Knocking down HIF-1α expression inhibits lung metastasis of breast cancer cell line 4T1 by attenuating EMT

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Research Article

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Abstract

Background: Globally, women's healthy and survival were threatened by breast cancer. Clarify the mechanism of distal metastasis of breast cancer will provide precise control and prolong patients' lifetime. It's well known that HIF-1α play important roles in breast cancer, however, few studies have directly examined the impact of knocking down HIF-1α on lung metastasis of breast cancer. In this study, HIF-1α was knocked down in mice breast cancer cell line 4T1 to explore its role on breast cancer lung metastasis.

Methods: HIF-1A knocked down lentivirus pHBL-U6-MSC-shHIF-1A-GFP-PURO was transfected to 4T1 cells to construct HIF-1α low expression cells model while pHBL-U6-MSC-GFP-PURO was used as control. The transfection efficiency of lentivirus was observed by fluorescence microscope and flow cytometry, HIF-1α knock down was tested by Q-PCR and western blot. Would healing and transwell migration were used to detect the migration ability of cells in vitro. Mouse lung metastasis model was used to verify cells metastasis in vivo.

Results: Lower expression of HIF-1α was detected in HIF-1A knocked down 4T1 cells. Decreased HIF-1α expression repressed migration of 4T1 cells in vitro and reduced lung metastasis in mice breast cancer model. Moreover, HIF-1α's down expression sponges E-cadherin up-regulation and Vimentin down-regulation to inhibit EMT, therefor reduced lung metastasis.

Conclusions: our study revealed that HIF-1α knocked down inhibited lung metastasis of 4T1 breast cancer in vivo by suppressed EMT, thus inspiring a new treatment for breast cancer.

Background

Breast cancer is the most prevalent malignancy worldwide, with the continuous improvement of treatment methods, the survival time of patients has been greatly improved[1, 2]. However, the survival time of patients with distal metastasis was significantly reduced[3], and more than 90% of breast cancer patients die because of metastasis[4]. Exploring the mechanism of distant metastasis will help breast cancer prevention and treatment[5].

EMT(epithelial mesenchymal transition) and angiogenesis were important factors that promotes tumor metastasis[6]. During EMT cells lose epithelial characteristics and obtain mesenchymal properties, promotes cancer cells metastasis from primary tumors[7]. EMT is an important process in tumor microenvironment[8].

Many research have identified tumor microenvironment play important roles in tumor progression including tumor migration, drug resistance and immunosuppression[9]. Central hypoxia is a common characteristic of solid tumors[10]. In response to hypoxia, the degradation of Hypoxia inducible factor 1a (HIF-1α) in tumor cells decreased, then HIF-1α binds to the hypoxia response elements of target genes to drive their transcription. It has confirmed that HIF-1α play important roles in tumor microenvironment.

Wang etal suggested extracellular matrix protein mindin inhibit the expression of HIF-1α to attenuates colon cancer progression[11].Samanta and groups demonstrated that chemotherapy induces breast cancer cells immune evasive through HIF-1α enriched CD47+/CD73+/PDL1 + cancers[12].

So far, there was few study on the effect of HIF-1α low expression at normal condition on the metastasis of breast cancer. In this paper, we knock down HIF-1α by lentivirus transfection. The effect of HIF-1α low expression on 4T1 metastasis was study by wound-healing and transwell experiment in vitro and mice lung metastasis model in vivo.

Materials And Methods

Cell lines and Lentivirus transfection

Mice breast cancer cell 4T1 was purchased from National Collection of Authenticated Cell Cultures, and maintained at RPMI1640 (Gibco) medium containing 10% FBS (Gibco) and 100U/ml penicillin, 0.1 mg/ml streptomycin (Solarbio). For knockdown of HIF-1A, pHBLV-U6-MCS-shRNA-HIF-1A-GFP-PURO or control shRNA-lentivirus were transfected to 4T1 cells at a density of 40%, using MOIs of 20 each other. 48 hours later, shRNA expressing cells were selected by 5ug/ml puromycin contained media for 15 days. The transfection ratio was confirmed by fluorescence microscope or flow cytometry, the expression of HIF-1α in 4T1 cells was detected by western-blot or quantitative real-time PCR.

Western-blot assay

Cells were lysed in RIPA containing Protease inhibitor for 15 min, the protein concentration was quantified by BCA protein Assay kit. For each test, 30ug total protein was used, protocol for western blot was carry out as described previously. Antibodies used are as following: β-actin (1:1000; proteintech), HIF-1α(1:1000; cell signaling technology), E-cadherin(1:1000, proteintech), Vimentin (1:1000; proteintech), horseradish peroxidase-conjugated anti-rabit IgG(H&L) (1:10000; proteintech) and horseradish peroxidase-conjugated anti-mouse IgG(H&L) (1:10000; proteintech). Electro chemiluminescence method was used to detection blots(millorpe), β-actin was used as internal standards and the expression of protein was semi quantitatived by ImageJ.

Wound-healing assay

For wound-healing assay, 1*105 cells were seeded in six-well plates, the next day, a straight line was scratched, suspending cells were removed by PBS and new media was added. The wound was observed and photographed at 24, 48 and 72 hours.

Transwell migration assay

For migration assay, chamber with 8um pore size was used. 3*104 cells in 150 ul medium were placed in chamber and 0.5ml medium containing 20% FBS was added to the lower chamber. 24 hours later, the

chambers were harvested, washed and fixed in 4% formaldehyde for 30 min, stained with 0.25% crystal violet for 30 min and photographed.

Mouse lung metastasis model

6 to 8 weeks BABL/C female mice were challenged by tail vein injection of 1×10^6 tumor cells. Euthanasia was carried out at days 3, 5, 7, 9, 11 and 13 and lung was obtained for photograph. The animal experiment was approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University.

Statistical analysis

Data were analyzed for significance in GraphPad Prism by unpaired Student's t tests or one-way ANOVA. Differences in values were considered significant if p values were < 0.05.

Results

Identification of HIF-1α knock down

HIF-1A shRNA containing lentivirus pHBLV-U6-MCS-shRNA-HIF-1A-GFP-PURO or control lentivirus pHBLV-U6-MCS-NC-GFP-PURO were transfected to 4T1 cells at a density of 40%. After 15 days Killing selection by puromycin the 2 cell lines were named 4T1-HIF-1A-KD and 4T1-NC. Under fluorescence microscope, green fluorescence was detected in 4T1-HIF-1A-KD and 4T1-NC for the GFP gene in lentivirus, and almost all the cells have green fluorescence (Fig 1A). Transfection ratio was assessed by Flow Cytometry, results showed that there were 95% or higher of GFP positive cells in both two cell lines (Fig 1B). q-PCR showed that compared with 4T1-NC HIF-1A gene in 4T1-HIF-1A-KD cell was lower (Fig 1C). These results suggested that pHBLV-U6-MCS-shRNA-HIF-1A-GFP-PURO lentivirus were successfully transfected and HIF-1A gene expression was knocked down.

HIF-1A know down inhibit wound-healing and migration of 4T1 cells

Cell migration ability of 4T1 cells after HIF-1A knock down was estimated by wound-healing and transwell migration. As shown in Fig 2A, when HIF-1A was known down the wound-healing ability was slower. The estimated healing rate displayed at Fig 2B, at each time point healing rate at 4T1-HIF-1A-KD group was less than 4T1-NC group. Crystal violet staining showed that there was more 4T1-NC cell migrated the well than 4T1-HIF-1A-KD cells (Fig 2C), statistical results were shown in Fig 2D. HIF-1A play important roles in 4T1 cells' wound-healing and migration were identified by these results.

HIF-1A knock down inhibit EMT of 4T1 cells

To explore the effect of HIF-1α low expression on Endothelial mesenchymal transformation (EMT) of 4T1 cells, the mRNA expression in 4T1-NC and 4T1-HIF-1A-KD cells were detected. As the results in Fig 3A, E-cadherin was upregulated in 4T1-HIF-1A-KD cells while Vimentin was down regulated. When detect the

protein expression in these two cells, same results were got, invasion suppressor protein E-cadherin have a higher level in 4T1-HIF-1A-KD cells than 4T1-NC cells; while cellular dynamic protein Vimentin's expression was lower in 4T1-HIF-1A-KD cells compared with 4T1-NC cells (Fig 3B and C). These results suggested that low expression of HIF-1A inhibit EMT of 4T1 cells.

HIF-1A knock down restrain lung metastasis of 4T1 tumor cells

In order to verify the migration ability of 4T1 cells in vivo, the lung tissue of BABL/C mice at different time points after tail vein injection of 4T1-NC or 4T1-HIF-1A-KD cells were harvested. On the third day post injection, small node was found in 4T1-NC group, and as time grown the node were bigger and bigger meanwhile the number of nudes was increasing. The small node was appeared in 4T1-HIF-1A-KD group at the eleventh day, and the size and quantity of node couldn't compare with 4T1-NC group (Fig 4A and B). These experimental data exhibited that HIF-1A known down prevent lung metastasis of 4T1 cell in mice.

Discussion

It has been reported that as a transcription factor the function of HIF-1α in tumor is priceless[13]. Respond to hypoxia, the degradation of HIF-1α decreased[14], Then HIF-1α enter the nucleus and bind to the genes containing hypoxia response elements (HRE)[15], over 200 genes are activated in response to HIF-1α that allow cells to survive and adapt to low oxygen tensions[16].

In this study, we demonstrated that knock down the expression of HIF-1α in normal condition inhibited breast cancer cells metastasis in vitro and in vivo. Low expression of HIF-1α reduced the migration promoting protein Vimentin and increased the adhesion protein E-cadherin both in mRNA level and protein level (Fig. 3).

As a cellular adhesion molecule E-cadherin regulates cell-cell adhesion and stimulates antigrowth signals, which is a key factor governing metastatic potential in the majority of epithelial cancers[17]. HIF-1α has been described as a critical factor for the regulation of E-cadherin in ovarian carcinoma and VHL-deficient renal cell[18, 19], here we demonstrated that HIF-1α also regulated E-cadherin expression in mice breast cancer cells.

Vimentin is extensively involved in multiple physiological activities and plays a significant role in regulating cell functions[20]. It participates in cell migration, differentiation, proliferation, adhesion and invasion[21, 22]. In this study, we show that low expression of vimentin is accompanied by low expression of HIF-1α, HIF-1α exercise its function through vimentin to prompt metastatic.

Interestingly, in mice lung metastatic model, normal 4T1 cells was colonized in the lungs on the third day, while until the eleventh day HIF-1α knocked down 4T1 cells appear in the lung.

In summary, our study shows that HIF-1α acted as a functional transcription factor in breast cancer cell, by promoting breast cancer metastatic. Reducing the expression of HIF-1α may be a feasible method to

inhibit metastasis of breast cancer.

Conclusions

In conclusion, we report that knock down HIF-1a inhibit breast cancer metastatic. These findings underlie the important of HIF-1a regulation EMT in breast cancer metastatic. Our study providing more evidence for targeting HIF-1a for breast cancer treatment.

Declarations

Authors' contributions

Jinjin Zhao performed all the data analysis and wrote the manuscript. Haiguang Zhang and Yaqian Liu carried out the relevant experiments. Guangjian Lu, Zhaohui Wang and Qingjiang Mo provided technical support. Luyang Jiao and Jinjin Zhao designed the study. The authors read and approved the final manuscript.

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Availability of data and materials

All raw data from in vitro experiments as well as further data supporting the chosen representative image presented in this published article can be made available from the corresponding author on request.

Ethics approval and consent to participate

This study was previously conducted according to Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (2020047). All research had been performed in accordance with the Declaration of Helsinki. All mouse experimental protocols were approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University. All tests were carried out with the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University. We confirm that all methods are reported in accordance with ARRIV guidelines for the reporting of animal experiments.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

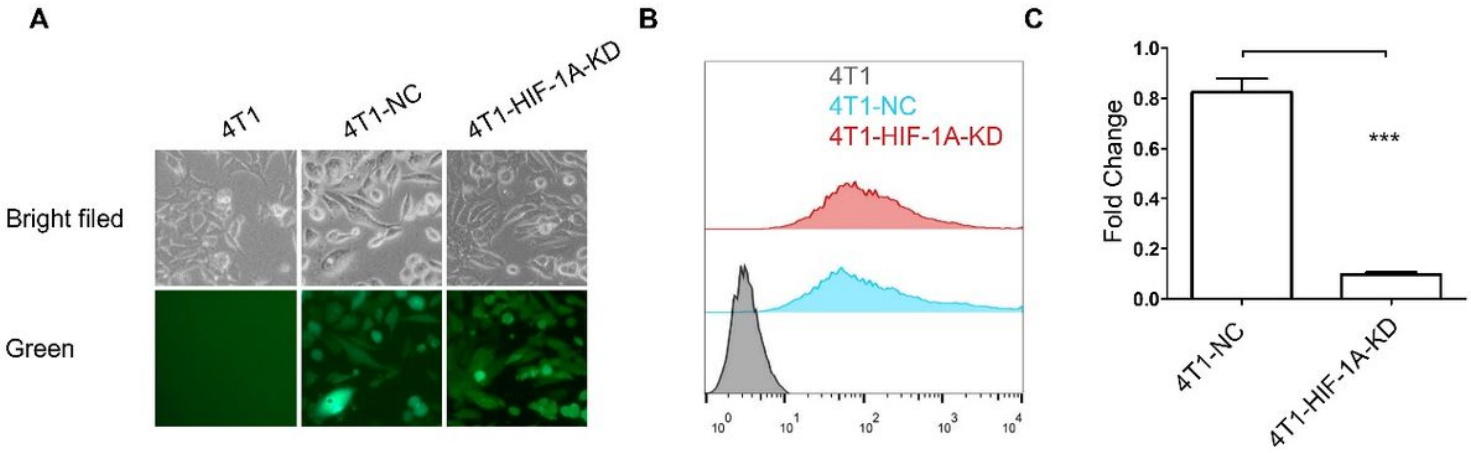

Figure 1

HIF-1a knocked down in 4T1 cells. pHBLV-U6-MCS-shRNA-HIF-1A-GFP-PURO and control lentivirus successfully transferred to 4T1 cells. After puromycin treatment HIF-1a low expression cell line 4T1-HIF-1A-KD and GPF expression control cell line 4T1-NC were constructed. A) Fluorescence microscope results shows the GFP expression in 4T1-NC and 4T1-HIF-1A-KD cell lines. B) Flow cytometry analysis shows

GFP+ cell ratio in 4T1,4T1-NC and 4T1-HIF-1A-KD groups. C) qPCR shows changes in HIF-1A mRNA levels relative to the 4T1-NC group. Data are the mean+ standard error of the mean from three independent experiments plated in triplicate with differences calculated using the delta-delta Ct method relative to the expression of reference gene GAPDH. Statistical tests are unpaired t tests performed in GraphPad Prism with p values represented as follows: ns=not significant, ***<0.001.

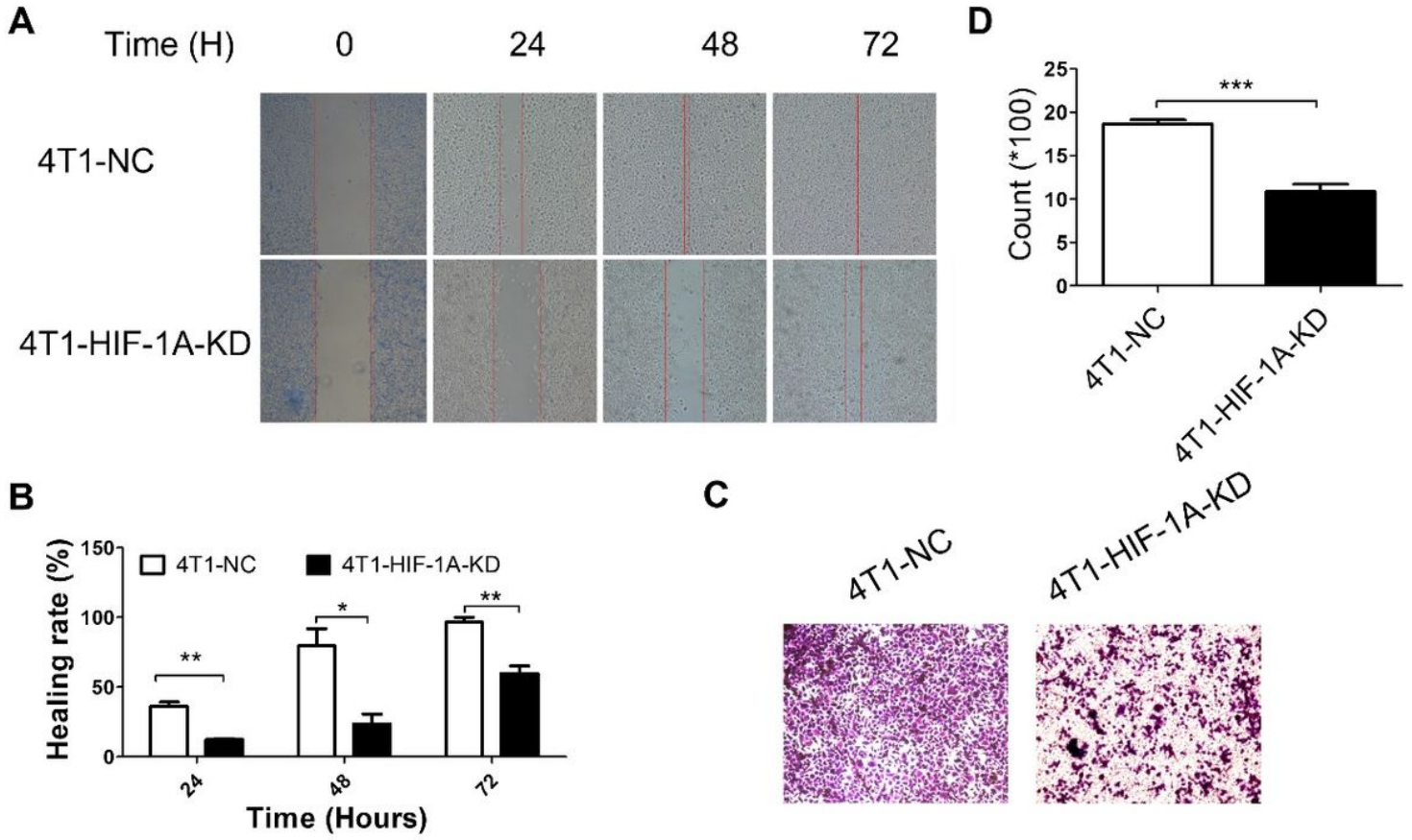

Figure 2

4T1 cell metastasis was attenuated when HIF-1a was low expression. A) 24, 48 and 72 hours later after scratch the cells by tips the wound were photographed. B) healing rate of A) were calculated. Data are the mean ± standard error of the mean from three independent experiments plated in triplicate with differences calculated using the ratio of different time points to 0 hour. C) transwell experiment of 4T1-NC and 4T1-HIF-1A-KD cells. D) statistical analysis of C), Data are the mean ± standard error of the mean from three independent experiments plated in triplicate with differences calculated using the number of cells on the member. Statistical tests are unpaired t tests performed in GraphPad Prism with p values represented as follows: ns=not significant, *<0.05, **<0.01, ***<0.005.

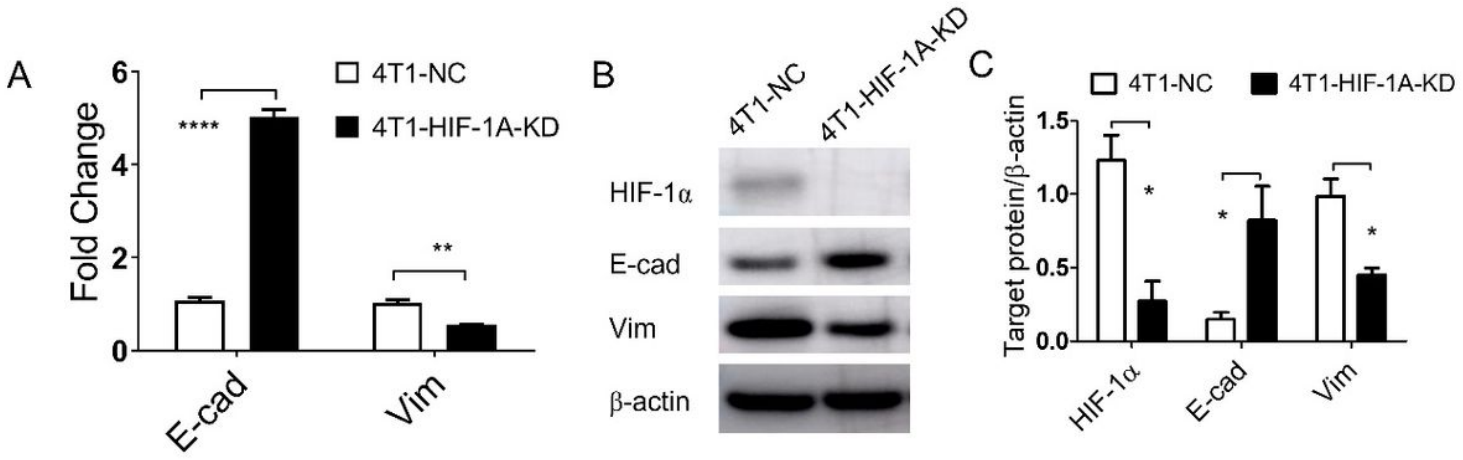

Figure 3

HIF-1a low expression attenuate the expression of EMT markers. A）q-PCR shows the changes of E-cadherin and Vimentin mRNA levels relative to 4T1-NC. Data are the mean+ standard error of the mean from three independent experiments plated in triplicate with differences calculated using the delta-delta Ct method relative to the expression of reference gene GAPDH. B) western blot shows the expression of HIF-1a, E-cadherin and Vimentin in 4T1-NC and 4T1-HIF-1A-KD cells, β-actin as an internal parameter. C) gray value in B) was calculated by ImageJ, data are the mean ± standard error of the mean from three independent experiments plated in triplicate with differences calculated using the ratio of target protein to β-actin. Statistical tests are unpaired t tests performed in GraphPad Prism with p values represented as follows: ns=not significant, *<0.05, **<0.01, ***<0.005, ***<0.001.

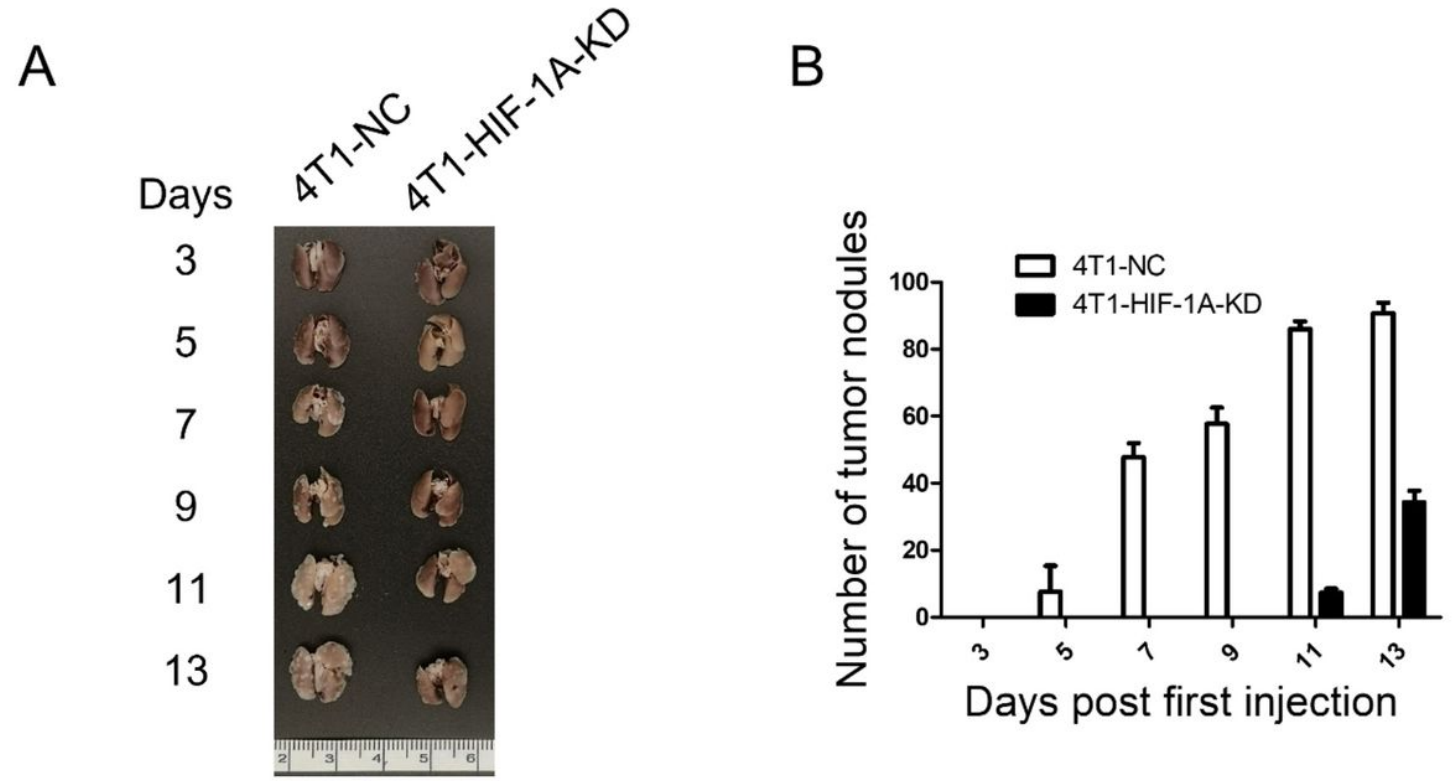

Figure 4

HIF-1a low expression inhibited 4T1 cells lung metastases. A) Photos of lung nodes after mice inoculation with 4T1-NC or 4T1-HIF-1A cells for different days. B) number of nodes were counted. Data are the mean ± standard error of the mean from three independent experiments plated in triplicate with differences calculated using the number of cells on the member. Statistical tests are unpaired t tests performed in GraphPad Prism with p values represented as follows: ns=not significant, *<0.05, **<0.01, ***<0.005.