HIF-1 is induced via EGFR activation and mediates resistance to anoikis-like cell death under lipid rafts/caveolae-disrupting stress

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The plasma membrane microdomains, lipid rafts, are involved in regulation of cellular functions such as cell survival and adhesion. Cholesterol is a critical component of lipid rafts in terms of their integrity and functions and rafts disruption by cholesterol depletion can induce detachment-induced cell death. Hypoxia inducible factor-1 (HIF-1α) is stabilized in hypoxia and transactivates numerous genes required for cellular adaptation to hypoxia. It is also induced by non-hypoxic stimuli and contributes to cell survival. Because hypoxia inhibits cholesterol synthesis and HIF-1α plays a role in this process, we here explored a possible connection between lipid rafts and HIF-1α. We investigated whether HIF-1α is regulated during cholesterol depletion/rafts disruption in A431 cells in normoxic conditions. Methyl-beta cyclodextrin (MβCD), which induces cholesterol depletion, upregulated HIF-1α even under normoxic conditions and this upregulation required epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase 1 and 2 activation, but not Akt activation. MβCD treatment induced HIF-1α upregulation at both the transcriptional and translational levels but not at the post-translational levels. In addition, MβCD robustly induced vascular endothelial growth factor production and stimulated an hypoxia response element-driven luciferase reporter activity under normoxic conditions, indicating that MβCD-induced HIF-1α is functionally activated. Both EGFR activity and HIF-1α expression were higher in the attached cells than in the detached cells after MβCD treatment. Furthermore, inhibition of HIF-1α by RNA interference accelerated cell detachment, thus increasing cell death, indicating that HIF-1α expression attenuates MβCD-induced anoikis-like cell death. These data suggest that, depending on cholesterol levels, lipid rafts or membrane fluidity are probably to regulate HIF-1α expression in normoxia by modulating rafts protein activities such as EGFR, and this connection between lipid rafts and HIF-1α regulation may provide cell survival under membrane-disturbing stress.

Introduction

Lipid rafts/caveolae are distinct cholesterol and sphingolipid-rich microdomains of the plasma membrane. Caveolae are a subset of rafts that are characterized by flask-like invaginations in cells expressing caveolin-1 (1–3). Rafts are enriched in proteins involved in transmembrane signal transduction in various cell types (3). Many receptors, including epidermal growth factor receptor (EGFR), Fas and the integrins are present in rafts, thereby rendering these membrane structures pivotal in the regulation of cell proliferation, death, adhesion and motility (3). Cholesterol is a critical lipid component for rafts integrity, and disruption of rafts by cholesterol depletion impairs a number of signal transduction pathways and results in deregulation of biological functions (1, 2). For example, we have demonstrated recently that cholesterol depletion induced a decrease in cell surface lipid rafts levels and cell detachment-induced cell death, indicating that the integrity and/or levels of rafts on the cell surface is essential for cell adhesion and survival (4).

Increasing evidence emphasizes that cholesterol levels are associated with tumor incidence and malignancy. A hospital-based case-control study has revealed that high levels of serum cholesterol correlate with the increased risk of prostate cancer (5). Interestingly, cholesterol and other fatty acid deposits accumulate in solid tumors and increased cholesterol levels have been associated with elevated levels of rafts in the plasma membrane (4, 6). In cancer cells, cholesterol accumulation might expand lipid rafts in which signals are processed that are essential to cell proliferation and cell survival (6).

The cellular cholesterol level is tightly regulated either through supply via endocytosis of low-density lipoprotein or de novo synthesis of cholesterol. The enzyme, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, is the rate-limiting enzyme of cholesterol synthesis. Synthesis of one molecule of cholesterol from one molecule of acetyl-CoA requires 11 molecules of O2, indicating that this process is O2-consuming (7). Therefore, the active cholesterol synthesis could aggravate hypoxic injury due to further depletion of O2. Indeed, a recent report revealed that hypoxia inhibits cholesterol synthesis and that the condition leads to downregulation of HMG-CoA reductase through hypoxia inducible factor-1α (HIF-1α)-mediated Insig-1 and Insig-2 induction. Therefore, there seems to be a connection between cholesterol synthesis and HIF-1α-mediated hypoxic responses (8).

HIF-1α is a transcription factor that mediates adaptive responses to hypoxia. It enhances O2 delivery and promotes cell survival under a low oxygen environment by upregulating numerous genes. In a view of tumor, HIF-1α acts as a tumor-promoting factor because it provides angiogenesis, metastasis and resistance against chemotherapy and radiotherapy (9). HIF-1α is a heterodimer consisting of a hypoxia-inducible α subunit and a constitutively expressed β subunit (10). Of these, HIF-1α functions as the prime transcription factor responsible for gene expression and HIF-1β helps HIF-1α bind to DNA. HIF-1α is upregulated in the posttranslational level through a decrease in protein degradation in response to hypoxia (O2-dependent mechanism). HIF-1α is also induced in the translational level, namely, by upregulation of its synthesis in response to growth factor stimulations and subsequent activations of phosphoinositols-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) or RAF/mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) pathways (O2-independent mechanism) (10). Of several HIF-1α stimuli, EGFR activation in prostate and breast cancers is regarded as a critical process that stimulates HIF-1α protein synthesis and thus promotes cell proliferation and resistance to apoptosis (11).

Especially, the non-hypoxic HIF-1α expression is an important mechanism underlying HIF-1α overexpression and hypervascularity found in some tumors (12). Therefore, therapeutic agents targeting HIF-1α showed marked effect on tumor growth (10).

Abbreviations: CHX, cycloheximide; EGFR, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HIF-1, Hypoxia inducible factor-1; HMG, 3-hydroxy-3-methylglutaryl; MEK, mitogen-activated protein kinase kinase; mRNA, messenger RNA; MβCD, methyl-beta cyclodextrin; MTS, 3-(4,5-dimethylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium; NF-κB, nuclear factor-kappaB; PI3K, phosphoinositols-3-kinase; SDS, sodium dodecyl; shHIF-1α, HIF-1α-specific shRNA; shRNA, small hairpin RNA; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor.

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Overexpressions of EGFR and HIF-1α have been associated with tumor malignancy and poor prognosis (12,13). Signaling events leading to cell transformation and cancer progression are known to be dependent on lipid rafts/caveolae (3). In addition, cholesterol levels have been linked to lipid rafts/caveolae levels on the cell surface (4,14). Previously, we have demonstrated that rafts disruption by cholesterol depletion induced anoikis-like cell death via focal adhesion kinase downregulation and rafts internalization and also that EGFR was activated during cholesterol depletion (4,15). Given that HIF-1α is expressed via EGFR activation and cholesterol synthesis is regulated by HIF-1α, we could hypothesize that rafts disruption influence HIF-1α expression and activity under normoxic conditions. Thus, we examined the effects of cholesterol depletion on HIF-1α and vascular endothelial growth factor (VEGF) expression and investigated the cellular signaling pathway leading to rafts disruption-induced HIF-1α upregulation. We also demonstrated the effect of HIF-1α induction on anoikis-like cell death induced by rafts disruption.

Materials and methods

Cell culture

Human epidermoid carcinoma cell lines, A431 and Hela, human lung carcinoma cell line A549 were purchased from the American Type Culture Collection (Rockville, MD). Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), and antibiotic-antimycotic (1×) were purchased from Gibco Laboratories (Grand Island, NY). A431 and Hela cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% FBS and antibiotic-antimycotic (1×). A549 cells were cultured in RPMI 1640 with 1-glutamine and antibiotic-antimycotic (100×) were purchased from Gibco Laboratories (Grand Island, NY). Human epidermoid carcinoma cell lines A431 and Hela were used in the study from the American Type Culture Collection (Rockville, MD). HIF-1α was determined on 293FT cells as transducing units using serial dilution with an envelope coding plasmid (VSVG) and vector constructs. Lentivirus was constructed in the pL-UGIP (17). The oligonucleotide sequences that generate double-stranded shRNAs for HIF-1α were adopted as described (18). Lentiviruses were produced as described previously (17). In brief, the lentiviral genome containing plasmid (pL-UGIP) was transfected into 293FT cells together with an envelope coding plasmid (pSVSVG) and vector constructs. Lentivirus was harvested 40 h after infection and concentrated by ultracentrifugation. Titers were determined on 293FT cells as transducing units using serial dilutions of vector stocks. When necessary, drug selections of cells were done with 1 μg/ml puromycin after a 24 h recovery in standard growth medium.

Reverse transcription–polymerase chain reaction

Total cellular RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Complementary DNA fragments were amplified with the following primer pairs: HIF-1α (anti-sense), 5’-ATGCAGCCA-GATCTCGGGCGACAA-3’ (sense), 5’-CTGGTCTGGCTATGCCTAACACTG-3’ (anti-sense); VEGF—5’-GTATGGCCACCTCTTGTCTT-3’ (sense), 5’-GAGT-GATGGCTCCCGTTT-3’ (anti-sense) and BNIP3—5’-GCTCCTGGGTA-GAACGTGAC-3’ (sense), 5’-TTCTCATTGAGCCTGCTGTTC-3’ (anti-sense). Polymerase chain reactions were performed in Unit Block Assembly for PTC DNA Engine system (Alpha2) for 28 cycles: 95°C for 30 s, 54°C for 30 s and 72°C for 1 min. Products were analyzed on a 1% agarose gel.

Luciferase assays

Serum-starved Hep3B cells were treated with MCreD and lysed in a lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N’,N’-tetracetic acid, 10% glycerol and 1% Triton X-100) and cells were scraped from the dishes. The supernatant was collected after centrifugation at 13 000 r.p.m. for 1 min. Luciferase activity was assayed using Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. Luciferase activity was measured using VICTOR Light Luminescence counter (Perkin Elmer, Shelton, CT) and the data were normal-
depletion using MβCD and analyzed HIF-1α levels. HIF-1α protein was noticeably upregulated under normoxic conditions by MβCD treatment in dose- and time-dependent manners (Figure 1A and B). Cholesterol supplementation after MβCD treatment abolished the HIF-1α induction, which supports that such the MβCD action is attributable to cholesterol depletion (Figure 1B). Given that EGFR activation mediates O2-independent HIF-1α expression (11) and that cholesterol depletion activates the EGFR through ligand-independent receptor dimerization (14), we investigated whether EGFR activation is involved in the MβCD-induced HIF-1α expression. Cholesterol depletion activated EGFR, as previously reported, and it also stimulated the mitogen-activated protein kinase pathways including extracellular signal-regulated kinase, p38 and JNK and Src, but not Akt (Figure 1C). Epidermal growth factor (EGF) treatment also increased HIF-1α along with activations of various signaling molecules including Akt (Figure 1D). To further elucidate the mechanism of HIF-1α induction, cells were cotreated with MβCD and various kinase inhibitors. EGFR inhibition using two different EGFR inhibitors abolished the MβCD-induced HIF-1α expression (Figure 2A and B). Previously, it was reported that extracellular signal-regulated kinase 1 and 2 is activated through EGFR in MβCD-treated cells (14) and that HIF-1α can be induced by the EGFR-Akt-mTOR pathway (12). Thus, we investigated whether these pathways are responsible for HIF-1α induction by rafts disruption. As a result, a MEK inhibitor could block HIF-1α induction in MβCD-treated cells, whereas a PI3K inhibitor failed (Figure 2C). In addition, Src and p38 inhibitors negligibly affected the MβCD-induced HIF-1α expression (Figure 2D). In contrast, when HIF-1α was induced by EGF treatment, Akt and p38 inhibitors somehow reduced HIF-1α expression (supplementary Figure S1 is available at Carcinogenesis Online).

Proposed mechanism underlying MβCD-induced HIF-1α expression

HIF-1α is regulated by oxygen-dependent posttranslational modifications such as prolyl hydroxylation. Under normoxic conditions, HIF-1α is hydroxylated, allowing binding to von Hippel-Lindau protein and the E3 ligase complex that leads to HIF-1α degradation by the ubiquitin/proteosomal pathway. Therefore, HIF-1α is maintained at low levels in normoxia. Under hypoxic conditions, however, the HIF-1α hydroxylation is inhibited, and thereby leading to HIF-1α stabilization and accumulation (12). Although many studies demonstrated a central role of HIF-1α in mammalian oxygen homeostasis, a series of studies reported HIF-1α induction under non-hypoxic conditions (10). For example, HIF-1α is upregulated by activation of oncogenes such as EGFR, Akt and ERBB2 and by inactivation or loss of tumor suppressor genes such as VHL and PTEN (10). In addition, EGFR, stem cell factor and osteopontin can induce HIF-1α accumulation through the PI3K/Akt and Ras-mitogen-activated protein kinase pathways (13,19,20). The results in our study showed that rafts disruption results in HIF-1α induction in an EGFR-activation dependent manner. However, Akt activation is unlikely to contribute to the MβCD-induced HIF-1α expression because Akt is inactivated in MβCD-treated cells (4,21) (Figure 1C) and Akt inhibition failed to prevent the HIF-1α induction (Figure 2C).

To examine how HIF-1α is upregulated in MβCD-treated cells, we first examined the messenger RNA (mRNA) levels of HIF-1α and found that HIF-1α is induced at the transcriptional levels (Figure 3A). Recent reports showed that HIF-1α mRNA expression can be induced under both hypoxia and growth signaling conditions by signal transducer and activator of transcription 3 (STAT3) or nuclear factor-kappaB (NF-κB) (22,23). To test these possibilities, we examined whether MβCD-treatment could activate NF-κB and STAT3. We found that there were inhibitor-xB degradation and STAT3 phosphorylation, indicating that HIF-1α expression possibly causes activation of both NF-κB and STAT3 (Figure 3B). Furthermore, both a STAT3 inhibitor (Figure 3C) and a NF-κB inhibitor (Figure 3D) could decrease HIF-1α induction in MβCD-treated cells. These data indicate that activation of NF-κB and STAT3 could be important for HIF-1α induction. Next,

Fig. 1. Effects of rafts disruption on HIF-1α expression and signaling events. (A) Serum-starved A431 cells were treated with 0.5, 1, 5 and 10 mM MβCD for 3 h and cell lysates were subjected to immunoblotting using anti-HIF-1α antibodies, and anti-β-actin antibodies for protein loading control. The levels of HIF-1α were quantified by densitometry and normalized with β-actin. The ratio of HIF-1α to the total β-actin in the control cells was set as 1. (B) Serum-starved A431 cells were treated 5 mM MβCD for 0–4 h. Some MβCD-treated cells were incubated with 1 mM cholesterol for 1 or 2 h. The cell lysates were analyzed by immunoblotting using anti-HIF-1α antibodies and anti-β-actin antibodies. (C and D) Serum-starved A431 cells were treated with either 5 mM MβCD (C) or 30 nM EGF (D) for indicated times and cell lysates were analyzed by immunoblotting using the indicated antibodies. These experiments were performed three independent times with comparable results.
Fig. 2. HIF-1α accumulation through EGFR activation induced by MJCD. (A–D) Serum-starved A431 cells were pretreated with the indicated inhibitors for 30–60 min and then treated with 5 mM MJCD for 2–3 h and then cell lysates were subjected to immunoblotting analysis using the indicated antibodies [AG1478 and PD153035, EGFR inhibitors; U0126, an extracellular signal-regulated kinase (ERK) 1 and 2 inhibitor; LY294002, a PI3K inhibitor and SB203580, a p38 inhibitor]. Similar results were observed in two independent experiments.

Fig. 3. Association of Stat3 and NF-κB with HIF-1α accumulation and a posttranscriptional mechanism induced by MJCD. (A) Serum-starved A431 cells were treated with 5 mM MJCD for 3 h and total RNA was extracted and subjected to reverse transcription–polymerase chain reaction to assess HIF-1α mRNA levels. The levels of HIF-1α were quantified by densitometry and normalized with β-actin. The ratio of HIF-1α to the total β-actin in the control cells was set as 1. (B) Serum-starved A431 cells were treated with 5 mM MJCD for 0–5 h and cell lysates were subjected to immunoblotting analysis using anti-HIF-1α, anti-phospho-Stat3, anti-IKKβ and anti-β-actin antibodies. (C and D) Serum-starved A431 cells were pretreated with S3I-201 (Stat3 inhibitor) for 9 h or SN50 (NF-κB inhibitor) for 0.5 h and then treated with 5 mM MJCD for 3 h. Cell lysates were subjected to immunoblotting analysis using the indicated antibodies. The levels of HIF-1α were quantified by densitometry and normalized with β-actin. The ratio of HIF-1α to the total β-actin either in the MJCD-treated cells (C) or in the control cells (D) was set as 1. (E) Serum-starved A431 cells were treated with 5 mM MJCD for 3 h and CHX was added the last 60, 30 or 15 min before harvest, followed by immunoblotting analysis for HIF-1α. (F) Serum-starved A431 cells were pretreated with CHX for 30 min and CHX was removed, followed by 5 mM MJCD treatment without or with MG132 for 3 h. Cell lysates were analyzed by immunoblotting using anti-HIF-1α antibodies. The levels of HIF-1α were quantified by densitometry and normalized with β-actin. The ratio of HIF-1α to the total β-actin in the MJCD-treated cells was set as 1. Similar results were observed in two independent experiments.
to examine whether MβCD induces HIF-1α expression in the protein synthesis step, cells were pretreated with cycloheximide (CHX), a protein synthesis inhibitor or MG132, a proteosome inhibitor. CHX treatment rapidly diminished HIF-1α accumulation, indicating ongoing protein synthesis is important for HIF-1α induction in MβCD-treated cells (Figure 3E). We blocked protein synthesis by CHX pretreatment to eliminate basal levels of HIF-1α and permitted protein synthesis by removal of CHX, followed by MβCD treatment in the absence or presence of MG132. As shown in Figure 3F, HIF-1α levels are comparable in MβCD- and MG132-treated cells, respectively, but MβCD and MG132 co-treatment further enhanced HIF-1α accumulation. These data indicate that the HIF-1α accumulation is due to an increase in protein synthesis rather than protein stability. Taken all together, the MβCD-induced HIF-1α expression is regulated in both transcriptional and translational levels, as reported in constitutively active RacG12V expressing cells (22).

Rafts disruption functionally activates HIF-1α

HIF-1α is a heterodimeric transcription factor that transactivates HIF-1α target genes such as VEGF, glucose transporters and enzymes of glycolysis (12). Nuclear localization of HIF-1α protein is known to be necessary for its transcriptional activation of HIF-1α-regulated genes (9,12). Therefore, to examine whether HIF-1α upregulation correlated with its increased activity, we determined nuclear localization of HIF-1α by immunoblotting after cell fractionation and found nuclear translocation of HIF-1α after rafts disruption (Figure 4A). Lamin B and α-tubulin were analyzed to verify nuclear and cytosolic fractions, respectively. HIF-1α activation leads to an increased transcription of a number of different genes including VEGF in response to non-hypoxic stimuli as well as hypoxia (9). To test whether HIF-1α activation by rafts disruption results in an increase of VEGF mRNA, total cellular RNA extracts were processed for reverse transcription–polymerase chain reaction for VEGF mRNA. Interestingly, rafts disruption induced VEGF transcription much greater than that of hypoxia (Figure 4B). In these experiments, because we treated cells with MβCD for 4 h, we included the sample of a 4 h-hypoxic condition as a positive control for HIF-1α induction. We also examined another protein BNIP3 that is upregulated by HIF-1α (24). As illustrated in Figure 4B, BNIP3 expression was slightly increased upon MβCD treatment. To further investigate whether this VEGF mRNA upregulation correlates with VEGF protein levels, cell culture supernatant was collected after MβCD treatment and VEGF levels were determined by enzyme-linked immunosorbent assay as described in the ‘Materials and Methods’. Data are expressed as the mean of triplicate ± SD; *, statistically significant difference as compared with the control (P < 0.001). CD-induced HIF-1α accumulation rapidly diminished HIF-1α and permitted protein synthesis by removal of CHX, followed by MβCD treatment in the absence or presence of MG132. As shown in Figure 3F, HIF-1α levels are comparable in MβCD- and MG132-treated cells, respectively, but MβCD and MG132 co-treatment further enhanced HIF-1α accumulation. These data indicate that the HIF-1α accumulation is due to an increase in protein synthesis rather than protein stability. Taken all together, the MβCD-induced HIF-1α expression is regulated in both transcriptional and translational levels, as reported in constitutively active RacG12V expressing cells (22).

Fig. 4. HIF-1α activity in MβCD-treated cells. (A) Serum-starved A431 cells were treated with 5 mM MβCD and then fractionated to obtain cytosol and nuclear fractions. Each fraction was subjected to immunoblotting analysis using HIF-1α, Lamin B and α-tubulin antibodies. (B) Serum-starved A431 cells were treated as in (A) and total RNA was extracted and subjected to reverse transcription–polymerase chain reaction to assess VEGF mRNA levels and BNIP3 mRNA levels. The levels of BNIP3 were quantified by densitometry and normalized with β-actin. The ratio of BNIP3 to the total β-actin in the control cells was set as 1. (C) After cells were treated as indicated, VEGF contents in supernatants were determined by enzyme-linked immunosorbent assay as described in the ‘Materials and Methods’. Data are expressed as the mean of triplicate ± SD; *, statistically significant difference as compared with the control (P < 0.001). (D) Serum-starved EPO-HEP3B cells were treated with 3 mM MβCD for 7 h and cell lysates were analyzed by immunoblotting using anti-HIF-1α antibodies. The levels of HIF-1α were quantified by densitometry and normalized with β-actin. The ratio of HIF-1α to the total β-actin in the control cells was set as 1. (E) After treatments, EPO-HEP3B cells were lysed and luciferase activity was measured as described in the ‘Materials and Methods’. *, statistically significant difference as compared with the control (P < 0.001). (F) Serum-starved HeLa and A549 cells were treated with 5 mM MβCD for 3–5 h and HIF-1α expression was detected by immunoblotting. These data are representative of at least three independent experiments.
measured using enzyme-linked immunosorbent assay. Consistent with the data in Figure 3B, VEGF secretion was enhanced by rafts disruption (Figure 4C). EGFR activation and subsequent PI3K/Akt activation pathway has been reported to be critical for both hypoxic and non-hypoxic HIF-1α induction (10,12). Because we observed that EGFR activation was critical for the HIF-1α induction (Figure 2), we further examined whether EGFR and MEK are involved in the MβCD-induced VEGF secretion. Both EGFR and MEK inhibitors significantly attenuated the MβCD-induced VEGF secretion (Figure 4C). We also examined the effect of rafts disruption on HIF-1 activity in another cell line, Hep3B (EPO-Luc) which is a stable transfectant harboring the EPO-enhancer-luciferase plasmid. MβCD treatment induced HIF-1α upregulation (Figure 4D) and increased luciferase activity in the Hep3B cell line (Figure 4E). In addition, we could observe the similar effects of MβCD on HIF-1α expression in HeLa and A549 cell lines, indicating that the MβCD-induced HIF-1α induction is not limited to A431 cells (Figure 4F). A recent study has demonstrated that non-hypoxic HIF-1α induced by proteosome inhibition is functionally inactivated (25), indicating that HIF-1α expression and function can be differentially regulated. However, in the present case, rafts disruption not only accumulates HIF-1α but also activates its transcriptional function.

**HIF-1α expression attenuates rafts disruption-induced anoikis**

Although cholesterol depletion results in ligand-independent EGFR activation (14), the consequence of its activation has not been...
addressed. Since EGFR activation is linked to integrin function (26) and rafts disruption enhances cell detachment (4,15), we examined the effect of EGFR inhibition on cell adhesion in cholesterol-depleted cells. Although AG1478 alone affects adhesion a little, the co-treatment of AG1478 and MJCD synergistically enhanced cell detachment compared with MJCD alone (Figure 5A). Next, we analyzed EGFR activity and HIF-1α levels in the detached and the attached cells after MJCD treatment. Compared with the detached cells, both EGFR activity and HIF-1α levels were higher in the attached cells (Figure 5B) and HIF-1α mRNA was also increased (supplementary Figure S2 is available at Carcinogenesis Online).

To further investigate whether HIF-1α expression is associated with cell adhesion, we reduced HIF-1α expression using shRNA and the HIF-1α reduction was confirmed in HIF-1α-specific shRNA (shHIF-1α) cells with MJCD treatment (Figure 5C). To test the effect of HIF-1α downregulation on cell adhesion in MJCD-treated cells, cell morphological changes were examined by time-lapse analysis. As shown in Figure 5D, rafts disruption accelerated cell detachment in shHIF-1α cells and consistently, more cells were detached in the MJCD-treated shHIF-1α cells compared with shRNA empty vector cells (Figure 5E). HIF-1α has been implicated as a mediator of cancer cell migration, invasion and metastasis. HIF-1α increases cell adhesion by inducing syndecan-4 and α5-integrin in various cell types (27,28). A recent report also discussed that HIF-1α-deficient gastric cancer cells are defective in cell migration, invasion and adhesion to endothelial cells (29). Therefore, it is possible that HIF-1α induction might regulate adhesion-related molecules, leading to sustained cell adhesion in the MJCD-treated cells.

Accumulating evidence has demonstrated that HIF-1α is induced by non-hypoxic stimuli but understanding of HIF-1α expression augmented anoikis (4,15). Because HIF-1α induction is associated with cell adhesion, we reduced HIF-1α expression using shRNA and the HIF-1α reduction was confirmed in HIF-1α-specific shRNA (shHIF-1α) cells with MJCD treatment (Figure 5C). To test the effect of HIF-1α downregulation on cell adhesion in MJCD-treated cells, cell morphological changes were examined by time-lapse analysis. As shown in Figure 5D, rafts disruption accelerated cell detachment in shHIF-1α cells and consistently, more cells were detached in the MJCD-treated shHIF-1α cells compared with shRNA empty vector cells (Figure 5E). HIF-1α has been implicated as a mediator of cancer cell migration, invasion and metastasis. HIF-1α increases cell adhesion by inducing syndecan-4 and α5-integrin in various cell types (27,28). A recent report also discussed that HIF-1α-deficient gastric cancer cells are defective in cell migration, invasion and adhesion to endothelial cells (29). Therefore, it is possible that HIF-1α induction might regulate adhesion-related molecules, leading to sustained cell adhesion in the MJCD-treated cells.

Supplementary data
Supplementary Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/.

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