ORIGINAL ARTICLE

CXCR7 mediates TGF β 1-promoted EMT and tumor-initiating features in lung cancer

Y-C Wu¹, S-J Tang², G-H Sun³ and K-H Sun¹

In the tumor microenvironment, chemokine system has a critical role in tumorigenesis and metastasis. The acquisition of stem-like properties by cancer cells is involved in metastasis and drug resistance, which are pivotal problems that result in poor outcomes in patients with lung cancer. Patients with advanced lung cancer present high plasma levels of transforming growth factor- β 1 (TGF β 1), which correlate with poor prognostic features. Therefore, TGF β 1 may be important in the tumor microenvironment, where chemokines are widely expressed. However, the role of chemokines in TGF β 1-induced tumor progression still remains unclear. In our study, TGF β 1 upregulated CXCR7 chemokine receptor expression, migration, invasion, epithelial–mesenchymal transition (EMT) and cancer stem cell (CSC) formation in lung adenocarcinoma. We found that CXCR7 was the most upregulated chemokine receptor induced by TGF β 1. CXCR7 knockdown resulted in reduction of migration, invasion and EMT induced by TGF β 1, whereas CXCR4 knockdown did not reverse TGF β 1-promoted EMT. CXCR7 silencing significantly decreased cancer sphere-forming capacity, stem-like properties, chemoresistance and TGF β 1-induced CSC tumor initiation *in vivo*. In clinical samples, high TGF β 1 and CXCR7 expression was significantly associated with the late stages of lung adenocarcinoma. Moreover, TGF β 1 and CXCR7 coexpression was positively correlated with the CSC marker, CD44, which is associated with lymph node metastasis. Besides, patients with high expression of both CXCR7 and TGF β 1 presented a significantly worse survival rate. These results suggest that the TGF β 1–CXCR7 axis may be a prognostic marker and may provide novel targets for combinational therapies to be used in the treatment of advanced lung cancer in the future.

Oncogene (2016) 35, 2123–2132; doi:10.1038/onc.2015.274; published online 27 July 2015

INTRODUCTION

Lung cancer is the leading cause of cancer mortality worldwide. Non-small-cell lung cancer is often diagnosed at a more advanced stage and has a poor prognosis.¹ The combination of platinum-based drugs and third-generation anticancer drugs or molecular targeting therapeutic drugs has been used in clinical treatment.² However, drug resistance leads to cancer recurrence. Therefore, the development of more effective molecular targeting agents is important to improve current therapies for lung cancer treatment.

In the clinic, high levels of transforming growth factor- β 1 (TGF β 1) have been found in the serum of patients with lung cancer as compared with healthy individuals. Furthermore, the TGF β 1 level was significantly correlated with angiogenesis, tumor progression and prognosis in patients with non-small-cell lung cancer.^{3,4} In advanced tumors, TGF β signaling pathways are severely deregulated and result in the loss of sensitivity to TGF β -induced growth arrest.^{5,6} Moreover, TGF β increases motility, invasion, epithelial–mesenchymal transition (EMT) and metastasis of cancer cells. Evidence also indicates that EMT leads to the emergence of cancer cells presenting stem-like characteristics.^{7,8} Cancer stem cells (CSCs) constitute a small number of neoplastic cells within a tumor and exhibit self-renewal and differentiation abilities.⁹ Studies have found that TGF β -induced EMT increased the pneumosphere-forming capacity and tumor-forming ability in

nonobese diabetic/severe-combined immunodeficiency (NOD/SCID) mice.^{7,10,11} Reports described that TGF β may stimulate the stromal cells in the tumor microenvironment and provide a favorable environment for tumor progression.^{12–14} However, the local and systemic influence of TGF β expression in the tumor microenvironment has not been clearly demonstrated. Thus, it is important to determine the functional role of TGF β signaling in modulating the tumor microenvironment.

In the tumor microenvironment, chemokines and chemokine receptors have critical roles in tumorigenesis and metastasis. Chemokines are chemoattractant cytokines that bind to G-protein-coupled receptors and mediate cell trafficking. Moreover, chemokines are regulators of the development, inflammation and homeostasis.¹⁵ Recent studies indicated that chemokines and chemokine receptors are overexpressed in various malignant tumors and are related to tumor progression, migration, invasion and metastasis.^{16,17} Reports demonstrated that increased secretion of TGF β stimulates cells in the tumor microenvironment and modulates the expression of chemokines or chemokine receptors, resulting in enhanced tumor metastasis and tumor progression. Bertran *et al.*¹⁸ showed that the increase in CXCR4 expression in response to TGF β is involved in mediating cell migration and survival in hepatoma. In addition, TGF β upregulates CXCR4 to enhance the response to CXCL12 and promote invasion in melanoma and breast cancer cells.^{19,20} However, much less is

¹Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Department of Education and Research, Taipei City Hospital, Taipei, Taiwan;

²Institute of Marine Biotechnology, National Taiwan Ocean University, Keelung, Taiwan and ³Division of Urology, Department of Surgery, Tri-Service General Hospital and National Defense Medical Center, Taipei City, Taiwan. Correspondence: Professor G-H Sun, Division of Urology, Department of Surgery, Tri-Service General Hospital and National Defense Medical Center, No. 325, Section 2, Chenggong Road, Neihu District, Taipei City 114, Taiwan or Professor K-H Sun, Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University; Department of Education and Research, Taipei City Hospital, No. 155, Section 2, Linong Street, Taipei 11221, Taiwan. E-mail: ghsun@ndmctsgh.edu.tw or khsun@ym.edu.tw

Received 23 January 2015; revised 1 June 2015; accepted 5 June 2015; published online 27 July 2015

known about the association between TGF β and chemokines in lung cancer. The precise mechanism of the chemokine system involvement in TGF β 1-induced tumor progression is unclear and requires further investigation.

In the present study, we found that CXCR7 was the chemokine receptor that showed the highest upregulation by TGF β 1. CXCR7 knockdown inhibited TGF β 1-induced cell motility and EMT. Furthermore, we found that CXCR7 is involved in TGF β 1-promoted CSC formation. TGF β 1 and CXCR7 coexpression correlated with advanced stages of lung adenocarcinoma and with the CSC marker, CD44. To our knowledge, this study is the first to demonstrate the role of CXCR7 in the control of EMT and CSC formation induced by TGF β 1 in lung cancer. Thus, the TGF β 1-CXCR7 axis may provide potential targeting molecules for advanced lung cancer therapy.

RESULTS

TGF β 1 enhanced migration, invasion, EMT and CSC formation in lung cancer

First, we examined whether TGF β 1 promotes lung cancer cell progression. Increasing concentrations of TGF β 1 significantly enhanced cell migration and invasion of A549 lung adenocarcinoma cells when compared with untreated control cells (Figure 1a). Moreover, TGF β 1 promoted the conversion from a

cuboidal epithelial phenotype into a spindle-shaped mesenchymal phenotype with the rearrangement of F-actin in cells (Supplementary Figure 1a). Furthermore, the expression of the EMT-associated molecule, E-cadherin, an epithelial marker, decreased, whereas the expression of the mesenchymal marker, N-cadherin, increased after TGF β 1 treatment (Figure 1b, left). The mRNA levels of EMT-associated transcriptional factors, *Snail*, *Slug* and *ZEB1*, were also upregulated in response to TGF β 1 (Figure 1b, right).

Tumor spheroids cultured in serum-free tumor sphere medium are known to be highly tumorigenic and enriched with CSCs. Therefore, sphere formation capacity of A549 cells was determined in the presence or absence of TGF β 1 in defined serum-free sphere medium. As shown in Figure 1c, untreated cells barely formed typical spheres, whereas the number of spheres in TGF β 1-treated cells was significantly higher in secondary and tertiary passage cultures. Moreover, the expression of stemness-related markers, Nanog, SOX-2 and Oct-4, was largely upregulated in TGF β 1-treated primary and secondary spheres (Figure 1c and Supplementary Figure 1b). In addition, secondary sphere cells were transfected with reporter constructs containing the promoter region of *OCT-4*, *SOX-2* and *NANOG*. The reporter luciferase activities of *OCT-4*, *SOX-2* and *NANOG* were significantly elevated in TGF β 1-treated cells compared with untreated control cells (Supplementary Figure 1c). We next determined the soft-agar

Figure 1. TGF β 1 enhanced migration, invasion, EMT and CSC formation in lung cancer. **(a, left)** A549 cells (8×10^5) were grown to confluence and scratched with a 100 μ l pipette tip. Cells were incubated with fresh medium in the presence or absence of TGF β 1 (0, 1, 2, 5 and 10 ng/ml). The migratory ability was examined at the indicated times. **(Middle and right)** A549 cells (2×10^4) were seeded onto the upper chamber of transwell inserts and incubated for 24 h with or without TGF β 1 in the lower chamber. Migration and invasion activities of the treated cells were determined after Liu's stain. Eight fields were counted per filter in each group. ** $P < 0.01$ compared with untreated control cells. **(b, left)** Cells were treated with or without TGF β 1 for 2 or 3 days and were examined by western blotting. **(Right)** A549 cells (2×10^5) were incubated with or without TGF β 1 for 24 h. The expression of EMT-associated transcriptional factors was detected by qRT-PCR. **(c)** A549 (2×10^2) cells were cultured in defined serum-free tumor sphere medium with growth factors in the presence or absence of TGF β 1 (10 ng/ml) for 30 days. Spheres were then serially passaged to form secondary and tertiary spheres. **(Bottom left)** Spheres $> 100 \mu$ m were counted in each well. ** $P < 0.01$ compared with untreated control cells. **(Bottom right)** Expression of stemness-related genes in spheres was determined by qRT-PCR. ** $P < 0.01$ compared with primary spheres; ## $P < 0.01$ compared with TGF β 1-induced primary spheres. **(d)** The expression of TGF β 1 in tissues from patient with lung adenocarcinoma was analyzed by IHC. Results are representative of three independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

clonogenicity of spheres, which is a surrogate assay for evaluating stemness. The colony formation ability of secondary spheres increased approximately fivefold in response to TGFβ1 (Supplementary Figure 1d).

Cisplatin and etoposide are the first-line agents in chemotherapy treatment of lung cancer. Therefore, we tested the drug of the spheres to cisplatin and etoposide. Compared with untreated control, TGFβ1-treated spheres become markedly less sensitive to cisplatin and etoposide and showed higher IC₅₀ (half-maximal inhibitory concentration; Supplementary Figure 1e). These data indicated that TGFβ1 promoted the formation of spheres with stemness properties. Furthermore, immunohistochemistry (IHC) staining of a lung cancer tissue microarray (TMA) indicated a higher TGFβ1 expression in stage III compared with that in stage I lung adenocarcinoma tissues (Figure 1d and Supplementary Figure 1f). These results suggest that TGFβ1 enhances tumor progression in lung adenocarcinoma.

TGFβ1 induced CXCR7 and CXCR4 expression in lung cancer cells. The mechanisms involved in the effects of TGFβ1 in the tumor microenvironment, contributing to tumorigenicity, remain unclear. To evaluate the effects of TGFβ1 on chemokine system, cells were incubated with TGFβ1 and quantitative reverse transcription-PCR (qRT-PCR) was performed. Among the chemokines and chemokine receptors expressed in A549 cells, chemokine CXCL8 and CXCL12 were upregulated by TGFβ1 treatment. Some chemokines and chemokine receptors were not detectable (CXCL2, CXCL4, CXCL6, CXCL7, CXCL9, CXCL10, CXCL11, CXCR1, CXCR3 and CXCR5). However, levels of chemokine receptor CXCR2, CXCR4, CXCR6 and CXCR7 increased in TGFβ1-treated cells (Figure 2a, left). Importantly, CXCR7 as well as its ligand CXCL12 was the most increased chemokine receptor and chemokine in response to TGFβ1. Moreover, CXCR4, which shares the same ligand with

CXCR7, was slightly upregulated after TGFβ1 stimulation. To further confirm the expression of chemokine receptors in response to TGFβ1, cells were treated with TGFβ1 and western blots were performed. We found that CXCR7 presented the highest expression level among these chemokine receptors in response to TGFβ1 (Figure 2a, right). This result is in agreement with our qRT-PCR results. Therefore, we hypothesized that CXCR7 is the most important chemokine receptor in TGFβ1-mediated tumorigenicity.

Next, we further confirmed that TGFβ1 induces CXCR7 expression. As expected, CXCR7 expression was increased in a time- and dose-dependent manner by TGFβ1 treatment (Figure 2b). In addition, CXCR7 protein level was markedly increased (Figure 2c). Moreover, screening of a panel of lung cancer cell lines revealed that TGFβ1 treatment largely enhanced CXCR7 expression in adenocarcinoma (AC), large-cell carcinoma (LCLC) and squamous cell carcinoma (SCC) cell lines (Figure 2d). Thus, TGFβ1-enhanced CXCR7 expression might be involved in TGFβ1-induced tumor progression of lung cancer.

CXCR7, but not CXCR4, knockdown repressed TGFβ1-induced migration and EMT

To investigate the role of CXCR7 and CXCR4 in TGFβ1-induced tumor progression, CXCR7 and CXCR4 were silenced by lentivirus infection, with luciferase being used as a control. After screening, we chose optimal shCXCR7 (shCXCR7, no. 300) and shCXCR4 (shCXCR4, no. 54) clones with efficient knockdown for further studies. As shown in Figure 3a, CXCR7 or CXCR4 expression levels were markedly decreased when A549 and EKVX cells were infected with shCXCR7 or shCXCR4 viruses. Both CXCR7 and CXCR4 levels were reduced in double-knockdown cells (shCXCR7/shCXCR4), indicating the high knockdown efficiency.

Figure 2. TGFβ1 induced CXCR7 and CXCR4 expression in lung cancer cells. **(a, left)** A549 cells (2×10^5) were incubated with or without TGFβ1 (5 ng/ml) for 24 h. The mRNA expression of chemokines and chemokine receptors was determined by qRT-PCR. **(Right)** A549 (2×10^5) cells were treated with or without TGFβ1 (5 ng/ml) for 2 days and western blotting was performed. **(b)** CXCR7 mRNA level was examined by qRT-PCR. **(Left)** Cells were treated with TGFβ1 for the indicated times. **(Right)** Cells were incubated with different concentrations of TGFβ1 (0, 1, 5 and 10 ng/ml) for 24 h. **(c)** CXCR7 protein expression levels were determined by flow cytometry (top) and western blotting (bottom) after cells were treated with TGFβ1 (5 ng/ml) for 2 days and indicated times, respectively. **(d)** FB, AC, SCC and LCLC cells were treated with or without TGFβ1. After 24 h, total RNA was extracted and qRT-PCR was performed. Results are representative of three independent experiments. ****** $P < 0.01$ compared with untreated control cells.

Figure 3. CXCR7, but not CXCR4, knockdown repressed TGFβ1-induced migration and EMT. (a–d) A549 and EKVX cells were infected with lentiviral shCXCR7, shCXCR4 or a combination of shCXCR7 and shCXCR4 to inhibit the expression of CXCR4 and CXCR7. shLuc was used as a control. (a) CXCR7 and CXCR4 mRNA and protein expression levels were examined by qRT-PCR and western blotting, respectively. (b) A549 and EKVX knockdown cells (8×10^5) were grown to confluence and scratched with a 100 μl pipette tip. Cells were then incubated with fresh medium in the presence or absence of TGFβ1 (5 ng/ml) for 18 h and the migratory ability was determined. (c) Migratory (top) and invasiveness (bottom) ability of A549 and EKVX knockdown cells were determined by transwell assay in the presence or absence of TGFβ1. (d) Knockdown cells (2×10^5) were incubated with TGFβ1 for 3 days. E-cadherin and N-cadherin expression was examined by western blotting. Results are representative of three independent experiments. ** $P < 0.01$ compared with untreated shLuc control cells; ## $P < 0.01$ compared with TGFβ1-treated shLuc cells.

Next, we identified the respective contribution of CXCR7 and CXCR4 in TGFβ1-induced migration and invasion. The TGFβ1-induced cell motility was significantly abolished in shCXCR7 and shCXCR7/shCXCR4-infected cells (Figure 3b). In the transwell assay, shCXCR7- and shCXCR7/shCXCR4-infected cells exhibited a decrease in cell motility in response to TGFβ1, whereas CXCR4 knockdown cells showed no difference in terms of cell migration as compared with the Luc control (Figure 3c, top). In contrast, TGFβ1-induced cell invasion was obviously attenuated in shCXCR7-, shCXCR4- and shCXCR7/shCXCR4-infected cells (Figure 3c, bottom). Next, we evaluated whether CXCR7 and CXCR4 were involved in the regulation of TGFβ1-mediated EMT. Obviously, both shCXCR7 and the combination of shCXCR7/shCXCR4 reversed the TGFβ1-mediated E-cadherin downregulation and N-cadherin upregulation. Interestingly, shCXCR4 did not reverse the effect of TGFβ1 on EMT marker expression (Figure 3d). Besides, TGFβ1 stimulation induced a spindle-shaped mesenchymal morphology in luciferase short hairpin RNA (shRNA) (shLuc)- and shCXCR4-infected cells, whereas shCXCR7- and shCXCR7/shCXCR4-infected cells exhibited a round-shaped epithelial phenotype as observed by immunofluorescence staining (Supplementary Figure 2a). Moreover, we used AMD3100, a well-

known CXCR4 antagonist, to further examine the role of CXCR4. Consistently, CXCR4 inhibition did not affect cell migration and expression of EMT markers, but significantly decreased TGFβ1-induced cell invasion (Supplementary Figures 2b and c). These data suggest that CXCR7 is the key chemokine receptor responsible for TGFβ1-induced EMT in lung cancer.

CXCR7 silencing impaired TGFβ1-induced sphere formation and stemness properties

Next, we investigated the importance of CXCR7 in TGFβ1-induced sphere formation and stemness properties. Knockdown cells were cultured in serum-free sphere formation medium with or without TGFβ1 treatment. Representative images presented in Figure 4a and Supplementary Figure 3a indicated that spheres formed from secondary passaged cells. Cells cultured in medium without TGFβ1 attached to the culture plate and barely formed spheres. In contrast, TGFβ1 treatment induced tumor sphere formation in primary and secondary passaged cells. shCXCR7, shCXCR4 and shCXCR7/shCXCR4 significantly inhibited TGFβ1-enhanced sphere formation when compared with shLuc-infected cells. Moreover, CXCR7 and CXCR7/CXCR4 knockdown had stronger inhibitory

Figure 4. CXCR7 silencing impaired TGFβ1-induced sphere formation and stemness properties. Knockdown cells were grown under defined serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 tumor sphere medium in the presence or absence of TGFβ1 (10 ng/ml) for 30 days. Spheres were then serially passaged to form secondary spheres and the number of spheres was counted. (a, top) Representative bright-field light microscopy images of spheres (magnification, ×40). (Bottom) Quantification of primary and secondary sphere numbers. ***P* < 0.01 compared with primary TGFβ1-induced shLuc cells; ##*P* < 0.01 compared with secondary TGFβ1-induced shLuc cells. (b and c) Expression of Nanog, SOX-2, Oct-4 and CD44 were determined by qRT-PCR (b) and flow cytometry (c). The number indicates the mean fluorescence intensity (MFI). ***P* < 0.01; ##*P* < 0.01; ++*P* < 0.01; ΔΔ*P* < 0.01 compared with TGFβ1-induced shLuc cells. (d) Secondary spheres (5 × 10²) were seeded as single cells in soft agar and cultured for 4 weeks in the presence or absence of TGFβ1 (5 ng/ml). Colonies > 100 μm were counted. ***P* < 0.01 compared with untreated shLuc cells; ##*P* < 0.01 compared with TGFβ1-treated shLuc cells. (e) Secondary spheres induced by TGFβ1 treatment (5 × 10³) were seeded in 96-well plates and treated with increasing concentrations of cisplatin or etoposide. Cell viability was analyzed 48 h after drug treatment by performing CellTiter-Glo Luminescent cell viability assay. Estimated half-maximal inhibitory concentration (IC₅₀) values for each group are indicated. (f) NOD/SCID mice were injected subcutaneously with different numbers of secondary spheres induced by TGFβ1 treatment. The tumor-initiating potential of shLuc-, shCXCR7- and shCXCR4-infected spheres were determined (*n* = 4 for each group). Experiments were performed in triplicate.

effects on sphere formation in comparison with CXCR4 knockdown (Figure 4a and Supplementary Figure 3a).

To study whether CXCR7 regulate stemness-related genes, secondary spheres were analyzed. Results obtained from qRT-PCR analysis showed that *NANOG*, *SOX-2*, *OCT-4* and *CD44* were largely upregulated in TGFβ1-induced spheres. shCXCR7, shCXCR4 and the combination of shCXCR7/shCXCR4 significantly inhibited the expression of *NANOG*, *SOX-2*, *OCT-4* and *CD44* (Figure 4b and Supplementary Figure 3b). A similar inhibitory effect was observed in flow cytometry analysis. CXCR7, CXCR4 or CXCR7/CXCR4 knockdown reduced the expression levels of Nanog, SOX-2 and Oct-4 induced by TGFβ1 (Figure 4c and Supplementary Figure 3c). In addition, to examine promoter activities of *OCT-4*, *SOX-2*, or *NANOG*, EKVX secondary spheres were transfected with *OCT-4*, *SOX-2* or *NANOG* reporter constructs. Luciferase reporter activities were reduced in CXCR7, CXCR4 and CXCR7/CXCR4 knockdown spheres (Supplementary Figure 3d). To further explore the functional effect of CXCR7 and CXCR4 in sphere cells, we conducted experiments to evaluate anchorage-independent

growth and drug resistance. In Figure 4d, the colony-forming ability induced by TGFβ1 was abolished in shCXCR7, shCXCR4 and shCXCR7/shCXCR4-infected secondary spheres. Furthermore, infection of TGFβ1-induced spheres with shCXCR7, shCXCR4 or shCXCR7/shCXCR4 significantly increased the cell sensitivity to cisplatin and etoposide treatment. In TGFβ1-induced A549 spheres, shCXCR7, shCXCR4 and shCXCR7/shCXCR4-infected cells exhibited the lowest resistance, with an average of 54.9%, 62.7% and 50.3% survival in the presence of 10 μM cisplatin, respectively, compared with 75.5% for the shLuc-infected control cells. Moreover, CXCR7, CXCR4 and CXCR7/CXCR4 silencing in TGFβ1-induced spheres also decreased the resistance to etoposide when compared with shLuc-infected spheres (Figure 4e). A similar result was also observed in EKVX sphere cells (Supplementary Figure 3e). In addition, we evaluated the tumor-initiating ability of spheres *in vivo* using a xenotransplantation assay. Different numbers of Luc, CXCR4 and CXCR7 knockdown sphere cells were subcutaneously injected into NOD/SCID mice. As shown in Figure 4f, in TGFβ1-induced spheres, Luc knockdown cells formed tumors with

as few as 10^3 cells and generated tumors in 100% of mice with 10^5 cells. However, CXCR7 and CXCR4 knockdown cells inefficiently formed tumors when 10^5 cells were injected. None of the mice injected with CXCR7 knockdown cells developed tumors. This indicated that CXCR7 knockdown was more efficient, when compared with CXCR4 knockdown, to inhibit the initiation of tumor *in vivo*. These results suggest that CXCR7 participates in TGFβ1-promoted stem-like properties of lung cancer.

To exclude the possibility that the effects of CXCR7 and CXCR4 were off-target effects, we used different shCXCR7 (shCXCR7; no. 205) and shCXCR4 (shCXCR4; no. 467) optimal sequences to knockdown the expression of CXCR7 and CXCR4. As shown in Supplementary Figures 4a–g, the results of migration, invasion, EMT and sphere formation were consistent with the results shown in Figures 3 and 4, indicating that the effect of CXCR7 and CXCR4 knockdown are not because of nonspecific or off-target effect.

CXCL12 involved in the maintenance of sphere formation of lung cancer cells

To evaluate whether the CXCL12–CXCR7 axis might have contributed to TGFβ1-mediated EMT, we examined the effect of the CXCL12 ligand on EMT and CSC phenotypes of knockdown cells. Stimulation of CXCL12 significantly promoted cell motility and invasiveness ability. CXCR4, CXCR7 and CXCR4/CXCR7 double knockdown attenuated CXCL12-induced migration and invasion (Supplementary Figure 5a and data not shown). In addition, CXCL12 increased the expression of *Snail* and *ZEB1*, whereas CXCR4, CXCR7 and CXCR4/CXCR7 double knockdown abolished the promotion effect of CXCL12 (Supplementary Figure 5b). Nevertheless, CXCL12 stimulation had no effect on cell morphology (data not shown). The EMT marker expression profiles in knockdown cells after CXCL12 treatment were similar to that of untreated cells (Supplementary Figure 5c). To examine the effect of CXCL12 on CSC phenotype, knockdown cells were incubated with CXCL12 and cultured in sphere medium to determine sphere formation ability. Although CXCL12 treatment did not promote sphere formation of A549 (Supplementary Figure 5d), CXCL12 signaling through CXCR4 and/or CXCR7 was important to maintain sphere formation ability in knockdown cells. In addition, the expression of stemness genes, *OCT-4*, *SOX-2* and *NANOG*, increased in CXCL12-enriched primary spheres (Supplementary Figure 5e). In summary, CXCL12 signaling through CXCR4 and/or CXCR7 did not promote sphere formation, but allowed the maintenance of sphere formation ability and stimulated the expression of stemness genes.

To further confirm the importance of CXCL12 in TGFβ1-promoted sphere formation, we knocked down CXCL12 in A549 cells and conducted a sphere formation assay. Interestingly, shCXCL12 knockdown significantly inhibited TGFβ1-induced sphere formation even in primary sphere culture (Supplementary Figure 5f). Moreover, we examined the expression levels of stemness genes in A549-derived primary spheres. As shown in Supplementary Figure 5g, TGFβ1-induced *OCT-4*, *SOX-2* and *NANOG* expression was abolished in CXCL12 knockdown cells. Therefore, we suggest that CXCL12 is involved in TGFβ1-promoted sphere formation.

TGFβ1 induces CXCR7 expression via the classical Smad signaling pathway

Evidence indicated that the canonical Smad pathway coexists with Smad-independent pathways in response to TGFβ1.⁵ Accordingly, we explored whether TGFβ1 regulates CXCR7 through Smad or Smad-independent pathways. A549 cells were pretreated with inhibitors of the Smad and Smad-independent pathways, including SB431542 (TGFβ1 type I receptor inhibitor), LY294002 (phosphoinositide 3-kinase inhibitor), PD98059 (mitogen-activated protein kinase kinase inhibitor), SP600125 (JNK inhibitor) and SB203580 (mitogen-activated protein kinase inhibitor) for 1 h and

Figure 5. TGFβ1 induces CXCR7 expression via the classical Smad signaling pathway. **(a)** A549 cells (6×10^5) were pretreated with SB431542 (10 mM), LY294002 (20 μM), PD98059 (20 μM), SP600125 (20 μM) or SB203580 (20 μM) for 1 h and then stimulated with TGFβ1 (5 ng/ml) for 24 h. CXCR7 mRNA level was detected by qRT-PCR. * $P < 0.05$; ** $P < 0.01$ compared with TGFβ1-treated cells. **(b)** After 1 h of inhibitor pretreatment, cells were incubated with TGFβ1 for 1 h (phosphoproteins) or 3 days (CXCR7). Target protein expression was evaluated by western blotting. **(c)** and **(d)** A549 cells (2×10^5) were infected with shSmad2 lentiviruses to knockdown the expression of Smad2. After puromycin selection, cells were used to extract RNA and proteins for qRT-PCR **(c)** and western blotting **(d)**. Results are representative of three independent experiments. ** $P < 0.01$ compared with TGFβ1-treated shLuc cells.

then stimulated with TGFβ1. As expected, TGFβ1 induced the phosphorylation of Smad2, Akt, ERK1/2, JNK and p38 kinase. However, CXCR7 expression induced by TGFβ1 was significantly attenuated by SB431542 both at the mRNA and protein levels (Figures 5a and b). We thus reasoned that CXCR7 expression induced by TGFβ1 was Smad-dependent. To further clarify the effect of Smad signaling on CXCR7 expression, cells were infected with shSmad2 to inhibit Smad2 expression. In the presence of TGFβ1, CXCR7 expression was lower in shSmad2-infected cells compared with in shLuc-infected cells (Figures 5c and d). Thus, TGFβ1 might be involved in signaling through the Smad pathway, resulting in upregulation of CXCR7 in lung cancer cells.

Expression levels of TGFβ1 and CXCR7 are positively associated with lung adenocarcinoma advanced stages and the CSC marker, CD44

To determine CXCR7 expression in human lung cancer, IHC was conducted on a commercial lung cancer TMA. As predicted, CXCR7 expression was higher in stage III as compared with stage I lung adenocarcinoma tissues (Figure 6a). Moreover, CXCR7 expression was positively correlated with TGFβ1 expression in 110 patients with various types of lung cancer (Figure 6b).

Figure 6. Expression levels of TGFβ1 and CXCR7 are positively associated with lung adenocarcinoma advanced stages and the CSC marker, CD44. **(a)** CXCR7 expression in 49 human lung adenocarcinoma tissues was analyzed by IHC. **(b)** Correlation between TGFβ1 and CXCR7 evaluated by the Spearman's correlation method. **(c, top)** CD44 expression in different stages of lung adenocarcinoma tissues. **(Bottom)** Analysis of CD44 levels in lymph node metastatic and non-metastatic tissues from human lung cancers. **(d)** Association of CD44 expression with TGFβ1/CXCR7 coexpression in patients with lung cancer ($n = 110$). **(Bottom)** Histogram representing the association between CD44 and the coexpression of TGFβ1 and CXCR7 from the data presented above. **(e)** Correlation between CXCR7/TGFβ1 coexpression and survival by using SurvExpress. Red and green curves indicated high and low CXCR7/TGFβ1 coexpression groups of patient death based on the Rousseau GSE30219 database. * $P < 0.05$; ** $P < 0.01$.

Notably, CD44 is an important marker of CSCs that is over-expressed in various cancer stem cells. Evidence indicates that it is involved in the control of metastasis, self-renewal and drug resistance of CSCs.²¹ Therefore, we assessed CD44 expression and compared it with that of CXCR7 and TGFβ1. CD44 expression was higher in stage III lung adenocarcinoma. In addition, CD44 correlated with lymph node metastasis (Figure 6c). Moreover, a positive correlation was demonstrated between CD44 expression and CXCR7/TGFβ1 coexpression in lung cancer tissues (Figure 6d). To further study the association between CXCR7/TGFβ1 expression and cancer patient prognosis, we analyzed the correlation between CXCR7/TGFβ1 coexpression and cancer patient survival using an online database SurvExpress analysis. We found that patients with high expression of both CXCR7 and TGFβ1 presented a significantly worst survival rate (Figure 6e). These results suggest that the TGFβ1-CXCR7 axis is a stemness and advanced-stage marker of patients with advanced lung cancer.

DISCUSSION

The interaction of cytokines and chemokines in the tumor microenvironment is vital for the acquisition of the metastatic potential and stemness properties of cancer cells. Based on data showing that TGFβ1 level are higher in the serum and tissue of patients with lung cancer,^{3,4} we aimed to identify the influence of TGFβ1 on the chemokine system in lung cancer. Our work

revealed that CXCL12 and CXCR7 were the highest induced chemokine and chemokine receptor in response to TGFβ1.

TGFβ1 has an important role in regulating cellular processes such as growth, differentiation, tissue homeostasis and immune suppression.⁵ Consequently, malfunction or inactivation of components of the TGFβ1 pathway is related to many conditions and processes, including tumorigenesis and tumor progression. Interestingly, TGFβ1 can be either a tumor promoter or tumor suppressor, depending on the cell context and tumor stage.²² Losing components of the TGFβ1 signaling pathway reduced cellular cytostatic program, providing the evidence of the TGFβ pathway being tumor suppressive.²³ On the contrary, TGFβ1 overexpression promoted motility, invasion and EMT of certain cancer cells, demonstrating the role for the TGFβ1 pathway in tumor progression. Cancer cells often evade TGFβ1 growth inhibition effects, while maintaining TGFβ1-mediated cellular responses that increase tumor progression. In lung cancer, a study indicated that reduced expression of TβRIII has been found in non-small-cell lung cancer cells compared with normal human bronchial epithelial cells.²⁴ Moreover, TβRII knockdown in A549 adenocarcinoma lung cancer cell resulted in the suppression of cell proliferation, invasion and metastasis.²⁵ These data indicated the importance of TGFβ1 in lung cancer.

Stromal-derived factor-1 (or CXCL12) is a broadly expressed chemokine that is secreted by marrow stromal cells and endothelial cells, and is found in nearly all organs.²⁶ It serves as a chemoattractant for hematopoietic cells and regulates

hematopoietic stem cell proliferation, survival and homing to the bone marrow.^{27,28} In addition, CXCL12 regulates the metastatic behavior of malignant cancers, including lung, breast and prostate cancers.^{29–31} For a long time, CXCR4 was considered as the only receptor for CXCL12. It is essential for hematopoiesis and vascularization. Moreover, it has been reported to be over-expressed in cancers, to mediate the metastasis of cancer cells and to promote cancer stem cells.³² Recently, studies showed that CXCL12 also binds to CXCR7, with 10 times higher affinity compared with that to CXCR4.^{33,34} The deregulation of CXCR7 is associated with inflammation, infection and cancer. Furthermore, CXCR7 has been found to increase proliferation of tumor cells, promote tumor growth in nude mice and exert prosurvival and antiapoptotic effects. CXCR7 was also demonstrated to promote angiogenesis and to mediate cancer metastasis.^{35,36}

It has been reported that CXCR7 and CXCR4 are highly expressed in lung cancer tissues and contribute to cancer metastasis.^{37–40} According to our findings, CXCR7 inhibition abolished TGFβ1-induced cell migration, invasion, EMT and tumor-initiating features, whereas CXCR4 may not be responsible for TGFβ1-induced EMT. Our data revealed that TGFβ1 induced CXCR7 expression in various lung cancer cell lines, including AC, LCLC and SCC cell lines (Figure 2d). In addition, CXCR4 expression was upregulated in AC cells after TGFβ1 treatment. However, unexpectedly, CXCR4 expression decreased in H460 LCLC and H226 SCC cell lines. Moreover, the increase in CXCR7 expression was higher compared with that of CXCR4 in response to TGFβ1 (data not shown). These results indicated that CXCR7 and CXCR4 may have different roles in TGFβ1-induced lung cancer progression.

The mechanism responsible for the reduction of cell migration may result from the increased cell–cell adhesion. In this study, cells knocked down for CXCR7 or CXCR4/CXCR7 showed decreased migration, invasion and N-cadherin in response to TGFβ1 (Figure 3). However, CXCR4 knockdown inhibited cell invasion, but not migration and EMT marker expression induced by TGFβ1. Schaeffer *et al.*⁴¹ demonstrated that migration and invasion can act discordantly during carcinoma-associated EMT. The cells that migrate less may exhibit a higher invasion capacity, which suggest that the limited decrease in migratory capacity in CXCR4 knockdown cells is still sufficient to inhibit invasion. Moreover, unexpectedly, CXCR4 knockdown cells exhibited elongated spindle-shaped morphology and displayed high levels of N-cadherin after TGFβ1 treatment (Supplementary Figure 2a and Figure 3d). Thus, we speculated that the morphologic differences might not be observed in cells cultured on two-dimensional (2D) culture system. A three-dimensional (3D) culture is a more optimal biological system to represent the complex architecture of a tissue or organ, and certain phenotypes can be observed only in a 3D environment.⁴² A study showed that the repression of *let-7i* altered the cellular morphology to allow mesenchymal movement in 2.5D or 3D cultures, but not in 2D cultures.⁴³ Hence, it is possible that the influence of CXCR4 knockdown on migration might only be visible in a 2.5D or 3D environment.

Most studies used CXCL12 as the chemoattractant to test the contribution of CXCR4 and CXCR7 to cancer cell chemotaxis. For instance, Choi *et al.*⁴⁴ pointed that CXCR4 expression, but not that of CXCR7, in lung cancer cells, was necessary for migration and invasion. However, the effects of CXCL12 addition were different from those of TGFβ1 treatment. We found that CXCL12 signaling through CXCR4 and/or CXCR7 did not promote sphere formation of lung cancer cells, but could maintain sphere formation ability and stimulate the expression of stemness genes. Nevertheless, CXCL12 knockdown significantly inhibited TGFβ1-induced sphere formation in lung cancer cells (Supplementary Figure 5). These data indicated that the CXCL12-CXCR7/CXCR4 axis participates in sphere formation induced by TGFβ1. In our study, TGFβ1

induced CXCL12 secretion by cancer cells (data not shown) and further boosted CXCR4 and CXCR7 expression. The TGFβ1-CXCL12-CXCR7/4 autocrine axis revealed a new sight about the different role of CXCR4 and CXCR7 in response to tumor progression induced by TGFβ1.

Interestingly, we observed morphologically distinct spheres in cells knocked down for CXCR7, CXCR4/CXCR7 and Luc. In TGFβ1-treated cells, shLuc-infected secondary spheres displayed a larger diameter, smooth defined edges and were perfectly compact and round. Compared with Luc control spheres, spheres enriched from shCXCR7- and shCXCR4/shCXCR7-infected cells appeared less compact and more irregularly shaped. Moreover, shCXCR7-infected cells adhered better to the culture plates, which resulted in a decrease in the spheroid-forming capacity. According to studies connecting EMT to the emergence of cancer stem cells,^{7,8} the adherent and irregular morphology of spheres enriched from shCXCR7-infected cells may result from the EMT inhibition effect of CXCR7 knockdown. CXCR7 knockdown cells exhibited a round-shaped epithelial morphology and the TGFβ1-induced increase in EMT markers was reversed (Figure 3d). However, CXCR4 knockdown had no significant effect on TGFβ1-induced EMT. This may explain why the size and number of spheres from CXCR7 knockdown cells were lesser compared with those observed for spheres from CXCR4 knockdown cells.

In the present study, we identified the distinct contribution of CXCR7 and CXCR4 in response to TGFβ1 in lung adenocarcinoma. The TGFβ1-CXCL12-CXCR7 autocrine axis increased EMT and CSCs formation, which further enhanced cell chemoresistance. To date, the precise role of CXCR7 in sphere formation and acquisition of stem-like properties remains unknown. To the best of our knowledge, this study is the first to demonstrate the critical role of CXCR7 in lung CSCs. Furthermore, our results suggested that CXCR7/TGFβ1 coexpression is positively correlated with CD44, which is a CSC marker associated with lymph node metastasis in lung cancer. These results raise the possibility of targeting the TGFβ1-CXCR7 axis and CD44 as a new strategy for combination therapy to suppress lung cancer chemoresistance and metastasis.

MATERIALS AND METHODS

Cell lines

Human lung adenocarcinoma A549 (ATCC, Manassas, VA, USA; no. CCL-185) cells were obtained from Bioresource Collection and Research Center (BCRC, Hsinchu City, Taiwan). Human lung cancer cell lines, including adenocarcinoma cells (EKVX, HOP62, NCI-H522 and NCI-H23), squamous cell carcinoma cell lines (NCI-H226 and NCI-H520) and large-cell lung cancer cell line (NCI-H460) were provided by Dr Sung-Liang Yu (National Taiwan University, Taipei City, Taiwan) and purchased from the National Cancer Institute (Bethesda, MD, USA). The human lung fibroblast cell line, MRC-5 (ATCC; no. CCL-171), was provided by Dr Yi-Chung Tung (Academia Sinica, Taipei, Taiwan). A549, EKVX, HOP62, NCI-H522, NCI-H23, NCI-H226, NCI-H520 and NCI-H460 were maintained in RPMI (Thermo Scientific Hyclone, South Logan, UT, USA) medium containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (P–S; Gibco BRL, Life Technologies, Carlsbad, CA, USA). H1299 cells were grown in Dulbecco's modified Eagle's medium (Thermo Scientific Hyclone) medium supplemented with 10% FBS and 1% P–S. MRC-5 cells were cultured in Eagle's minimum essential medium (Thermo Scientific Hyclone) containing 10% FBS and 1% P–S.

Lentivirus-mediated knockdown of CXCR4 and CXCR7

pCMV-ΔR8.91 and pMD.G, used as the lentiviral packing vector, and pLKO.1 plasmid with shRNA targeting human CXCR4 (shCXCR4), CXCR7 (shCXCR7) or Smad2 (shSmad2) were purchased from National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Lentiviruses were produced by cotransfection of H293T cells with pCMV-ΔR8.91, pMD.G and pLKO.1 vectors. For knockdown, A549 and EKVX cells were infected with the collected viruses in the presence of 8 μg/ml protamine sulfate and then selected using 2 μg/ml of puromycin (Sigma-Aldrich, St Louis, MO, USA).

The vector with the shRNA against luciferase (shLuc) was used as a control. The shRNA sequences are listed in Supplementary Table 1.

Migration and invasion assay

Transwell assay inserts (24 mm diameter, 8 μm pores, Costar, Corning, NY, USA) were placed in 24-well plates. For invasion assay, each insert was first coated with Matrigel (BD Biosciences, San Jose, CA, USA). Cells (2×10^4) were resuspended in 0.5% FBS-RPMI medium and placed in the upper chamber, whereas 10% FBS-RPMI alone or containing 5 ng/ml TGFβ1 (Peprotech, Rocky Hill, NJ, USA) was added to the lower chamber. After incubation for 24 h, the inserts were fixed by methanol and stained with Liu's stain (Muto Pure Chemical, Tokyo, Japan). Matrigel and non-invading cells were mechanically wiped using cotton swabs. The invading cells were counted under light microscopy ($\times 100$ magnification). In the migration assay, the inserts were not coated with Matrigel.

Wound healing assay

Cells were grown to ~80% confluence and scratched with a pipette tip. The cells were incubated with fresh 10% FBS-RPMI medium in the presence or absence of TGFβ1 (5 ng/ml) for the indicated time. Cell migration images were obtained and cell mobility was quantified by using the Image J software (NIH, Bethesda, MD, USA).

Reverse transcription and quantitative PCR

Total cellular RNA was extracted using the TRIzol reagent (Ambion, Life Technologies, Carlsbad, CA, USA). The extracted RNA sample was used for reverse transcription with the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cDNA was used for qRT-PCR with Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) on the StepOnePlus Real-Time PCR System (Applied Biosystems). All values were normalized against the glyceraldehyde-3-phosphate dehydrogenase. The specific primers are shown in Supplementary Table 2.

Western blot

Total cellular proteins were extracted in RIPA Lysis buffer (Millipore, Temecula, CA, USA) and were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore). After incubating with 5% skim milk, the membrane was probed with the indicated antibodies and horseradish peroxidase-conjugated secondary antibodies. The immunoblots were revealed by using an ECL Kit (Millipore). For inhibitor treatment, cells were pretreated with LY294002 (Merck, Darmstadt, Germany), SB431542, PD98059, SP600125 and SB203580 (Abcam, Cambridge, UK) for 1 h and incubated with TGFβ1 for 1 h or 3 days. The antibodies used in this study are listed in Supplementary Table 3.

Spheroid formation assay

For CSC enrichment, cells were cultured in serum-free Dulbecco's modified Eagle's medium/F12 (Gibco BRL, Life Technologies, Grand Island, NY, USA) tumor sphere medium consisting of N2 supplement (Gibco BRL, Life Technologies), 20 ng/ml human recombinant basic fibroblast growth factor (Gibco BRL, Life Technologies), and 20 ng/ml EGF (Gibco BRL, Life Technologies) in the presence or absence of TGFβ1 (10 ng/ml). After 30 days of incubation, the primary spheres were passaged under the same culture conditions as those used for the initial growth of the spheres. Briefly, primary spheres were dissociated into single cells and seeded for secondary sphere formation. The spheres larger than 100 μm were counted in each well.

Flow cytometry

The secondary CSCs were harvested and suspended in BD Cytofix/Cytoperm solution (BD Biosciences) for 20 min. After washing by $1 \times$ BD Perm/wash buffer, cells were stained with antibodies for 1 h at 4 °C, followed by fluorescein isothiocyanate-conjugated secondary antibodies. Cells were analyzed using a FACS Calibur (BD Biosciences).

Anchorage-independent growth assay

CSCs (5×10^3) were suspended in 0.33% Bacto-agar (Sigma-Aldrich) with or without 5 ng/ml TGFβ1 and layered over 0.5% Bacto-agar. Cells were

incubated for 30 days, fixed and then stained with Giemsa stain to examine colony formation.

Cell viability

Enriched CSCs (5×10^3) were treated with the indicated concentrations of cisplatin (Sigma-Aldrich) or etoposide (Sigma-Aldrich) for 2 days. Cell viability was then examined by CellTiter-Glo Luminescent cell viability assay according to the manufacturer's protocol (Promega, Madison, WI, USA).

Immunohistochemistry

TMA slides were commercially obtained from Biomax (US Biomax Inc., Rockville, MD, USA). The TMA slides were deparaffinized in xylene and rehydrated through serial dilutions of alcohol. Heat-induced epitope retrieval was performed in 10 mM citrate buffer (pH 6.0) in a microwave for two 5-min sessions. After blocking with 3% H₂O₂, the treated TMA slides were then incubated with CXCR7 (GeneTex Inc., Hsinchu City, Taiwan), CXCR4 (Abcam), CD44 (R&D System, Minneapolis, MN, USA) or TGFβ1 (R&D System) primary antibody at 4 °C overnight. TMA slides were washed and subsequently processed using an LSAB+System-HRP Kit (Dako North America Inc., Carpinteria, CA, USA). In addition, TMA slides were counter-stained with hematoxylin, rinsed and mounted. The slides were analyzed using the Aperio ImageScope (Aperio Technologies Inc., Vista, CA, USA) and a digital-stained cell score was obtained. The final score is the sum of the percentage of stained area (scored as 0, 1⁺, 2⁺, 3⁺ and 4⁺) and intensity (0, 1, 2 and 3 were designated as negative expression, weak expression, intermediate expression and strong expression, respectively) of stained cells. The expression levels were divided into weak category (score below the average) and high category (score above the average). The information with regard to the clinicopathologic features of the patients included in the TMA is listed in Supplementary Table 4.

Xenograft model

The NOD/SCID mice (NOD.CB17-Prkdcscid/lcrCrlBltw; 6–8 weeks of age, male) were obtained from BiloLASCO (Taipei, Taiwan). The mice were raised in the Animal Center of National Yang-Ming University according to the regulations of the Animal Care Committee of National Yang-Ming University. Different numbers of secondary sphere cells were subcutaneously injected into NOD/SCID mice (10^3 – 10^5 per 100 μl cells). Tumor volume was measured with a caliper every week. The mice were randomly separated into each groups.

Statistical analysis

Statistical analyses were performed by using an SPSS software program (Version 22.0; SPSS Inc., Chicago, IL, USA). The results are reported as the mean $\pm$ s.d. Differences between two groups were carried out using Student's *t*-test. Mann–Whitney *U*-test was used to identify the significance of proteins expression at different stages of lung cancer. Spearman's correlation was applied to evaluate the linear relationship between CXCR7 and TGFβ1 expression. A two-tailed χ^2 test was used to determine the association between protein expression and clinicopathologic characteristics.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Science and Technology (MOST 103-2320-B-010-039-MY3, 103-2321-B-010-029), the Ministry of Education (Aim for the Top University Plan), the Tri-Service General Hospital and the National Defense Medical Center (TSGHC-C103-062, TSGH-104-507) and the Taipei City Hospital, Taiwan.

REFERENCES

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69–90.
- Bareschino MA, Schettino C, Rossi A, Maione P, Sacco PC, Zeppa R *et al*. Treatment of advanced non small cell lung cancer. *J Thorac Dis* 2011; **3**: 122–133.

- 3 Hesegawa Y, Takanashi S, Kanehira Y, Tsushima T, Imai T, Okumura K. Transforming growth factor-beta1 level correlates with angiogenesis, tumor progression and prognosis in patients with non-small cell lung carcinoma. *Cancer* 2001; **91**: 964–971.
- 4 Hou YL, Chen H, Dong ZH, Xue CJ, Wu YF, Luo HX et al. Clinical significance of serum transforming growth factor-beta1 in lung cancer. *Cancer Epidemiol* 2013; **37**: 750–753.
- 5 Massague J. TGFbeta in cancer. *Cell* 2008; **134**: 215–230.
- 6 Principe DR, Doll JA, Bauer J, Jung B, Munshi HG, Bartholin L et al. TGF-beta: duality of function between tumor prevention and carcinogenesis. *J Natl Cancer Inst* 2014; **106**: djt369.
- 7 Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY et al. The epithelial–mesenchymal transition generates cells with properties of stem cells. *Cell* 2008; **133**: 704–715.
- 8 Singh A, Settleman J. EMT cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* 2010; **29**: 4741–4751.
- 9 Lobo NA, Shimono Y, Qian D, Clarke MF. The biology of cancer stem cells. *Annu Rev Cell Dev Biol* 2007; **23**: 675–699.
- 10 Pirozzi G, Tirino V, Camerlingo R, Franco R, La Rocca A, Liguori E et al. Epithelial to mesenchymal transition by TGFbeta-1 induction increases stemness characteristics in primary non small cell lung cancer cell line. *PLoS One* 2011; **6**: e21548.
- 11 Sakaki-Yumoto M, Katsuno Y, Derynck R. TGF-beta family signaling in stem cells. *Biochim Biophys Acta* 2013; **1830**: 2280–2296.
- 12 Kuperwasser C, Chavarria T, Wu M, Magrane G, Gray JW, Carey L et al. Reconstruction of functionally normal and malignant human breast tissues in mice. *Proc Natl Acad Sci USA* 2004; **101**: 4966–4971.
- 13 Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 2004; **303**: 848–851.
- 14 Brier B, Moses HL. Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat Rev Cancer* 2006; **6**: 506–520.
- 15 Lazennec G, Richmond A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol Med* 2010; **16**: 133–144.
- 16 Balkwill FR. The chemokine system and cancer. *J Pathol* 2012; **226**: 148–157.
- 17 Vandercappellen J, Van Damme J, Struyf S. The role of CXC chemokines and their receptors in cancer. *Cancer Lett* 2008; **267**: 226–244.
- 18 Bertran E, Caja L, Navarro E, Sancho P, Mainez J, Murillo MM et al. Role of CXCR4/SDF-1 alpha in the migratory phenotype of hepatoma cells that have undergone epithelial–mesenchymal transition in response to the transforming growth factor-beta. *Cell Signal* 2009; **21**: 1595–1606.
- 19 Bartolomé R, Gálvez B, Longo N, Baleux F, Van Muijen G, Sánchez-Mateos P et al. Stromal cell-derived factor-1alpha promotes melanoma cell invasion across basement membranes involving stimulation of membrane-type 1 matrix metalloproteinase and Rho GTPase activities. *Cancer Res* 2004; **64**: 2534–2543.
- 20 Zhao XP, Huang YY, Huang Y, Lei P, Peng JL, Wu S et al. Transforming growth factor-beta1 upregulates the expression of CXC chemokine receptor 4 (CXCR4) in human breast cancer MCF-7 cells. *Acta Pharmacol Sin* 2010; **31**: 347–354.
- 21 Zoller M. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer* 2011; **11**: 254–267.
- 22 Meulmeester E, Ten Dijke P. The dynamic roles of TGF-beta in cancer. *J Pathol* 2011; **223**: 205–218.
- 23 Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003; **3**: 807–821.
- 24 Jiang X, Liu R, You J, Zhou Q, Zhang H. Defective expression of TGFBR3 gene and its molecular mechanisms in non-small cell lung cancer cell lines. *Zhongguo Fei Ai Za Zhi* 2010; **13**: 451–457.
- 25 Xu CC, Wu LM, Sun W, Zhang N, Chen WS, Fu XN. Effects of TGF-β signaling blockade on human A549 lung adenocarcinoma cell lines. *Mol Med Rep* 2011; **4**: 1007–1015.
- 26 Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 1996; **382**: 635–638.
- 27 Sun X, Cheng G, Hao M, Zheng J, Zhou X, Zhang J et al. CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression. *Cancer Metast Rev* 2010; **29**: 709–722.
- 28 Guo Y, Hangoc G, Bian H, Pelus LM, Broxmeyer HE. SDF-1/CXCL12 enhances survival and chemotaxis of murine embryonic stem cells and production of primitive and definitive hematopoietic progenitor cells. *Stem Cells* 2005; **23**: 1324–1332.
- 29 Kucia M, Reca R, Miekus K, Wanzeck J, Wojakowski W, Janowska-Wieczorek A et al. Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1–CXCR4 axis. *Stem Cells* 2005; **23**: 879–894.
- 30 Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan M et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001; **410**: 50–56.
- 31 Hartmann T, Burger J, Glodek A, Fujii N, Burger M. CXCR4 chemokine receptor and integrin signaling co-operate in mediating adhesion and chemoresistance in small cell lung cancer (SCLC) cells. *Oncogene* 2005; **24**: 4462–4471.
- 32 Furusato B, Mohamed A, Uhlen M, Rhim JS. CXCR4 and cancer. *Pathol Int* 2010; **60**: 497–505.
- 33 Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z et al. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med* 2006; **203**: 2201–2213.
- 34 Balabanian K, Lagane B, Infantino S, Chow KY, Harriague J, Moepps B et al. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem* 2005; **280**: 35760–35766.
- 35 Maksym RB, Tarnowski M, Grymula K, Tarnowska J, Wysoczynski M, Liu R et al. The role of stromal-derived factor-1–CXCR7 axis in development and cancer. *Eur J Pharmacol* 2009; **625**: 31–40.
- 36 Sanchez-Martin L, Sanchez-Mateos P, Cabanas C. CXCR7 impact on CXCL12 biology and disease. *Trends Mol Med* 2013; **19**: 12–22.
- 37 Miao Z, Luker KE, Summers BC, Berahovich R, Bhojani MS, Rehemtulla A et al. CXCR7 (RDC1) promotes breast and lung tumor growth *in vivo* and is expressed on tumor-associated vasculature. *Proc Natl Acad Sci USA* 2007; **104**: 15735–15740.
- 38 Iwakiri S, Mino N, Takahashi T, Sonobe M, Nagai S, Okubo K et al. Higher expression of chemokine receptor CXCR7 is linked to early and metastatic recurrence in pathological stage I nonsmall cell lung cancer. *Cancer* 2009; **115**: 2580–2593.
- 39 Su L, Zhang J, Xu H, Wang Y, Chu Y, Liu R et al. Differential expression of CXCR4 is associated with the metastatic potential of human non-small cell lung cancer cells. *Clin Cancer Res* 2005; **11**: 8273–8280.
- 40 Suzuki M, Mohamed S, Nakajima T, Kubo R, Tian L, Fujiwara T et al. Aberrant methylation of CXCL12 in non-small cell lung cancer is associated with an unfavorable prognosis. *Int J Oncol* 2008; **33**: 113–119.
- 41 Schaeffer D, Somarelli JA, Hanna G, Palmer GM, Garcia-Blanco MA. Cellular migration and invasion uncoupled: increased migration is not an inexorable consequence of epithelial-to-mesenchymal transition. *Mol Cell Biol* 2014; **34**: 3486–3499.
- 42 Even-Ram S, Yamada KM. Cell migration in 3D matrix. *Curr Opin Cell Biol* 2005; **17**: 524–532.
- 43 Yang WH, Lan HY, Huang CH, Tai SK, Tzeng CH, Kao SY et al. RAC1 activation mediates Twist1-induced cancer cell migration. *Nat Cell Biol* 2012; **14**: 366–374.
- 44 Choi YH, Burdick MD, Strieter BA, Mehrad B, Strieter RM. CXCR4 but not CXCR7, discriminates metastatic behavior in non-small cell lung cancer cells. *Mol Cancer Res* 2014; **12**: 38–47.

Supplementary Information accompanies this paper on the Oncogene website (<http://www.nature.com/onc>)