Cannabinoid receptors promote chronic intermittent hypoxia-induced breast cancer metastasis via IGF-1R/AKT/GSK-3β

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The progression of breast cancer is closely related to obstructive sleep apnea-hypopnea syndrome (OSAHS). Low concentrations of cannabinoids promote tumor proliferation. However, the role of cannabinoid receptors (CBs) in chronic intermittent hypoxia (CIH)-induced breast cancer has not been reported. The migration and invasion of breast cancer cell lines (MCF-7 and T47D) were measured by scratch assay and transwell assay. Gene and protein expressions were analyzed by qPCR and western blotting. Tumor xenograft mice model were established to evaluate the function of CBs. We observed that chronic hypoxia (CH) and CIH increased CBs expression and promoted migration and invasion in breast cancer. Mice grafted with MCF-7 exhibited obvious tumor growth, angiogenesis, and lung metastasis in CH compared with CH and control. In addition, CIH induced CBs expression, which subsequently activated insulin-like growth factor-1 receptor (IGF-1R)/AKT/glycogen synthase kinase-3β (GSK-3β) axis. Knockdown of CBs alleviated CIH-induced migration and invasion of breast cancer in vitro. Furthermore, CIH exaggerated the malignancy of breast cancer and silencing of CBs suppressed tumor growth and metastasis in vivo. Our study contributed to understanding the role of CIH in breast cancer development modulation.

INTRODUCTION

Breast cancer is the second leading cause of death among all types of cancers in women, and it tends to spread lymphatically and hematologically, leading to distant metastasis and poor prognosis. Current treatment strategies for breast cancer are surgery and chemotherapy. However, the initial response of chemotherapy is poor, and patients receiving it will develop multidrug resistance gradually. Recent studies demonstrated that nearly 50% of locally advanced breast cancer exhibits hypoxia and/or anoxic tissues. Hypoxia can be recognized as a mortality predictor in various tumors. Under the hypoxic conditions, numerous physiological processes are impaired and cancer cells take strategies to adapt to this hostile environment, which enable them to better survive. Thus, understanding the role of hypoxia-induced breast cancer progression will provide a powerful prevention and treatment tool for breast cancer treatment and failure control.

Obstructive sleep apnea-hypopnea syndrome (OSAHS) is characterized as random hypoxemia at night, hypercapnia, snoring with paused or shallow breathing, and disordered sleep. It is such a highly prevalent condition throughout our life that it has been recognized as a major healthy issue in the world. Chronic intermittent hypoxia (CIH) is the main pathogenesis of OSAHS, whose hallmark features can be recapitulated by CIH. Numerous evidences demonstrated that OSAHS is associated with metabolic and cardiovascular disease, as well as behavioral and cognitive dysfunctions. In the past decades, OSAHS is associated with higher rates of cancer and increased cancer mortality given the well-known role of hypoxia in cancer progression. Tumor hypoxia induces cellular processes, leading to adaptation and survival, such as angiogenesis, metastasis, and resistance to radio- and chemotherapy. Based on a cohort of approximately 5.6 million individuals in the United States, Gozal et al. found that the incidence of all cancer diagnoses combined are similar in OSAHS and retrospectively matched cases. Payne et al. indicated that, independent of the size of the primary malignancy, there is a strong association between OSAHS and the prevalence of cancer of oral cavity.
and oropharynx. Intermittent hypoxia has also been reported to alter the expression of pro-metastatic genes through nuclear factor κB (NF-κB) in inflammatory breast cancer cells.\textsuperscript{12} However, the effects of OSAHS on breast cancer and the underlying mechanisms remain elusive.

Cannabinoid receptors (CBs), are members of the G protein coupled receptor family, expressed throughout the body.\textsuperscript{13} CB1 and CB2, two known subtypes of CBs, are reported to participate in a variety of physiological processes, such as learning, memory, pain sensation, etc.\textsuperscript{14} And the role of CB1 and CB2 has been extensively investigated, and their contradictory role in various cancers has attracted increasing interest in the past decades.\textsuperscript{15} Several evidences validated the anti-tumor efficacy of CBs, but studies reported that low concentrations of cannabinoids can promote proliferation of breast cancer.\textsuperscript{16} However, little is known about their role in tumor generation and progression. Moreover, in central nervous system (CNS), CIH is reported to induce the cognitive impairment through CB1 and CaMKII,\textsuperscript{7} suggesting that CB1 and CB2 may represent an important cause of CIH-induced breast cancer malignancy. However, it remains elusive whether CB1 and CB2 play an important role in the proliferation and metastasis of breast cancer cells exposed to CIH.

In the present study, we aimed to explore the role of CBs in breast cancer and the underlying mechanism of pro-malignancy efficacy. We found that CIH promoted the migration and invasion of breast cancer cells through enhancing CB1 and CB2 expressions. Overexpression of CBs increased the expression of proteins, including Ki-67, cyclin D1, N-cadherin, and vimentin, via insulin-like growth factor-1 receptor (IGF-1R)/V-akt murine thymoma viral oncogene homolog (AKT)/glycogen synthase kinase-3β (GSK-3β) signaling pathway. In addition, silencing of CBs inhibited CIH-induced proliferation and metastasis of breast cancer \textit{in vitro} and \textit{in vivo}. Our study revealed that CBs might be a new therapeutic strategy in breast cancer treatment.

RESULTS

\textbf{CH and CIH promoted cells migration and invasion of breast cancer \textit{in vitro}}

MCF-7 cells were used to investigate the effects of hypoxia on breast cancer. The scratch assay and transwell assay were employed to study the cell migratory and invasive capability. The relative migration rate of MCF-7 cells under CIH condition was about 80%, which was significantly higher than that of control and CH (Figures 1A and 1B). And transwell assay results demonstrated that hypoxia enhanced the tumor cell invasion of CH and CIH group compared to the control and even more in CIH than CH (Figures 1C and 1D). Subsequently, the protein expression of Ki-67, cyclin D1, N-cadherin, and vimentin, via insulin-like growth factor-1 receptor (IGF-1R)/V-akt murine thymoma viral oncogene homolog (AKT)/glycogen synthase kinase-3β (GSK-3β) signaling pathway. In addition, silencing of CBs inhibited CIH-induced proliferation and metastasis of breast cancer \textit{in vitro} and \textit{in vivo}. Our study revealed that CBs might be a new therapeutic strategy in breast cancer treatment.
poor prognosis in various cancers. And we further detected the expression of CBs in MCF-7 cells under hypoxic conditions. The qPCR and western blotting results showed that CB1 and CB2 were upregulated in CH and CIH compared to control in MCF-7 cells (Figures 1G–I), and the levels of CBs were higher in CIH group than CH group. Taken together, CH and CIH facilitated proliferation, cell migration, and invasion of MCF-7 cells.

**CH and CIH promoted metastasis of breast cancer in vivo**

Next, we investigated the effects of CH and CIH on breast cancer with tumor xenografts nude mice model. Results revealed that the tumor volume was dramatically increased in CH and CIH group compared to control, and the difference between CIH and control was more significant than that of CH and control (Figures 2A and 2B). Meanwhile, more tumor nodules were observed in lungs of mice exposed to CH and CIH and even more in the latter (Figures 2C and 2D). Tumor tissues sections were subjected to hematoxylin and eosin (H&E) staining and immunohistochemical staining for CD31. The results showed that blood vessel formation was increased in CH and CIH group, especially in CIH group (Figure 2E). Western blotting displayed that, similar to the results in vitro, under CH and CIH conditions, Ki-67, cyclin D1, N-cadherin, vimentin, and Snail1 expressions increased and E-cadherin expression decreased (Figures 2F and 2G). Furthermore, we found that the expressions of CB1 and CB2 were enhanced in CH and CIH compared with the control group in breast cancer tissues of mice housed (Figures 2H–J). And the induction efficacy of CIH was higher than CH. Thus, in the following study, we would focus on the CIH treatment. Collectively, these results suggested that CH and CIH accelerated metastasis of breast cancer in vivo.

**Knockdown of CBs suppressed the migration and invasion of MCF-7 cells under CIH condition**

To investigate the role of endogenous CBs in metastasis of breast cancer, CB1 and CB2 were knocked down with corresponding short hairpin RNA (shRNA) in MCF-7 cells to detect cell migratory and invasive capability. As shown in Figures 3A and 3B, scratch assay results demonstrated that knockdown of CB1 and CB2 suppressed migration of MCF-7 cells compared to the control group. The invasive capability was also compromised in MCF-7 cells transfected with sh-CB1 or sh-CB2 as trans-membrane cell number decreased.

**Figure 2. CH and CIH promoted metastasis of breast cancer in vivo**

(A) Representative images of tumors from mice in control, CIH, or CH over 22 days following MCF-7 xenograft. (B) The tumors’ volumes from mice in control, CIH, or CH over 22 days following MCF-7 xenograft are shown. (C and D) Metastatic nodules in the lungs of mice in control, CIH, or CH over 22 days following MCF-7 xenograft are shown. (E) Angiogenesis in tumor tissue sections from mice in control, CIH, or CH over 22 days following MCF-7 xenograft was analyzed by H&E staining and immunohistochemical staining for CD31. (F and G) The protein levels of Ki-67, cyclin D1, E-cadherin, N-cadherin, vimentin, and Snail1 in tumor tissues from mice in control, CIH, or CH over 22 days following MCF-7 xenograft were analyzed by western blotting. (H) The mRNA levels of CB1 and CB2 in tumor tissues from mice in control, CIH, or CH over 22 days following MCF-7 xenograft were determined by qPCR. (I and J) The protein levels of CB1 and CB2 in tumor tissues from mice in control, CIH, or CH over 22 days following MCF-7 xenograft were evaluated by western blotting. At least three biological repeats were performed for all data. "p < 0.05; **p < 0.01."
dramatically (Figures 3C and 3D). And the trans-membrane cell number was even fewer in dual knockdown of CB1 and CB2, suggesting stronger inhibitory efficacy in cell invasion. Western blotting indicated that knockdown of CB1 or CB2 reduced the expression levels of Ki-67, cyclin D1, N-cadherin, vimentin, and Snail1 and increased the expression level of E-cadherin in MCF-7 cells exposed to CIH (Figures 3E and 3F). And co-transfection of sh-CB1 and sh-CB2 further amplified these expressions. To unravel the mechanism, we investigated the effects of CB1 and CB2 on the expression of proteins that regulate breast cancer progression by western blotting. We found that knockdown of CB1 and CB2 dramatically reduced the activation of IGF-1R/AKT/GSK-3β signaling axis as the ratio of p-IGF-1R/IGF-1R, p-AKT/AKT, and p-GSK3β/GSK3β decreased compared to the control group (Figures 3G and 3H). Collectively, these data suggested that knockdown of CB1 and CB2 suppressed the migration and invasion of breast cancer cells in vitro.

Overexpression of CBs enhanced migration and invasion of MCF-7 cells under normoxia condition
To further confirm the role of CBs in migration and invasion of breast cancer, MCF-7 cells were cultured under normoxia condition. The scratch assay results showed that overexpression CB1 or CB2 promoted cell migration compared to the control group (Figures 4A and 4B). In contrast with the knockdown results, MCF-7 transfected with overexpression-CB1 (OE-CB1) and overexpression-CB2 (OE-CB2) plasmids demonstrated stronger invasive capability (Figures 4C and 4D). In addition, western blotting demonstrated that overexpression of CB1 or CB2 increased the expression levels of Ki-67, cyclin D1, N-cadherin, vimentin, and Snail1 and decreased the expression level of E-cadherin (Figures 4E and 4F). Furthermore, the results of western blotting showed that overexpression of CB1 and CB2 potentiated the activation of IGF-1R/AKT/GSK-3β signaling axis compared to the control group (Figures 4G and 4H). To summarize, these data indicated that overexpression CBs induced breast cancer migration and invasion under normoxia condition.

CBs mediated breast cancer metastasis via IGF-1R/AKT/GSK-3β signaling pathway
To further investigate whether CBs mediated promotion of breast cancer metastasis via IGF-1R, tyrphostin AG1024, one selective inhibitor of IGF-1R, was used to evaluate effects on migration and invasion in MCF-7 cells. In contrast with the effects of CB1 or CB2 overexpression on cell migration, AG1024 treatment could inhibit migration of MCF-7 cells (Figures 5A and 5B). Consistently, transwell assay results showed that AG1024 could attenuate the tumor cell invasion (Figures 5C and 5D). Furthermore, AG1024 attenuated the expression levels of Ki-67, cyclin D1, N-cadherin, vimentin, and Snail1 and increased the expression level of E-cadherin in MCF-7 cells cultured under normoxia condition (Figures 5E and 5F). And western blotting showed that AG1024 could inhibit the activation of CBs-induced IGF-1R/AKT/GSK-3β signaling pathway compared to overexpression of CB1 and CB2 in MCF-7 cells (Figures 5G and 5H). Taken together,
these data indicated that CBs mediated promotion of breast cancer metastasis via IGF-1R.

**Knockdown of CBs attenuated metastasis of T47D cells via IGF-1R/IKB/GSK-3β signaling pathway under CIH**

To investigate whether endogenous CBs mediated promotion of breast cancer metastasis via IGF-1R, we detected cell migratory and invasive capability of T47D cells with CB1 and CB2 knockdown. As shown in Figures 6A–6C, qPCR and western blotting results showed that the expression levels of CB1 and CB2 were downregulated after silencing CB1 or CB2 compared to the control group in T47D cells. Scratch and transwell assay results demonstrated that knockdown of CB1 and CB2 suppressed in T47D cells migration and invasion compared to the control group under CIH condition (Figures 6D–6G). Furthermore, western blotting showed that knockdown of CB1 or CB2 decreased the expression levels of Ki-67, cyclin D1, N-cadherin, vimentin, and Snail1 and increased the expression level of E-cadherin in T47D cells treated with CIH, and both knockdown of CB1 and CB2 further amplified these alterations (Figures 7A and 7B). We also found that knockdown of CB1 and CB2 obviously reduced the activation of IGF-1R/IKB/GSK-3β signaling as the ratio of p-IGF-1R/IGF-1R, p-AKT/AKT, and p-GSK3β/GSK3β decreased compared to the control group in T47D cells treated with CIH, and both knockdown of CB1 and CB2 further amplified these alterations (Figures 7C and 7D). Together, these data suggested that knockdown of CB1 and CB2 suppressed migration and invasion of T47D cells in vitro via IGF-1R/IKB/GSK-3β signaling pathway under CIH condition.

**Knockdown of CBs inhibited breast cancer proliferation and metastasis in vivo**

To confirm the role of CBs in breast cancer proliferation and metastasis in vivo, we used tumor xenografts nude mice model to detect proliferation and metastasis. We observed that knockdown of CB1 and CB2 efficiently inhibited the growth of breast cancer under CIH and non-hypoxic conditions, as the tumor volume decreased dramatically (Figures 8A, 8B, S1A, and S1B). Moreover, the number of tumor nodules in lungs of mice transplanted with MCF-7 cells transfected with sh-CB1 and sh-CB2, followed by CIH exposure or non-hypoxic conditions, was reduced (Figures 8C, 8D, S1C, and S1D). The results of western blotting showed that decreased Ki-67, cyclin D1, N-cadherin, vimentin, and Snail1 and increased E-cadherin were observed in the tumor tissues of mice engrafted with MCF-7 cells transfected with sh-CB1 and/or sh-CB2 followed by CIH exposure or non-hypoxic conditions (Figures 8E, 8F, S1E, and S1F). And consistent with in vitro data, knockdown of CB1 and CB2 restrained CIH or non-hypoxic-conditions-induced activation of IGF-1R/IKB/GSK-3β signaling pathway (Figures 8G, 8H, S1G, and S1H). Collectively, these results indicated that inhibition of CB1 and CB2 suppressed breast cancer cell proliferation and metastasis in vivo.

**DISCUSSION**

Hypoxia plays a vital role in the development of solid tumor. CIH is the hallmark of sleep apnea, or OSAHS. Recently, OSAHS had been...
implicated in higher prevalence and adverse outcomes in patients diagnosed with solid tumor. It was reported that OSAHS is present in 76% patients with head and neck cancer. In a large multicenter Spanish cohort study, Campos-Rodriguez et al. found that it is the overnight hypoxia induced by obstructive sleep apnea that contributes to the increased cancer incidence. Patricia and his colleagues demonstrated that the metastatic ability of breast cancer is enhanced by hypoxia through upregulation of CXCR4, suggesting OSAHS may be implicated in the breast cancer progression as well. As a key feature of OSAHS, CIH compromises various physiological processes through disrupting oxygen supply. For example, CIH induces cognitive impairment by affecting the hippocampus structure through increasing the expression of CB1 and calcium/calmodulin-dependent protein kinase II. But how CIH regulates cannabinoid receptors in breast cancer is not fully understood. Here, we validated that CIH, as a surrogate of OSAHS, facilitated proliferation and migration of breast cancer cells by upregulating CB1 and CB2 in vitro and in vivo, revealing a putative novel mechanism for the metastatic potential of breast cancer.

Several papers had reviewed current knowledge on hypoxic pathobiology of breast cancer metastasis, and emerging novel mechanisms were reported to explain the role of cannabinoid receptors in cancer progression. For example, Esther et al. reported that CB2 agonists promote proliferation of colon cancer cells and enhance the aggressive molecular feature through the activation of AKT/GSK-3β pathway. Mukhopadhyay et al. displayed that CB1 activation promotes hepatocyte proliferation by inducing cell cycle proteins, including Forkhead Box M1 (FOXM1), and upregulates numerous tumor-promoting genes, such as indoleamine 2,3-dioxygenase. Moreover, genetic silencing of CB1 can attenuate these changes, resulting in suppressed growth of hepatocellular carcinoma. Low-dose CB2 promotes the proliferation and invasion in colon cancer cells, and CB2-specific agonists JWH-133 (CB2 agonist) and HU-308 (CB2 agonists) promote the increase of cell proliferation rate by activating AKT pathway in colon cancer in vitro and in vivo. It was reported that SR 141716A (CB1 inverse agonist) blocks IGF-1R-mediated mitogen-activated protein kinase (MAPK) activation in Chinese hamster ovary cells, suggesting CB1 may activate IGF-1R signaling. A previous study reported that CB2-specific agonist (JWH-015)-induced CB2 activation inhibits epidermal growth factor (EGF) and/or IGF-1-induced migration and invasion in estrogen receptor α− (ERα−) and ERα+ breast cancer cells. JWH-015 suppresses epidermal growth factor receptor (EGFR) and IGF-1R activation and their downstream targets extracellular signal-regulated kinase (ERK), AKT, signal transducer and activator of transcription 3 (STAT3), NF-κB, and matrix metalloproteinases. In the present study, we reported that knockdown of CB1 and CB2 efficiently

Figure 5. CBs mediated breast cancer metastasis via IGF-1R/AKT/GSK-3β signaling pathway
(A and B) Migratory capability of MCF-7 cells treated with AG1024 was detected by wound healing assay. (C and D) Invasive capability of MCF-7 cells treated with AG1024 was measured by transwell. (E and F) The protein levels of Ki-67, cyclin D1, E-cadherin, N-cadherin, vimentin, and Snail1 in MCF-7 cells treated with AG1024 were analyzed by western blotting. (G and H) Expressions of p-IGF1R, IGF1R, p-AKT, AKT, p-GSK3β, and GSK3β in MCF-7 cells treated with AG1024 were analyzed by western blotting. At least three biological repeats were performed for all data. *p < 0.05; **p < 0.01; ***p < 0.001.
suppressed the progress of breast cancer and manual upregulation of CB1 and CB2 could amplify the malignant change of breast cancer. qPCR and western blotting results showed that CB1 and CB2 were upregulated in MCF-7 cells under CH and CIH. Moreover, we also found that the expression levels of CB1 and CB2 were increased in breast cancer tissues of mice housed in hypoxia conditions. Knockdown of CB1 and CB2 attenuated the migration and invasion of breast cancer cells in vitro, whereas overexpression of CB1 and CB2 had opposite effects. However, whether CBs regulate the initiation of breast cancer needs further study. It is noting that CBs play a dual role in cancer progression. We found that upregulated CBs contributed to enhanced cell migration and invasion, which is consistent with what was observed in esophageal squamous cell carcinoma (ESCC), hepatocellular carcinoma, and fibrosarcoma. And in clinical study, patients with renal cell carcinoma (RCC) whose CB2 is upregulated tend to perform poor clinical outcomes in survival. However, the expression level of CB1 was reported to induce cell apoptosis and proliferation inhibition in cancers, including prostatic cancer, colorectal cancer, and intestinal tumor. It suggested that the role of CBs in cancer differs in various cancer types.

The IGF-1/IGF-1R signaling is the major signal-transducing pathway in IGF family, and its activation mediates cell survival, proliferation, differentiation, and metabolism. In the past decades, mounting evidence also suggested that IGF-1/IGF-1R signaling is involved in epithelial-mesenchymal transition (EMT)-associated tumor metastasis. The EMT is a change in cell adhesion molecule expression and cell migration ability. The fundamental event in EMT is the decrease of epithelial marker E-cadherin and gain of mesenchymal marker vimentin. E-cadherin breaks down the adherent junctions between cells and depolarizes cells, triggering invasive mesenchymal phenotypes. This process is regulated by various signals. It was reported that CB1 promotes IGF-1R-mediated MAPK activation in Chinese hamster ovary cells. AKT acts as an important target gene in the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and IGF-1R can activate the PI3K/AKT pathway in breast cancer cells by promoting AKT phosphorylation level. Therefore, we hypothesized that there is an interaction between CBs pathway and IGF-1R/AKT pathway. In this study, we identified the important molecular regulatory relationship between CBs and IGF-1R/AKT/GSK-3β signaling in breast cancer under hypoxic. We found that CBs promoted chronic intermittent hypoxia-induced breast cancer malignancy via IGF-1R/AKT/GSK-3β signaling in breast cancer under hypoxic. We found that CBs promoted chronic intermittent hypoxia-induced breast cancer malignancy via IGF-1R/AKT/GSK-3β signaling in breast cancer under hypoxic. Furthermore, overexpression of CB1 and CB2 also promoted the activation of IGF-1R/AKT/GSK-3β signaling. Consistently, AG1024 downregulated the expression levels of Ki-67, cyclin D1, N-cadherin, and Snail1 and decreased E-cadherin expression in MCF-7 cultured in normoxia condition.

Figure 6. Knockdown of CBs suppressed metastasis of T47D cells under CIH

T47D cells were divided into 5 groups: (1) cells were transfected with empty vector of shRNA (sh-NC); (2) cells were transfected with CB1 shRNA (sh-CB1); (3) cells were transfected with CB2 shRNA (sh-CB2); (4) cells were co-transfected with sh-CB1 and sh-CB2; and (5) T47D normal control. (A) The expression levels of CB1 and CB2 in CB1 and CB2 knocked down T47D cells were detected by qPCR. (B and C) The expression levels of CB1 and CB2 in CB1 and/or CB2 knocked down T47D cells were detected by western blotting. (D and E) Migratory capability of CB1 and/or CB2 knocked down T47D cells was detected by scratch assay. (F and G) Invasive capability of CB1 and/or CB2 knocked down T47D cells was measured by transwell assay. At least three biological repeats were performed for all data. *p < 0.05; **p < 0.01; ***p < 0.001.
vimentin, and Snail1 and increased the expression level of E-cadherin compared to overexpressed CB1 and CB2 of MCF-7 cells cultured in normoxia condition. And AG1024 could inhibit the activation of IGF-1R/AKT/GSK-3b signaling pathway compared to overexpressed CB1 and CB2 in MCF-7 cells. Based on these data, we postulated a model to explain the role of CIH in breast cancer progression. CIH induced CB1 and CB2 expression, which subsequently activated IGF-1R and AKT expression. Activation of AKT pathway leads to the inactivation of GSK-3β via Ser9 phosphorylation. Kim et al.28 validated that GSK-3β negatively regulates Snail1 through its E3 ligase activity. Thus, CIH promoted the expression of Snail1, an EMT-induced transcription factor that binds to E-boxes of E-cadherin promoter and negatively regulates the transcription of E-cadherin. As a result, CIH promotes the malignancy of breast cancer.

In conclusion, we validated that, in hypoxic breast cancer, CIH increased the expression of CB1 and CB2, which promoted tumor growth, angiogenesis, and lung metastasis through activating IGF-1R/AKT/GSK-3β signaling. Knockdown of CB1 and CB2 could inhibit the migration and invasion in MCF-7 cells and T47D cells under CIH conditions by inactivating the IGF-1R/AKT/GSK-3β axis. Furthermore, silencing of CB1 and CB2 suppressed malignancy of breast cancer in vivo under CIH condition or normoxia condition. Thus, taking into consideration the effects of sleep disorders on breast cancer progression might allow us to develop novel approaches for breast cancer treatment.

MATERIALS AND METHODS

Cell culture and treatment

Human breast cancer cell lines MCF-7 and T47D were purchased from American Type Culture Collection (ATCC) (VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 80 U/mL penicillin, and 0.08 mg/mL streptomycin at 37°C in humidified atmosphere with 5% CO2. Cell culture reagents were all purchased from Invitrogen (Carlsbad, CA, USA).

Construction of CIH and CH cell model. For studies in CH, MCF-7 and T47D cells were grown at 37°C in an atmosphere of 1.5% O2, 5% CO2, and 93.5% N2 for 48 h in a humidified incubator inside a sealed workstation. And for CIH, MCF-7 and T47D cells were exposed to 10 cycles of hypoxia and reoxygenation. Each cycle consisted of a period of 12 h in 1.5% hypoxia followed by 12 h recovery under normoxia (21% O2, 74% N2, and 5% CO2). During the reoxygenation period, the medium was changed.

Construction of animal models

8-week-old female nude BALB/c mice were used in this study. The mice were acclimatized to the laboratory conditions and provided free access to ad libitum food and water. The mice were acclimated for 1 week before use and maintained throughout the study in a controlled environment: 24°C ± 2°C; 50% ± 10% relative humidity; and a 12-h light/dark cycle. All experiments were conducted in accordance with the protocols approved by the Animal Care and Use Committee of the Shanxi Medical University.

CIH and CH model of mice were established as previous reported.29 MCF-7 cells were suspended in phosphate-buffered saline (PBS) at a final concentration of 1 × 10⁵ cells/mL. A volume of 0.1 mL of suspended cells was subcutaneously injected into the second mammary fat pad on the left side. After cell injection for 5 days, tumor-bearing mice were randomly divided into 3 groups (5 animals per group) for

In Figure 7, Knockdown of CBs attenuated IGF-1R/AKT/GSK-3β signaling pathway under CIH. T47D cells were divided into 5 groups: (1) cells were transfected with empty vector of shRNA (sh-NC); (2) cells were transfected with CB1 shRNA (sh-CB1); (3) cells were transfected with CB2 shRNA (sh-CB2); (4) cells were co-transfected with sh-CB1 and sh-CB2; and (5) T47D normal control. (A and B) The protein levels of Ki-67, cyclin D1, E-cadherin, N-cadherin, vimentin, and Snail1 of CB1 and/or CB2 knocked down T47D cells were analyzed by western blotting. (C and D) Expressions of p-IGF-1R/IGF-1R, p-AKT/AKT, and p-GSK3β/GSK3β of CB1 and/or CB2 knocked down T47D cells were analyzed by western blotting. At least three biological repeats were performed for all data. *p < 0.05; **p < 0.01; ***p < 0.001.
CH treatment, CIH treatment, or control (normoxia). A gas-control delivery system was designed to regulate air, nitrogen, and oxygen flow into the cages. Mice in the control group were persistently supplied with air consisting of 21% O₂, 74% N₂, and 5% CO₂. For CH group, the air consisted of 5% O₂, 90% N₂, and 5% CO₂. Mice in the CIH group received air at the hypoxia level for 30 s, followed by reoxygenation to normoxia levels within the subsequent 90 s. Exposure proceeded for 8 h daily, and the duration of exposure was 4 weeks. During the CIH/CH/normoxia period, tumor sizes were measured in two dimensions using a caliper, and the tumor volume was calculated with the following formula: tumor volume (cm³) = 0.5 × ab² (a and b refer to the longest and the shortest diameters of the tumor, respectively). The tumors were formalin fixed and paraffin embedded for H&E staining or homogenized to prepare the tissue lysates for western blotting analysis. The lungs were fixed in Bouin’s solution or homogenized to prepare the tissue lysates.

**Lentiviral vectors and lentivirus infection**

The shRNAs of CB1 (sh-CB1) and CB2 (sh-CB2) were purchased from Genepharma (Shanghai, China). Overexpression plasmids that clone wild-type human CB1/CB2 into the lentiviral vector TRC-pLKO.1 system (Open Biosystem) were obtained from cDNA Resource Center. Lentiviral vectors were produced, concentrated, and titrated according to standard protocols. Then, the breast cancer cells in the exponential growth phase were planted into 24-well plates, and the virus supernatant with pLKO.1 shRNA plasmid, pCMVdr-8.91 packaging plasmid, and pMD2.G envelope plasmid were added to the MCF-7 and T47D cells. After 72 h, the transfection rate of the cells was measured.

**Real-time quantitative PCR (qPCR)**

Total RNA was extracted from breast cancer cells or mouse tissues using Trizol Reagent (Invitrogen, Paisley, UK), and cDNA was synthesized by reverse transcription using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Subsequently, qPCR was performed according to the direction of the SYBR Green PCR Master Mix (Thermo, Waltham, MA, USA) according to the manufacturer’s instructions. The gene expression was measured by qPCR with Applied Biosystems 7500 Fast Real Time qPCR machine. GAPDH was used as the reference gene. The relative expression levels were calculated using the 2⁻ΔΔCt method. qPCR primers were synthesized by Invitrogen, and sequences were as follows:

**Figure 8. Knockdown of CBs inhibited breast cancer proliferation and metastasis in vivo**

(A) Representative images of tumors from mice housed in CIH over 20 days following grafting with MCF-7 cells that transfected with sh-CB1 and/or sh-CB2 are shown. (B) The volume tumors from mice housed in CIH conditions over 20 days following grafting with MCF-7 cells that transfected with sh-CB1 and/or sh-CB2 are shown. (C and D) Metastatic nodules in the lungs of breast cancer mice housed in CIH following grafting with MCF-7 that transfected with sh-CB1 and/or sh-CB2 are shown. (E and F) The protein levels of Ki-67, cyclin D1, E-cadherin, N-cadherin, vimentin, and Snail1 from tumor tissues of breast cancer mice housed in CIH following grafting with MCF-7 that transfected with sh-CB1 and/or sh-CB2 were analyzed by western blotting. (G and H) Expressions of p-IGF-1R, IGF-1R, p-AKT, AKT, p-GSK3β, and GSK3β from tumor tissues of breast cancer mice housed in CIH following grafting with MCF-7 that transfected with sh-CB1 and/or sh-CB2 were detected by western blotting. At least three biological repeats were performed for all data. *p < 0.05; **p < 0.01; ***p < 0.001.
and Snail1 were purchased from Abcam (Cambridge, MA, USA). Ki-67 antibodies. Primary antibodies against CB1, CB2, E-cadherin, and the bands was quantified using an optical microscope.

**Western blotting**

Total protein was isolated and then quantified via Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, USA). Subsequently, the sample was separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) and blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. The membranes were then incubated overnight at 4°C with the primary antibodies. Primary antibodies against CB1, CB2, E-cadherin, and Snail1 were purchased from Abcam (Cambridge, MA, USA). Ki-67 and β-actin antibodies were purchased from Sigma-Aldrich (Miamisburg, OH, USA). Cyclin D1, N-cadherin, vimentin, p-IGF-1R (Tyr1135/1136), IGF-1R, p-AKT (Ser473), AKT, p-GSKβ (Ser9), and GSKβ were purchased from Cell Signaling Technology (Boston, MA, USA). The membranes were then washed with Tris Buffered Saline-Tween 20 (TBST) buffer and incubated with horse-radish peroxidase (HRP)-conjugated secondary antibody for 1 h. Protein bands were then visualized using the chemiluminescence detection system (Bio-Rad, Hercules, CA, USA). The intensity of the bands was quantified using ImageJ software tools.

**Transwell assay**

Transwell plates were pre-coated with Matrigel Matrix (BD Biosciences, CA, USA), 1 × 10^4 MCF-7 and T47D cells were inoculated into the upper chamber with serum-free DMEM, and 500 μL DMEM containing 10% FBS was added to the lower chamber. Incubated over 24 h in humidified incubator with 5% CO₂ at 37°C, cells on the lower membrane surface were fixed with 4% paraformaldehyde for 15 min, stained with crystal violet, and photographed under microscope.

**Scratch assay**

Scratch assay was utilized to evaluate the migration ability of MCF-7 and T47D cells. Briefly, cells were seeded in 12-well plates (2 × 10^5) overnight. Then, an artificial scratch was created in cells, and cell debris was removed by washing with 1 × PBS. Cell migration was observed, and the width of the wound was measured before and after the treatment. The cell migration is based on (T₀ – Tₜ)/T₀ × 100%.

**Tumor xenografts in BALB/c nude mice**

To investigate the effect of CBs on breast cancer metastasis, we carried out tumor xenografts in BALB/c nude mice. MCF-7 cells were divided into four groups: (1) transfected with empty vector of shRNA; (2) transfected with CB1 shRNA; (3) transfected with CB2 shRNA; and (4) transfected with CB1 and CB2 shRNA. Then, cells were suspended and subcutaneously injected as mentioned above. The final concentration in PBS was 1 × 10^6 cells/mL. A volume of 0.1 mL of suspended cells was subcutaneously injected into the second mammary fat pad on the left side. Mice with MCF-7 tumors had their drinking water supplemented with 10 mg/mL water-soluble β-Estradiol (Sigma-Aldrich, Miamiusburg, OH, USA). Mice were subjected for 8 h under CIH condition every day and last for 3 to 4 weeks, where tumor sizes were measured in different groups.

**Tissues samples and H&E staining**

At the end of treatment, mice were anesthetized and perfused with 4% paraformaldehyde for 15 min. Lung and tumor tissues were isolated, embedded in paraffin, and cut into 5-μm sections. The dried slices were soaked in alcohol, dewaxing for 10 min, rehydrated, hematoxylin rinse for 5 min and washed, followed by differentiation with 1% hydrochloric acid alcohol. Then, slides were stained with eosin for 30 s, dehydrated with gradient alcohol, soaked in xylene 3 times, and mounted with neutral gum. Finally, the slides were observed and photographed using an optical microscope.

**Immunohistochemistry (IHC) staining**

The tissues were fixed in 4% paraformaldehyde for 24 h and dehydrated with a gradient of ethanol (100%, 95%, 80%, and 70%). The tissues were embedded in paraffin and sectioned in 3-μm thickness. The thickness was soaked in 3% H₂O₂ for 10 min and blocked with non-immune goat serum at room temperature for 30 min. The thickness was stained with diaminobenzidine tetrahydrochloride (DAB) reagent (Sigma-Aldrich, Miamiusburg, OH, USA) for 5 min and then stained with hematoxylin for 2 min. Then, we observed the histomorphological changes under the microscope (LEI-CADMLB2, Germany).

**Statistics**

Data are expressed as mean ± standard deviation (SD). Student’s t test was employed to compare the differences between two groups. The statistical analysis between multi-groups was carried out using one-way ANOVA with post hoc contrasts by Tukey test. p < 0.05 was considered statistically significant. All statistical analyses were performed by Graphpad Prism 5 (GraphPad Software, La Jolla, CA, USA).

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.09.007.
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AUTHOR CONTRIBUTIONS
L.-T.L., F.-F.Z., Z.-M.J., and X.-L.G. contributed to the conception and study design. Y.-Y.L., L.-Q.Q., X.-Z.Z., and L.Z. conducted the experiment and collected data. J.-J.Y., S.-J.W., and H.L. analyzed the data. L.-T.L., C.-H.L., and D.-D.A. drafted the manuscript. And L.-T.L., Y.-Q.H., and X.-L.G. were responsible for revising and correcting the manuscript. All authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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