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Dihydroartemisinin Prevents Distant Metastasis of Laryngeal Carcinoma by Inactivating STAT3 in Cancer Stem Cells

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Background: Accumulating evidence indicates that cancer stem cells (CSCs) are a minor subpopulation of cancer cells that may be the primary source of cancer invasion, migration, and widespread metastasis.

Material/Methods: We investigated the effects of dihydroartemisinin (DHA) on distant metastasis of laryngeal carcinoma and the relevant mechanism. *In vitro*, we used the Hep-2 human laryngeal squamous carcinoma cell line (Hep-2 cells) to assemble CSCs, using CD133 as the cell surface marker. Our data demonstrate that the $CD133^+$ subpopulation of Hep-2 cells has greater invasion and migration capabilities than $CD133^-$ cells. We also evaluated the effects of DHA, a newly defined STAT3 inhibitor, on the invasion and migration of $CD133^+$ Hep-2 cells under hypoxia and IL-6 stimulation, both of which can activate STAT3 phosphorylation.

Results: CSCs exhibited a significant decrease in the ability of migration and invasion upon the application of DHA, along with simultaneous alterations in related proteins, both in cultured cells and in xenograft tumors. The associated signaling proteins included phosphorylated STAT3 (p-STAT3), matrix metalloproteinase-9 (MMP-9), and E-cadherin, which are closely involved in cancer invasion and metastasis. *In vivo*, we found that DHA can reduce lung metastasis formation caused by CSCs and prolong survival in mice, and can inhibit STAT3 activation, downregulate MMP-9, and upregulate E-cadherin in lung metastatic tumors.

Conclusions: Taken together, our findings indicate that CSCs possess stronger invasive and metastatic capabilities than non-CSCs, and DHA inhibits invasion and prevents metastasis induced by CSCs by inhibiting STAT3 activation.

MeSH Keywords: **Laryngeal Neoplasms • Neoplasm Invasiveness • Neoplasm Metastasis • Neoplastic Stem Cells • STAT3 Transcription Factor**

Abbreviations: **DHA** – dihydroartemisinin; **STAT3** – signal transducer and activator of transcription; **CSCs** – cancer stem cells; **HNSCC** – head and neck squamous cell carcinoma; **MMP-9** – matrix metalloproteinase-9; **EMT** – **0**epithelial-mesenchymal transition; **ALDH1** – aldehyde dehydrogenase 1; **ECM** – extracellular matrix

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Background

Laryngeal cancer is a common malignant neoplasm in head and neck cancers. In 2018, the estimated number of new cases of laryngeal cancer was 13 150 and 3710 were likely to die from laryngeal cancer in the United States [1]. The prognosis of the disease depends on the size of the tumor lesion, the level of local invasion, the spread of cervical lymph nodes, and the presence of distant metastasis [2]. It is clear that metastasis to the lymph nodes and distant organs is a major cause of treatment failure in head and neck cancers [3]. To date, the emergence of cancer stem cells (CSCs) and the occurrence of epithelial-mesenchymal transition (EMT) are considered to be the primary causes of invasion and metastasis [4]. It has been shown that the activation of signal transducer and activator of transcription 3 (STAT3) is a crucial cell signaling event that regulates CSCs, EMT, and metastasis of head and neck cancers [5].

Evidence from many studies indicates that a minor subpopulation of tumor cells, CSCs, is responsible for tumor initiation, proliferation, progression, and metastasis [6]. CSCs have been identified in various human malignancies, such as liver carcinoma, breast cancer, leukemia, glioblastoma [7], and head and neck squamous cell carcinomas (HNSCCs), including oral squamous cell cancer [8], laryngeal carcinoma [9], and nasopharyngeal carcinoma [10]. There are a variety of methods that can be used to isolate and characterize CSCs, including fluorescence-activated cell sorting (FACS), which identifies corresponding specific cell surface markers [6]. Several cell surface molecules, such as CD44, CD133, and aldehyde dehydrogenase 1 (ALDH1), have been used to characterize the stem cell nature of CSCs in HNSCC [11]. For instance, CD133 has been proposed as a potential marker of CSCs in the human laryngeal squamous carcinoma cell line [12]. $CD133^+$ cells possess tremendous proliferative, invasive, and tumorigenic capabilities *in vivo*, compared to $CD133^-$ cells [11].

EMT is a biological process that occurs in embryogenesis and has been implicated in cancer progression, invasion, and metastasis [13–15]. EMT is induced by activity of growth and transcription factors, leading to the absence of cell-cell junctions and apical-basal polarity, as well as the acquisition of a mesenchymal phenotype [16]. Tumor cells then acquire invasive mesenchymal phenotype motility [17,18]. In addition, the loss of the epithelial marker E-cadherin and the overexpression of mesenchymal markers, such as matrix metalloproteinase-9 (MMP-9) and vimentin, are the principal molecular indicators of EMT [19]. E-cadherin is a hyplotype of cadherin protein expressed by epithelial cells and is encoded by the CDH1 gene [20]. These cadherin proteins are glycoproteins consisting of intracellular, transmembrane, and extracellular portions [21]. In addition to the calcium ion-dependent control of cellular adhesion, they are also involved in tissue morphogenesis, the identification and grouping of appropriate cells, the maintenance of tissue continuity, and the harmonization of cell translocation, playing an important role in cell migration [22,23]. MMP-9, another EMT-related protein with collagenase activity, belongs to a family of zinc-containing metalloendopeptidases and is involved in cell adhesion [24]. Its activity is essential for breaching the extracellular matrix (ECM) barrier. The vital modulatory effect of MMP-9 in the invasion and metastasis of malignant tumors has been clearly established [25,26].

In recent years, a link between EMT and the induction of the stem cell-like properties of tumor cells has been reported for solid tumors [27]. EMT is associated with invasion and metastasis. CSCs can exhibit typical characteristics of EMT [28], causing CSC proliferation and differentiation [29], especially in solid breast tumors. Moreover, EMT can also induce the expression of stem cell markers on cancer cells and endow these cells with stem cell-like properties, including the formation of mammospheres and invasion [30–32]. Therefore, EMT is associated with the formation of stem cell properties in various cancer cells. EMT inhibition can help restrain CSC formation and subsequent cancer progression events, including cancer cell invasion, migration, and metastasis.

DHA has been widely applied in clinical practice as an antimalarial agent, with a high cure rate and low toxicity. Recent evidence suggests that DHA has antineoplastic capability in a variety of tumor types [33]. The potential antitumor activities of DHA as a new type of putative STAT3 inhibitor are not thoroughly understood. As demonstrated in the present study, the activation and overexpression of STAT3 are closely related to the metastasis and survival of laryngeal cancer patients. Moreover, DHA was shown to counter the invasion and migration of laryngeal CSCs *in vitro* and *in vivo* and improve the survival time of animal models of metastasis. Our study provides insight into the roles of STAT3 activation in the metastasis of laryngeal cancer and possible mechanisms of DHA actions, suggesting an effective way to reduce the invasion and metastasis of laryngeal cancer.

Material and Methods

Test compounds and reagents

The PE-conjugated mouse anti-human monoclonal antibody CD 133 was purchased from Miltenyi Biotechnology Corporation (Bergisch Gladbach, DE). Bovine serum albumin (BSA) was acquired from Roche Corporation. Epidermal growth factor (EGF), β-FGF, and IL-6 were obtained from PeproTech (Rocky Hill, NJ, USA). Monoclonal antibodies against phosphorylated Stat3 (Tyr705), Stat3, MMP-9, β-catenin, and E-cadherin were obtained from Cell Signaling Technologies (Cambridge, MA).

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DHA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 mmol/L and stored at –20°C.

Tumor specimens and immunohistochemical staining

We collected 124 primary cancer specimens from patients who underwent surgery for laryngeal cancer. Among these patients, 24 developed distant metastasis during follow-up. All specimens were subjected to immunohistochemical examinations for expression of p-STAT3 using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturers' instructions. Protein expression levels were evaluated according to scores of positively stained cells and intensity of the specific immunostaining of the associated proteins on all immunostained slides. All patients were regularly followed up for survival status and tumor progression. The present study was approved by the Ethics Committee of Bethune International Peace Hospital, and with the informed consent of all included subjects (Ethics NO. 2017-KY-02). Tumor specimens were obtained and used with the written and informed consent of all patients, whose ages were over 18 years old, following the principles of the Helsinki Declaration.

Isolation of CD133$^+$ cells

The trypsinized Hep-2 cells were washed twice with 0.01 M phosphate-buffered saline (PBS). Cells were then incubated with PE-conjugated CD133 at 4°C for 30 min. Subsequently, cells were washed with PBS and prepared for the sorting of CD133$^+$ and CD133$^-$ cell subpopulations by using a flow cytometer (BD Bioscience). The sorted CD133$^+$ and CD133$^-$ cells were used for the following experiments.

Cell line culture

Hep-2 cells were acquired from the American Type Culture Collection (Manassas, VA). The cells were revitalized and cultured in RPMI-1640 medium (HyClone, Logan, UT) supplemented with 10% fetal calf serum (Sijiqing Co., China), 100 µg/ml penicillin, and 100 µg/ml streptomycin in an incubator at 37°C with 5% CO_2. The selected cells with high expression levels of CD133 were cultured in serum-free RPMI-1640 supplemented with 0.5% BSA, 40 ng/ml β-FGF, 100 ng/ml epidermal growth factor (EGF), 5 µg/ml insulin, 100 µg/ml penicillin, and 100 µg/ml streptomycin. After 1 week, the CD133$^+$ cells readily proliferated and formed spheres.

Migration and invasion assays

The migration and invasion of cells were determined by using a 24-well polycarbonate transwell chamber with an 8-µm diameter pore size. The sorted cells were resuspended in serum-free RPMI-1640 medium and plated into the upper chamber at a density of 2.5×10^4/well. The lower chamber was filled with 500 µL medium containing 10% FBS as the attractive substance. After incubation for 24 h at 37°C, the chambers were fixed with methanol and stained with Giemsa according to the manufacturer's instructions and all the noninvaded (or nonmigrated) cells were removed. The migratory cells were counted in 3 random fields per chamber under a microscope.

Western blot analysis

Total protein extraction was performed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with 1% phenylmethylsulfonyl fluoride (PMSF; Beyotime). Protein concentrations of each sample were determined by BCA assays. Lysates containing 40 µg of protein were electrophoresed by 8–12% SDS/PAGE, and then transferred to polyvinylidene difluoride membranes. GAPDH was used as a reference protein. After the membranes were blocked with skimmed milk for 1 h at room temperature (RT), the membranes were incubated and blotted with the corresponding primary anti-rabbit antibodies overnight at 4°C. Membranes were washed with TBS plus 0.1% Tween-20. After incubation with a horseradish peroxidase-conjugated antibody (ZhongShan Biotechnology Co., Beijing, China) for 1 h at RT, immunoreactive bands were detected using chemiluminescence reagents.

Establishment of a lung metastasis mouse model and treatment of animals

The murine experiments were approved by the Ethics Committee of Bethune International Peace Hospital and maintained according to institutional guidelines. To assess the capability of CD133$^+$ cells to form lung metastatic tumors, male BALB/c nude mice (Vital River Laboratory Animal Technology Co., Beijing, China), 3–4 weeks old, weighing 12–18 g, were randomly divided into 2 groups – the experimental group (n=7) and the control group (n=7). Each mouse assigned to the experimental group was injected with 5×10^5 CD133$^+$ cells suspended in 200 µl culture medium into the tail vein. In the control group, all mice were injected with the same number of CD133$^-$ cells. To test the effects of DHA on preventing distant metastasis induced by CSCs, 14 mice were injected with 5×10^5 CD133$^+$ cells through the tail vein. The animals were randomly divided at into a control group and a treatment group. On the second day after injection, the mice in the treatment group were given DHA dissolved in DMSO at a dose of 50 mg/kg (body weight). The mice in the control group were treated with DMSO [34]. The same injections were performed once daily thereafter until termination of the *in vivo* experiments.

The mental state, body weight, or death status of the animals were evaluated every day. Any mouse demonstrating a 20%

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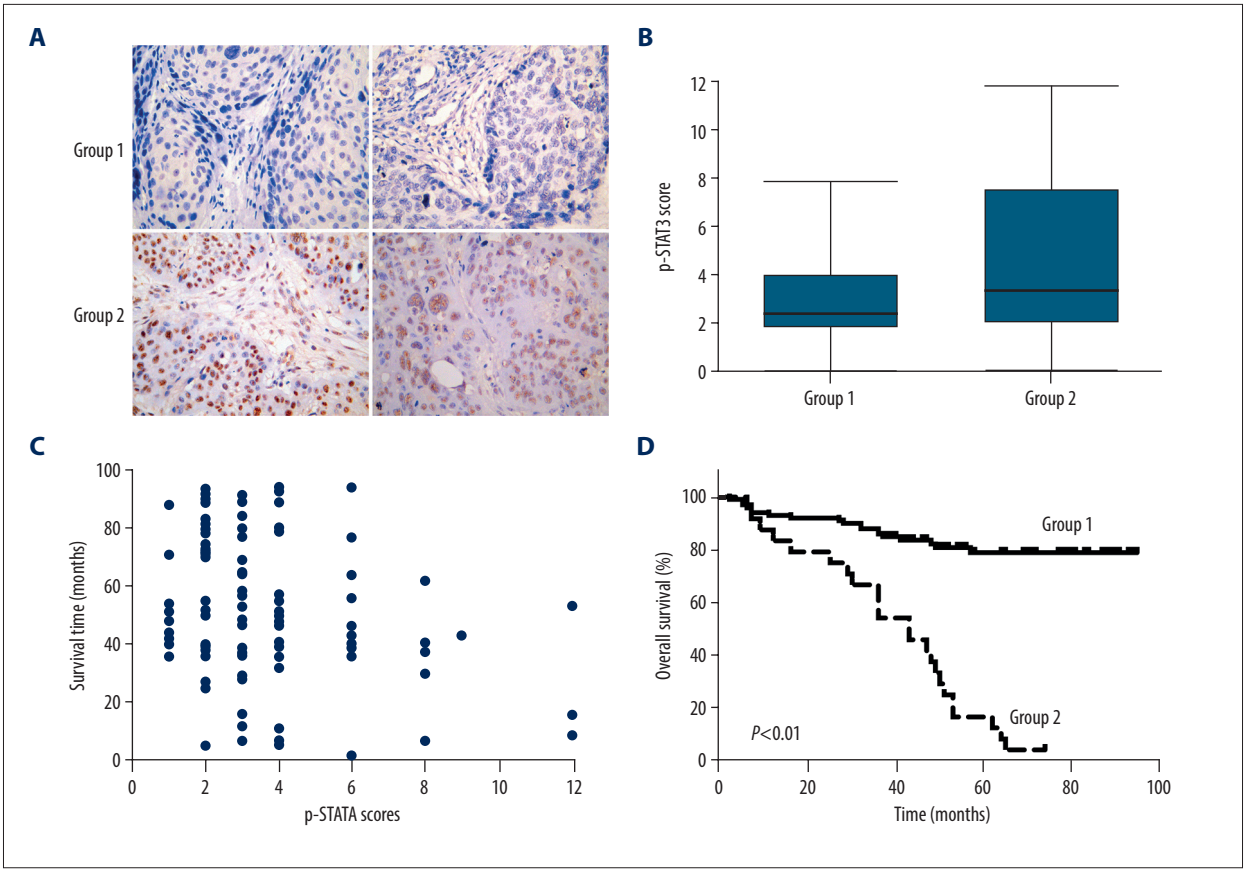

Figure 1. Activation and expression of STAT3 are associated with metastasis and prognosis in patients with laryngeal carcinoma (group 2 and group 1 represent patients with and without distant metastasis, respectively). (**A**) Immunohistochemical staining of p-STAT3 in patients with laryngeal carcinoma. (**B**) Comparison of p-STAT3 expression levels between group 1 and group 2. (**C**) Scatter plot of p-STAT3 expression levels in relation to individual patient survival in 124 laryngeal cancer patients. The Mann-Whitney U test was used to determine differences in the p-STAT3 score between the distant metastasis group and non-distant metastasis group. p-STAT3 scores between distant metastasis group (mean rank=79.75) and non-distant metastasis group (mean rank= 58.36) were significantly different (U=786, z=–2.665, p=0.008). (**D**) Kaplan-Meier analysis of overall survival in 124 laryngeal cancer patients. Group 1 included 100 patients who did not have distant metastasis, and the median survival time was 80.846 months, (95% CI: 75.042, 86.649); Group 2 included 24 patients with distant metastasis, and the median survival time was 39.333 months, (95% CI: 31.733, 46.934). The overall survival of the 2 groups was significantly different (chi-square=50.317, *P*<0.01).

to 25% loss of its original body weight or cachexia and self-injurious behaviors as well as immobility, was considered to be dead. All animals were euthanized at the end of the experiment. The lungs were surgically resected, and the suspected metastatic foci were identified, located, and counted. Metastatic lesions were confirmed by pathological examinations of tissue sections of the suspected nodules in the lung.

Statistical analysis

SPSS statistical software was used to perform statistical analysis. Data for analysis were expressed as the mean±SD, and comparisons between/among groups were performed using the *t* test or one-way analysis of variance (ANOVA). The Mann-Whitney U test was used to determine differences in the p-STAT3 score between the distant metastasis group and the non-distant metastasis group. Tukey's test was used for multiple comparisons between groups. Kaplan-Meier analysis with the log-rank test was used to determine survival differences [35]. A P-value <0.05 was considered statistically significant.

Results

Activation and overexpression of STAT3 are associated with distant metastasis and survival in laryngeal carcinoma patients

As shown in Figure 1A, p-STAT3 is mainly located in the nuclei of cancer cells. The expression of p-STAT3 was significantly

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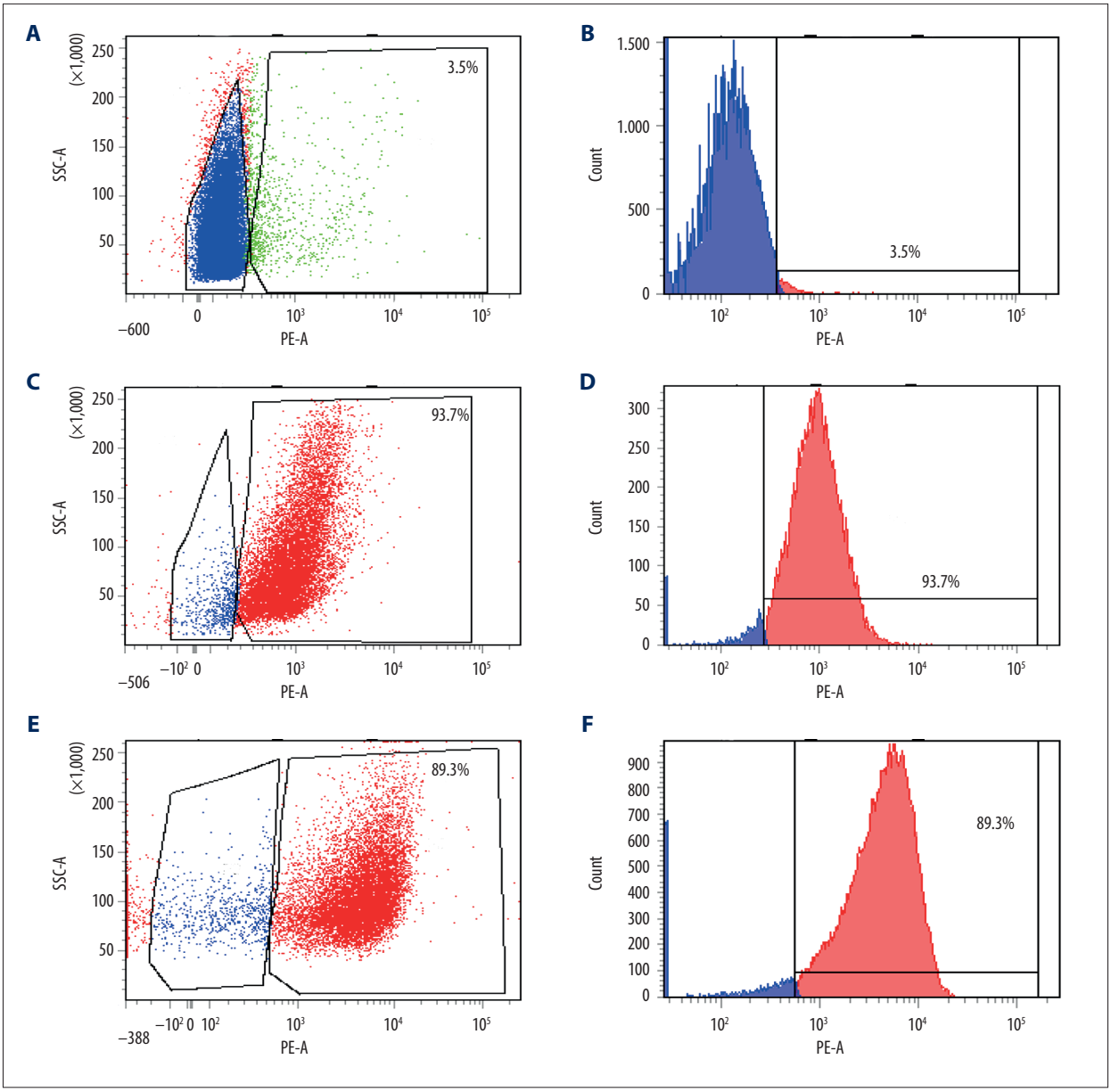

Figure 2. Sorting CD133⁺ cells from the Hep-2 laryngeal squamous carcinoma cell line (Hep-2 cells). (**A**) Scatter diagram and (**B**) corresponding peak chart displaying the purity of CD133⁺ cells sorted from Hep-2 cells by flow cytometry, as shown by the percentage of CD133⁺ cells. (**C**) Scatter diagram and (**D**) corresponding peak chart showing the percentage of CD133⁺ cells detected by flow cytometry after separation from Hep-2 cells. (**E**) Scatter diagram and (**F**) corresponding peak chart displaying the purity of CD133⁺ cells cultured in SFM supplemented with growth factor for 1 week after preliminary cell sorting.

higher in metastatic patients (group 2) than in nonmetastatic patients (group 1) (Figure 1B). The expression of p-STAT3 had a great impact on the survival of laryngeal cancer patients (Figure 1C). ROC curves of the p-STAT3 expression level were used to predict distant metastasis in patients with laryngeal carcinoma. As calculated, the area under the curve (AUC) was 0.6760. The cutoff value of the p-STAT3 expression level for predicting distant metastasis in laryngeal cancer was 3.0. Quite expectedly, distant metastasis had a significant influence on survival of laryngeal cancer patients (Figure 1D).

Purity of CD133⁺ cells after cell sorting and *in vitro* expansion

The separation and enrichment of CSCs were performed based on differences in cell surfaces. Here, we used CD133⁺

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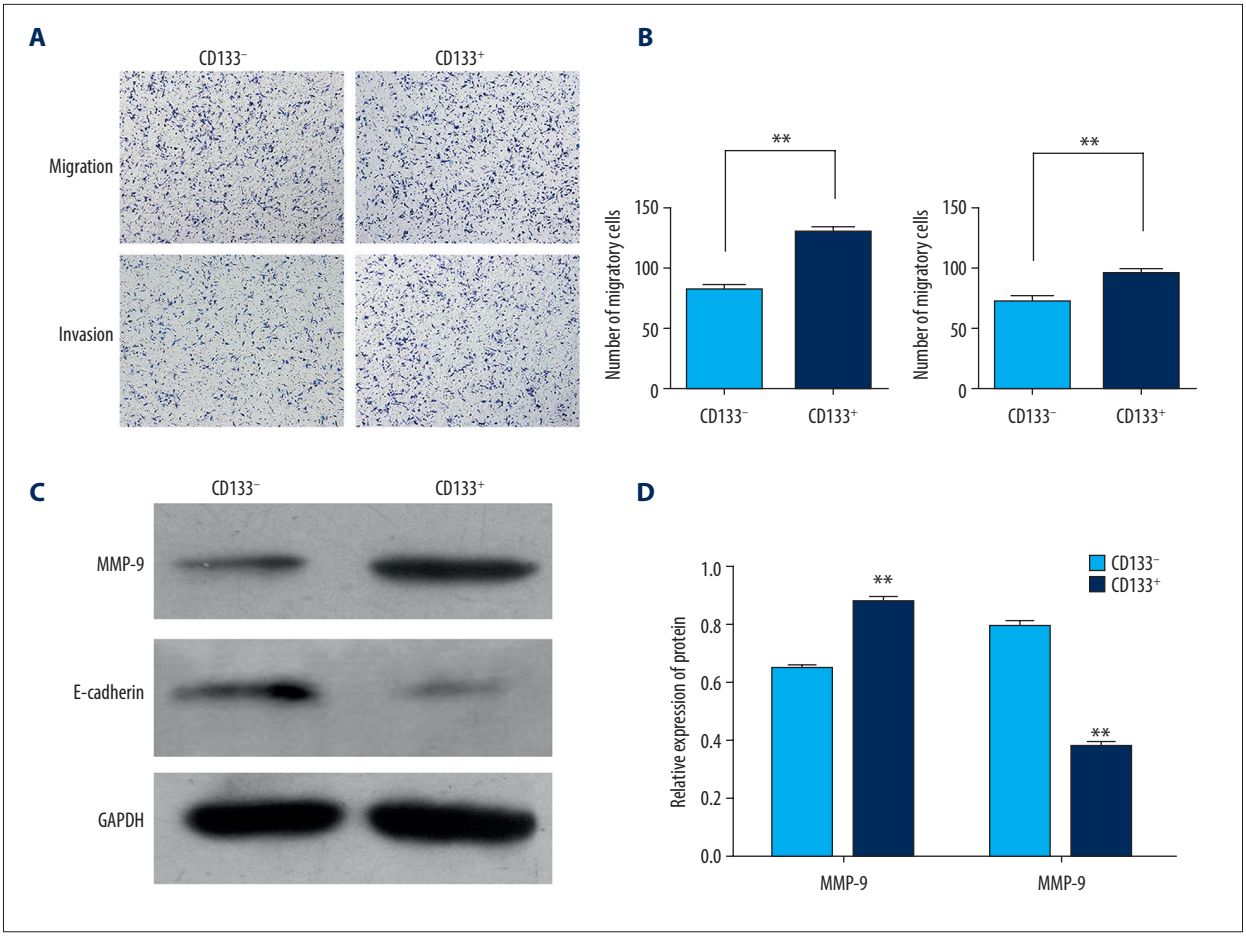

Figure 3. Comparison of migration/invasion between CD133⁻ and CD133⁺ Hep-2 cells. (**A**) CD133⁺ and CD133⁻ cells were seeded onto a upper wells of the transwell chamber with SFM, and medium containing FBS was added into the lower wells. After incubation for 24 h, migration/invasion ability was assessed by counting the number of cells crossing the chamber (original magnification, ×100). (**B**) Corresponding histogram showing the migration/invasion ability of the tested cells. Data are from 3 independent experiments and are shown as the mean±SD. ** $p<0.01$. (**C**) Expression of EMT-related markers MMP-9 and E-cadherin was measured in CD133⁻ and CD133⁺ cells by Western blot. (**D**) The relative gray value of the 2 groups is shown as the mean±SD, ** $p<0.01$.

cells, which accounted for 3.5% of all Hep-2 cells, as shown in Figure 2A (scatter diagram) and 2B (corresponding peak chart), consistent with previous reports [36–38]. The proportion of CD133⁺ cells in the selected cells was identified by flow cytometry, as shown in Figure 2C (scatter diagram) and 2D (corresponding peak chart). The 2 sets of selected cells were cultured in SFM for approximately 1 week. CD133⁺ cells formed typical spheres, but CD133⁻ cells were scattered in the culture medium. Thus, CD133⁺ cells possess higher colony-forming capability than CD133⁻ cells. The purity of CD133⁺ cells after amplification for 1 week was maintained at approximately 89.3%, as shown in Figure 2E (scatter diagram) and 2F (corresponding peak chart), showing that the purity of CSCs was adequate for subsequent experiments.

CD133⁺ cells exhibit stronger migration and invasion abilities than CD133⁻ cells

Findings from several other studies suggested that the expression of CD133 results in increased invasion and metastasis [39–41]. To assess the difference in migration and invasion between CD133⁺ and CD133⁻ subpopulations in Hep-2 cells, we counted the number of cells crossing the membrane after 24-h incubation *in vitro*. CD133⁺ cells revealed significantly enhanced invasion and migration abilities compared to CD133⁻ cells (Figure 3A, 3B). Additionally, CD133⁺ cells exhibited increased expression of MMP-9 and decreased expression of E-cadherin (Figure 3C, 3D). These findings suggest that changes in EMT-associated protein expression patterns promote the invasion/migration of CD133⁺ cells. Overall, MMP-9 and E-cadherin regulated migration and invasion in these 2 kinds of cells.

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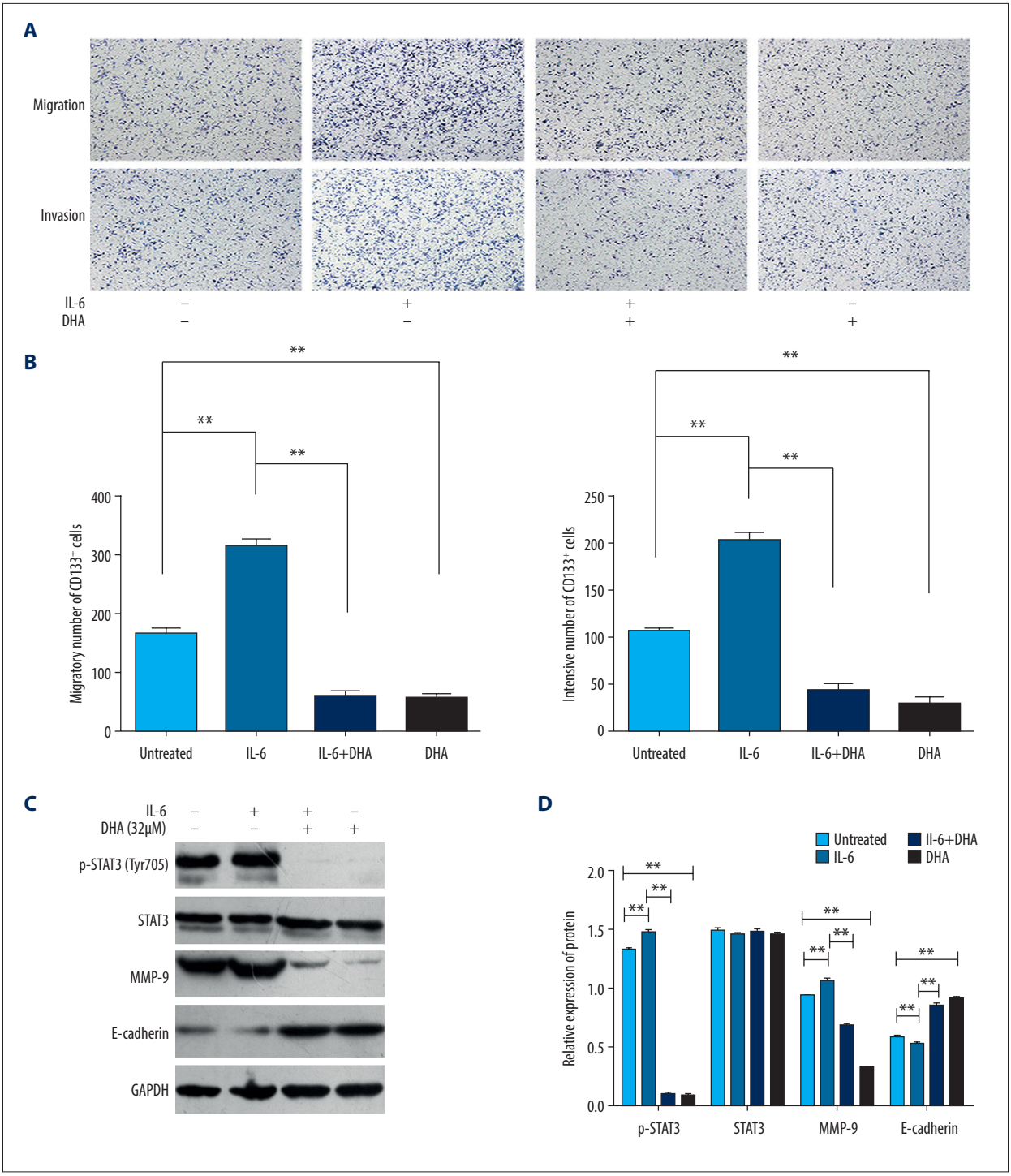

Figure 4. DHA inhibited IL-6-induced migration/invasion in CD133⁺ cells. (**A**) DHA restrained the migration/invasion of CD133⁺ cells after IL-6 stimulation. CD133⁺ cells were pretreated with IL-6 (20 ng/mL) for 1 h, and treated with DHA (32 μM) for 24 h. The concentration of DHA was established by concentration-escalation assay (data not shown). Transwell assay was performed to detect the migration/invasion of CD133⁺ cells under various conditions (original magnification, ×100). (**B**) The number of cells that exhibited migration/invasion was calculated and is expressed in the indicated histograph. Data are shown as the mean±SD, ** $p<0.01$. (**C**) DHA inhibited STAT3 activation and affected the expression of STAT3 downstream proteins. All indicated proteins were analyzed by Western blot. (**D**) The relative gray value of every group is shown as the mean±SD, ** $p<0.01$.

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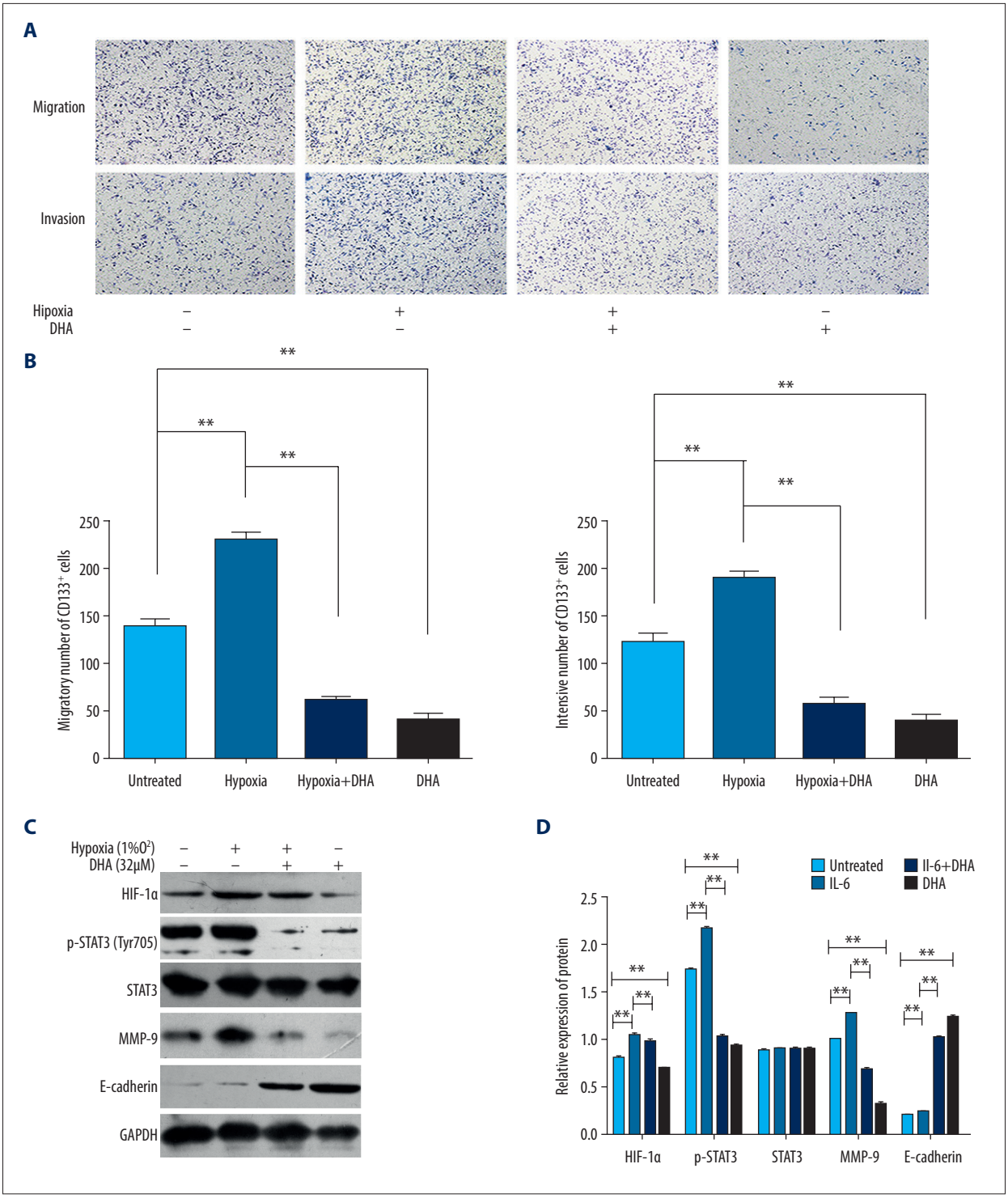

Figure 5. DHA inhibited migration/invasion induced by hypoxia in CD133⁺ cells. (**A**) DHA suppressed the migration/invasion of CD133⁺ cells under hypoxia. The invasive and metastatic ability of CD133⁺ cells were measured by transwell assays after DHA treatment (32 μM) under hypoxic conditions for 24 h. (**B**) The comparison of invasion and metastasis is presented in the histograms. The migrating/invading cells were counted under a microscope at 100× magnification. Data are displayed as the mean±SD, ** $p<0.01$. (**C**) DHA inhibited activation of STAT3 induced by hypoxia. The expression of HIF-1α and p-STAT3 and downstream proteins related to EMT was examined by Western blot. (**D**) The relative gray value of every group is shown as the mean±SD, ** $p<0.01$.

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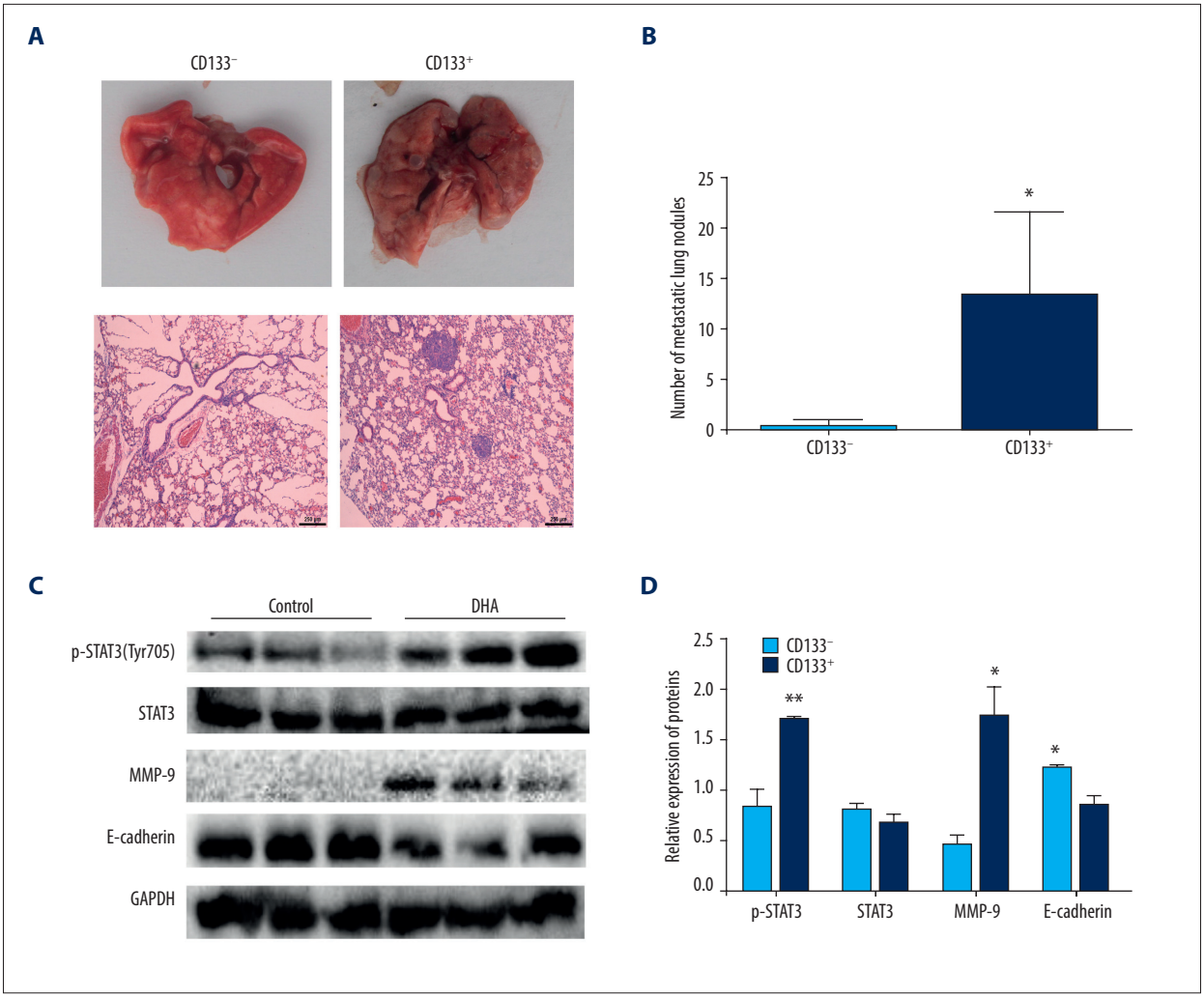

Figure 6. CD133⁺ cells are more likely to form lung metastasized foci than CD133⁻ cells *in vivo*. (**A**) Lung morphology from metastatic mouse models induced by CD133⁻ and CD133⁺ cells. Representative image of lung tissue following histological H&E staining of the 2 groups is shown in the inset. (**B**) The number of lung nodules in mice injected with CD133⁻ and CD133⁺ cells through the tail vein was calculated, and the data are expressed as the mean±SD (n=7), * $p<0.05$. (**C**) Expression of STAT3 and its downstream proteins was correlated with invasion and metastasis in metastatic tumors from the CD133⁺ and CD133⁻ laryngeal cancer cell established model of lung metastatasis, as assessed by Western blotting. (**D**) The relative gray value of every group is shown as the mean±SD, * $p<0.05$, ** $p<0.01$.

DHA inhibits the migration and invasion of CD133⁺ cells in response to IL-6 stimulation by blocking STAT3 activation

Increased circulating IL-6 levels and the downstream effector STAT3 are associated with metastatic progression of CSCs [42]. As shown in Figure 4A, IL-6 increased the migration/invasion of CD133⁺ cells, suggesting that blocking the IL-6/STAT3 axis can prevent the spread of CD 133⁺ cells. We first tested the effect of DHA, a putative STAT3 inhibitor, on the spread of laryngeal CSCs. We found that DHA significantly inhibited the migration and invasion of CD133⁺ cells in the presence or absence of IL-6 (Figure 4A). Invasion and migration for every group were determined using ImageJ software. The number of cells displaying invasion/metastasis was counted and the corresponding histograms are shown in Figure 4B. To further understand the potential mechanisms, we examined the changes in the expression of the corresponding EMT-associated proteins. When IL-6 was administered, STAT3 was activated. The active form of STAT3 is phosphor-STAT3 (p-STAT3), and this form was efficiently inhibited by DHA. Changes in the expression levels of the tow EMT-correlative proteins, MMP-9 and E-cadherin, were also noticed. IL-6 increased the expression of MMP-9 and decreased the expression of E-cadherin. However, when DHA was added, the expression of MMP-9 was significantly decreased, but that of E-cadherin was markedly increased (Figure 4C, 4D), with decreased invasion and migration capabilities in CD133⁺

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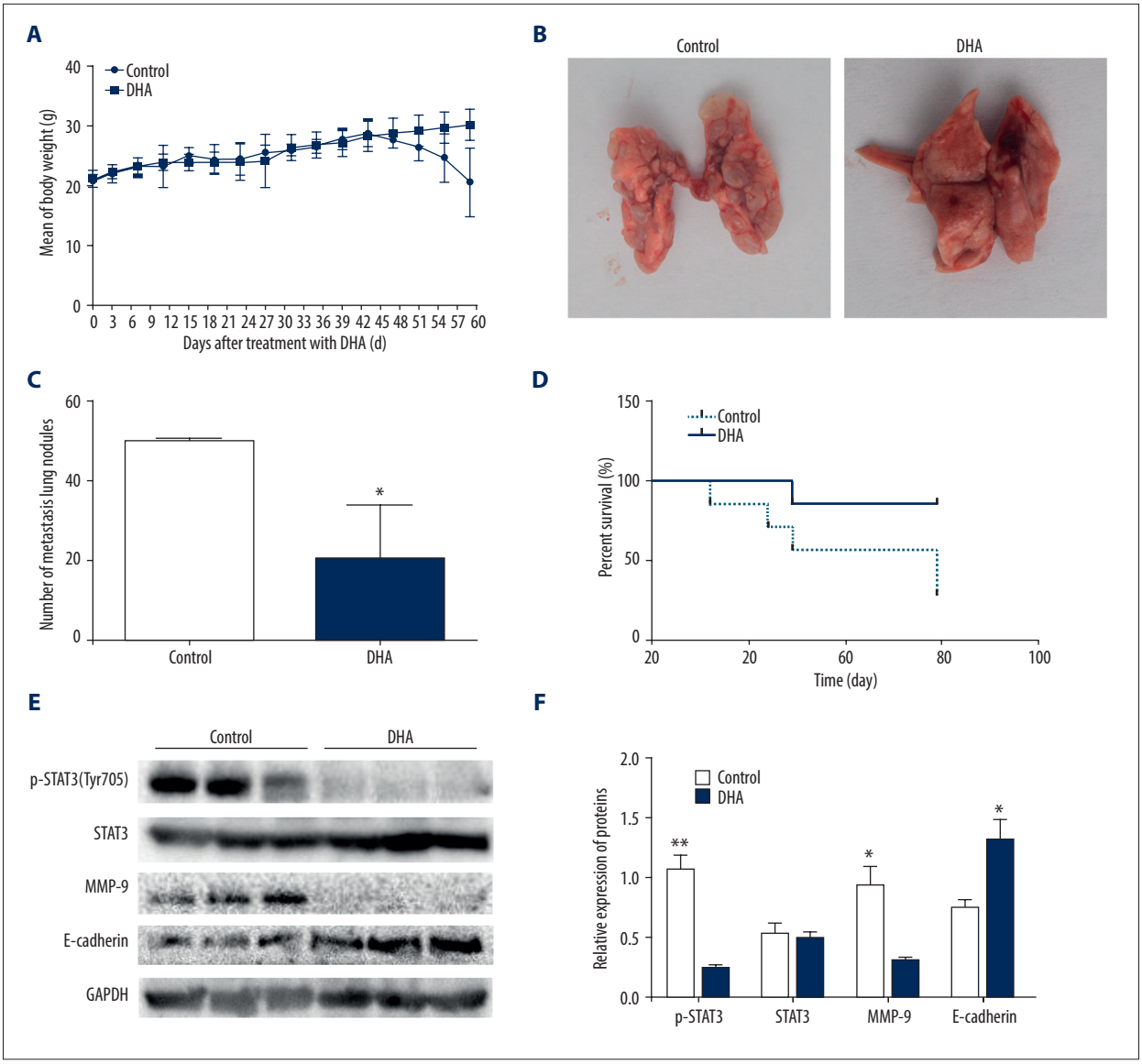

Figure 7. DHA inhibits laryngocarcinoma metastasis and promotes the survival of metastatic mice *in vivo*. (**A**) Dynamic changes in body weight of a mouse model of metastasis during DHA treatment. (**B**) CD133⁺ Hep-2 cells were used to establish a metastasis model, and animals were treated with DMSO or DHA. Representative whole specimens of the 2 groups. (**C**) The number of metastatic lung nodules is shown as the mean±SD, * $p<0.05$. (**D**) DHA can improve the survival of CD133⁺ cell-induced metastatic mice, and DHA signficantly prolonged the survival of mice compared to the control group, $p<0.05$. (**E**) DHA selectively blocked the activation of STAT3 and its downstream proteins and is correlated with metastasis. (**F**) The relative gray value of every group is shown as the mean±SD, *$p<0.05$, ** $p<0.01$.

cells, as evidenced by the transwell assay. These results suggest that DHA inhibit IL-6-induced STAT3 activation and resultant CSC migration and invasion by regulating of the expression of MMP-9 and E-cadherin.

DHA effectively inhibits the migration and invasion of CSCs induced by hypoxia by preventing the activation of STAT3

In a variety of tumor types, hypoxia is a vital condition for tumor development. HIF-1α, generated under oxygen-deficient condition, is a major protein that promotes aggressiveness and metastasis. Hypoxia-induced EMT promotes metastasis [43]. In agreement with these findings, our observations indicated

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that the migration/invasion abilities of CD133+ cells were increased in response to hypoxia, and this effect was substantially countered by DHA (Figure 5A). We counted the number of cells displaying invasion/metastasis, and the corresponding histograms are shown in Figure 5B. The status of hypoxia was indicated by the increased expression of HIF-1α as demonstrated by Western blot. Hypoxia-induced STAT3 activation resulted in the upregulation of HIF-1α. DHA inhibited HIF-1α and p-STAT3 and regulated 2 downstream proteins related to EMT. In addition, the expression of MMP-9 was significantly decreased by treatment with DHA, with a simultaneous increase in the expression of E-cadherin (Figure 5C, 5D). Taken together, our results suggest that DHA reverses the hypoxia-induced effects on migration and invasion in CSCs by regulating the activation status and altering the expression of proteins related to EMT.

The CD133+ cell population possesses a greater propensity to form lung metastatic tumors than the CD133− cell population in mice

Based on the findings that CD133+ cells exhibit stronger migration and invasion abilities than CD133− cells, we further examined the metastatic potential of these 2 cell populations in a mouse model. After injection into the tail vein, CD133+ cells demonstrated a stronger ability to form lung metastasis foci than CD133− cells (Figure 6A). The lung tissues of mice were subjected to immunohistochemistry and hematoxylin and eosin (H&E) staining. The total number of metastatic nodules in the CD133− control group was less than that in the CD133+ group (Figure 6B), and the difference was statistically significant. The expression of STAT3 along with its downstream proteins related to metastasis, MMP-9 and E-cadherin, was also examined by Western blot analysis (Figure 6C, 6D).

DHA prevents tumor metastasis in the lungs and improves the overall survival of animals

To determine whether DHA has the potential to prohibit the metastasis of laryngocarcinoma *in vivo*, we treated mice with DHA after injecting CD133+ cells through the tail vein. At the termination of the experiments, the lung tissues were obtained and stained with H&E, and pulmonary nodules were counted under a microscope. The application of DHA resulted in no significant changes in the general condition or body weight of the experimental animals, which indicates that DHA has no obvious toxic effects (Figure 7A). However, DHA significantly decreased the number of metastatic lung nodules compared to vehicle (DMSO) (Figure 7B, 7C) in mice injected with CD133+ cells. The mice that received DHA treatment survived significantly longer than the mice in the control group (Figure 7D). Consistent with these findings, DHA treatment significantly inhibited the expression of p-STAT3 and MMP-9 and upregulated E-cadherin in metastatic tumors (Figure 7E, 7F).

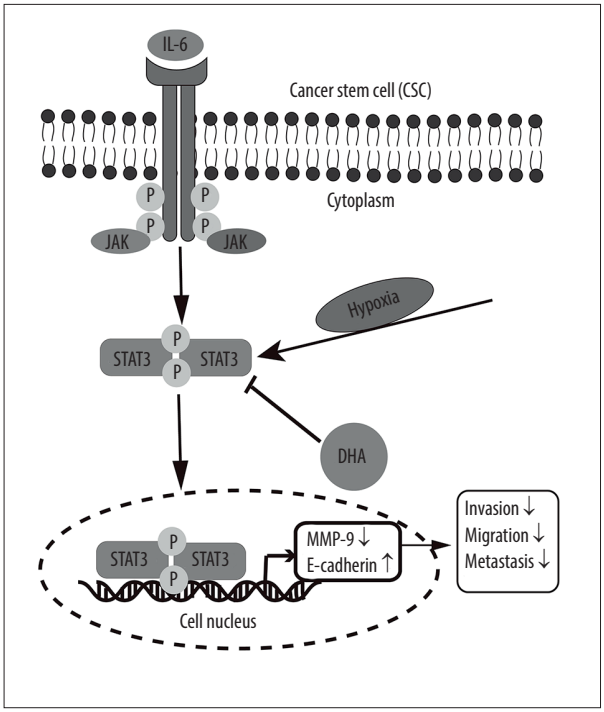

Figure 8. A diagram of the possible mechanism by which DHA inhibits STAT3 activation in CSCs and prevents the invasion, migration, and metastasis of laryngeal carcinoma induced by IL-6 and hypoxia in the tumor microenvironment.

Discussion

Distant metastasis is very difficult to predict and remains a major cause of poor prognosis in HNSCC. Once distant metastasis occurs, there is almost no cure for this unsatisfactory situation, and most such cases have lethal outcomes. Studies have demonstrated that the hyperphosphorylation and activation of STAT3 are associated with the clinicopathologic characteristics and prognoses of cancer patients [44]. A previous study in our laboratory found that DHA can inhibit the proliferation of HNSCC and trigger autophagy in HeLa cells [45,46]. Based on the above experiments, we further studied the effect of DHA on the invasion and metastasis of CSCs. In the present study, we found that the constitutive activation and overexpression of STAT3 are closely related to distant metastasis and poor prognosis of laryngeal cancer patients. In this regard, the expression of activated STAT3 can be used as a predictor for distant metastasis and poor prognosis. Targeting STAT3 activation could serve as a potential strategy for preventing distant metastasis in laryngeal carcinoma.

The activation of STAT3 and execution of its functions involves a series of extra- and intracellular events, which include the phosphorylation of STAT3 by specific cytokines and growth factors such as IL-6 and nonspecific stimuli such as hypoxia, followed

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by the dimerization of phosphorylated STAT3 (p-STAT3), and its translocation to the nucleus and binding to the transcription regions of its target genes, regulating the biological characteristics of certain cells, such as CSCs. In view of the importance of STAT3 activation in the pathogenesis and progression of cancers, STAT3 has been considered a cancer gene, and inhibition of its activation has become a promising strategy for cancer therapy.

CSCs, also known as tumor-initiating cells, are considered the "seeds" of tumors. CSCs account for only a small percentage of all cancer cells but play a pivotal role in metastasis/invasion of cancer [47]. We also demonstrated these results *in vitro* and *in vivo* (Figures 3A, 6A). CSCs are gradually becoming a new focus for exploring treatment strategies for tumor metastasis. To facilitate tumor progression, some CSCs undergo EMT to acquire the capacity to permeate through surrounding tissues and induce invasion and metastasis, suggesting that EMT is required for invasion and metastasis [48–50]. STAT3 activation causes changes in the expression of EMT marker proteins, indicating that STAT3 signaling may play an important part in the process of EMT [51]. It is highly likely that the inhibition of STAT3 can prevent EMT and thus the migration and invasion of cancers. Based on the accumulated findings, we postulate that CSCs play significant roles in cancer metastasis; therefore, targeting the CSC population may be an effective way to block migration and invasion in HNSCC. The identification of an effective STAT3 inhibitor that targets CSCs, a specific subpopulation of cancer cells that exhibit the excessive activation of STAT3, is essential for the overall inhibition of invasion and metastasis in HNSCC, such as laryngeal carcinoma.

To date, various inhibitors capable of suppressing the activation of STAT3[38], including peptides, peptidomimetics, and nonpeptide small-molecule compounds, have been studied. The peptides and peptidomimetics include ISS610 [52], BP-PM279G, BP-PM6 [53], and acetyl-pYLKTKF [54], which suppress the binding of STAT3 to a target gene by anchoring the SH2 domain of STAT3. Nonpeptide small-molecule compounds, including STA21, STX-0119, and S31-201, interact with certain *loci* associated with STAT3 phosphorylation or dimerization. Although many chemicals have been shown to be effective STAT3 inhibitors with high biological activities, almost all remain under investigation. For some products, the adverse effects and toxicities, as well as low bioactivity caused by metabolic inactivation within the human body, have prevented routine use in the clinic. Therefore, the identification of agents with fewer adverse effects and toxicities with strong inhibition of STAT3 activation is being conducted.

Research interest in identifying a STAT3 inhibitor has focused on natural agents [34]. For instance, DHA, originally developed as an antimalarial drug, is now being tested as a possible anticancer agent, and shows significant potential for use in the treatment of tumors [55]. Although DHA has certain curative effects with few adverse effects and considerable clinical safety, its effectiveness for the treatment of uveal melanoma [56] and non-small cell lung cancer [57], as well as its underlying antitumor mechanisms, is far from clear. We recently defined the conceivable targets and underlying mechanisms of DHA in suppressing STAT3 activation in parental HNSCC cells [58]. However, it is unknown whether DHA can suppress the invasion and metastasis of CSCs in human laryngeal carcinoma. Here, we proved for the first time that DHA treatment results in a significant decrease in the invasion and metastasis of laryngeal CSCs *in vitro* and *in vivo*. Metastasis is associated with poor prognosis and is an important factor when selecting appropriate treatments [59].

In the present investigation, CSCs from Hep-2 cells displayed higher metastasizing and invading capacities than cells with negative CSC surface markers, both *in vitro* and *in vivo*, consistent with findings from previous studies, especially for $CD133^+$ cells [60], $CD44^+$ cells [61], $ALDH^+$ cells [62], and side population [37] cells. Some studies have indicated that CD133 is one of the best markers that can be used to characterize CSCs and is an important marker for metastasis and prognosis [63]. EMT-associated events, including the increased expression of MMP-9 and the simultaneous loss of E-cadherin expression, were also observed in this study, indicating that CSCs of laryngocarcinoma must undergo EMT to achieve invasion and migration abilities. As a specific event in cancer progression, EMT is accompanied by a loss of cell-to-cell adhesion and connections between epithelial cells, and is characterized by the downregulation of E-cadherin. This downregulation of E-cadherin allows cells to separate from their adjacent cells and begin migration toward the circulatory or lymphatic system to spread to new locations [64]. In addition, increased MMP-9 expression functions to degrade the ECM, facilitating invasion by CSCs into surrounding tissue [65].

In the cancer microenvironment, STAT3 can be activated by various factors, such as certain cytokines and hypoxic conditions within solid tumors. Specifically, the present study found that STAT3 activation via IL-6 and hypoxia can increase invasion and metastasis abilities in $CD133^+$ cells, suggesting that the IL-6/STAT3 and hypoxia/STAT3 axis play vital roles in promoting the invasion/migration of laryngocarcinoma by regulating of CSCs. Blockage of the IL-6/STAT3 and hypoxia/STAT3 signaling pathways may be a promising strategy to decrease or prevent the invasion/metastasis of laryngeal carcinoma. Apart from invasion and migration of CSCs, angiogenesis is a crucial step in cancer metastasis, during which endothelial cells play very important parts [66]. Because DHA also inhibits endothelial cell function by repression of STAT3 signaling [67], the preventive effects of DHA on distant metastasis found *in vivo* in

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the present study are possibly due at least in part to the inhibition of angiogenesis [68]. The effects of DHA on angiogenesis-mediated tumor metastasis in laryngeal carcinoma and the underlying mechanisms remain to be further elucidated.

Conclusions

DHA is a potent STAT3 inhibitor that mediates its antimetastatic effects via the blockade of STAT3 phosphorylation and activation, which is at least in part due to the regulation of its downstream factors, MMP-9 and E-cadherin. In particular, DHA can effectively suppress the invasion and migration of laryngeal CSCs by inhibiting STAT3 activation and thus affecting the EMT of CSCs, as well as improve the survival of animals with distant metastasis (Figure 8). Due to its negligible toxicities and few adverse effects, and because it has already been proven in the clinical treatment of malaria, DHA may be an ideal agent for the chemoprevention and treatment of HNSCCs, and it also may prolong the survival time of patients with advanced tumors. Further in-depth and broad-based experimental and preclinical studies must be performed to carefully assess the feasibility and efficacy of DHA in clinical practice.

Acknowledgment

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Conflict of interest

None.

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