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Identification of the *KAI1* metastasis suppressor gene as a hypoxia target gene

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ABSTRACT

KAI1 is a metastasis suppressor gene known to inhibit cancer metastasis without affecting primary tumorigenicity. Although KAI1 expression has been reported to undergo transcriptional regulation, how its expression is up- or down-regulated by specific upstream signaling pathways has not been studied in detail. In this study, we characterized the regulatory elements within the 500 bp upstream region of mouse *KAI1* gene and identified a functional hypoxia-response element (HRE) within the promoter region. Hypoxia-dependent induction of KAI1 was directly mediated by hypoxia-inducible factor (HIF)-1α binding on the promoter, which subsequently caused increased recruitment of RNA polymerase II for transcriptional activation. The failure of HIF-1α recruitment to the *KAI1* promoter was observed in *Hif-1α* knockout mouse embryonic fibroblasts. Furthermore, KAI1 protein synthesis was markedly increased in ischemic tissues, suggesting that *KAI1* is a hypoxia target gene *in vivo*.

Introduction

Under hypoxic conditions, a mismatch between oxygen supply and its demand occurs at the cellular level. Oxygen deficiency in the body as a whole affects not only physiological processes including embryonic development, wound healing, and inflammation, but also pathological conditions such as tumor progression, ischemic disease, and atherosclerosis [1,2]. To overcome the local depletion of oxygen and nutrients, cells use adaptive measures that involve genetic changes [3,4]. One of the well-known genetic changes in tumor hypoxia is the loss of *von Hippel–Lindau* (*VHL*) gene [5], which is a tumor suppressor gene that is responsible for the ubiquitination of HIF. It has been reported that *VHL* gene loss is linked to the progression of retinal hemangioblastoma and clear-cell renal carcinoma via HIF activation [6].

HIF is a key transcription factor in several hypoxia-mediated transcription processes [7]. It consists of one of the three HIF-α (HIF-1α and HIF-2α) subunits and a common β-subunit (HIF-1β). Under normoxic conditions, HIF-α subunit is modified by prolyl hydroxylases (PHDs) that are activated at a high oxygen concentration [8], and the hydroxylated HIF-α binds to VHL E3 ligase and undergoes proteasomal degradation. However, under hypoxic conditions, PHD activity is reduced, thereby stabilizing HIF-α [9]. Following the stabilization, HIF-α translocates into the nucleus and binds to HIF-β. The HIF-α/β heterodimer binds to the HRE that contains ACGTG as a core sequence. Hypoxia target genes containing functional HRE affect diverse cellular processes including apoptosis (*NIX*, *RTP801*), erythropoiesis (*EPO*), angiogenesis (*VEGF*), glucose metabolism (*GLUT1*), and transcriptional regulation (*NUR77*) [2].

KAI1 is a metastasis suppressor gene that inhibits cancer metastasis without affecting primary tumorigenesis [10]. KAI1 has been shown to function as a metastasis suppressor in breast, prostate, liver, and lung cancers [11]. Structurally, KAI1 belongs to the tetraspanin family, most of which having four transmembrane domains [12]. It has been reported that KAI1 suppresses cancer metastasis by inhibiting integrin-mediated cell migration or by attenuating the EGF signaling pathway through facilitating EGFR internalization [13]. Cell surface interactions between KAI1 and the decoy cytokine receptor DARC on vascular cells has been shown to promote tumor cell senescence [14].

Previously, we have reported the transcriptional regulation of KAI1 mediated by Tip60/pontin coactivator and β-catenin/reptin corepressor complexes [15]. In metastatic cancer cells, IL-1β treatment failed to induce KAI1 expression because the reptin/β-catenin repressor complex, which is present at high levels, competes with the Tip60/pontin coactivator complex. In addition, the overexpression of p53 has been shown to increase *KAI1* promoter activity [16]. In sarcomas, the level of KAI1 is regulated by proteasomal degradation, and this degradation process is mediated by the E3 ubiquitin ligase, gp78 [17]. In this study, we report that the *KAI1* gene pro-

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moter encompasses a functional HRE and that the KAI1 protein synthesis is induced in ischemic tissues, suggesting that *KAI1* is a hypoxia target gene *in vivo*.

Materials and methods

Cell culture and hypoxia treatment. HEK293, NIH3T3, and mouse embryonic fibroblast (MEF) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin at an atmospheric CO_2 concentration of 5% at 37 °C. The calcium phosphate method (Invitrogen) and Effectene transfection reagent (QIAGEN) were used for the transfection of HEK293 and NIH3T3 cells, respectively. For the hypoxic challenge, cells were placed in a hypoxia work station (Ruskinn) which reduces O_2 and CO_2 tension to approximately 0.1% and 5%, respectively, for the indicated time periods. For HIF-1α activation, cells were treated with 100 μM desferrioxamine mesylate (DFO, Sigma).

Antibodies. The commercially available antibodies used were as follows: anti-HIF-1α, anti-CBP, anti-p300, and anti-KAI1 (Santa Cruz Biotechnology); anti-HIF-1α (Cayman); and anti-RNA polymerase II (Berkeley Antibody Company).

Luciferase reporter assay. For the luciferase reporter construct, a 597-bp DNA fragment was cloned into a pGL2-basic reporter vector (Promega) by using the PCR method. The primers used were KAI1p-KpnI-F (5′-CAGGTACCCCACGCCATTCCCGGGTTG-3′) and KAI1p-XhoI-R (5′-CACTCGAGCGCCACGCCCCCCAAGAC-3′). The nPfu-forte DNA Polymerase Kit (Enzynomics) was used to introduce mutations into the HRE region. For the luciferase assay, NIH3T3 and HEK293 cells were transfected by the Effectene transfection reagent and the calcium phosphate method, respectively. After 24 h of transfection, NIH3T3 or HEK293 cells were placed into the hypoxia work station or treated with DFO for an additional 24 h. Luciferase activity was measured using the Luciferase Reporter Assay System (Promega) and normalized with β-galactosidase activity.

Real-time RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen), and cDNA was synthesized with RevertAid™ M-MuLV Reverse Transcriptase (Fermentas). Semi-quantitative real-time reverse transcription (RT)-PCR was performed using 50–100 ng total RNA by the SYBR Green method. The primer sequences were as follows: sense, 5′-TGCTCCTGCGAGAAGATCAA-3′ and antisense, 5′-TGACAGCAACACCAGCACAC-3′ for KAI1; sense, 5′-TAGCCATCCAGGCTGTGCTG-3′ and antisense, 5′-CAGGATCTTCATGAGGTAGTC-3′ for β-actin; sense, 5′-CTGTGCAGGCTGCTGTAACG-3′ and antisense, 5′-GCTCATTCTCTCTATGTGCTGGC-3′ for VEGF-A.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation assay was performed as previously described in Ref. [15], with the average size of sheared fragments being approximately 0.5–1 kb. For PCR, 2 μl out of 35 μl eluted DNA was used and 30–35 cycles of amplification were performed. The primer sequences were as follows: KAI1-HRE-F, 5′-GGAGACCATAGGGGGTGAGACT-3′ and KAI1-HRE-R, 5′-ACACTGAGCTGGCTACCCTTTG-3′, which generated a 201-bp fragment; VEGF-A-HRE-F, 5′-GCCAGACTACACAGTGCATA-3′ and VEGF-A-HRE-R, 5′-GCTTATCTGAGCCCTTGTCTG-3′.

Induction of myocardial infarction. All of the procedures were performed in accordance with the Institutional Animal Care and Use Committee of Seoul National University Hospital, and the investigators conformed to the National Research Council's "Guide for the Care and Use of Laboratory Animals" (revised 1996). Female Sprague–Dawley rats aged 8 weeks were anesthetized with ketamine hydrochloride (100 mg/kg, Yuhan Corp) and xylazine (10 mg/kg, Bayer) by intraperitoneal injection. Rats were intubated and artificially ventilated with a mechanical ventilator (model 683; Harvard Apparatus). A left thoracotomy was performed at the fourth intercostal space and the pericardium was opened. The left anterior descending artery (LAD) was ligated using 6–0 silk sutures as previously described [18,19]. After ligation of the proximal LAD, the middle and apical portion of the left ventricle was observed for the evidence of myocardial blanching and akinesia indicating the interruption of coronary flow, and then the chest was closed in layers.

Immunohistochemical analysis. After 3 days of myocardial infarction, rats were euthanized, hearts were perfused retrogradely through the right carotid artery with PBS and formaldehyde, and the tissues were embedded in paraffin. The tissue sections were deparaffinized and antigen retrieval was performed using citrate buffer. KAI1 expression was evaluated in the center of infarction, peri-infarct border zone, and contralateral remote zone by immunohistochemical staining. Rabbit polyclonal anti-KAI1 antibody was applied. Biotinylated anti-rabbit IgG and Vectastain Elite ABC kit (Vector Laboratories) were used for detection. Mayer's hematoxylin was applied for counterstaining. The sham-operated rat hearts were used as a control.

Results

KAI1 promoter contains functional hypoxia-response element

We have previously reported that the *KAI1* metastasis suppressor gene is regulated by Tip60/pontin coactivator and β-catenin/reptin corepressor complexes [15]. In order to understand how *KAI1* gene expression is up- or down-regulated by upstream signaling pathways, we focused on the regulatory elements within the 0.5-kb upstream non-coding region of the mouse *KAI1* gene. Using the PromoSer software available on the Internet (http://biowulf.bu.edu/zlab/PromoSer/), we searched for the promoter region of the *KAI1* gene for potential binding sites of transcription factors. Interestingly, we found the potential HRE sequence ACGTG within the 0.2-kb upstream region of the *KAI1* gene (Fig. 1A).

In order to determine whether this 0.5-kb DNA segment is sufficient to drive reporter gene expression, the 0.5-kb upstream promoter region encompassing the HRE was sub-cloned into a pGL2-basic luciferase reporter plasmid. We found that the presence of 0.5-kb upstream promoter region encompassing the HRE was sufficient to drive luciferase activity by hypoxia, whereas the one with HRE mutation failed to stimulate luciferase activity which could otherwise be induced by hypoxia (Fig. 1B). We treated cells with a hypoxia-mimicking reagent DFO to confirm that the HRE on the *KAI1* promoter was indeed functional. DFO treatment stimulated *KAI1* promoter reporter activity, whereas DFO treatment had little or no activation of the *KAI1* promoter reporter containing the mutated HRE (Fig. 1C). Furthermore, overexpression of HIF-1α activated *KAI1* transcription, whereas *KAI1* promoter reporter containing mutated HRE failed to be induced regardless of HIF-1α presence (Fig. 1D). Together, these data support the idea that the *KAI1* promoter contains a functional HRE, of which its activity can be induced by hypoxia, DFO treatment, or HIF-1α overexpression.

KAI1 promoter occupancy by HIF-1α and CBP coactivator induced by hypoxia

To prove further that HIF-1α is the DNA-binding transcription factor that directly binds to the *KAI1* promoter, we examined the binding affinity using the ChIP assay in NIH3T3 cells. Since *KAI1* mRNA expression is induced by hypoxic conditions (Fig. 2A), we examined whether the CBP coactivator, a well known HIF-1α coactivator, is recruited to the *KAI1* promoter along with HIF-1α. As ob-