

cAMP-response Element-binding Protein (CREB) and NF- κ B Transcription Factors Are Activated during Prolonged Hypoxia and Cooperatively Regulate the Induction of Matrix Metalloproteinase *MMP1*^{*[5]}

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Background: Changes in gene expression mediate adaptation to hypoxia.

Results: *MMP1* was induced during prolonged hypoxia, and this induction was blocked after depletion of CREB and/or NF- κ B.

Conclusion: CREB and NF- κ B mediate the up-regulation of *MMP1* during prolonged hypoxia.

Significance: CREB- and NF- κ B-mediated gene transcription may play a major role in the cellular response to prolonged hypoxia.

Responses to low levels of oxygen (hypoxia) are essential to maintain homeostasis. During the hypoxic response, gene expression is altered by various transcription factors. The transcription factor, hypoxia-inducible factor (HIF), plays a central role in the hypoxic response. The α subunit of HIF, which is actively degraded during normoxia, becomes stabilized during hypoxia, which leads to HIF activation. A microarray analysis of HeLa cells showed that expression of matrix metalloproteinase 1 (*MMP1*) was markedly induced during prolonged hypoxia. CREB and NF- κ B binding sites were identified in the *MMP1* promoter region between 1945 and 1896 nucleotides upstream of the transcription start site. Assays with luciferase reporters demonstrated that HIF activity was induced during the early phase of hypoxia, whereas CREB and NF- κ B were activated during the later (prolonged) phase. Depletion of CREB and/or NF- κ B reduced *MMP1* induction during prolonged hypoxia both at the mRNA and protein levels. A chromatin immunoprecipitation assay demonstrated binding of CREB and NF- κ B to the *MMP1* promoter. Finally, cell migration and invasion on a collagen matrix and pulmonary metastasis in nude mice were inhibited after depletion of CREB and NF- κ B in MDA-MB-231 cells. Taken together, these results suggest that the cooperative action of CREB and NF- κ B plays an important role to induce *MMP1* expression during prolonged hypoxia and regulates cell migration and invasion in cancer cells.

Organisms utilize oxygen to efficiently produce energy. Homeostasis is maintained during exposure to low levels of oxygen (hypoxia) by the hypoxic response. The transcription factor hypoxia-inducible factor (HIF)² plays a key role in the hypoxic response (1). HIF consists of two subunits, HIF- α and - β . Whereas the β subunit is constitutively expressed, the α subunit is mainly expressed during hypoxia, and it is actively degraded during normoxia by the ubiquitin-proteasome system. The von Hippel-Lindau (pVHL) ubiquitin ligase complex ubiquitinates HIF- α when the proline residues of the α subunit are hydroxylated (2). This prolyl-hydroxylation of HIF- α is mediated by prolyl-hydroxylases (PHD, also called HPH (HIF-prolyl hydroxylase) or EGLN (egg-laying abnormal nine)), which belong to the 2-oxoglutarate-dependent dioxygenase family and require co-factors such as 2-oxoglutarate, Fe²⁺, ascorbic acid, and oxygen for their enzymatic activity (3). PHD activity is attenuated during hypoxia, leading to a reduction in HIF- α ubiquitination and stabilization of the protein. Consequently, a heterodimer of HIF- α and - β is able to form, which induces the expression of a number of hypoxia-regulated genes.

Regulation of gene expression during hypoxia is also mediated by other transcription factors, such as CREB and NF- κ B. NF- κ B is activated during acute hypoxia in macrophages and pulmonary artery smooth muscle cells (4, 5). In macrophages the activation of NF- κ B during hypoxia precedes the stabilization of HIF-1 α ; NF- κ B (p50/p65) binds the promoter region of *HIF-1 α* and up-regulates *HIF-1 α* mRNA. In contrast, HIF-1 α appears to regulate NF- κ B expression in neutrophils (6). These results suggest there is a regulatory loop between the two factors. CREB, which mediates a cAMP-dependent gene expression, is activated when it is phosphorylated by kinases, such as protein kinase A or Ca²⁺/calmodulin-dependent kinase (7).

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[5] This article contains a supplemental table and Figs. 1–5.

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² The abbreviations used are: HIF, hypoxia-inducible factor; HUVEC, human umbilical vein endothelial cells; PRP, pre-mRNA processing factor; MMP, matrix metalloproteinase; PHD, prolyl-hydroxylase domain containing; HRE, hypoxia response element; CRE, cAMP-responsive element; CREB, CRE-binding protein; norm, normoxia; hypo, hypoxia; qPCR, quantitative PCR; IKK, I κ B kinase.

However, precisely when CREB and NF- κ B is activated during hypoxia still remains unknown.

Pre-mRNA processing factor 19 (PRP19) is a component of the spliceosome. We previously demonstrated that the PHD enzyme, PHD3, interacts with PRP19 and inhibits cell death during hypoxia (8). To further examine the role of PRP19 during hypoxia, we performed microarray analysis of PRP19-depleted cells that had been cultured in prolonged hypoxic conditions. The matrix metalloproteinase 1 (*MMP1*) gene was highly up-regulated in these cells, whereas there was little change in the level of MMP1 protein. Nevertheless, *MMP1* was clearly induced independently of PRP19 after prolonged hypoxic treatment. Because it remains largely unknown how gene expression is regulated during prolonged hypoxia, we studied how the expression of *MMP1* is up-regulated during prolonged hypoxic treatment.

MMP1 is a member of the matrix metalloproteinase family and requires zinc for its activity (9). MMP1 is subgrouped with MMP8 and MMP13, and all three proteins have collagenase activity. Through its enzymatic activity, MMP1 plays roles in various processes including tumor invasion, blood vessel remodeling, and wound healing (9). Previous reports indicate that NF- κ B, p38, and JNK pathways are involved in *MMP1* expression by cytokine stimulation in fibroblast or chondrocytes (10, 11). It was also reported that induction of *MMP1* is mediated by HIF-1 during hypoxia in fibroblasts (12, 13) or by HIF-2 in chondrocytes upon IL-1 β stimulation (14). Although HIF is a key regulator of the hypoxic response, it remains unclear if *MMP1* is induced by these factors during prolonged hypoxia or if any other factor(s) is involved in *MMP1* induction. In this study it is reported that *MMP1* is specifically induced during prolonged hypoxia in multiple cancer cells and that this process is cooperatively regulated by CREB and NF- κ B.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells and the breast tumor cell lines MCF7 and MDA-MB-231 were cultured in Dulbecco's modified Eagle's medium (high glucose) (Wako, Japan) containing 10% fetal bovine serum and antibiotics. Human umbilical vein endothelial cells (HUVECs) were cultured in complete endothelial cell growth medium (211K-500) (Cell Applications inc., San Diego, CA).

Hypoxia Treatment—Cells (HeLa, MCF7, MDA-MB231, or HUVEC) were treated under hypoxic conditions (1% or 5% O₂, 5% CO₂, and the rest were balanced with N₂) in a hypoxia workstation (Hirasawa Works, Tokyo, Japan). An oxygen sensor was used to ensure the oxygen concentration inside the workstation, which was maintained at 1 or 5% throughout the experiment (MC-8G-S, Iijima Electrics, Gamagori, Japan).

siRNA Treatment—Initially, three kinds of siRNA for each gene were tested, and the most effective ones were selected. HeLa or MDA-MB-231 cells were transfected with negative control siRNA (#45-2001, Invitrogen), HIF-1 α siRNA (HSS #179231 from Invitrogen), HIF-2 α siRNA (HSS #103261 from Invitrogen), Rel A (NF- κ B) siRNA (SASI Hs01 00171091 from Sigma), CREB siRNA (SASI Hs01 00116988 from Sigma), and MMP1 siRNA (HSS #106611 from Invitrogen) using LipofectamineTM RNAiMAX transfection reagent according to

manufacturer's instructions (Invitrogen). The day after transfection, cells were plated onto collagen-coated dishes or plates and subjected to the scratch and invasion assays, respectively. For the luciferase assay, cells were transfected with luciferase vectors the day after siRNA transfection and were cultured in normoxic or hypoxic conditions for 24 or 48 h. A portion of the cells were collected, and total RNA or protein was extracted. For Western blotting and quantitative real-time PCR (qPCR), cells were cultured in normoxic or hypoxic conditions for up to 48 h, and protein or RNA was extracted, respectively. The efficiency of gene silencing was determined by qPCR or Western blotting.

Reagents and Antibodies—The following antibodies were used: anti- β -actin antibody (Sigma); anti-HIF-1 α antibody (Novus, Littleton, CO); anti-HIF-2 α antibody (Novus); anti-NF- κ B p65 antibody (Cell Signaling Technology, Danvers, MA); anti-CREB antibody (Cell Signaling Technology); anti-phosphoSer-133-CREB antibody (Cell Signaling Technology); anti-I κ B α antibody (Cell Signaling Technology). For the chromatin immunoprecipitation (ChIP) assay, control rabbit IgG (Millipore Upstate, Temecula, CA), anti-CREB antibody (Millipore Upstate), and anti-NF- κ B p65 antibody (Santa Cruz, CA) were used. An anti-MMP1 antibody was generated by immunizing rabbits with a mixture of peptides corresponding to amino acids 131–144 and 375–388 of human MMP1. Its specificity to recognize MMP1 was characterized by Western blotting (Fig. 3, D and E). Staurosporine was purchased from Wako Pure Chemical (Osaka, Japan).

Western Blotting—Cells (HeLa, MCF7, MDA-MB231, or HUVEC) were harvested on ice and lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1 μ g/ml PMSF, and 2 μ g/ml leupeptin). Total cell lysates were subjected to SDS-PAGE (50 μ g of total lysate/lane) and were transferred onto a nitrocellulose membrane (PALL, East Hills, NY).

qPCR—Total RNA was isolated from cultured cells (HeLa, MCF7, MDA-MB231, or HUVEC) using an RNeasy[®] kit (Qiagen, Valencia, CA). First strand cDNA synthesis was performed with 2 μ g of total RNA using the PrimeScriptTM II kit (Takara Bio). Synthesized cDNA was used for PCR analysis using SsoFastTM EvaGreen[®] Supermix (Bio-Rad) with a Chromo4TM real-time PCR detecting system (Bio-Rad). Relative expression levels were calculated using the Δ Ct method and normalized against β -actin or cyclophilin internal control. Primer sequences are listed in the supplemental table.

Luciferase Assay—HeLa or MCF7 cells were plated on 24-well culture plates. The *MMP1* promoter luciferase vector and its deletion mutants HRE-luc, κ B-luc, or CRE-luc (500 ng) were transiently transfected with the Renilla control pRL-null vector (160 ng) using the FuGENE[®] transfection reagent (Promega, Madison, WI). Cells were cultured in normoxia or hypoxia, and each well was lysed in 100 μ l of passive lysis buffer (Promega) 2 days after transfection. The luciferase assay was performed using 30 μ l of cell lysate with the Dual-Luciferase[®] Reporter Assay (Promega). Luciferase activity was measured using a Microplate reader Centro LB960 (Berthold, Bad Wildbad, Germany). The relative luciferase activity was calculated

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by dividing the firefly luciferase activity with that of the Renilla control.

ChIP Assay—HeLa cells were cultured in normoxia or hypoxia (1% O₂) for 24 h, and cross-linking was performed using 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped by the addition of 150 mM glycine. Cells were lysed and sonicated 5 times to obtain 200–800-bp chromatin fragments. The protein-chromatin complex was immunoprecipitated by incubating the cell extract with 20 μ g of antibodies overnight and then with 30 μ l of protein G magnetic beads (Cell Signaling Technology) for 2 h at 4 °C. After eight washes, cross-links of the protein/chromatin complex were resolved, and chromatin was purified using phenol-chloroform extraction followed by ethanol precipitation. DNA was prepared and used as a template in PCR analyses using the specific primer sets listed in the supplemental table.

MMP1 ELISA—MMP1 activity in the cell culture medium was measured using the SensoLyte[®] MMP1 assay kit (Anaspec, Fremont, CA). Briefly, cell culture medium of MDA-MB-231 cells containing the secreted form of MMP1 is incubated with a substrate that fluoresces when cleaved by MMP1. The fluorescence was measured by ARVO-MX plate reader (PerkinElmer Life Sciences).

Pulmonary Metastasis Studies—MDA-MB-231 cells were transfected with siRNA (control or CREB and NF- κ B). One day after the transfection, cells were suspended in PBS at 5 \times 10⁶ cells/ml, and 100 μ l of cell suspension was injected to the tail vein of BALB/cAJcl-*nu/nu* mice (4-week-old female, Japan CLEA, Inc.). Mice were sacrificed after 6 weeks of injection, and the lungs were collected and fixed with Bouin's fixative. Metastatic nodules on the lung surface were counted under the dissection microscope. All the animal experiments have been performed in accordance with a protocol approved by the Tokyo Medical and Dental University Animal Care Committee.

Cell Migration Assay—MDA-MB-231 cells were transfected with control, CREB, NF- κ B, or MMP1 siRNAs. One day later cells were re-plated on collagen-coated dishes (coated with collagen type I, Asahi Glass Co. Ltd., Tokyo, Japan) and grown until fully confluent. The cell layer was scratched using a 1-ml pipette tip, and cells were cultured in normoxic or hypoxic conditions for up to 24 h in the presence of mitomycin C (5 μ g/ml). Cells were imaged 0, 12, and 24 h after the scratch wound was introduced, and the width of gap was measured. Results presented are the means of three independent experiments.

Invasion Assay—A cell invasion assay was performed using a CytoSelect[™] 96-well Cell Invasion assay kit (Cell Biolabs, San Diego, CA). MDA-MB-231 cells were transfected with siRNA, and 1 day later cells were detached and 2 \times 10⁵ cells were placed in the upper chamber of the assay plate that was covered with a layer of collagen. The assay plate was cultured in hypoxic conditions for 48 h, and cells that migrated toward the lower chamber were collected and quantified using CyQUANT[®] GR fluorescent dye. Each experiment was performed at least three times, and the representative data is shown.

RESULTS

Up-regulation of MMP1 during Hypoxia—MMP1 was initially identified in a microarray analysis as a gene induced in

PRP19-depleted cells after 24 h of hypoxic treatment. Although the level of MMP1 mRNA was found to be increased in PRP19-depleted cells, the level of MMP1 protein was unaffected (data not shown). Alternatively, it is identified that MMP1 was markedly induced after prolonged hypoxia, and the level of MMP1 induction was greater after 48 h of hypoxia than after 24 h in MCF7 cells (Fig. 1A). Thus, MMP1 induction during prolonged hypoxia is further characterized. In contrast to MMP1, the induction of VEGF, a typical HIF target gene, was highest after 24 h of hypoxia, after which the level of induction fell (Fig. 1A). Induction of MMP1 during prolonged hypoxia was detected in several cell types, including HUVECs, the breast cancer cell line MDA-MB-231, and HeLa cells (Fig. 1B). These results suggest that MMP1 is a hypoxia-inducible gene that is mainly up-regulated during the prolonged phase of hypoxia. The induction of MMP1 was observed both in 5% O₂ (mild hypoxia) and in 1% O₂ (Fig. 1C). In contrast, whereas VEGF was clearly induced in 1% O₂, it was not significantly induced in 5% O₂. The induction of VEGF was similar to the expression of HIF-1 α and HIF-2 α , which were greatly increased in 1% O₂ and moderately increased in 5% O₂ (Fig. 1C).

Characterization of the MMP1 Promoter Region Required for Hypoxic Induction—The promoter of MMP1 was analyzed to determine the region that is responsible for the induction of MMP1 during prolonged hypoxia. The full-length promoter region of the MMP1 gene, defined as the 2995-bp upstream of the transcription start site, was cloned into the pGL3 luciferase vector (pGL3-MMP1 promoter (–2995 to –1 bp), referred to as “full-length”) (Fig. 2A). The luciferase vector containing the hypoxia response element (HRE) efficiently induced luciferase activity after hypoxic treatment for 24 h (Fig. 3F). Under the same conditions, the full-length vector induced luciferase activity in a hypoxia-dependent manner (Fig. 2A). The two halves of the full-length promoter were cloned; the upstream half (–2995 to –1497 bp) and the downstream half (–1496 to –1 bp). Only the upstream region increased luciferase activity in response to hypoxia (Fig. 2A). These results suggest that the upstream region of the MMP1 promoter contains the sequence responsible for the induction of MMP1 expression in response to hypoxia. Next, we generated a series of deletion mutants of the upstream construct that lacked 300, 600, 900, or 1200 bp starting from the first nucleotide of the promoter (position –2995), which were named Δ 1, Δ 2, Δ 3, and Δ 4, respectively (Fig. 2B). The Δ 1, Δ 2, and Δ 3 mutants were activated during hypoxia, whereas the Δ 4 mutant was not (Fig. 2B). These results indicate that the 300 bp region from –2095 to –1796 bp in the MMP1 promoter is responsible for the hypoxic induction. A database search detected conserved binding sequences for CREB, NF- κ B, and C/EBP β within this region (15, 16). To further narrow down the region required for promoter activity, three deletion mutants of the full-length construct were generated that each lacked 50 bp. These mutants were named Δ –2045 to –1996, Δ –1995 to –1946, and Δ –1945 to –1896 (the numbers indicate the deleted regions). The induction of MMP1 during hypoxia was significantly reduced with the Δ –1945 to –1896 mutant (Fig. 2C). The consensus binding sequences for CREB, NF- κ B, and C/EBP β locate in this region.

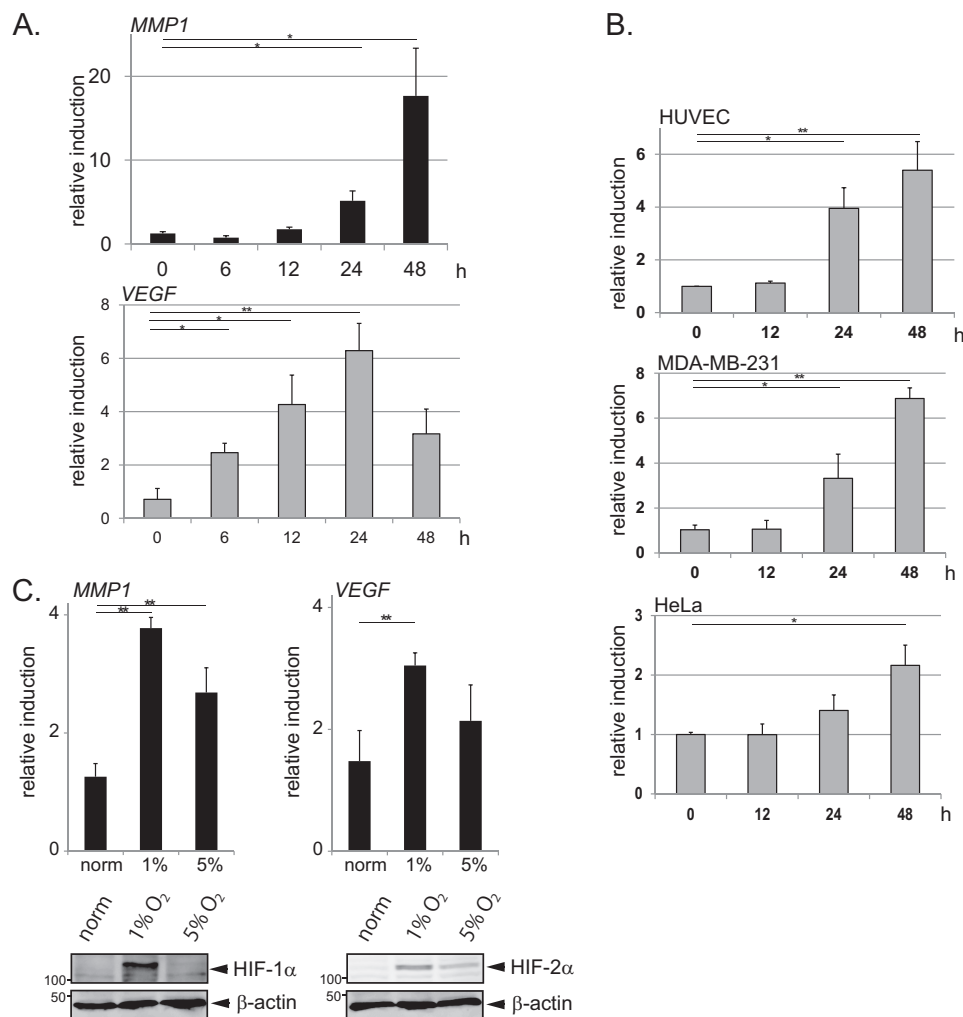


FIGURE 1. MMP1 gene expression is induced during prolonged hypoxia. A, shown is induction of *MMP1* and *VEGF* during hypoxia. MCF7 cells were cultured in 1% O₂ for the indicated times, and expression of *MMP1* and *VEGF* was determined by qPCR analyses ($n = 3$). Error bars indicate S.D., and significance was statistically analyzed with a t test (*, $p < 0.05$; **, $p < 0.02$) (A–C). B, shown is induction of *MMP1* in different cell lines during hypoxia. HUVEC, MDA-MB-231, and HeLa cells were cultured in 1% O₂ for the indicated times, and expression of *MMP1* was determined by qPCR analyses ($n = 3$). C, shown is the effect of mild hypoxia on *MMP1* and *VEGF* expression. MCF7 cells were cultured in 1 or 5% O₂ for 24 h. Expression of *MMP1* and *VEGF* was determined by qPCR analyses ($n = 4$). HIF-1 α and HIF-2 α were detected by Western blotting.

Activation of MMP1 and CREB- and NF- κ B-dependent Promoters during Prolonged Hypoxia—Next, activity of the *MMP1* promoter constructs at different time points during hypoxic treatment was compared in HeLa cells. The full-length construct and the upstream construct were activated in a time-dependent manner during hypoxia; they were moderately activated (around 5-fold) after 24 h of hypoxia and were activated further (up to 20-fold) after 48 h of hypoxia (Fig. 3A). *MMP1* protein expression was also found to be up-regulated during prolonged hypoxia in MDA-MB-231 and MCF7 cells as well as in HUVECs (Fig. 3B). Similarly, *MMP1* secreted in the culture medium, which was measured by its activity, increased during hypoxia, which corresponds to the increase of *MMP1* in cell lysate (Fig. 3C). Specificity of the *MMP1* antibody was assured by the depletion of *MMP1* band with three independent siRNAs against *MMP1* and its ability to detect purified GST-*MMP1* in Western blotting (Fig. 3, D and E). Cancerous MCF7 and MDA-MB-231 cells grew efficiently in high glucose medium compared with non-cancerous HUVECs (supplemental Fig. 1), yet the induction profile of *MMP1* was comparable.

Of note, overall expression level of *MMP1* in MDA-MB-231 cells was higher than that in MCF7 cells. To determine the activities of CREB, NF- κ B, and HIF, luciferase assays using vectors driven by HRE (HRE-luc), κ B-response element (κ B-luc), and CRE (CRE-luc) were performed at different time points during hypoxic treatment. Whereas the activity of HRE-luc peaked at 24 h and began to decrease at 48 h, the activities of κ B-luc and CRE-luc were greater after 48 h of hypoxia than after 24 h (Fig. 3F and supplemental Fig. 2). These results suggest that HRE, κ B-response element, and CRE are maximally active at different time points during hypoxia. Based on this result, prolonged hypoxia is defined in this report as being more than 24 h of hypoxic treatment, after which the activity of HIF-1 started to decrease.

The activation profiles of κ B-luc or CRE-luc were similar to that of the *MMP1* promoter (Fig. 3, A and F, and supplemental Fig. 2). Next, the CREB, NF- κ B, and HIF signaling pathways were examined by Western blotting. CREB activation was examined by monitoring the phosphorylation of Ser-133 using a phospho-specific antibody. CREB phosphorylation was

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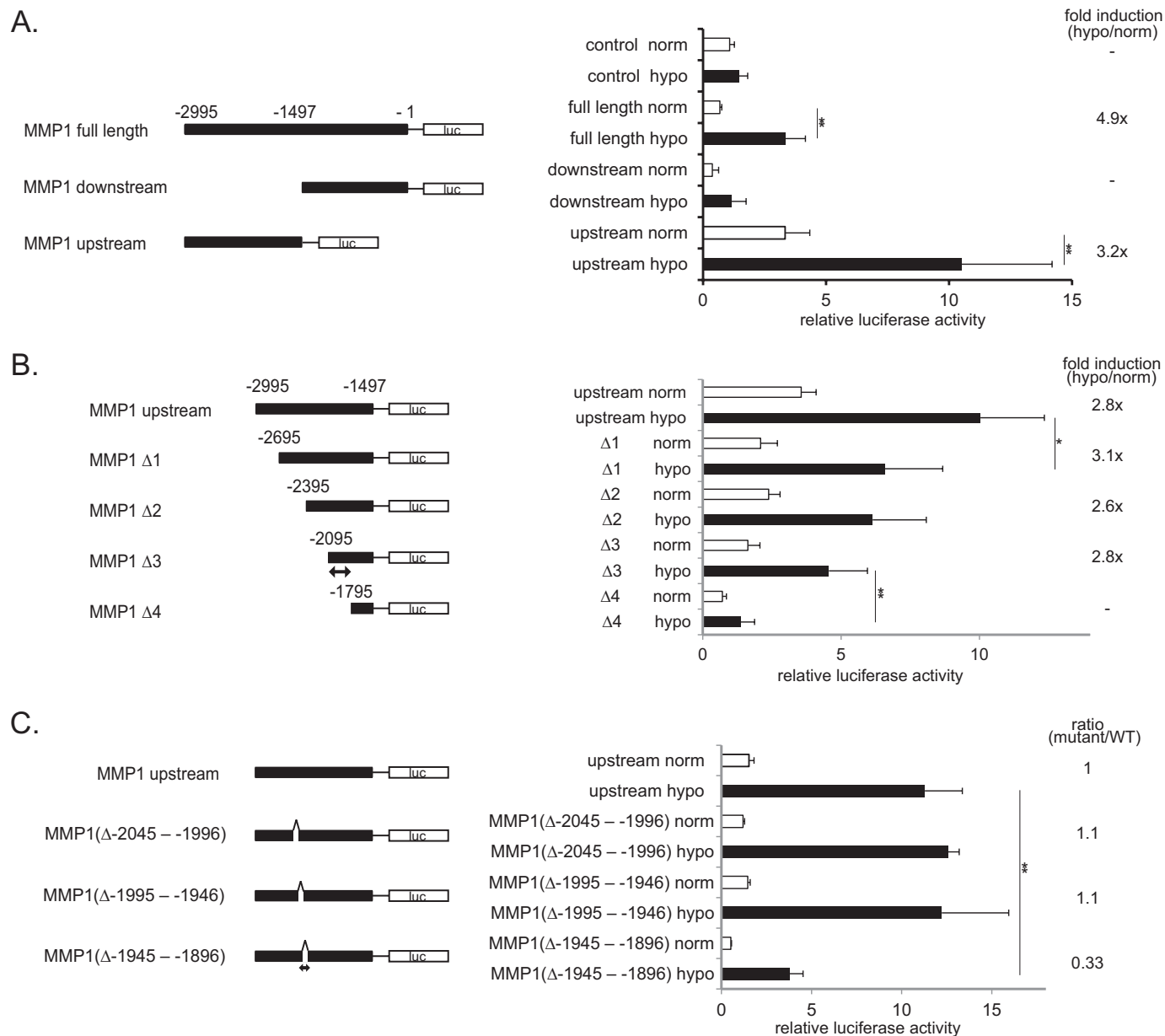


FIGURE 2. Identification of the region of the *MMP1* promoter required for *MMP1* induction during prolonged hypoxia. *A*, shown is the requirement of -2995 to -1497 bp for the hypoxic activation of the *MMP1* promoter. The region of the *MMP1* promoter required for induction of the *MMP1* during hypoxia (24 h) was characterized by performing a luciferase assay in HeLa cells using the deletion constructs indicated ($n = 6$). The ratio between the firefly luciferase activity (*MMP1* promoter) and the Renilla luciferase activity (pRL-null internal control) is shown (*A–C*). Error bars indicate S.D., and significance was statistically analyzed with a *t* test (*, $p < 0.05$; **, $p < 0.02$) (*A–C*). -Fold induction upon hypoxic treatment (hypo/norm) is shown. Promoter activity below the activity of control (basal promoter) was considered to be background and is indicated as a dash (*A* and *B*). *B*, shown is the requirement of -2095 to -1796 bp for the hypoxic activation of the *MMP1* promoter. The activities of a series of *MMP1* promoter deletion constructs were monitored using a luciferase assay in HeLa cells after 24 h of hypoxia ($n = 6$). The position of the sequence relevant for the promoter activation is marked with a double-headed arrow. *C*, shown is the requirement of -1945 to -1896 bp for the hypoxic activation of the *MMP1* promoter. A series of deletion mutants of the upstream construct was generated. Their activities were monitored using a luciferase assay in HeLa cells after 48 h of hypoxia ($n = 3$). The position of the sequence relevant for the promoter activation is marked with a double-headed arrow. Relative activity of mutant promoters to the wild type promoter (mutant/wild type) is shown as ratio.

induced after 24 h of hypoxic treatment in HeLa, MCF7, and MDA-MB-231 cells (Fig. 3G). To evaluate NF- κ B activity, the expression of its inhibitory subunit I κ B α was monitored. The level of I κ B α protein decreased after hypoxic treatment and was lowest after 48 h of hypoxia, suggesting that NF- κ B activity had increased (Fig. 3G). In contrast, the protein expression of HIF-1 α and HIF-2 α in most cases peaked at earlier time points and decreased during prolonged hypoxia (Fig. 3G), which is similar to the pattern of HRE luciferase activity observed (Fig. 3F).

*Inhibition of the CREB and NF- κ B Signaling Pathways Suppresses *MMP1* Expression during Hypoxia*—Because the timing of *MMP1* promoter activation was synchronized with CREB and NF- κ B activation and consensus binding sequences for CREB and NF- κ B were found in the *MMP1* promoter, it was examined whether CREB and/or NF- κ B are involved in up-regulating *MMP1* during prolonged hypoxia. Western blotting confirmed that the protein levels of CREB and NF- κ B were effectively depleted after transfection of siRNAs targeting these molecules

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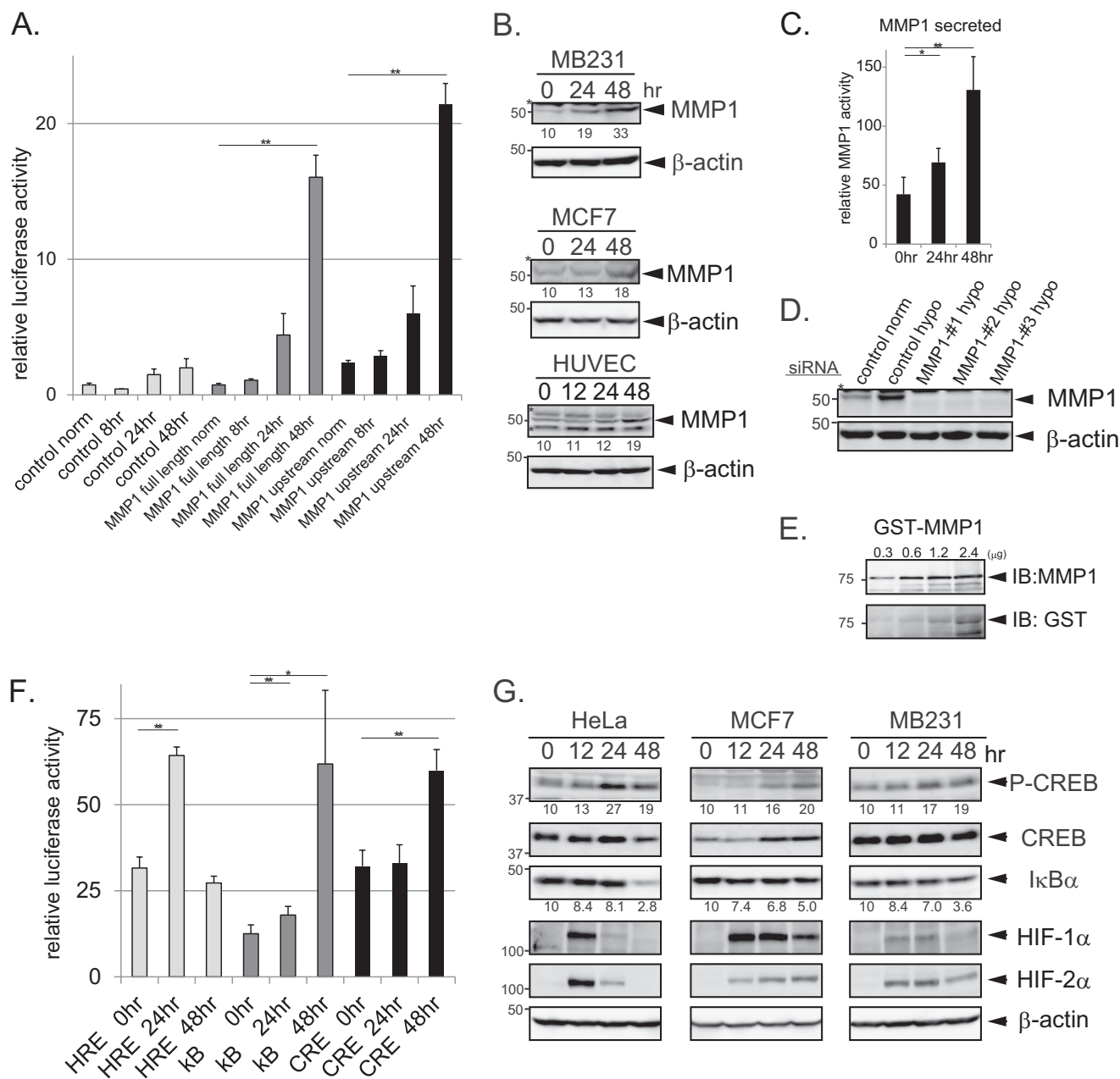


FIGURE 3. Increased activity of NF- κ B and CREB during prolonged hypoxia coincides with the induction of MMP1. *A*, shown is enhancement of MMP1 promoter activity during prolonged hypoxia. The luciferase activities of the full-length and upstream MMP1 promoter constructs were measured in HeLa cells during hypoxia ($n = 3$). The ratio between the firefly luciferase activity (MMP1 promoter) and the control Renilla luciferase activity (pRL-null internal control) is shown (*A* and *F*). Error bars indicate S.D., and significance was statistically analyzed with a *t* test (**, $p < 0.02$). *B*, shown is induction of MMP1 protein expression during prolonged hypoxia. MDA-MB-231, MCF7, and HUVECs were cultured in 1% O₂ for the indicated times. MMP1 expression was detected by Western blotting. The numbers below the bands represent the relative intensities quantified using densitometric analyses. Asterisks indicate nonspecific bands detected by the antibody. *C*, shown is increased MMP1 activity in the cell culture medium during hypoxia. An ELISA assay was performed on cell culture medium collected from MDA-MB-231 cells incubated with hypoxia for different times to measure MMP1 activity ($n = 3$). The graph shows relative MMP1 activity. Error bars indicate S.D., and significance was statistically analyzed with a *t* test (*, $p < 0.05$; **, $p < 0.02$). *D*, shown is decreased expression of MMP1 by MMP1 siRNA. MDA-MB-231 cells transfected with control or three kinds of MMP1 siRNA were treated with hypoxic condition for 24 h, and cell lysate was subjected to Western blotting. The antibody detected the expression of MMP1, which was induced by hypoxia and depleted by the three siRNAs. The asterisk indicates nonspecific bands detected by the antibody. *E*, shown is detection of GST-MMP1. GST-MMP1 (75 kDa) purified from *Escherichia coli* was subjected to Western blotting (IB). Different amounts of the protein were loaded to the lanes as indicated in the figure. The blot was detected with anti-MMP1 antibody (upper panel) and anti-GST antibody (lower panel). *F*, increased luciferase activity of HIF, NF- κ B (kB), and CREB target sequences during prolonged hypoxia is shown. The luciferase activities of the typical target sequences of HIF, CREB, and NF- κ B were monitored in HeLa cells during hypoxia ($n = 3$). Error bars indicate S.D., and significance was statistically analyzed with a *t* test (*, $p < 0.05$; **, $p < 0.02$). *G*, shown is activation of CREB and NF- κ B signaling pathways during hypoxia. Phosphorylation of CREB and the protein expression levels of CREB and I κ B α were monitored at the indicated time points by Western blotting after hypoxic treatment in HeLa, MCF7, and MDA-MB-231 cells. The numbers below the bands represent the relative intensities quantified using densitometric analyses. Immunoblots of HIF-1 α , HIF-2 α , and β -actin are shown in the bottom panels.

(Fig. 4A). Massive cell death was observed when C/EBP β -depleted cells were cultured in hypoxic conditions, and so the effects of C/EBP β could not be studied. The activation of the MMP1 pro-

motor during hypoxia was significantly inhibited after depletion of CREB and NF- κ B in HeLa cells (Fig. 4A, left and middle). When CREB or NF- κ B was depleted separately, luciferase activity was

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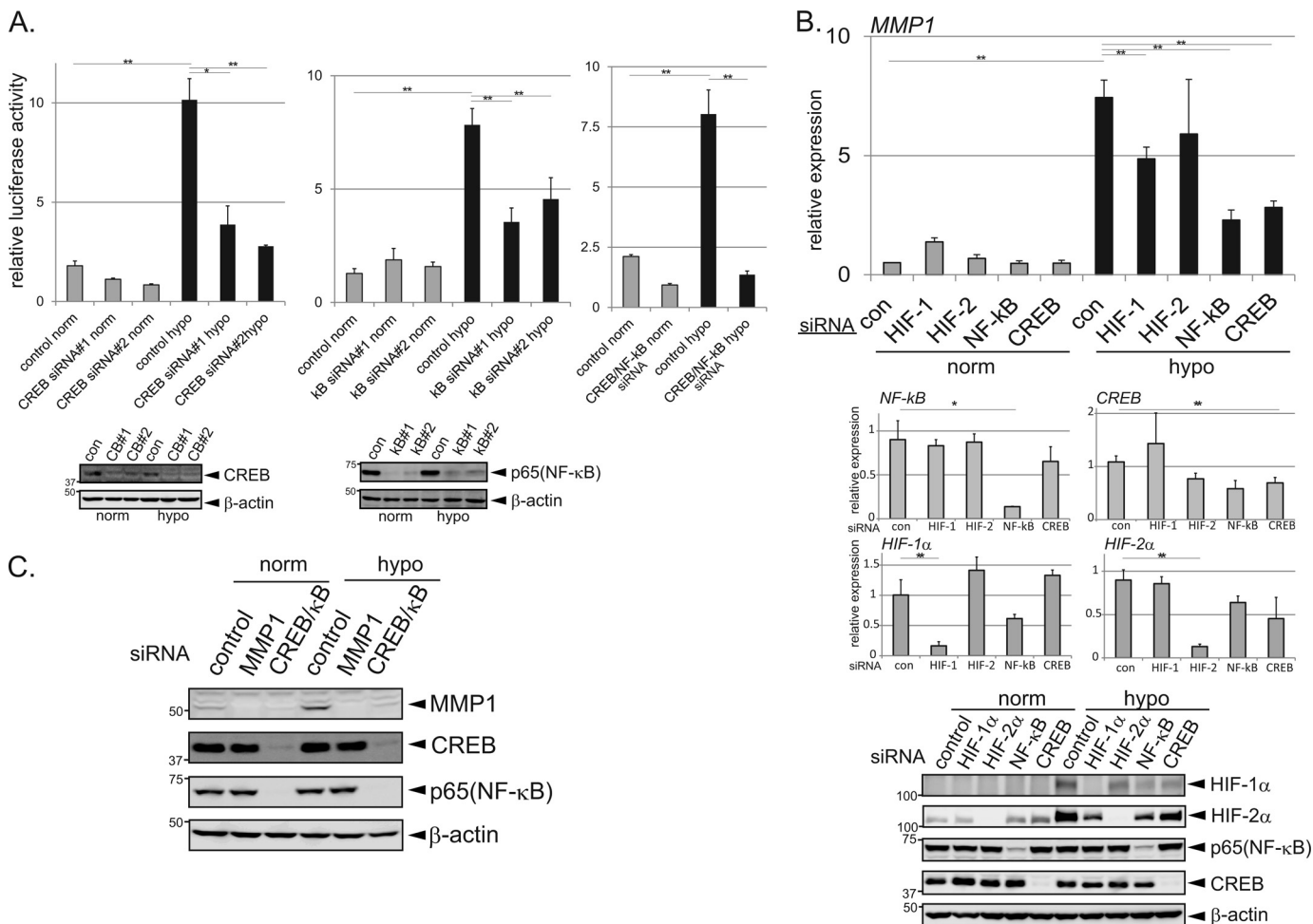


FIGURE 4. CREB and NF- κ B regulate the induction of *MMP1* during prolonged hypoxia. *A*, mediation of *MMP1* promoter activation during prolonged hypoxia by CREB and NF- κ B is shown. *MMP1* promoter activity was monitored after depletion of CREB and/or NF- κ B (left, CREB; middle, NF- κ B; right, CREB and NF- κ B) in HeLa cells ($n = 3$). Two siRNAs targeted against each gene were used, and the depletion efficiency was monitored by Western blotting (bottom panels). Error bars indicate S.D., and significance was statistically analyzed with a *t* test (*, $p < 0.05$; **, $p < 0.02$). *B*, induction of *MMP1* during prolonged hypoxia is mainly dependent on CREB and NF- κ B. HeLa cells treated with control, HIF-1 α , HIF-2 α , NF- κ B, or CREB siRNAs were cultured in normoxic or hypoxic conditions for 48 h. Expression of *MMP1* was monitored by qPCR analyses ($n = 3$). Middle graphs confirm the inhibition of gene expression after siRNA transfection. Error bars indicate S.D., and significance was statistically analyzed with a *t* test (*, $p < 0.05$; **, $p < 0.02$). Depletion of each protein by the corresponding siRNAs was confirmed by Western blotting (bottom). *C*, shown is inhibition of *MMP1* induction after depletion of CREB and NF- κ B. Western blotting was used to monitor *MMP1* protein expression in MDA-MB-231 cells depleted of *MMP1* or CREB and NF- κ B and grown in normoxic or hypoxic conditions for 24 h.

reduced by $\sim 50\%$; however, simultaneous depletion of CREB and NF- κ B reduced luciferase activity by up to 80% (Fig. 4A, right). These results suggest that CREB and NF- κ B cooperatively activate the *MMP1* promoter during prolonged hypoxia. It is possible that the 20% activity remaining after depletion of CREB and NF- κ B is due to the activity of another transcription factor(s) (e.g. C/EBP β). Of note, depletion of CREB moderately reduced the basal activity of *MMP1* luciferase reporter (Fig. 4A, left), which implicates the involvement of CREB in the basal expression of *MMP1*. The effect of CREB and NF- κ B depletion on *MMP1* gene expression was examined by qPCR in HeLa cells. Depletion of HIF-1 α and HIF-2 α was also performed to examine the previously reported involvement of the HIF pathway in *MMP1* expression (14). Although expression of *MMP1* was enhanced in control cells after 48 h of hypoxia, this increase was significantly inhibited after depletion of CREB or NF- κ B (Fig. 4B, top). Depletion of HIF-1 α or HIF-2 α also inhibited *MMP1* induction, although to a lesser extent than CREB or NF- κ B depletion (Fig. 4B, top). The efficient depletion of each

molecule after siRNA treatment was confirmed by qPCR and Western blotting (Fig. 4B, middle four panels and bottom). Taken together, these results suggest that the induction of *MMP1* during prolonged hypoxia mainly depends on the CREB and NF- κ B signaling pathways but is also affected by HIF-1 α and HIF-2 α . *MMP1* protein expression after depletion of CREB and NF- κ B was examined by Western blotting in MDA-MB-231 cells (Fig. 4C). Simultaneous depletion of CREB and NF- κ B efficiently decreased *MMP1* protein levels during both normoxia and hypoxia; however, the effect was less pronounced than that observed after transfection of *MMP1*-targeting siRNA (Fig. 4C). These results indicate that whereas the CREB and NF- κ B signaling pathways play a certain role in the regulation of basal *MMP1* expression during normoxia, they play a major role in up-regulating *MMP1* expression during prolonged hypoxia.

Recruitment of CREB and NF- κ B to the *MMP1* Promoter—To determine if CREB and NF- κ B interact with the promoter region of *MMP1*, ChIP assays were performed using lysates

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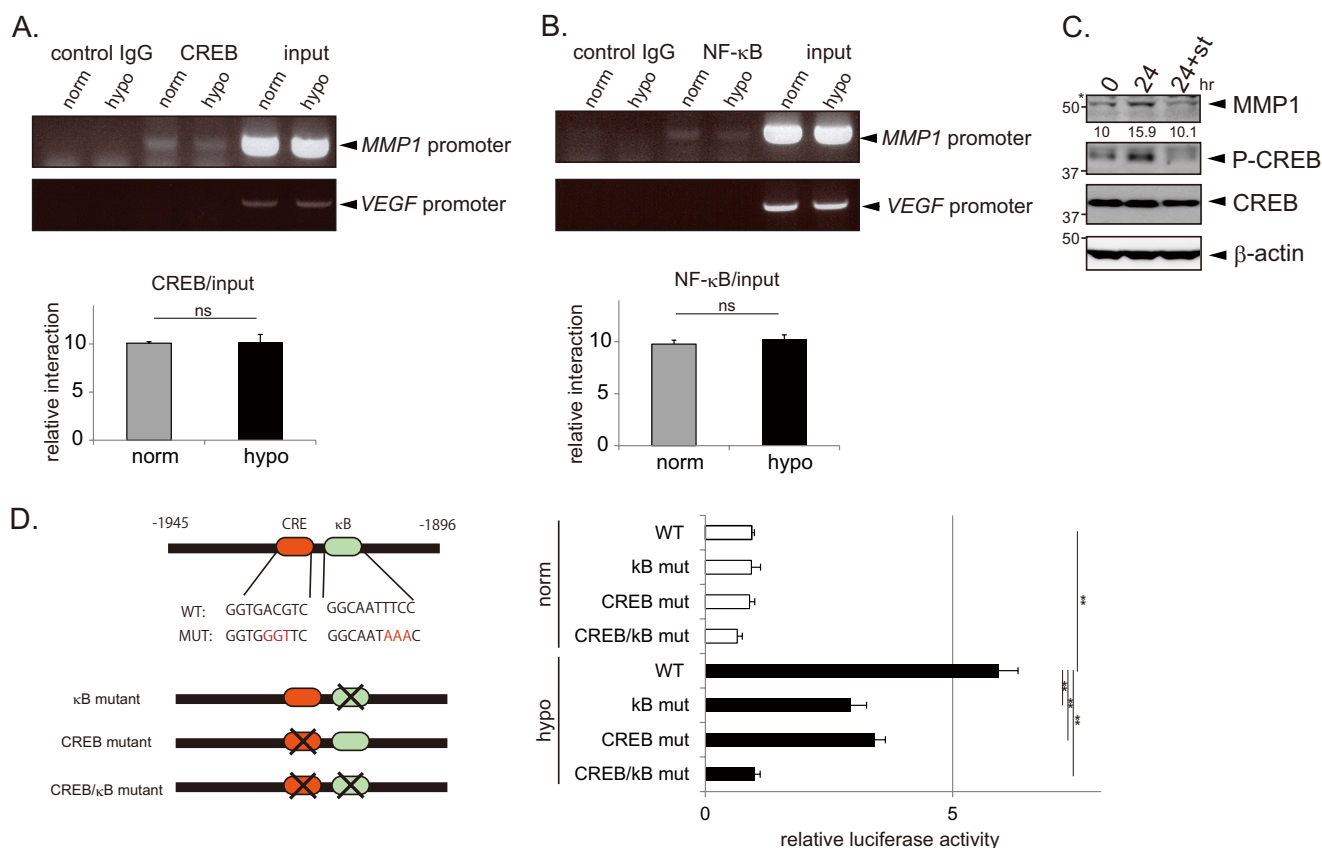


FIGURE 5. Binding of CREB and NF- κ B to the *MMP1* promoter. *A* and *B*, CREB and NF- κ B bind the *MMP1* promoter between -2095 and -1796 bp. A ChIP assay was performed using lysates prepared from HeLa cells cultured in normoxic or hypoxic conditions for 24 h using an anti-CREB (*A*) or an anti-NF- κ B (*B*) antibody. The promoter region was specifically amplified from the immunoprecipitates of the CREB and NF- κ B antibodies and was not amplified from the immunoprecipitate of the control IgG. *VEGF* promoter was monitored as a negative control. The ratio of ChIP/input was calculated, and an average of three experiments is shown in the graph ($n = 3$). Error bars indicate S.D., and significance was statistically analyzed with a *t* test (*ns*, significant difference was not found). *C*, hypoxic induction of *MMP1* is inhibited by staurosporine. MDA-MB-231 cells were incubated under hypoxic condition for 24 h in the presence or absence of staurosporine (st, 10 nM). Expression of *MMP1* and CREB and status of CREB phosphorylation was monitored by Western blotting. The numbers below the bands represent the relative intensities quantified using densitometric analyses. The asterisk indicates a nonspecific band detected by the antibody. *D*, the binding sites of CREB and NF- κ B (*kB*) on the *MMP1* promoter are important for the hypoxic activation of the *MMP1* promoter. The activities of *MMP1* promoter luciferase constructs containing point mutations in the NF- κ B or CREB binding sites (shown in the left figure) were compared with that of the wild type (WT) full-length vector using the luciferase assay in HeLa cells ($n = 3$). Error bars indicate S.D., and significance was statistically analyzed with a *t* test (****, $p < 0.02$).

prepared from HeLa cells grown under normoxic or hypoxic conditions. The *MMP1* promoter region (-2095 to -1796 bp) was amplified from chromatin immunoprecipitated with the CREB and NF- κ B antibodies (Fig. 5, *A* and *B*, top). Both transcription factors bound to the *MMP1* promoter, and the binding efficiency between normoxia and hypoxia was found not to be significantly different. Because hypoxic-treated samples grew slower and had less cell numbers, the signals obtained in the ChIP PCR were normalized against the input and shown in the graph (Fig. 5, *A* and *B*, bottom). Inhibition of CREB phosphorylation by staurosporine treatment attenuated the hypoxic induction of *MMP1* (Fig. 5*C*). These results suggest that the hypoxic induction of *MMP1* may depend on activation of CREB and NF- κ B, which is mediated by post-translational modification(s) or recruitment of co-factors to the promoter region through protein-protein interaction and is not due to enhanced binding of them to the promoter. *MMP1* promoter luciferase constructs were generated that had mutations in the NF- κ B-binding site, the CREB binding site, or in both sites. Hypoxic-dependent activation of the *MMP1* promoter was reduced in all mutants, and the largest reduction was observed with the CREB/NF- κ B double mutant (Fig. 5*D*).

Increased Expression and Activity of *MMP1* during Hypoxia—Because the hypoxic induction of *MMP1* gene expression appears to be regulated by CREB, NF- κ B, and HIF-1/2, the role of these molecules in the hypoxic induction of *MMP1* protein expression was examined in MDA-MB-231 cells. *MMP1* protein levels increased during hypoxia, and this was partially blocked after depletion of NF- κ B or CREB (Fig. 6*A*). This inhibition was observed after 24 and 48 h of hypoxic treatment. However, single knockdown of NF- κ B or CREB did not completely block *MMP1* expression, suggesting that another factor(s) also contributes to the induction of *MMP1*. Depletion of CREB/NF- κ B or HIF-2 α efficiently blocked *MMP1* expression in MDA-MB-231 cells, whereas depletion of HIF-1 α blocked it moderately (Fig. 6*B*). Moreover, simultaneous depletion of NF- κ B/HIF-2 α or CREB/HIF-2 α further enhanced the degree of *MMP1* inhibition, indicating that CREB, NF- κ B, and HIF-2 function in parallel to regulate *MMP1* expression during prolonged hypoxia (Fig. 6*C*). Next, the secreted form of *MMP1* was monitored in the cell culture medium derived from CREB- and/or NF- κ B-depleted cells. A moderate but significant increase in *MMP1* activity in the culture medium was observed in MDA-MB-231 cells grown in hypoxic conditions (Fig. 6*D*).

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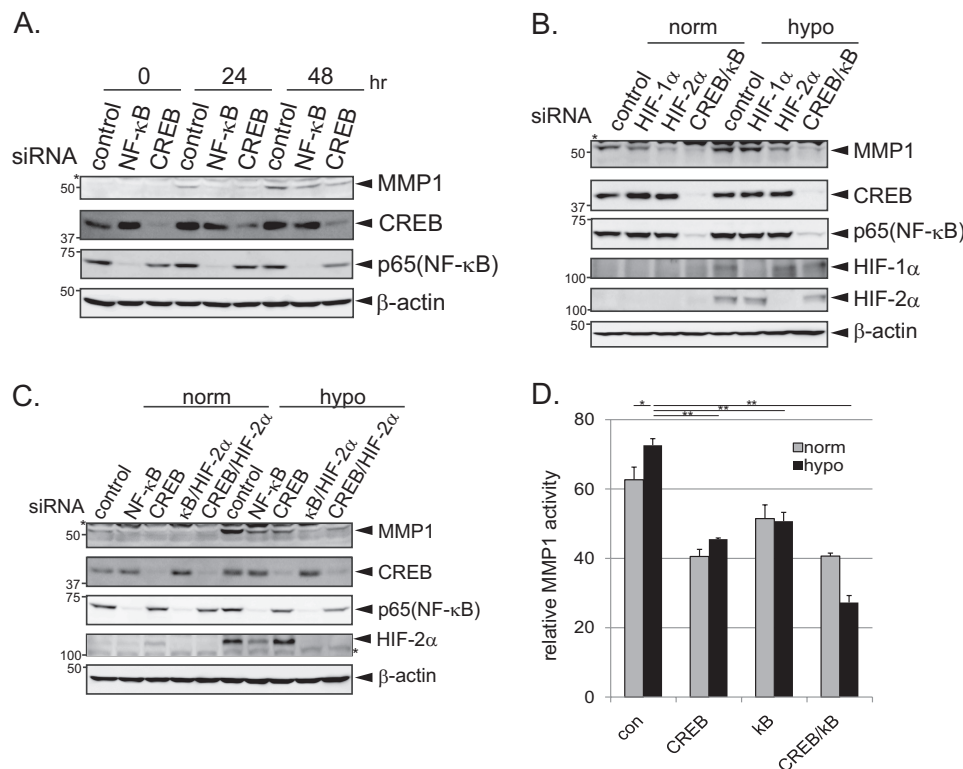


FIGURE 6. CREB and NF- κ B signaling pathways play an important role in the induction of MMP1 expression during prolonged hypoxia. *A*, shown is inhibition of MMP1 induction after depletion of NF- κ B or CREB. MDA-MB-231 cells were transfected with control, NF- κ B, or CREB siRNA and were cultured in hypoxic conditions for up to 48 h. Cell lysates were subjected to Western blotting. The asterisk indicates a nonspecific band detected by the antibody. *B*, shown is the effect of HIF-1 α , HIF-2 α , and CREB/NF- κ B depletion on MMP1 induction. MDA-MB-231 cells transfected with HIF-1 α , HIF-2 α , or CREB/NF- κ B siRNAs were cultured in normoxic or hypoxic conditions for 24 h, and cell lysates were subjected to Western blotting. The asterisk indicates a nonspecific band detected by the antibody. *C*, shown is the effect of NF- κ B/HIF-2 α and CREB/HIF-2 α siRNA on MMP1 induction. MDA-MB-231 cells transfected with corresponding siRNAs were cultured in normoxic or hypoxic conditions for 24 h, and cell lysates were subjected to Western blotting. Asterisks indicate nonspecific bands detected by the antibodies. *D*, shown is decreased MMP1 activity in cell culture medium after depletion of CREB and/or NF- κ B (*kb*). An ELISA assay was performed on cell culture medium collected from MDA-MB-231 cells transfected with CREB and/or NF- κ B siRNA and treated with normoxia or hypoxia (24 h) to measure MMP1 activity ($n = 3$). The graph shows relative collagenase activity. Error bars indicate S.D. Significance was statistically analyzed with a *t* test (*, $p < 0.05$; **, $p < 0.02$).

MMP1 activity was diminished after depletion of NF- κ B and/or CREB, further indicating that the hypoxic induction of MMP1 is dependent on these transcription factors (Fig. 6D).

Inhibition of the CREB and NF- κ B Signaling Pathways Reduces Cell Migration, Invasion, and Metastasis—Next, cell migration and invasion assays were performed to characterize the biological significance of the hypoxic induction of *MMP1*. MDA-MB-231 cells transfected with control, NF- κ B, CREB, CREB/NF- κ B, or MMP1 siRNAs were plated on collagen-coated dishes, and a single scratch was introduced. Depletion of CREB and/or NF- κ B reduced the ability of cells to migrate into the gap throughout the entire duration of the experiment (24 h) (Fig. 7A). Depletion of MMP1 also reduced the cell migration, yet the effect was moderate compared with CREB and/or NF- κ B depletion (Fig. 7A). Reduced migratory activity of CREB/NF- κ B-depleted cells was partly recovered when cultured in conditioned medium derived from MDA-MB-231 cells treated with hypoxia but not by that derived from MMP1 siRNA cells (supplemental Fig. 3). Significant reduction of migratory activity was only found on the collagen-coated dishes but not on fibronectin or poly-L-lysine surfaces (supplemental Fig. 4). Furthermore, the invasive activity of MDA-MB-231 cells was monitored using the transwell migration assay with collagen-coated wells. Control cells actively passed through the

collagen layer and moved to the lower well. This movement was inhibited after depletion of CREB and NF- κ B or HIF-2 α to a level comparable to that observed after MMP1 depletion (Fig. 7B). Similar inhibition was also observed in MDA-MB-231 cells stably expressing shRNA of CREB, NF- κ B, or MMP1 (supplemental Fig. 5). These results indicate that MMP1 is one of the critical factors to control cell migration and invasion on a collagen layer during prolonged hypoxia and whose expression is regulated by CREB/NF- κ B and HIF-2. Finally, the requirement of CREB and NF- κ B for the lung metastasis was examined in the mouse pulmonary metastasis model using MDA-MB-231 cells. Whereas control cells formed an average of 72 metastatic nodules in the lung, CREB- and NF- κ B-depleted cells formed significantly reduced numbers of nodules compared with the control cells (Fig. 7C). This result further indicates the importance of CREB and NF- κ B, which induces MMP1, for tumor metastasis under pathophysiological conditions.

DISCUSSION

In the present study, it has been demonstrated that CREB and NF- κ B are activated during prolonged hypoxia and cooperatively induce *MMP1* expression. Binding sites for CREB and NF- κ B were located adjacently in the *MMP1* promoter, and these sites are important for the induction of *MMP1* after pro-

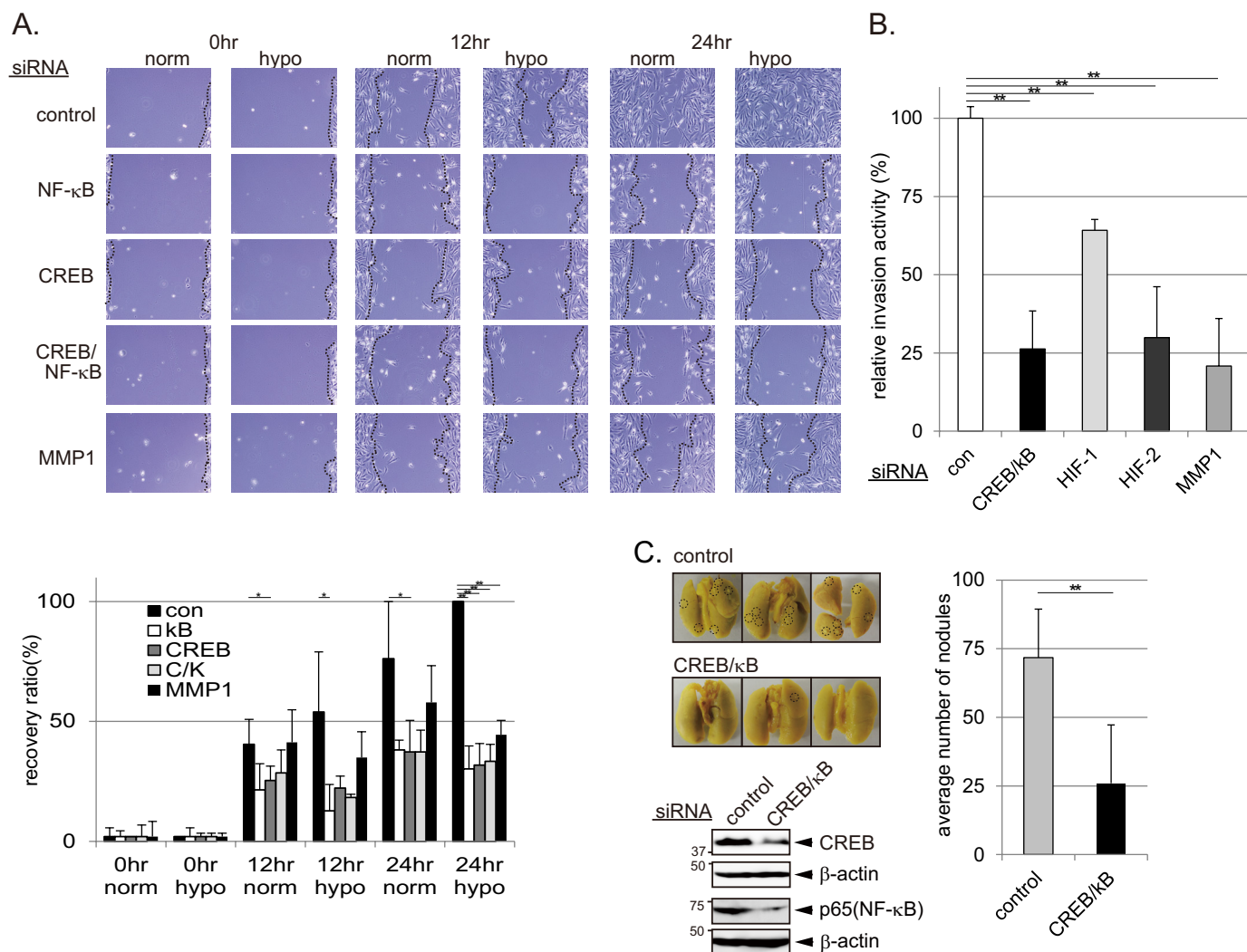


FIGURE 7. Inhibition of CREB and NF- κ B impairs cell migration, invasion, and metastasis. *A*, cell migration on collagen-coated dishes decreased after depletion of MMP1, CREB, and/or NF- κ B (*kb*). MDA-MB-231 cells transfected with siRNAs (as indicated in the figure) were plated on collagen-coated dishes and cultured in normoxic or hypoxic conditions for the indicated times. Representative images for each time point are shown. Recovery ratio (%) during the time course is shown in the graph (*A*, bottom) ($n = 3$). Error bars indicate S.D., and significance was statistically analyzed with a *t* test (*, $p < 0.05$; **, $p < 0.02$). *B*, cell invasion decreased after depletion of CREB and NF- κ B. MDA-MB-231 cells transfected with siRNAs (as indicated in the figure) were subjected to the transwell migration assay. Cells were cultured in hypoxic conditions for 48 h, and the average number of cells that passed through the collagen-coated membrane was determined ($n = 3$). Error bars indicate S.D., and significance was statistically analyzed with a *t* test (**, $p < 0.02$). *C*, lung metastasis was decreased by depletion of CREB and NF- κ B. Control or CREB/NF- κ B depleted MDA-MB-231 cells were injected to the tail vein of athymic *nu/nu* mice. Representative images of the lungs and the graph of the average number of metastatic nodules formed on the lung surface are shown ($n = 7$). Error bars indicate S.D., and significance was statistically analyzed with a *t* test (**, $p < 0.02$). Inhibition of CREB and NF- κ B in MDA-MB-231 cells was examined by Western blotting.

longed hypoxia. Although the hypoxic activation of HIF is mainly mediated by stabilization of its α subunit, the molecular mechanism underlying the hypoxic activation of CREB and NF- κ B is not well understood. NF- κ B is activated after the IKK-dependent phosphorylation and degradation of I κ B α (17). It has been shown that hypoxia activates NF- κ B signaling and that this is decreased in IKK- $\beta^{-/-}$ mice (5), indicating that the hypoxic activation of NF- κ B occurs through a canonical pathway mediated by the IKK complex (IKK- β , IKK- α , and NF- κ B essential modulator (NEMO)) (17). IKK- β is a PHD substrate (mainly PHD1), and prolyl-hydroxylation of IKK- β negatively regulates its kinase activity (18). PHD-dependent prolyl-hydroxylation may regulate IKK- β activity and thereby NF- κ B signaling during hypoxia. Because NF- κ B signaling was found to be mainly activated during prolonged hypoxia in MCF7, MDA-MB-231, and HeLa cells, it is possible that PHD activity is

maximally inhibited during this phase. However, it has been shown that PHD2 and PHD3 become reactivated during prolonged hypoxia, leading to the down-regulation of HIF-1 α (19). This is thought to be due in part to the up-regulation of PHD2 and PHD3 during prolonged hypoxia. In contrast, PHD1 is not up-regulated during hypoxia (20); therefore, it is possible that the PHD1 activity remains inhibited during prolonged hypoxia leading to activation of IKK- β and thereby NF- κ B signaling.

One of the hallmarks of CREB activation is phosphorylation of Ser-133, and this is significantly increased during prolonged hypoxia. This phosphorylation is mediated by several kinases, including protein kinase A, protein kinase C, Akt, Ca²⁺/calmodulin-dependent kinase, and MAPKAP (MAPK-activated protein) kinase 2 (7). Some of these kinases, including Akt, Ca²⁺/calmodulin-dependent kinase, and MAPKAP kinase are activated in hypoxic/ischemic conditions, and may, therefore,

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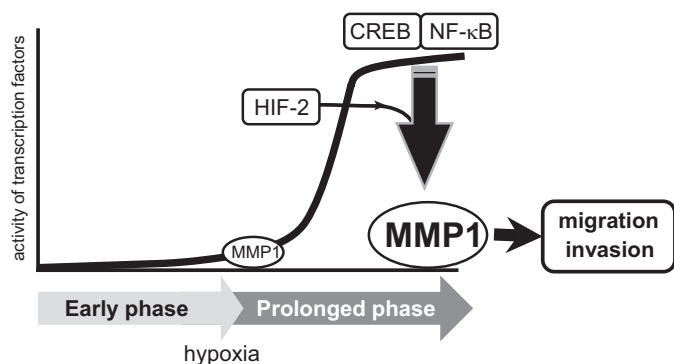


FIGURE 8. **Schematic diagram of the study.** Activity of CREB and NF- κ B is increased during prolonged phase of hypoxia. CREB and NF- κ B together with HIF-2 induces the expression of MMP1 under such condition. Induction of MMP1 is involved in enhanced migration and invasion of cancer cells.

contribute to the hypoxic activation of CREB (4, 21, 22). However, it is also reported that CREB activation in PC12 cells is mediated by none of these kinases (23), and the kinase responsible for regulating CREB activity during prolonged hypoxia still remains unclear. In addition to the phosphorylation-dependent activation of CREB, the stability of CREB is also regulated during hypoxia. CREB is reported to be ubiquitinated and degraded during the acute phase of hypoxia (24). However, it has been reported that SUMOylation of CREB increases during prolonged hypoxia, causing CREB to be stabilized and activated, and thereby leading to the induction of various genes such as *amphiregulin* (25, 26). Thus, CREB activation during prolonged hypoxia is mediated by at least two mechanisms, phosphorylation and stabilization.

There are several examples of CREB and NF- κ B acting cooperatively. For example, simultaneous activation of CREB and NF- κ B occurs in endothelial cells and astrocytes by phospholipase activation and β 2-adrenergic receptor signaling (27, 28). Moreover, protein kinase A expression is induced by NF- κ B, leading to activation of CREB and cooperation between these molecules (29). Although HIF signaling was induced/activated during acute hypoxia, CREB and NF- κ B signaling pathways were maximally activated during prolonged hypoxia (Fig. 3, F and G). When CREB or NF- κ B were activated separately, the activity of the *MMP1* promoter increased by \sim 3-fold, and this was increased to 6-fold when CREB and NF- κ B were activated together (Fig. 5D). Additionally, inhibition of CREB phosphorylation by staurosporine decreased the hypoxic induction of MMP1 (Fig. 5C). Importantly, possible roles of other kinases and their substrates besides the CREB pathway cannot be excluded in this experiment, as staurosporine inhibits multiple kinases. Yet, the synchronized activation of CREB and NF- κ B might be an important regulatory mechanism to enhance gene expression during prolonged hypoxia.

Generally, when cells are exposed to hypoxic conditions, HIF-1 α is immediately stabilized and induces the expression of various hypoxia-responsive genes (1). It is also reported that the expression of HIF-1 target genes is maintained during prolonged hypoxia (30). However, because HIF-1 α expression tends to decrease during prolonged hypoxia (30), expression of some HIF-1 target genes may similarly decrease, such as *VEGF* observed in this study (Fig. 1A). Therefore, maintenance of the

gene expression during prolonged hypoxia may be one of the important roles for prolonged hypoxic signaling, which involves CREB and NF- κ B (Fig. 8). Additionally, the prolonged hypoxic signaling induces a specific set of genes including *MMP1*, which may regulate changes in cell shape, migration, and invasion that are often observed during prolonged hypoxia (31). Thus, it is likely that the combinatory effect of genes regulated by CREB and NF- κ B as well as another transcription factor(s) determines the migratory and invasive activity of cancer cells under such condition. The time window during which HIF-1 activity decreases and the activities of CREB and NF- κ B increase may signify the shift from acute to prolonged hypoxic responses. Changes in the activities of signaling pathways would be responsible for alterations in global gene expression at different stages of hypoxia.

Finally, this study demonstrates that MMP1 expression is up-regulated during prolonged hypoxia. Because MMP1 possesses type I collagenase activity, its expression is linked to the invasion and metastasis of cancer cells (9). NF- κ B is reported to also induce several MMPs upon cytokine activation, including MMP9, MMP13, and MT1-MMP (10, 32, 33). Similarly, CREB induces MMP2, MMP9, and MMP13 (34, 35). Therefore, synchronized activation of CREB and NF- κ B during prolonged hypoxia might control the timing of metastasis, which is mediated by the orchestration of MMP1 and other MMPs (9). Because induction of MMP1 was observed in both cancerous MCF7/MB231 cells and non-cancerous HUVECs, which have a different metabolic status, hypoxic induction of MMP1 would be involved in various physiological processes besides tumor invasion and metastasis. In articular cartilage cells, IL-1 β increases the level of HIF-2 α and induces MMP1, leading to the destruction of osteoarthritic cartilage (14). This is consistent with our results showing that HIF-2 is an important regulator of MMP1 during prolonged hypoxia, although HIF-1 also appears to be involved in this process moderately. HIF-1 also regulates *MMP1* expression in different cell lines, such as mesenchymal stem cells and lung adenocarcinoma cells (36, 37). Thus, the requirement of HIF-1 or HIF-2 to induce MMP1 might also depend on cell types. Similarly, induction profile of MMP1 may also be different between the cell types depending on the activity of CREB and NF- κ B. It has been reported that the activity of HIF-2 is higher than that of HIF-1 during prolonged hypoxia in certain cell lines (38, 39). Therefore, CREB, NF- κ B, and HIF-2 might constitute a network that regulates gene expression during prolonged hypoxia (Fig. 8). Further study is needed to identify the target genes regulated by the interaction of these transcription factors during prolonged hypoxia.

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