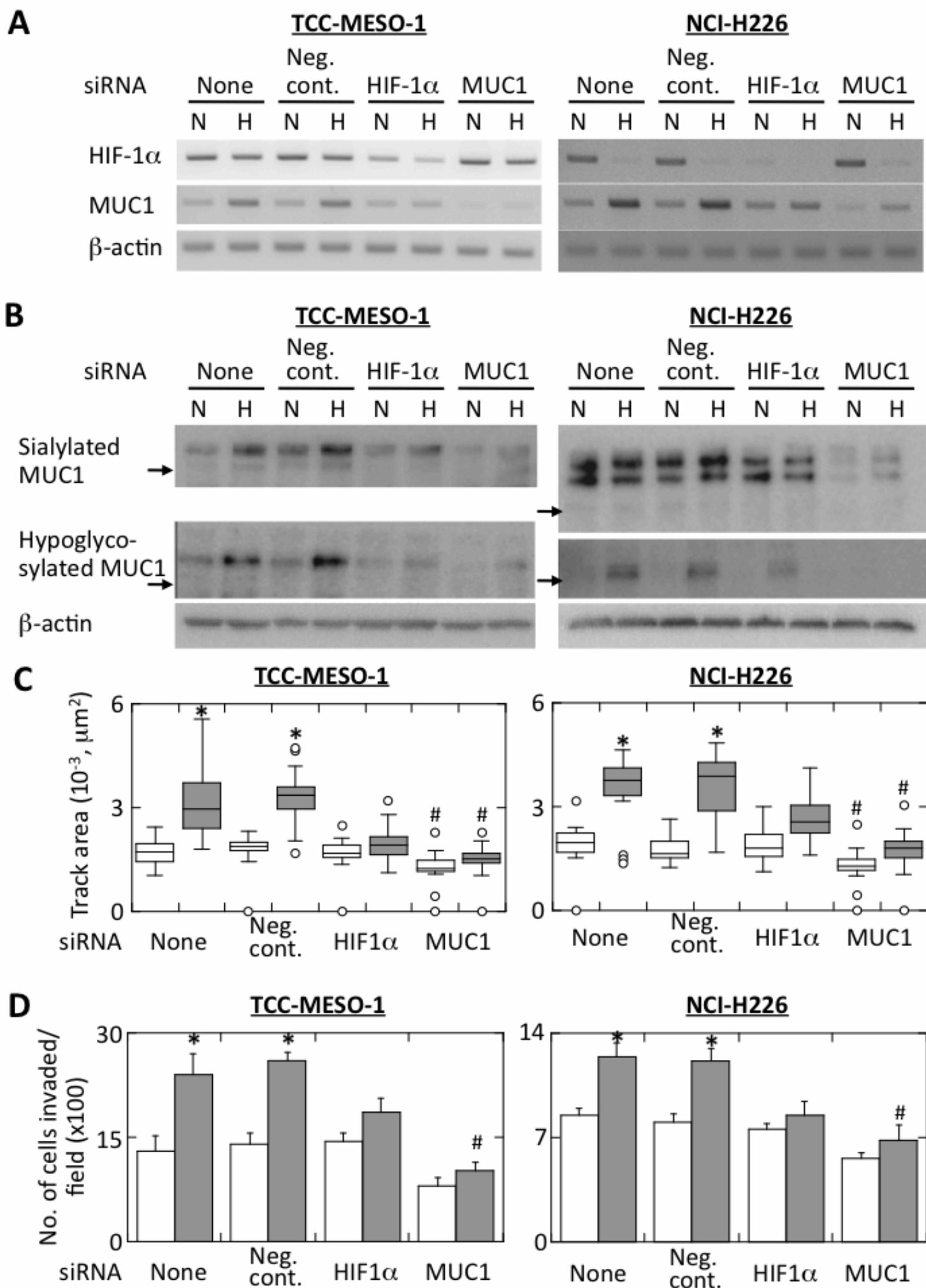


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Table 1. Primer sets for RT-PCR analysis

Gene names	Forward primers (5'-3')	Reverse primers (5'-3')
<i>SNAI1 (Snail)</i>	GCCTTCAACTGCAAATACTGC	CCTGGCACTGGTACTTCTTGA
<i>VIM (Vimentin)</i>	GCAGGCTCAGAITCAGGAAC	GCTTCAACGGCAAAGTTCTC
<i>SNAI2 (Slug)</i>	CCTGGTCAAGAAGCATTTCAA	GCCCCAAAGATGAGGAGTATC
<i>ZEB1 (Zeb1)</i>	CAGCCCTGCAGTCCAAGAAC	CCTGCCCTTCCCTGCTCTGT
<i>ZEB2 (Zeb2)</i>	AGCAAAGGAGAAAGTACCAGCG	GAGTGAAGCCTTGAGTGCTCG
<i>TWIST1 (Twist)</i>	AGCTGAGCAAGATTCAGACCCCTC	CCCCTCTGGGAATCACTGTC
<i>MMP9</i>	CACGACGCTCTCCAGTACCAGAGA	CATAGGTCACCGTAGCCCACTTGG
<i>MMP2</i>	GTTCAATTGGCGGACTGTGACG	ATTCATTCCCTGCAAAGAACACAGCC
<i>CDH1 (E-cadherin)</i>	AACGCCCCCATACCAGAACC	AACAGCAAGAGCAGCAGAATCAGA
<i>CDH2 (N-cadherin)</i>	GAATGGATGAAAAGACCCCATCC	AGCTTCTGAATGCTTTTTGGG
<i>HIF1A (HIF1<math>\alpha</math>)</i>	GTGTACCCTAACTAAGCCGAG	GCACTGTGTTGAGAAATTCCTTGG
<i>EPAS1 (HIF2<math>\alpha</math>)</i>	AGGTGAAAGTCTACAACAACACTGCC	CTCGGAGTCTAGCGCATGGT
<i>ARNT (HIF1<math>\beta</math>)</i>	TACGCATGGCAGTTTCTCAC	TGGGTGCACCTGATCATAGA
<i>VEGFA</i>	ACCCTGGTGACATCTTCCA	CTTGCAACGCCGAGTCTGTGT
<i>SLC2A1 (GLUT-1)</i>	GGCTCCGGTATCGTCAACA	CAGCTCCTCGGGTGTCTTG
<i>MUC1</i>	ACCTACCATCCTATGAGCGA	ACAACACTTCCCAAGGCATGA
<i>HGF</i>	CTCCTGCTCCCCATCGCCAT	TGTTCCCTTGTAGCTGCGTCT
<i>GPI (AMF)</i>	CGTGTGACCACCAGACAGG	GCTTTCCCAAGCTCCACTCCC
<i>CTGF</i>	CGAGCCCAAAGGACCAAACCG	TTGAACTCCAACCGGCAAGGT
<i>MET</i>	AGCTTTGCAGCGGTTGAC	CCATGTCCCGCTCAGGCAT
<i>AMFR</i>	ACCTCCAGCTGACACGCTCA	GGCAGCCAGCATCTTCGAC
<i>ACTB (<math>\beta</math>-actin)</i>	TTGCCGACAGGATGCAGAA	GCCGATCCACACGGAGTACT