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TGFβ1 Promotes Breast Cancer Local Invasion and Liver Metastasis by Increasing the $CD44^{high}/CD24^{-}$ Subpopulation

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Abstract

Objective: Previous studies have shown that the transforming growth factor β1 pathway plays an important role in breast cancer metastasis to the liver. However, the mechanism of this metastasis has not been fully clarified. Cancer stem cells are essential for the initiation and propagation of tumor metastasis. The objective of our current study was to define the role of cancer stem cells in transforming growth factor β1-mediated breast cancer hepatic metastases. **Method:** Hematoxylin and eosin staining was used to assess the formation of breast cancer liver metastases and local invasion. Cancer stem cells surface markers (CD44, CD24, and Epithelial cell adhesion molecule [EpCAM]), luminal/mesenchymal markers (keratin8 and alpha smooth muscle actin), and proliferation markers (Ki-67 and cyclinD1) were detected by immunohistochemistry assays. Flow cytometry was used to evaluate the effect of transforming growth factor β1 on the $CD44^{+}/CD24^{-}$ cancer stem cell population. Quantitative real-time polymerase chain reaction was employed to assess the gene expression of the stem cell self-renewal markers nanog, pou5f1 (coding for Oct4), and sox2. **Results:** Transforming growth factor β1 increased the formation of liver metastases by the MDA-MB231 (MDA) breast cancer cell line but did not affect the liver metastasis of $CD44^{+}/CD24^{+}$ noncancer stem cells. Transforming growth factor β1 treatment did not significantly affect tumor proliferation *in vitro* or *in vivo*. Transforming growth factor β1 promoted mammary tumor local invasion. Furthermore, the $CD44^{high}/CD24^{-}$ cancer stem cell population was also significantly increased by transforming growth factor β1 treatment. Besides, the gene expression of the stem cell self-renewal markers (nanog, pou5f1, and sox2) and another stem cell surface marker (EpCAM) was increased by transforming growth factor β1 treatment. Finally, clusters of CD44-positive breast cancer cells were observed in the livers of mice from the control and transforming growth factor β1 pretreatment groups. **Conclusion:** Our results indicate that transforming growth factor β1 increases the local invasive capacity and liver metastasis of breast cancer cells by inducing the $CD44^{high}/CD24^{-}$ cancer stem cell population.

Keywords

TGFβ1, breast cancer, invasion, liver metastasis, cancer stem cell

Abbreviations

CSCs, ecancer stem cells; EMC, extracellular matrix; EMT, epithelial–mesenchymal transition; H&E, hematoxylin and eosin; KRT8, keratin8; TGF, transforming growth factor

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Introduction

Worldwide, breast cancer is the most commonly diagnosed cancer and it is the second most common cause of cancer-related death in women.[1] As diagnosis strategies and systemic cancer therapies improve, primary cancer is no longer the dominant cause of cancer-related death; instead, metastasis is primarily responsible for the poor clinical outcome of patients with cancer. Statistically, the 5-year survival rate of patients

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with localized breast cancer is as high as 98.6%, but the survival rate of patients with distant metastases is only 25.9%.[2] The liver is one of the most frequent metastasis sites of breast cancer; 60% of patients with breast cancer will ultimately develop liver metastases, and 10% of patients will have metastasis to the liver only.[2] Breast cancer metastases in the liver are always insensitive to traditional chemotherapy and are associated with a worse prognosis than lung or bone metastasis.[3] Therefore, the development of new targeted therapeutic strategies is expected to reduce the incidence of breast cancer hepatic metastasis and to improve the clinical outcome of patients with breast cancer.

Metastasis is a complicated multistep process. It begins with the proliferation and detachment of cancer cells from the primary tumor and is followed by regional invasion into the extracellular matrix (EMC). Then, migratory cancer cells enter into the circulatory system, arrest at a secondary site, and eventually take over the host organ.[4,5] Most cancer cells perish during the metastatic process; only a minority of cancer cells survive in the circulatory system and eventually settle in distant organs.[6,7] Cancer stem cells (CSCs) are a small cell population in solid tumors that possesses self-renewal and unlimited differentiation capacity.[8-10] This cell population plays an essential role in initiating tumor metastasis and promoting tumor cell metastasis to remote anatomical sites. In addition, these cells are also closely associated with tumor relapse and patient prognosis. An increasing amount of evidence shows that eliminating CSCs may help effectively reduce tumor metastasis and recurrence. Therefore, studying the CSC regulatory mechanism is beneficial for reducing breast cancer metastasis to distant organs, such as the liver.

The transforming growth factor β1 (TGFβ1) family is involved in various cell processes including cell proliferation, metastasis, apoptosis, and wound healing.[11] It has been reported that blocking the TGFβ1 pathway induces hepatic metastasis in colorectal and breast cancer.[12,13] Transforming growth factor β1 is also a well-known crucial regulator of epithelial–mesenchymal transition (EMT) and induces the transformation of epithelial cancer cells into the mesenchymal morphology.[14] Since mesenchymal cancer cells always display stem-like features and possess a high capacity for invasion and migration,[15] we hypothesized that TGFβ1 may increase breast cancer metastasis to the liver by potentiating the stem-like breast cancer cell population. In our study, we used nude mice to investigate the role of $CD44^{high}/CD24^{-}$ CSCs in TGFβ1-mediated breast cancer liver metastasis.

Materials and Methods

Cell Lines

The MDA-MB231 cell line was kindly provided by Dr Zhao, Zhejiang University. Breast cancer MDA-MB231 (MDA) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 2 mM L-glutamine. All cell lines were grown at 37°C in 5% CO_2.

Flow Cytometry

Adherent cells were dissociated into single cells, and 5×10^5 cells were washed with phosphate buffer saline (PBS) containing 0.5% bovine serum albumin (BSA) (Fluorescence Activted cell sorter [FACS] buffer) and were incubated with Allophycocyanin (APC)-conjugated anti-CD44 (APC mouse anti-human CD44 clone G44-26 [RUO] 560890; BD Biosciences, San Jose, California) and phycoerythrin (PE) conjugated anti-CD24 (PE mouse anti-human CD24 clone ML5 [RUO] 560991; BD Biosciences) for 30 minutes on ice. All antibodies were from BD Biosciences. Isotype-matched conjugated nonimmune antibodies were used as negative controls. After incubating with antibody, the cells were washed with FACS buffer 3 times and then were analyzed with a flow cytometer (BD Biosciences).

Orthotopic Breast Cancer Model

The animal study and the use of MDA cells in the mouse model were approved by the ethics committee of the Second Affiliated Hospital of Zhejiang Medical University (code: ZSLL-2015-196). Three-week-old female Balb/c nude mice were purchased from Shanghai SLAC Laboratory Animal Co, Ltd (Shanghai, China) and were allowed to adapt to the environment at the Zhejiang Chinese Medical University for 1 week. Fifty thousand MDA or TGFβ1-pretreated MDA cells in 100 μL of saline (20% Matrigel) were injected into the mouse mammary fat pads using a 30-gauge needle. PBS was injected into the mammary fat pad as a negative control. Tumor growth and size were measured weekly. The mice were killed 5 weeks later, and the mammary tumors, surrounding tissues, and livers were fixed in 10% neutral-buffered formalin for 1 day.

Hematoxylin and Eosin Staining

Mammary tumor sections were formalin fixed and paraffin embedded, then the sections were stained with hematoxylin and eosin (H&E) to assess for locally advanced cancer, including skeletal muscle and mammary fat pad invasion. Images of the tumors were photographed by a light microscope using 5× and 40× objectives. Ten slices were randomly chosen for analysis, and the number of lesions and the percentage of liver metastasis area were quantified using ImageJ software (version 1.51k).

Immunohistochemistry

Immunohistochemical analysis was performed on tissue samples with the EnVision 2-step method according to the following procedure. Conventional dewaxing and antigen microwave thermal retrieval were performed on 4-μm-thick serial sections of formalin-fixed and paraffin-embedded tissues. The sections were incubated in a 3% hydrogen peroxide solution to block endogenous peroxidase activity. Primary antibodies CD44

(ab51037, rabbit monoclonal to CD44, specific to human; Abcam, Cambridge, Massachusetts), Epithelial cell adhesion molecule (EpCAM) (ab20160, mouse monoclonal to EpCAM, specific to human; Abcam), CD24 (ab64064, rat monoclonal to EpCAM, specific to human; Abcam), Ki67 (GB13030-2; Servicebio, Wuhan, China), cyclinD1 (GB13079; Servicebio), and alpha smooth muscle actin (α-SMA, GB13044; Servicebio) were applied to the sections and incubated at 4°C overnight. Next, the sections were incubated for 20 minutes at room temperature. Polymerase auxiliary agent was then added, and the sections were incubated for another 30 minutes at room temperature. Subsequently, secondary antibodies (Bioss, Beijing, China) were applied. The reaction product was visualized after incubating the sections with the substrate/chromogen 3, 3′-diaminobenzidine (Sigma-Aldrich, St Louis, Missouri). Finally, the sections were counterstained with 0.02% hematoxylin (Sigma-Aldrich).

Quantitative Real-Time Polymerase Chain Reaction

MDA cells were lysate in TRIzol reagents (Invitrogen, Carlsbad, California) and total RNA was extracted according to the manufacturer's protocol. Reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen) and random primers as per the manufacturer's instructions. Quantitative real-time polymerase chain reactions were carried out using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, California) in a Rotor Gene 6000 PCR detection system (MBI Lab Equipment; Montreal Biotech Inc, Kirkland, Quebec, Canada). Polymerase chain reaction conditions were as follows: 95°C for 30 seconds, 40 cycles (95°C for 5 seconds and 60°C for 20 seconds). The primer sequences were as follows: SOX2 forward primer, TGGACAGTTACGCGCACAT; reverse primer, CGAGTAGGACATGCTGTACGT; pou5f1 (coding for Oct4) forward primer, GCTCGAGAAGGATGTGGTCC; reverse primer, CGTTGTGCATAGTCGCTGCT; NANOG forward primer, CATGAGTGTGGATCCAGCTTC; reverse primer, CCTGAATAAGCAGATCCATGG.

Statistical Analysis

The Bresalier semiquantitative scoring formula[16] was used to evaluate the staining results. Ten views were randomly selected from each slice, and the cell staining intensity was scored as follows: negative cells (−) had no color (0); weakly positive cells (+) presented as a yellow color (1); moderately positive cells (++) were a light brown color (2); and strongly positive cells (+++) were identified by a deep brown color (3). Within each field of view, the number of cells presenting with each staining intensity was counted, and the average staining intensity for each slice was calculated according to the following formula: Intensity score $= \Sigma[(0 \times F0) + (1 \times F1) + (2 \times F2) + (3 \times F3)]$, where $F = \% \times 10$ views. CD44-positive cells included those with weakly positive (+), moderately positive, or strongly positive (+++) staining. $CD24^-$ cells were negative or weakly positive. The differences between groups were analyzed using Student t test, and $P < .05$ was considered statistically significant.

Results

Transforming Growth Factor β1 Increased the Formation of Breast Cancer Liver Metastases

Previous studies have shown that the TGFβ1 pathway plays an essential role in modulating the hepatic metastasis of breast cancer and colorectal cancer. To investigate the function of TGFβ1 pretreatment on breast cancer liver metastasis, a breast cancer liver metastasis model was established by culturing MDA breast cancer cells either with or without 10 ng/mL TGFβ1 for 7 days and then injecting the cells into the mammary fat pad of nude mice. After 5 weeks of inoculation, mice were killed, and the whole liver of each mouse was collected and fixed; H&E staining of serial sections was used to assess the number of mice with breast cancer liver metastasis. As shown in Figure 1A and B, the number of mice with liver metastasis was increased 3-fold and the metastatic liver burden was increased 4-fold in the group injected with TGFβ1-pretreated cells compared to the animals receiving the vehicle control (Figure 1C and D).

To determine whether the hepatic metastases originated from the primary tumor, Ki-67, cyclinD1, and α-SMA expressions were examined by immunohistochemical staining. We observed that the Ki-67, cyclinD1, and α-SMA staining in the liver lesions were similar to that of the *in situ* carcinoma (Figure 1E), suggesting the liver metastases probably originated from the primary breast cancer.

Transforming Growth Factor β1 Did Not Significantly Affect Tumor Proliferation In Vitro or In Vivo

Since primary tumor proliferation, detachment, and local invasion are key steps in cancer metastasis, we wanted to better understand how pretreating breast cancer cells with TGFβ1 increased the formation of liver metastases. Breast cancer MDA cells (2×10^5) were cultured either with or without TGFβ1 stimulation for 4 days, then the cell number was counted and graphed. We observed no significant difference between the proliferation rate of untreated and TGFβ1-treated cancer cells (Figure 2A).

In line with our *in vitro* findings, after mice were injected with cancer cells *in vivo*, tumor size was measured weekly, and tumor volumes were calculated. Although the tumor size was slightly larger in mice injected with TGFβ1-treated cells (Figure 2B and C), the difference was not significant, as evidenced by a P value > .05. Together, our results suggested that TGFβ1 did not significantly affect tumor proliferation *in vitro* or *in vivo*.

Transforming Growth Factor β1 Promoted Mammary Tumor Local Invasion

To test the effect of TGFβ1 pretreatment on tumor local invasion, primary tumors and their surrounding tissues were fixed and subjected to H&E staining. We observed that the tumor

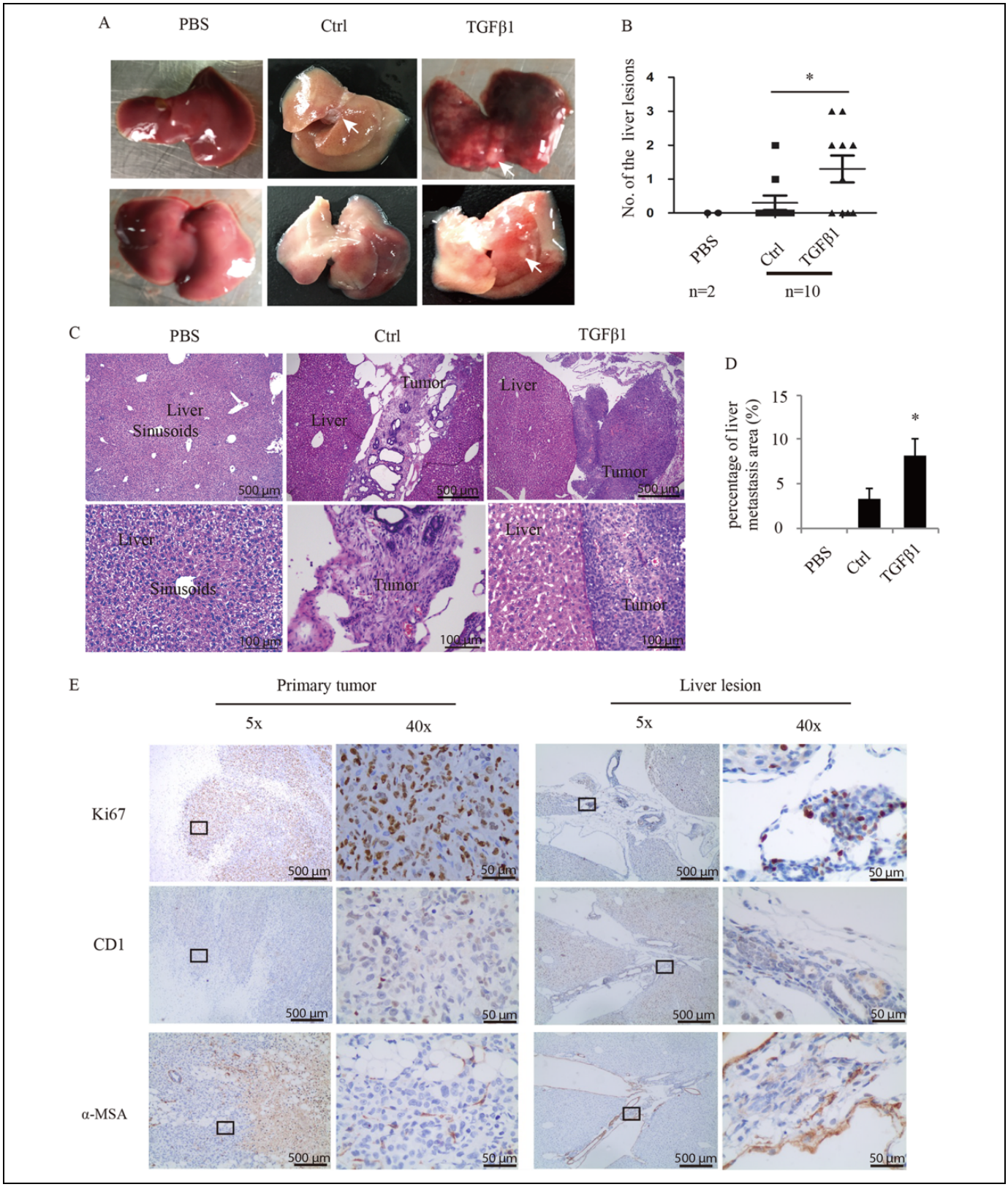

Figure 1. Transforming growth factor β1 (TGFβ1) increased the formation of breast cancer liver metastases. A, Liver metastasis formation in mice injected with PBS or MDA cells treated either with or without TGFβ1. B, The number of liver metastases and the metastatic burden was counted and graphed. *$P \leq .05$. C, Representative images of hematoxylin and eosin staining of the mammary glands of mice from different groups. D, The percentage of the liver metastasis area was analyzed and graphed by ImageJ (error bars indicate SEM; *$P \leq .05$). E, Immunohistological analysis of cyclinD1 (CD1) and alpha smooth muscle actin (α-SMA) in primary tumor and liver metastases derived from animals in the control and TGFβ1 pretreatment groups. Two independent experiments were performed.

APC: Allophycocyanin; EpCAM: Epithelial cell adhesion molecule; MDA: MDA-MB231; PBS: phosphate buffer saline; SEM: Standard Error of Mean.

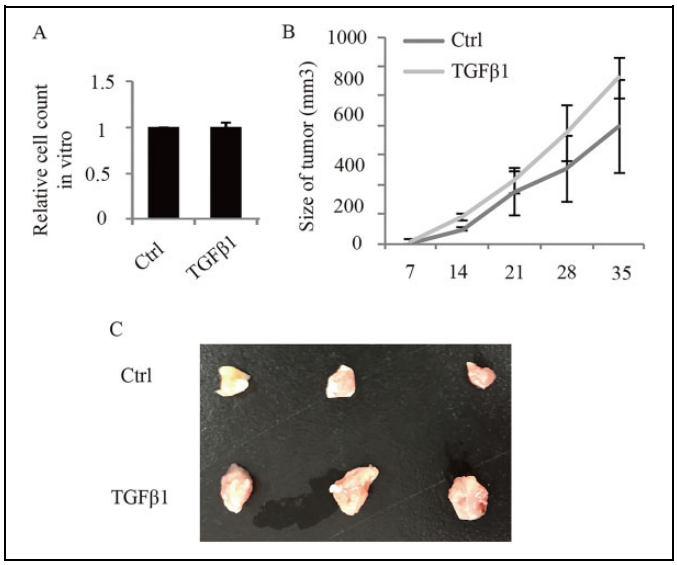

Figure 2. Transforming growth factor β1 (TGFβ1) did not significantly affect tumor proliferation *in vitro* or *in vivo*. A, MDA breast cancer cells (2×10^5) were cultured either with or without 5 ng/mL TGFβ1 for 4 days, and then the cell number was counted and graphed (error bars indicate SEM; n = 3 independent experiments). B, MDA cells from the control or TGFβ1 pretreatment group were implanted into the mammary fat pad of 3-week-old female Balb/c nude mice. Mammary tumor growth was measured from 2 sets of mice, and tumor size was quantified at the indicated times (3 tumors per group; error bars indicate SEM). C and D, Representative photos of primary tumors from mice in the control and TGFβ1 pretreatment groups. Three independent experiments were performed.
APC: Allophycocyanin; EpCAM: Epithelial cell adhesion molecule; MDA: MDA-MB231; PBS: phosphate buffer saline; SEM: Standard Error of Mean.

margins in animals injected with control cells were well encapsulated with a noninvasive nature. However, the deep tumor margins of the TGFβ1-pretreatment group were less distinct and invaded nearby structures, including skeletal muscles and the mammary fat pad (Figure 3A).

It is well known that mesenchymal cells are always found in the invasive front of advanced cancer and promote tumor invasive capacity. We performed immunohistochemistry on primary tumors arising from mice either with or without TGFβ1 pretreatment injections and assessed the level of the epithelial marker keratin8 (KRT8) and the mesenchymal marker α-SMA. We found that the expression of KRT8 was clearly higher in control tumors than in tumors from TGFβ1-pretreated cells, but α-SMA expression was higher in the TGFβ1 pretreatment group (Figure 3B), further confirming that the TGFβ1 pretreatment group tumors displayed more invasive features.

$CD44^+/CD24^-$ CSC May Be Responsible for TGFβ1-Mediated Breast Cancer Liver Metastasis

Cancer stem cells are a small population of cells in the tumor mass that are responsible for tumor migration, invasion, and metastasis. To elucidate the role of CSCs in TGFβ1-mediated breast cancer hepatic metastases, we examined the expression levels of CSC markers CD44, CD24, and EpCAM in secondary breast tumors to the those of the liver. Livers from animals with or without injections of TGFβ1-pretreated MDA cells were analyzed by immunohistochemistry, and CD44 expression was detected in the cells of the secondary breast tumor. As shown in Figure 4A, in the liver lesions of both the control and TGFβ1 pretreatment group, clusters of CD44-positive breast cancer cells were observed. The CD44 expression intensity was not markedly different between the 2 groups (Figure 4E). CD24 expression was barely detectable in the hepatic metastases of both groups (Figure 4B). EpCAM expression was slightly increased in the TGFβ1 pretreatment group compared to the control group (Figure 4C). Moreover, proliferative indices were further examined in the resulting lesions. Although the hepatic metastases from mice injected with TGFβ1-pretreated cells showed a slight increase in the degree of tumor cell proliferation (Ki67), no statistically significant differences in cell proliferation were observed among the liver metastases derived from these cohorts (Figure 4D). These results indicated that the $CD44^+/CD24^-$ CSC population may be responsible for the hepatic metastasis of breast cancer.

Since $CD44^+/CD24^-$ CSC account for more than 95% of MDA cells and CD44- non-CSC were less than 0.1%, non-CSC $CD44^+/CD24^+$ were sorted using a FACSAria cell sorting system to further investigate the TGFβ1-mediated breast cancer liver metastasis. $CD44^+/CD24^+$ cells were cultured with or without TGFβ1 for 7 days; then, these cells were injected into the mammary fat pad of nude mice. We found that mice from both the control and TGFβ1 pretreatment groups had minimal tumor development *in situ*, and after 5 weeks, when the mice were killed, hepatic metastases were not found. The results suggested $CD44^+/CD24^-$ CSC may be responsible for TGFβ1-mediated breast cancer liver metastasis.

Transforming Growth Factor β1 Increased the $CD44^{high}/CD24^-$ CSC Population in Breast Cancer In Vitro

To study TGFβ1 function on breast cancer $CD44^+/CD24^-$ CSC population, *in vitro* flow cytometry assays were used to assess the expression of the CSC markers CD44 and CD24 in breast cancer MDA cells in the presence or absence of TGFβ1 treatment. We found that repeated TGFβ1 stimulation increased the $CD44^+/CD24^-$ stem-like cell population from 94% to 98% (Figure 5A and B).

CD44 has been identified as a functional CSC marker in colon cancer,[17] and it is closely associated with CSC migration, invasion, and metastases. For these reasons, we used FACS analyses to assess the mean florescence intensity of CD44 in MDA cells with or without TGFβ1 stimulation. As Figure 5C shows, TGFβ1 significantly increased CD44 mean florescence intensity in MDA cells. The $CD44^{high}/CD24^-$ cell population was increased from 8.9% to 32% by TGFβ1 treatment (Figure 5D). Interesting, with the increased $CD44^{high}/CD24^-$ cell population proportion, the gene expression of the stem cell self-renewal markers nanog, pou5f1 (coding for Oct4), and sox2

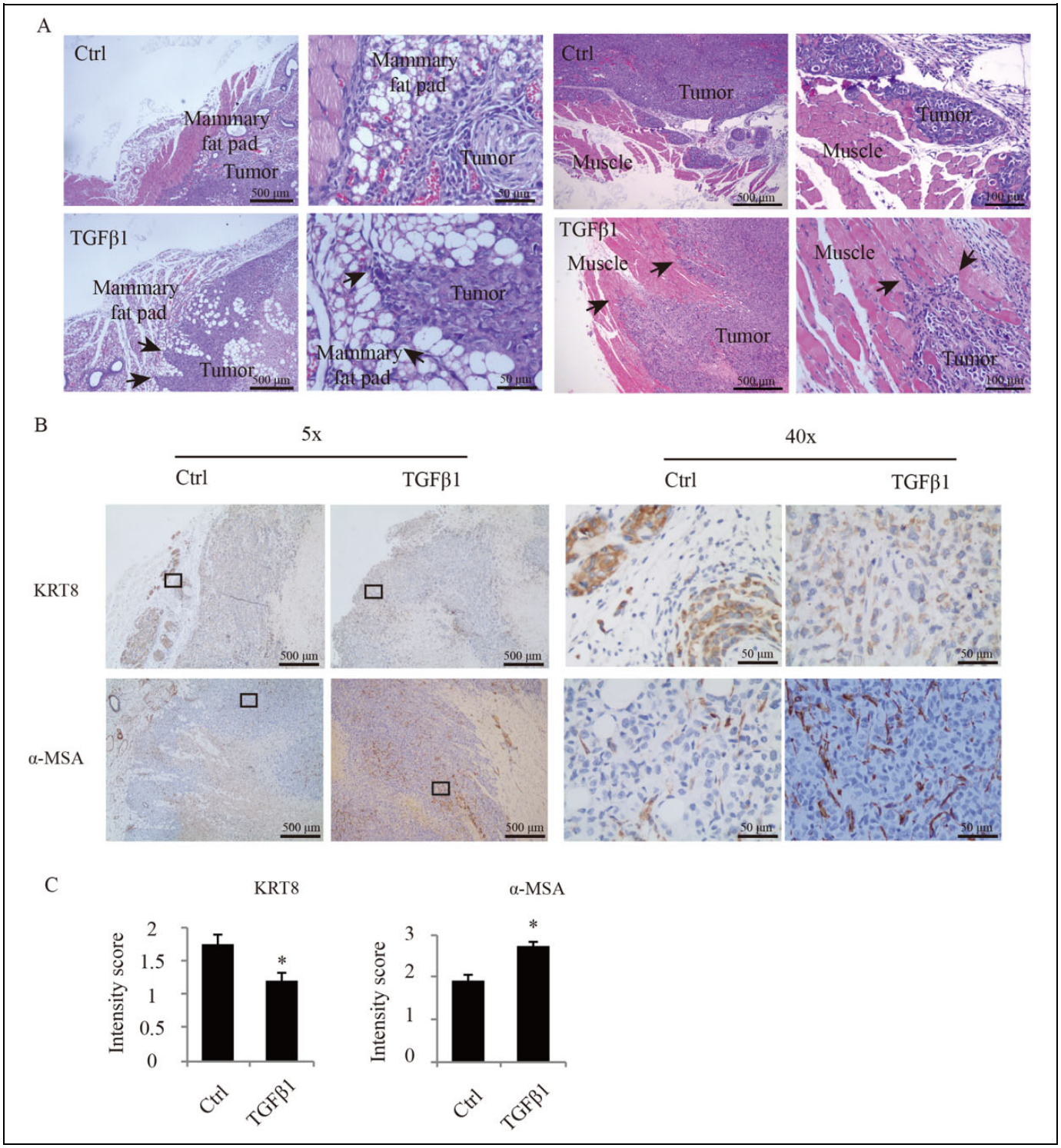

Figure 3. Transforming growth factor β1 (TGFβ1) promoted mammary tumor local invasion *in vivo*. A, Primary tumors and their surrounding tissues from the control and TGFβ1 pretreatment groups were fixed and subjected to hematoxylin and eosin staining. B and C, Immunohistological analysis of the epithelial marker keratin8 (KRT8) and mesenchymal marker α-SMA in primary tumors from animals in the control and TGFβ1 pretreatment groups. Three independent experiments were performed (error bars indicate SEM; $*P \leq .05$). α-SMA indicates alpha smooth muscle actin.

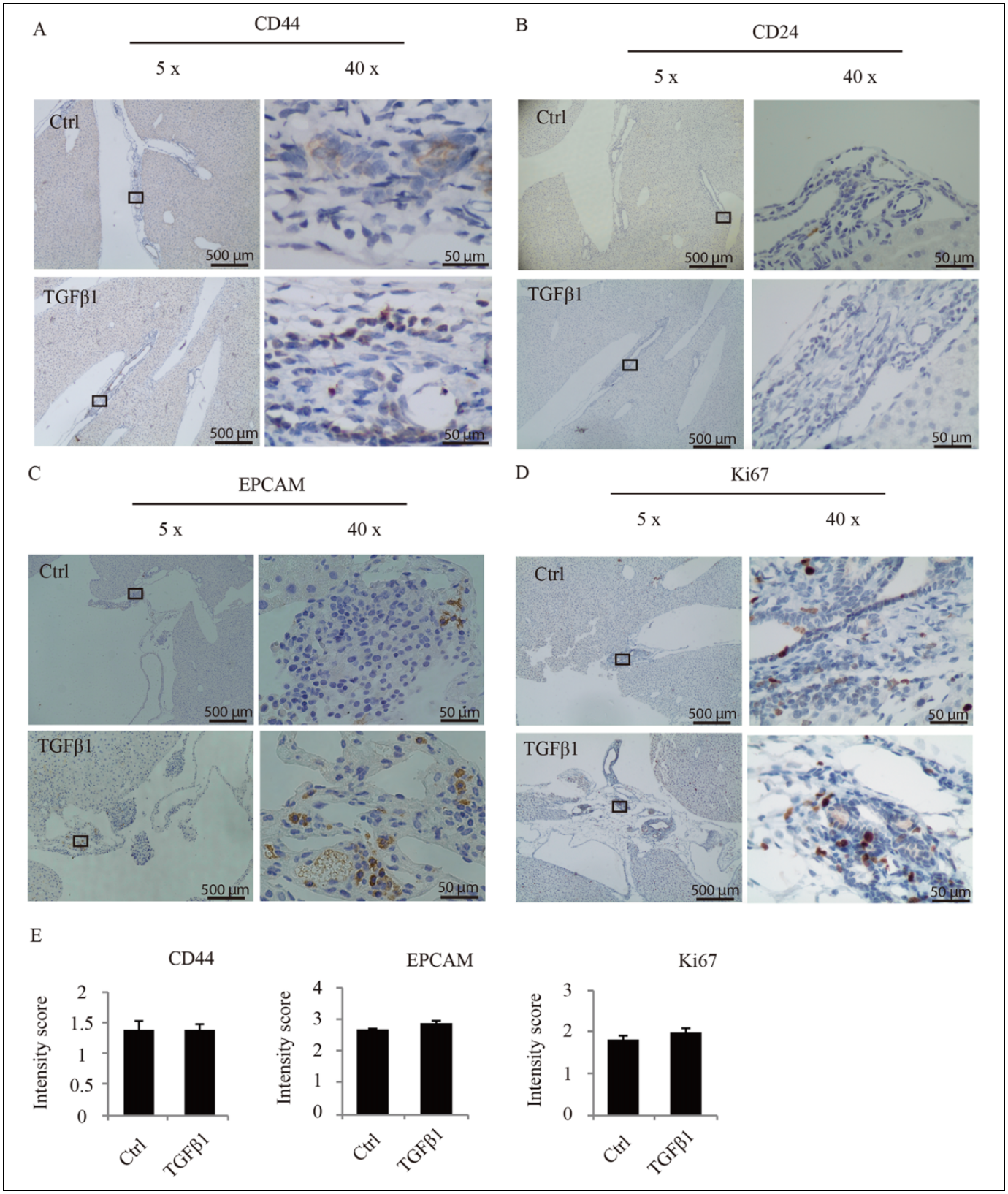

Figure 4. Expression of CD44, CD24, EpCAM, and Ki67 in hepatic metastases. Immunohistological analysis of CD44, CD24, EpCAM, and Ki67 expression in liver metastases derived from animals in the control and transforming growth factor β1(TGFβ1) pretreatment groups (error bars indicate SEM).

APC: Allophycocyanin; EpCAM: Epithelial cell adhesion molecule; MDA: MDA-MB231; PBS: phosphate buffer saline; SEM: Standard Error of Mean.

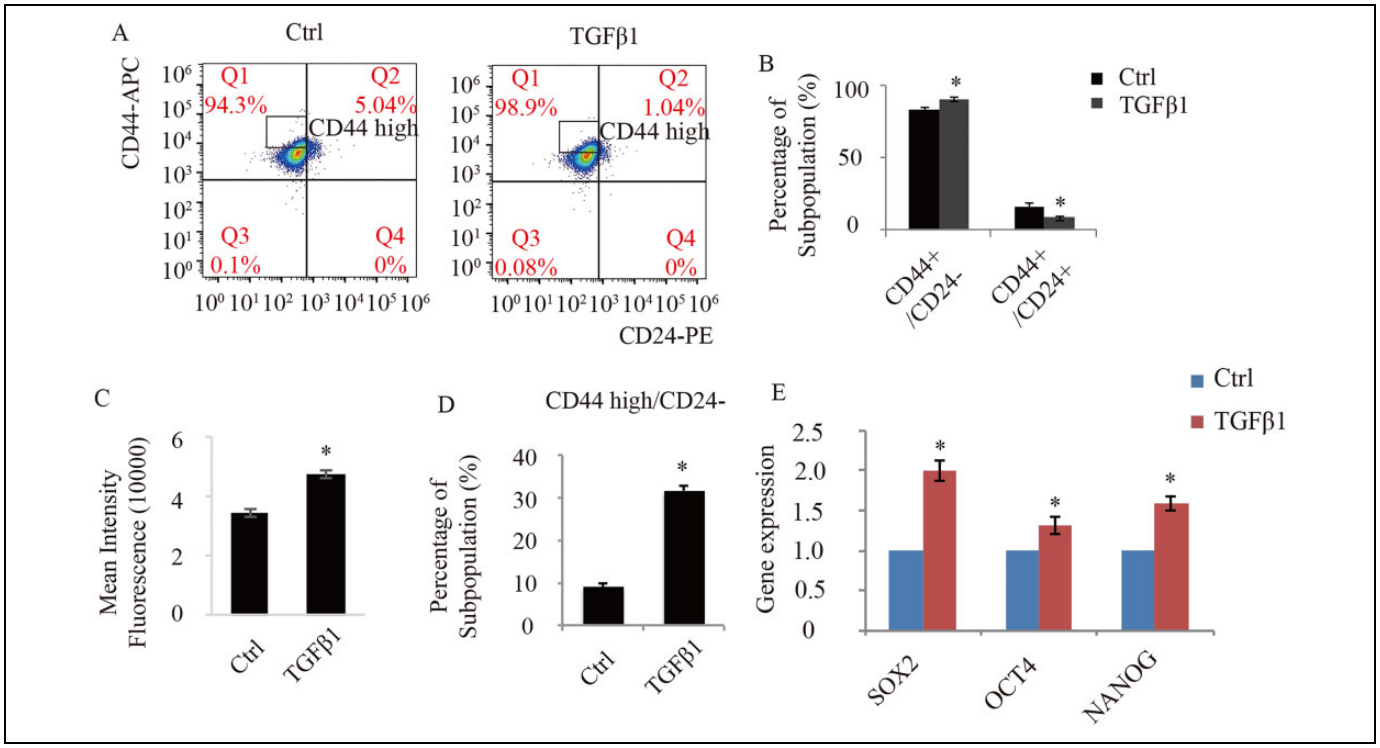

Figure 5. Transforming growth factor β1 (TGFβ1) increased the $CD44^{high}/CD24^{-}$ CSC population in breast cancer *in vitro*. A, MDA breast cancer cells (2×10^5) were cultured either with or without 5 ng/mL TGFβ1 stimulation for 4 days and were labeled with anti-CD44-APC and anti-CD24-PE antibodies. Then, the cells were analyzed by flow cytometry, and the mean fluorescence intensity and the proportions of the different $CD44^{high/low}/CD24^{-/low}$ subpopulations were graphed and quantified using FlowJo software (v10.4.1) (B, C, and D). E, Quantitative real-time polymerase chain reaction was used to evaluate the gene expression of the stem cell self-renewal markers nanog, pou5f1 (coding for Oct4), and sox2. Three independent experiments were performed (error bars indicate SEM).
APC: Allophycocyanin; EpCAM: Epithelial cell adhesion molecule; MDA: MDA-MB231; PBS: phosphate buffer saline; SEM: Standard Error of Mean.

was increased by TGFβ1 treatment (Figure 5E). These results indicate that TGFβ1 increased the $CD44^{high}/CD24^{-}$ cell population, which possesses greater "stemness" capacity and is closely associated with increased breast cancer local invasion and liver metastasis.

Transforming Growth Factor β1 Increased $CD44^{high}$ CSC Population in Breast Cancer In Vivo

To test whether this observation was also true *in vivo*, CD44 and CD24 expression levels were assessed by immunohistochemical analysis of the primary tumor mass from mice injected with or without TGFβ1-pretreated cancer cells, and the staining intensity was quantified using the Bresalier semi-quantitative scoring method. Compared to the control group, cells pretreated with TGFβ1 presented with a significant increase in CD44-positive cell number and staining intensity (Figure 6A-D). However, CD24 expression was barely detectable in both the control and TGFβ1 pretreatment groups (Figure 6A-D). Interestingly, in mice injected with TGFβ1-pretreated breast cancer cells, cancer cells with high CD44 expression were located at the invasive front of the primary tumor. Furthermore, along with the increased $CD44^{high}$ cell population, the expression of another stem cell surface marker, EpCAM, was also upregulated (Figure 6E). Together, our results suggested that TGFβ1 may increase the local invasive capacity and liver metastasis of breast cancer cells by inducing the $CD44^{high}/CD24^{-}$ CSC population.

Discussion

The liver is an important metastatic site of breast cancer; approximately 60% of patients with untreated breast cancer will develop liver metastases. Liver metastasis is considered an independent prognostic factor in patients with breast cancer. Transforming growth factor β1 has a wide range of influence on a large number of physiological and pathophysiological processes. Previous studies have shown that the knockdown of smad7, one of the key regulators of the TGFβ pathway, increased breast cancer metastasis to the liver. In our study, TGFβ1 pretreatment increased breast cancer hepatic metastasis, reiterating the crucial role of the TGFβ1 pathway in the regulation of breast cancer metastasis. However, the underlying mechanism has not fully delineated.

Breast cancer hepatic metastasis is mainly hematogenous. The first key steps include the expansion of the primary tumor,

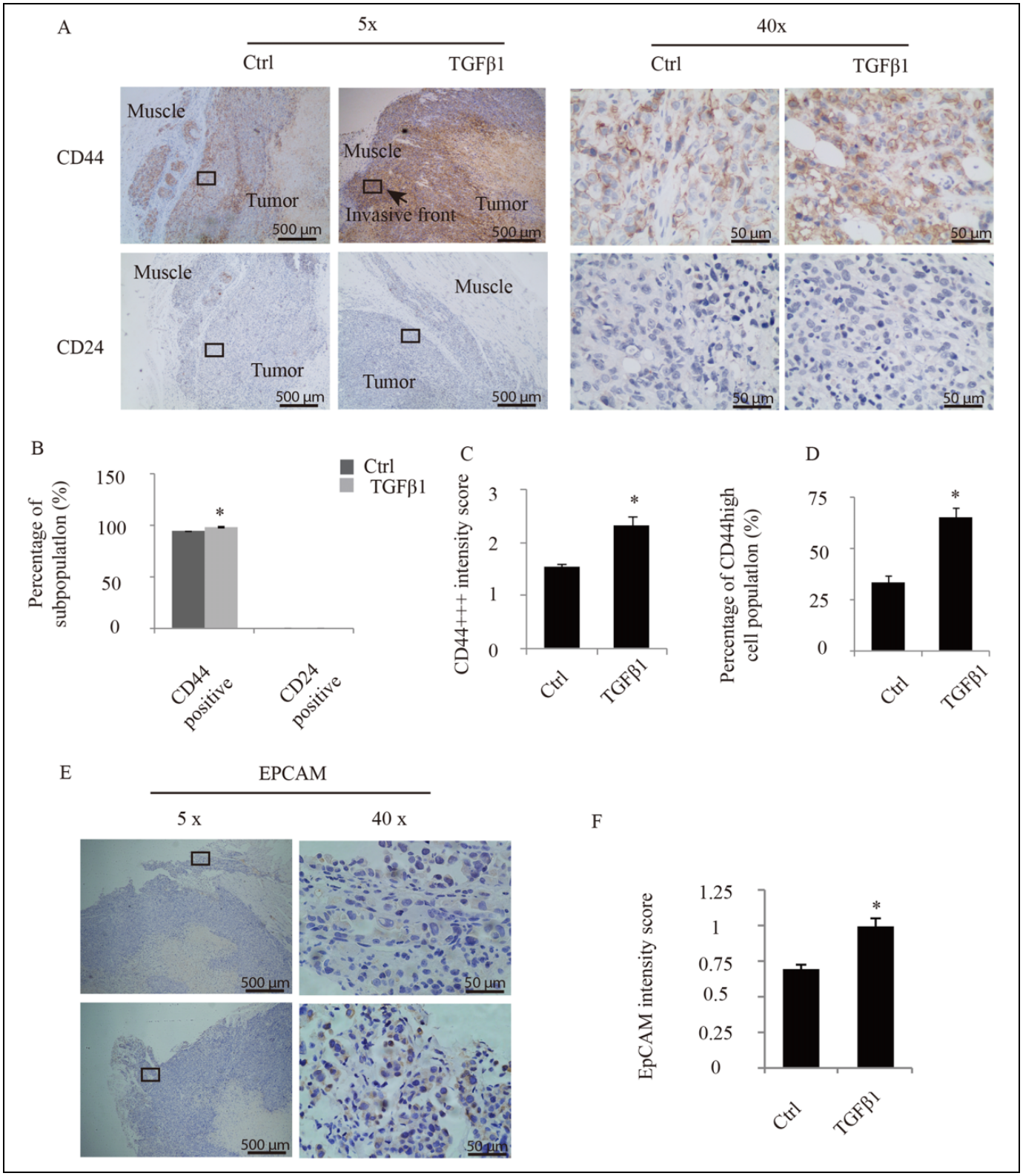

Figure 6. Transforming growth factor β1 (TGFβ1) increased the CD44^{high} CSC population in breast cancer *in vivo*. A, Immunohistological analysis of CD44 and CD24 expression in primary tumors from animals in the control and TGFβ1 pretreatment groups; CD44 and CD24 intensity was analyzed and graphed by ImageJ (B, C, and D). E, Immunohistological analysis of EpCAM expression in primary tumors from animals in the control and TGFβ1 pretreatment groups; EpCAM intensity was analyzed and graphed by ImageJ (F; error bars indicate SEM).

APC: Allophycocyanin; EpCAM: Epithelial cell adhesion molecule; MDA: MDA-MB231; PBS: phosphate buffer saline; SEM: Standard Error of Mean.

regional invasion into the EMC, and then penetration into the circulatory system. Transforming growth factor β1 is a well-known, potent inhibitor of epithelial cell proliferation, including breast cancer epithelial cells. However, our study did not show a significant effect of TGFβ1 treatment on MDA cell number *in vitro* or on tumor growth *in vivo*, suggesting that cancer cell proliferation is not influenced by TGFβ1 stimulation. This result is consistent with that of the *in vivo* experiment by Zugmaier *et al*, which demonstrated that treating mammary tumors derived from MDA-MB231 cells with TGFβ1 did not suppress tumor growth.[18] Furthermore, TGFβ1 is a well-known crucial regulator for inducing EMT and for mediating cancer migration and invasion. In our study, the cell invasive capacity was significantly increased by TGFβ1 stimulation, with more mesenchymal morphology cells. These results indicated that a proportion of mesenchymal MDA cells may have increased migratory and invasive behaviors, and these cells may be closely associated with TGFβ1-mediated breast cancer liver metastasis.

Recent studies have shown that a small proportion of cells, defined as CSCs, utilize their self-renewal properties to initiate and promote cancer cell metastasis to distant organs.[19] In 2003, Al-Hajj *et al* was the first to find in breast cancer a minority subpopulation with cell surface markers epithelial-specific antigen $(ESA)^+/CD44^+/CD24^-$ that had the ability to form new tumors *in vivo*.[20] Since then, distinct CSCs with different surface markers have been discovered and have been successfully isolated from different cancers. In our study, the $CD44^+/CD24^-$ breast cancer CSCs subpopulation was observed in hepatic metastases in both TGFβ1 and control groups. Besides, TGFβ1 did not affect the liver metastasis of $CD44^+/CD24^+$ non-CSCs. Since the $CD44^+/CD24^-$ cell population presents with a mesenchymal phenotype and always displays a high invasive and migratory capacity, we speculated that $CD44^+/CD24^-$ CSC subpopulation was mainly responsible for the formation of TGFβ1-mediated breast cancer liver metastases.

Further studies showed that the number of $CD44^{high}/CD24^-$ breast cancer cells was significantly increased by TGFβ1 stimulation *in vivo* and *in vitro*. With the increased $CD44^{high}/CD24^-$ CSC population, CSC self-renewal markers (nanog, pou5f1 [coding for Oct4], sox2) and CSC surface marker EpCAM were also significantly increased by TGFβ1 treatment. Moreover, immunohistological analysis demonstrated that the $CD44^{high}/CD24^{low/-}$ cell population was located in the margin and invasive front of primary tumors and that this population displayed a more aggressive capacity for invading peripheral tissues. All together, these results indicate that TGFβ1 increased the $CD44^{high}/CD24^-$ cell population, which possesses greater "stemness" capacity and displays a high invasion and metastatic capacity. The cell surface adhesion molecule CD44 is involved in cell–cell and cell–matrix interactions and is a functionally CSC marker in colon cancer. CD44 also plays an important role in facilitating cancer cell invasion and metastasis to distant organs. A previous study showed that *in vivo*, CD44 promotes breast tumor invasion and metastasis to the liver,[21] which was consistent with our results.

Combined with our results above, we speculate that TGFβ1 may increase breast cancer cell metastasis to the liver by promoting $CD44^{high}/CD24^-$ CSCs. However, our study has not shown how $CD44^{high}/CD24^-$ cells interact with the liver microenvironment to eventually take over the host organ, but in subsequent studies, we will further investigate this interaction.

Declaration of Conflicting Interests

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