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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-

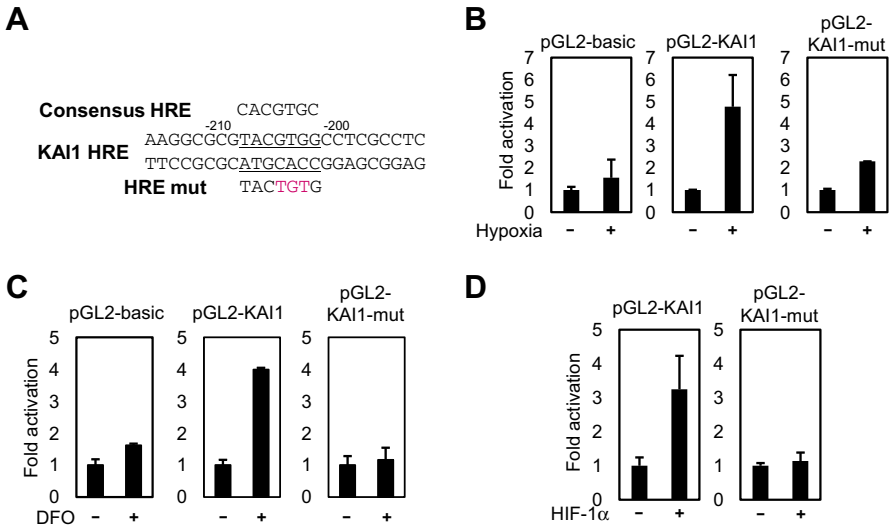

Fig. 1. Identification of functional hypoxia-response element in the *KAI1* promoter. (A) Schematics of the HRE located in the *KAI1* promoter in mice. (B,C) NIH3T3 cells were transfected with *KAI1* promoter-luciferase (pGL2-KAI1), *KAI1* promoter-luciferase with a mutated HRE (pGL2-KAI1 mut), or control vector (pGL2-basic). Luciferase activity was measured 24 h after hypoxic or normoxic conditions (B), in the presence or absence of 100 μM DFO for 24 h (C). The data are represented as the mean ± SD and are representative of three separate experiments. (D) Luciferase activity was measured in the absence or presence of HIF-1α in HEK293 cells. The data are represented as the mean ± SD and are representative of three separate experiments.

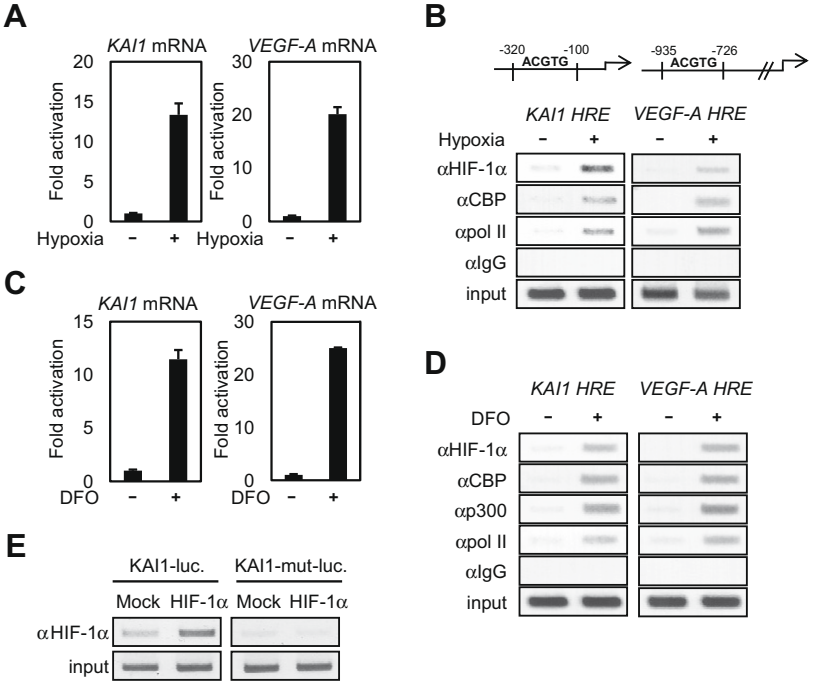

Fig. 2. *KAI1* promoter occupancy by HIF-1α and CBP coactivator under hypoxic conditions. (A) Real-time RT-PCR analysis of *KAI1* and *VEGF-A* transcripts under hypoxic condition. NIH3T3 cells were grown in hypoxic or normoxic conditions for 12 h. *VEGF-A* was used as a positive control. The data are represented as the mean ± SD and are representative of three separate experiments. (B) ChIP analysis was conducted on the *KAI1* and *VEGF-A* promoters from NIH3T3 cells grown in hypoxic or normoxic conditions for 6 h. The *VEGF-A* HRE region was used as a positive control. Occupancy of the promoters by HIF-1α, CBP, and RNA polymerase II is indicated. (C) Real-time RT-PCR analysis of *KAI1* and *VEGF-A* transcripts in NIH3T3 cells in the absence or presence of 100 μM DFO for 12 h. (D) ChIP analysis was conducted on the *KAI1* and *VEGF-A* promoters from NIH3T3 cells with treatment of 100 μM DFO for 6 h. Occupancy of the promoters by HIF-1α, CBP, and RNA polymerase II is indicated. (E) ChIP assay was performed 48 h after transfection of pGL2-*KAI1* promoter-luciferase in the absence or presence of HIF-1α in HEK293 cells. HIF-1α occupancy of the *KAI1* promoters, but not the *KAI1* promoter with mutated HRE, is indicated.

served in Fig. 2B, the hypoxic conditions induced HIF-1α occupancy on the *KAI1* and *VEGF-A* promoters. As expected, the CBP coactivator and RNA polymerase II were recruited to the *KAI1* and *VEGF-A* promoters (Fig. 2B).

To determine whether DFO treatment brings the same effect as does hypoxia, we treated cells with DFO to stabilize HIF-1α. Indeed, DFO treatment increased the *KAI1* and *VEGF-A* transcript levels (Fig. 2C). Moreover, the ChIP assay conducted in NIH3T3 cells after DFO treatment revealed that HIF-1α, p300, and CBP were recruited to the promoter followed by increased recruitment of RNA polymerase II for transcriptional activation (Fig. 2D).

To verify whether HIF-1α is the DNA-binding protein that tethers CBP to the *KAI1* promoter, we carried out the ChIP assay to test the HIF-1α binding to the 0.5-kb upstream *KAI1* promoter with

HRE as well as to the *KAI1* promoter containing the mutated HRE as a negative control. As expected, ChIP assay revealed that localization of HIF-1α was inhibited on the *KAI1* promoter containing the mutated HRE (Fig. 2E). Taken together, these data extend our findings for *KAI1* promoter encompassing a functional HRE and reveal an important contribution of hypoxia on the regulation of *KAI1* gene expression, which is mediated by direct HIF-1α binding on the functional HRE.

Failure of KAI1 induction in Hif-1α knockout MEFs

We used *Hif-1α* knockout (KO) (*Hif-1α*$^{-/-}$) MEFs to examine whether hypoxia-dependent *KAI1* induction is abolished in *Hif-1α*$^{-/-}$ MEFs. In wild-type (WT) MEFs, a hypoxic challenge for 12 h resulted in approximately 4-fold induction of *KAI1* gene expression, whereas hypoxia-dependent *KAI1* induction was almost completely abolished in *Hif-1α*$^{-/-}$ MEFs (Fig. 3A). In parallel, HIF-1α stabilization by DFO treatment increased the *KAI1* transcript level in WT MEFs, whereas *KAI1* induction was significantly diminished in *Hif-1α*$^{-/-}$ MEFs (Fig. 3B). These results support the idea that HIF-1α is a crucial transcription factor whose activity is sufficient to confer the induction of *KAI1* under hypoxic conditions.

Further, we performed the ChIP assay to monitor HIF-1α recruitment and the activation of *KAI1* and *VEGF-A* promoters in WT or *Hif-1α*$^{-/-}$ MEFs under hypoxic conditions (Fig. 3C). In *Hif-1α*$^{-/-}$ MEFs, failure of HIF-1α recruitment resulted in diminished recruitment of RNA polymerase II, indicating the transcriptional repression of *KAI1* and *VEGF-A* under hypoxic conditions. In parallel, DFO treatment resulted in little or no induction of *KAI1* and *VEGF-A* in *Hif-1α*$^{-/-}$ MEFs (Fig. 3D). These data strongly demonstrate that *KAI1* is a direct HIF-1α target gene induced by hypoxia.

KAI1 expression is up-regulated in the ischemic heart in vivo

Since *KAI1* turned out to be a direct HIF-1α target gene, we next examined whether KAI1 induction is observed under hypoxic conditions *in vivo*. We decided to implement the myocardial infarction (MI) model in rats by coronary ligation to verify hypoxia-induced KAI1 expression under ischemic and hypoxic conditions *in vivo*. Three days after MI, we extracted heart muscles and evaluated KAI1 expression by immunohistochemical staining using the anti-KAI1 antibody (Fig. 4A, $n = 4$). The sham-operated rat hearts were used as a control (Fig. 4B, $n = 2$). In the control group, a small portion of endothelial cells within coronary capillaries expressed KAI1, and none of the cardiomyocytes expressed KAI1. However, in the experimental group, KAI1 expression was strongly induced in cardiomyocytes, vascular smooth muscle cells, endothelial cells, fibroblasts, and infiltrated inflammatory cells in the center of the infarct region as well as in the peri-infarct border zone. Furthermore, the remote zone from the site of infarction showed mild upregulation of KAI1. Together, these findings indicate that KAI expression is markedly induced by ischemic and hypoxic conditions *in vivo*.

Discussion

Hypoxia affects diverse cellular processes including apoptosis, erythropoiesis, angiogenesis, and glucose metabolism. Identifying the direct hypoxia target genes and their positive and negative regulators for HIF-1 is a highly active research area. In this study, we newly identified a functional HRE in the *KAI1* promoter located in the 0.2-kb upstream region of the mouse *KAI1* gene, and confirmed its function by both luciferase and ChIP assays. The hypoxia-dependent activation of the *KAI1* gene was almost completely abolished in *Hif-1α*$^{-/-}$ MEFs, demonstrating that hypoxia-dependent KAI1 induction is directly mediated by the HIF-1α and that its recruitment on the promoter with the CBP coactivator is crucial for increased KAI1 expression under hypoxic conditions.

It has been reported that HIF-α has two contrasting functions, which are activation and inhibition of tumor progression [20,21]. Generally, it is considered that hypoxic conditions are favorable for tumor progression. High level of HIF-α expression correlates with poor prognosis since the unlimited proliferation of tumor cells causes hypoxic conditions in solid tumors. In turn, the persis-

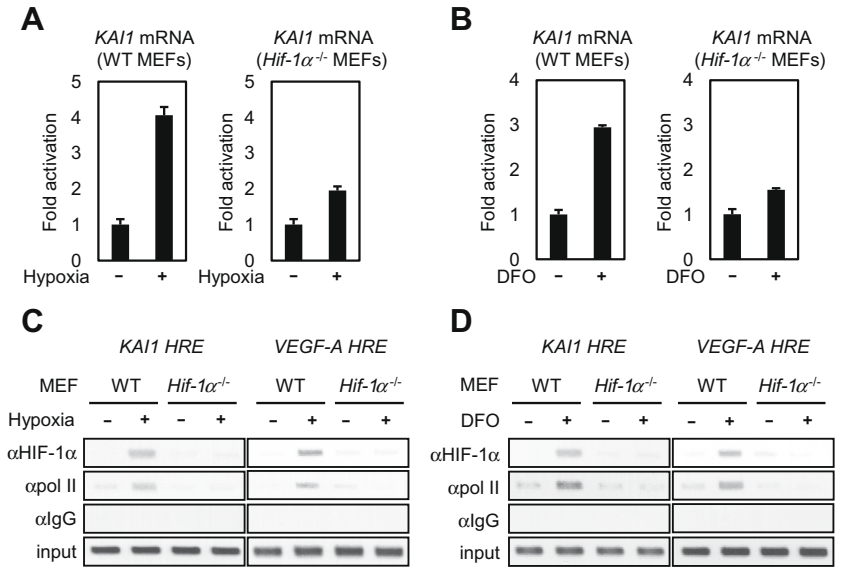

Fig. 3. Failure of hypoxia-dependent induction of *KAI1* in *HIF-1α* knockout MEFs under hypoxia. (A,B) Relative transcript levels of *KAI1* in WT or *Hif-1α*$^{-/-}$ MEFs under hypoxia. MEFs were grown under hypoxic conditions for 12 h (A) or with treatment of 100 μM DFO for 12 h (B), and real-time RT-PCR analysis of *KAI1* was performed. The data are represented as the mean ± SD and are representative of three separate experiments. (C,D) ChIP assay was performed on the *KAI1* and *VEGF-A* promoters in WT and *Hif-1α*$^{-/-}$ MEFs. MEFs were grown in hypoxic or normoxic conditions for 6 h (C) or in the presence of 100 μM DFO for 6 h (D). The *VEGF-A* HRE region was used as a positive control.

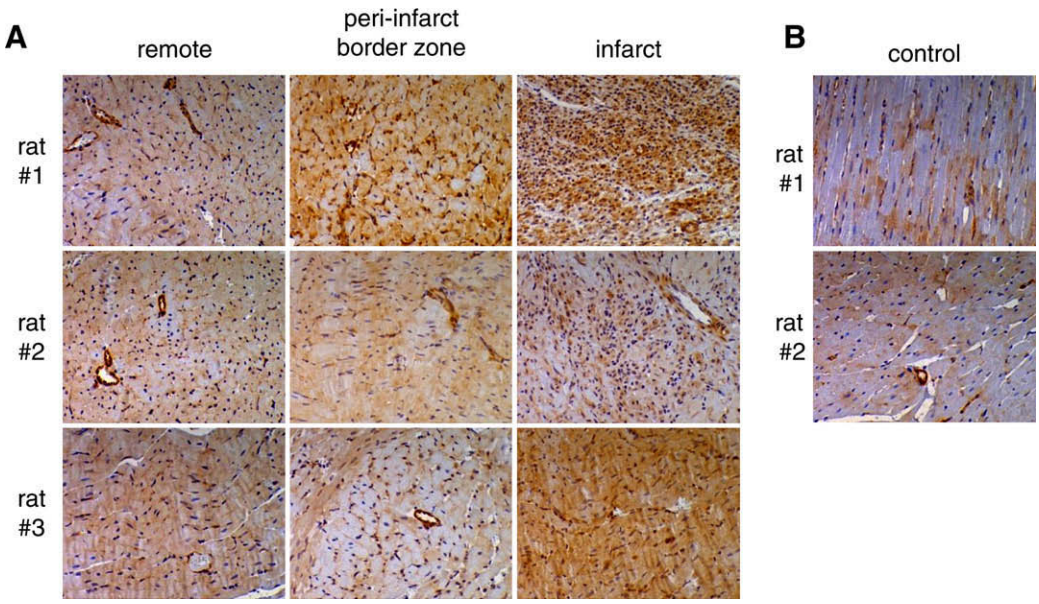

Fig. 4. KAI1 expression is up-regulated in the ischemic heart *in vivo*. (A,B) Myocardial infarction (MI) experiments in rats by coronary ligation to verify hypoxia-induced KAI1 expression under ischemic/hypoxic conditions *in vivo*. Three days after MI, the hearts were harvested and KAI1 expression was evaluated by immunohistochemical staining using anti-KAI1 antibody (a, $n = 4$). The sham-operated rat hearts were used as a control (b, $n = 2$).

tence of hypoxic conditions increases HIF-α levels, which leads to the activation of certain hypoxia target genes related to angiogenesis, metabolism, and proliferation [22].

Given that *KAI1* is a metastasis suppressor gene that inhibits cancer metastasis, it is tempting to speculate that hypoxia-induced *KAI1* protein might function to inhibit tumor metastasis through a negative regulatory circuit under hypoxic conditions. There have been reports suggesting that HIF-α acts as an inhibitor of cancer progression [23]. Tumors derived from *Hif-1α*$^{-/-}$ embryonic stem (ES) cells grow larger tumors than those derived from WT ES cells do [24]. Moreover, in glioblastomas and VHL-deficient fibrosarcomas, HIF activation inhibits tumor progression by decreasing cell proliferation and increasing apoptosis [25].

Considering that there exist putative negative regulators in hypoxic conditions that enhance the ability of cells to survive and adapt [26], it is possible that KAI1 is able to negatively regulate adaptive cell survival by controlling certain target genes under hypoxic conditions. Our model of myocardial infarction linked to the induction of KAI1 expression *in vivo* supports the role of KAI1 as a metastasis suppressor under hypoxic conditions. Alternatively, hypoxia-induced KAI1 might function to regulate a subset of hypoxia target genes under certain physiological conditions such as wound healing and inflammation. Collectively, our findings address the significance of hypoxia-induced KAI1 in the context of human cancer and shed light on possible development of therapeutic strategies for human cancer.

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