

Follistatin Suppresses the Production of Experimental Multiple-Organ Metastasis by Small Cell Lung Cancer Cells in Natural Killer Cell – Depleted SCID Mice

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Abstract Purpose: Follistatin (FST), an inhibitor of activin, regulates a variety of biological functions, including cell proliferation, differentiation, and apoptosis. However, the role of FST in cancer metastasis is still unknown. Previous research established a multiple-organ metastasis model of human small cell lung cancer in natural killer cell – depleted SCID mice. In this model, i.v. inoculated tumor cells produced metastatic colonies in multiple organs including the lung, liver, and bone. The purpose of this study is to determine the role of FST in multiple-organ metastasis using this model.

Experimental Design: A human FST gene was transfected into the small cell lung cancer cell lines SBC-3 and SBC-5 and established transfectants secreting biologically active FST. The metastatic potential of the transfectants was evaluated using the metastasis model.

Results: FST-gene transfection did not affect the cell proliferation, motility, invasion, or adhesion to endothelial cells *in vitro*. I.v. inoculated SBC-3 or SBC-5 cells produced metastatic colonies in multiple organs, including the lung, liver, and bone in the natural killer cell – depleted SCID mice. FST transfectants produced significantly fewer metastatic colonies in these organs when compared with their parental cells or vector control clones. Immunohistochemical analyses of the liver metastases revealed that the number of proliferating tumor cells and the tumor-associated microvessel density were significantly less in the lesions produced by FST transfectants.

Conclusions: These results suggest that FST plays a critical role in the production of multiple-organ metastasis, predominantly by inhibiting the angiogenesis. This is the first report to show the role of FST in metastases.

A number of growth factors, including members of the transforming growth factor- β (TGF- β) superfamily, such as activin, are involved in the regulation of cell proliferation, differentiation, and apoptosis (1). These biological functions

are regulated by several molecules, such as follistatin (FST), which is a critical regulator of activin activity in many adult tissues (2). FST was originally identified from ovarian follicular fluid as a suppressor of follicle-stimulating hormone secretion by pituitary cells (3, 4). Subsequently, FST has been reported to be able to neutralize the activin bioactivity by binding activin with high affinity (50-500 pmol/L) to form biologically inactive complexes (5). FST-deficient mice exhibit numerous phenotypes, including musculoskeletal and cutaneous abnormalities, and they die within hours of birth because they fail to breathe, thus suggesting that FST is a mediator of cell growth, development, and differentiation in many tissues and organs (6). Recently, several reports showed that the activin/FST system plays important roles in the progression of malignant disease by regulating cell proliferation or angiogenesis (1). However, the precise mechanisms of activin/FST system activity on cancer progression, especially cancer metastasis, are still unknown.

Small cell lung cancer (SCLC) accounts for 15% to 20% of lung cancer and presents aggressive clinical behavior characterized by rapid growth and metastatic spread to the distant organs (7). The production of clinically undetectable micro-metastasis during the early stages frequently makes the prognosis of this disease poor. In addition, SCLC frequently metastasizes to the brain, lung, liver, and bone, indicating a unique organ tropism of SCLC to these organs.

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Metastasis, the spread of cancer from the primary site of tumor growth to other organs is the leading cause of cancer-related morbidity and mortality. Therefore, novel effective therapies to control cancer metastasis are necessary to improve the prognosis of malignant disease. Recently, several molecules have been reported to play important roles throughout the multiple steps of metastasis, such as detachment from the primary tumor mass, microinvasion into stromal tissue, intravasation into blood vessels, survival in the circulation, attachment to endothelial cells, and extravasation and growth with neovascularization in secondary sites (8). However, the precise mechanisms of metastasis remain to be elucidated.

To investigate the molecular and biological mechanisms of metastasis and to develop novel therapeutic modalities against cancer metastasis, a multiple-organ metastasis model of human SCLC cells was previously established in natural killer (NK) cell-depleted SCID mice (9, 10). In this model, *i.v.* inoculated tumor cells produced metastatic colonies in multiple organs, including the lung, liver, and bone. This experimental metastasis model has several advantages. It yields patient-like pattern of metastasis; the formation of metastasis is reproducible (all mice produce metastatic lesions) and the number of metastatic lesions is consistent. Therefore, this model is suitable for analyzing the molecular mechanisms of metastasis.

FST has been recently reported to be 1 of 11 genes, overexpressed in derivative subpopulations that showed a highly metastatic ability in breast cancer cell lines (11). However, the role of FST on cancer metastasis is still totally unknown. The goal of this study is to determine the role of FST in the production of metastasis by SCLC. A human FST expression vector (FS-315) was transfected into two SCLC cell lines, SBC-3 and SBC-5, and examined whether the FST gene transfection affected the production of experimental metastasis in a NK cell-depleted SCID mouse model.

Materials and Methods

Cell lines. Human SCLC cell lines, SBC-3 and SBC-5, kindly provided by Drs. M. Tanimoto and K. Kiura (Okayama University; ref. 12), were maintained in Eagle's MEM, and human erythroleukemia cell line K562 was maintained in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 units/mL) and streptomycin (50 μ g/mL). All cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

Reagents. Antimouse interleukin-2 receptor β -chain monoclonal antibody TM- β 1 (IgG2b) was supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University; ref. 13). Recombinant human activin A was purchased from R&D Systems.

FST gene transfection. HA-tagged human FST (FS-315) cDNA was ligated into the pcDNA3 plasmid vector (Invitrogen). The cDNA-containing or empty vector was transfected into SBC-5 or SBC-3 using LipofectAMINE 2000 (Invitrogen). Transfected cells were selected for 3 weeks in medium containing 1 mg/mL G418 (Sigma), and G418 resistant cells were subcloned and maintained in medium containing 1 mg/mL G418. FST expression was confirmed by ELISA (R&D Systems) with cell culture supernatant. Two clones and a vector control clone were established in each cell line, which were designated SBC-5/Mock, SBC-5/FST1, and SBC-5/FST9 or SBC-3/Mock, SBC-3/FST10, and SBC-3/FST12.

FST bioactivity assay. The bioactivity of FST, produced by transfectants, was measured by hemoglobin synthesis in K562 erythroleukemia cells by activin A (14). Briefly, 50 μ L of the assay medium containing 2.5% fetal bovine serum were plated into each well of the 96-well plate,

and 100 μ L of cell culture supernatant of each clone were added. Human activin A solution (50 μ L) in the assay medium (80 ng/mL) was added to all wells. After incubation at 37°C for 1 h, 5.0×10^3 K562 cells were added and then incubated at 37°C for 96 h. At the end of incubation, the cells were washed twice with PBS. After removing the supernatant, 70 μ L dH₂O was added to the well. The plate was kept at -80°C for 30 min and incubated at 37°C for 30 min to lyse the cells. The plate was centrifuged to pellet the cell debris, and 50 μ L of supernatant were transferred from each well to a new plate. The substrate reagents (R&D Systems) were added to each well and incubated at room temperature for 20 min. The absorbance was measured at 600 nm.

Cell proliferation assay. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction method (15). Tumor cells (2×10^3 cells/200 μ L) were plated into each well of the 96-well plate and incubated for 24, 48, 72, and 96 h. After incubation, 50 μ L of stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (2 mg/mL; Sigma) was added to all wells, and the cells were then further incubated for 2 h at 37°C. The media containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution were removed, and 100 μ L of DMSO were added. The absorbance was measured with the microplate reader at test and reference wavelengths of 550 and 630 nm, respectively.

Wound healing assay. The wound healing assays were done as reported previously (16). Specific areas for measurement were also marked, and digital images were taken using an inverted microscope immediately after scraping at 6, 12, and 24 h. After alignment, the images were superimposed, and the migration distances were measured.

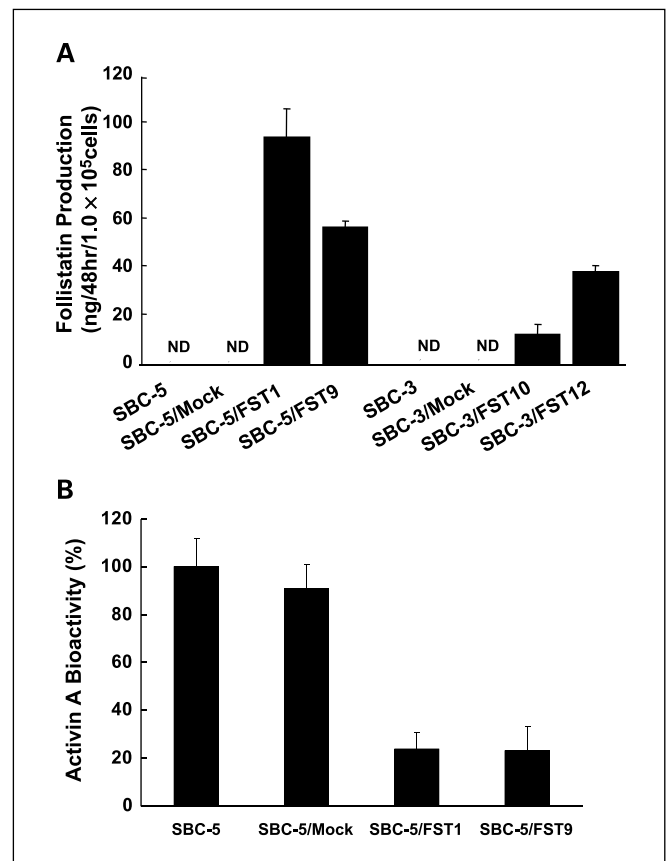


Fig. 1. Transfection of FST gene into SCLC cells. **A**, the expression of exogenous FST protein in stable transfectants in SBC-5 SBC-3 determined by an ELISA analysis. **B**, FST bioactivity assay. The bioactivity of FST, produced by FST transfectants, was measured by hemoglobin synthesis in K562 erythroleukemia cells by activin A. Cell culture supernatants of FST transfectants inhibited activin A bioactivity >70% compared with the parental cell or vector control clone.

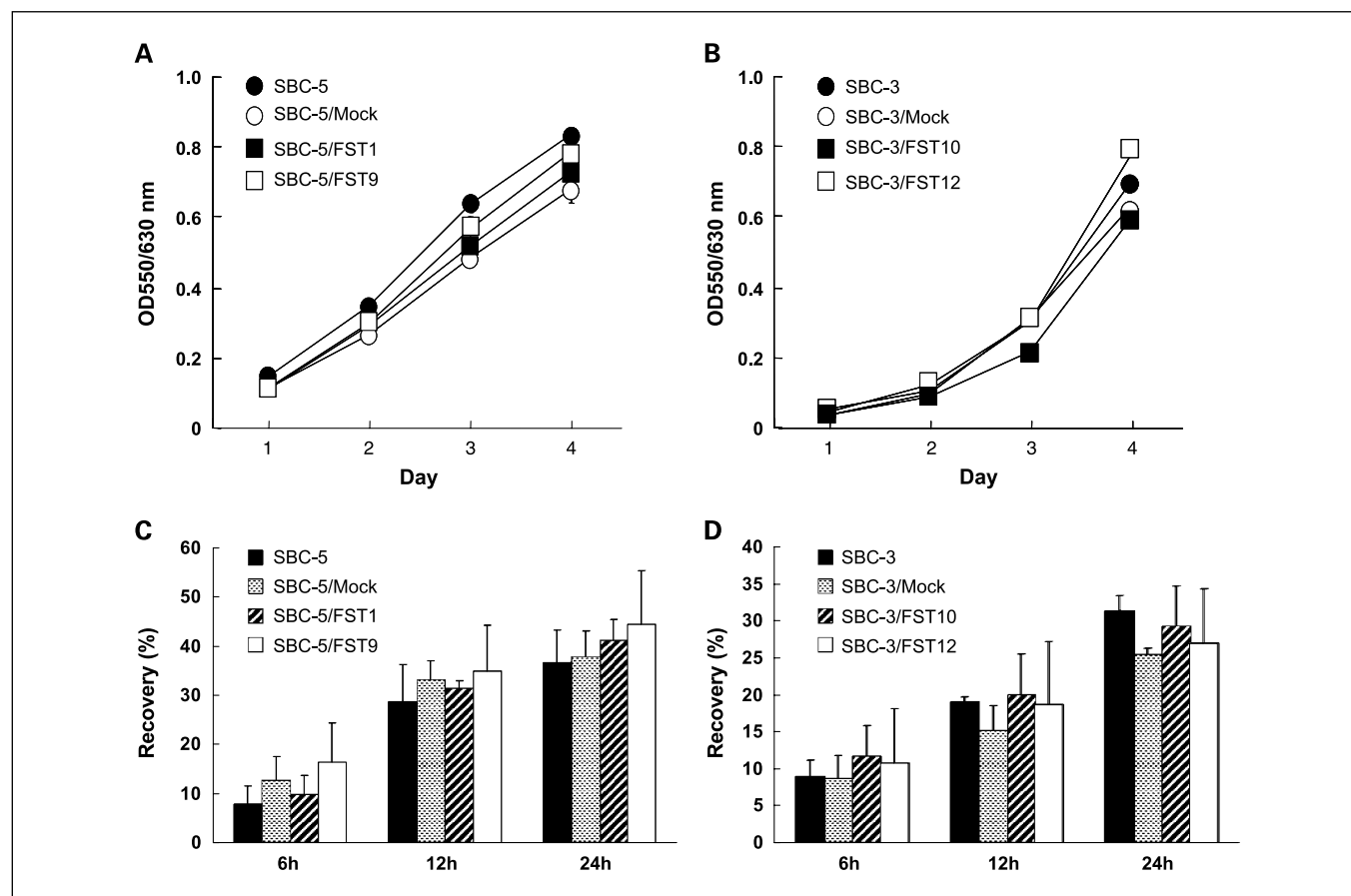


Fig. 2. The effect of FST gene transfection on the growth and migration of cancer cells *in vitro*. *A* and *B*, the anchorage-dependent growth determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; *C* and *D*, the cell motility determined by scratch assay. The recovery of a scratch wound was determined 6, 12, and 24 h later.

Three random positions per three different specific areas were measured. The motility was expressed as the percentage of recovery of the scratched wound. The percentage of recovery (% recovery) was calculated by the following formula:

$$\% \text{ recovery} = [1 - (a - b)/a] \times 100$$

wherein *a* is the distance at 0 h and *b* is the distance at 6, 12, or 24 h.

Animals. Male SCID mice, 5 to 6 weeks old, were obtained from CLEA Japan and maintained under specific pathogen-free conditions throughout this study. All experiments were done in accordance with the guidelines established by the Tokushima University Committee on Animal Care and Use.

In vivo metastasis models. To facilitate metastasis formation, SCID mice were pretreated with antimouse interleukin-2 receptor β -chain antibody to deplete NK cells (9, 10). Two days later, the mice were inoculated with SBC-3, SBC-5, or other transfected clones (1.0×10^6 per mouse) into the tail vein. Five weeks (SBC-5 and subclones) or 7 weeks (SBC-3 and subclones) after tumor cell inoculation, the mice were anesthetized by i.p. injection of pentobarbital and X-ray photographs of the mice were taken to determine bone metastasis. The mice were killed humanely under anesthesia, the major organs were removed and weighed, and the number of metastatic colonies on the surface of the organs was counted. The lungs were fixed in Bouin's solution (Sigma) for 24 h. The number of osteolytic lesions on X-ray films was counted by two investigators independently (H.O. and S.Y.).

Histologic analyses. For histologic analyses, the major organs with metastasis were fixed in 10% formalin. The bone specimens were decalcified in 10% EDTA solution for 1 week and then embedded in

paraffin. Paraffin-embedded tissues (4 μ m thick), stained with H&E, were used to count the number of the microscopic metastatic foci. The small metastatic foci that were <1 mm in diameter were defined as the microscopic metastatic foci. The paraffin-embedded tissues were also used to quantitate *in vivo* cell proliferation using mouse anti-human Ki-67 monoclonal antibody (MIB1; 1:50 dilution; DAKO) and apoptosis using the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling method with the Apoptosis Detection System (Promega). Frozen tissue section (8 μ m thick) were fixed with cold acetone and used for identification of endothelial cells using rat anti-mouse CD31/PECAM-1 monoclonal antibody (1:100 dilution; PharMingen). In each analysis, the five areas containing the highest number of staining within a section were selected for histologic quantification under light microscopy or fluorescent microscopy with a 400-fold magnification. The results were independently evaluated by two investigators (H.O. and S.Y.).

Statistical analysis. The statistical significance of difference in *in vitro* and *in vivo* data was analyzed by one-way ANOVA. When the *P* values for the overall comparisons were <0.05, post-hoc pairwise comparisons were done by Newman-Keuls multiple comparison test. *P* values of <0.05 were considered to be statistically significant. The statistical analysis was done using the GraphPad Prism program Ver. 4.01.

Results

Generation of cell lines stably overexpressing FST. In the first set of experiments, FST gene or vector control were transfected into SBC-3 and SBC-5 cells. After the cloning, the FST expression

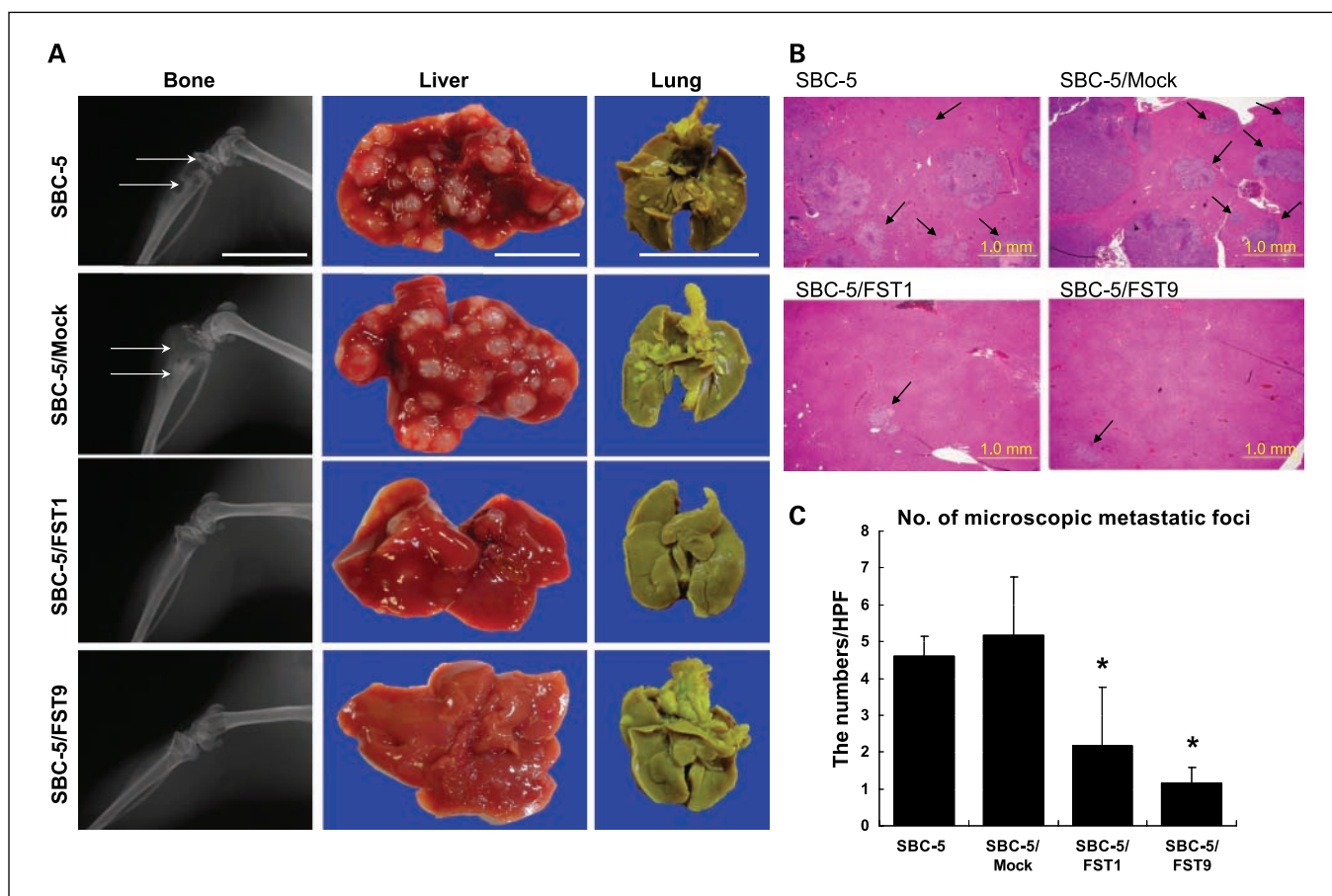


Fig. 3. The production of experimental multiple-organ metastasis by FST-transfected cells. SBC-5 cells transfected with or without FST gene (1×10^6) were injected i.v. into NK cell – depleted SCID mice. From 4 to 5 wk after tumor cell inoculation, the metastatic burden was determined as described in Materials and Methods. **A**, SBC-5 cells produced large number of metastatic colonies in the liver, lung, and bone. FST transfectants produced much less number of metastatic colonies in the each organ; *bar*, 10 mm. **B**, histology of the metastatic lesions in the liver (magnification, 20 \times). The small metastatic foci (*arrow*; <1 mm in diameter) were defined as the microscopic metastatic foci; *bar*, 1 mm. **C**, the numbers of the microscopic metastatic foci by the FST transfected cells also significantly decreased compared with the parental SBC-5 or mock control cells. *, $P < 0.01$ compared with SBC-5 tumors and SBC-5/mock tumors (one-way ANOVA).

level was assessed by ELISA. All of the FST gene transfectants, but not the parental or mock SBC-3 or SBC-5 cells, overexpressed FST protein (Fig. 1A). Next, the bioactivity of FST produced by these clones was examined using the bioassay with K562 cells.

Cell culture supernatants of the FST transfectants inhibited activin A bioactivity of >70% compared with the parental cells or the vector control clone, suggesting that the FST secreted by transfectants were biologically active (Fig. 1B).

Table 1. Production of metastasis by SBC-5 cells with or without FST in the NK cell–depleted SCID mice

SBC-5 cell line	FST production (ng/48 h/10 ⁵)	Liver weight (g)	No. metastatic colonies*		
			Liver	Lung	Bone
SBC-5	<0.25	1.5 (1.4-1.7)	57 (52-68)	41 (38-52)	5 (4-8)
SBC-5/mock	<0.25	1.2 (1.1-2.2)	34 (25-62)	15 (11-27)	5 (4-7)
SBC-5/FST1	93.3	1.2 (0.9-1.7)	6 (2-6) ^{†,‡}	2 (0-3) ^{‡,§}	1 (0-2) ^{†,‡}
SBC-5/FST9	56.1	1.4 (1.3-1.5)	5 (4-8) ^{†,‡}	3 (2-7) ^{‡,§}	3 (3-5)

NOTE: SBC-5 cells (1×10^6) with or without FST gene were inoculated i.v. into NK cell – deleted SCID mice on day 0 (five mice per group). The mice were killed on day 31, and the formation of metastases was evaluated. Data shown are representative of three independents with similar results. The statistical significance of difference was analyzed by one-way ANOVA, and post-hoc pairwise comparisons were done by Newman-Keuls multiple comparison test.
 *Values are the median (minimum - maximum).
[†] Statistically significant difference compared with SBC-5 ($P < 0.001$).
[‡] Statistically significant difference compared with SBC-5/mock ($P < 0.001$).
[§] Statistically significant difference compared with SBC-5/mock ($P < 0.01$).

FST gene transfection did not affect the behavior of cancer cells in vitro. To explore whether FST gene transfection affected cell behavior related with metastasis, the effect of FST on cell growth was examined *in vitro*. There was no discernible difference between the FST transfectants and the parental cells or the vector control in both cell lines on the anchorage-dependent or anchorage-independent growth, irrespective of the presence (data not shown) or absence (Fig. 2A and B and data not shown) of activin A. The results of the wound healing assay, two-chamber migration assay, and Matrigel invasion assay showed that FST gene transfection did not affect cell motility, migration, invasiveness, and adhesion to endothelial cells in both cell lines (Fig. 2C and D and data not shown). Recently, several chemokine receptors, such as CXCR4 and CCR7, expressed on cancer cells, have been shown to play an important role in cancer growth and metastasis (17). FST gene transfection, however, did not change the expression level of such chemokine receptors (data not shown).

FST gene transfection resulted in the suppression of experimental multiple organ metastasis in vivo. To study the tumorigenicity and growth rate *in vivo*, SBC-5 and its subclones (1.0×10^6 cells per mouse; $n = 5$) were s.c. inoculated into NK cell-depleted SCID mice. The volumes of the s.c. tumors were measured every 3 days. The results showed that FST gene transfection did not affect the tumor growth rate *in vivo* (data not shown).

The metastatic potential of FST gene transfectants was next evaluated in the multiple-organ metastasis model with NK cell-depleted SCID mice. In this model, highly bone metastatic SBC-5 cells produced metastatic colonies predominantly into the liver, lung, and bone (10, 18). SBC-5 cells transfected with FST gene developed significantly fewer metastases in the liver and lung compared with the parental cell or vector control clone (Fig. 3A; Table 1). We also counted the microscopic metastatic foci in the liver using archived tissue specimens. Surprisingly, the numbers of the micrometastatic foci (<1 mm in diameter) by the FST-transfected cells were also significantly decreased compared with the parental SBC-5 or mock control cells (Fig. 3B and C). The number of osteolytic bone metastasis was also significantly less in SBC-5/FST1, an FST-high

producing clone. In SBC-5/FST9, an FST-low production clone, although the number of bone metastasis was not reduced significantly, the size of metastatic colonies tended to be smaller than parental or vector control.

Low bone metastatic SBC-3 cells produced metastasis predominantly in the liver and kidney (10, 18). The number of metastatic colonies produced by FST transfectants in the liver was significantly smaller than that produced by parental or mock control cells (Table 2). In the other organs, such as the kidney, lymph nodes, or bone, the number of metastatic colonies by FST transfectants tended to be lower than that of parental cell or vector control clone, whereas the difference was statistically not significant. These results suggest that FST has the potential to suppress the metastasis to multiple organs, at least against SCLC cell lines.

The number of proliferating tumor cells and microvessel density were significantly less in the liver metastatic lesions produced by FST transfectants. To explore the mechanism by which FST gene transfection resulted in the inhibition of multiple-organ metastasis by SCLC cells *in vivo*, histologic analysis was done using metastatic tissue from the liver by parental SBC-5, mock control, and FST-gene transfectant. There was no significant difference in the number of apoptotic tumor cells, determined with the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling staining, among the three groups. However, Ki-67 or CD31 staining showed that the number of proliferating tumor cells and the tumor-associated microvessel density were significantly less in the lesions produced by FST transfectants, compared with the parental SBC-5 or mock control (Fig. 4A-D). These results suggest that FST inhibited multiple steps of the metastasis, such as colonization and proliferation supported by angiogenesis, in the liver.

Discussion

Molecular interactions between cancer cells and their micro-environments play important roles throughout the multiple steps of metastasis (19, 20). Over a century ago, Paget compared metastatic tumor cells to widely disseminated seeds,

Table 2. Production of metastasis by SBC-3 cells with or without FST in the NK cell-depleted SCID mice

SBC-3 cell line	FST production (ng/48 h/10 ⁵)	Liver weight (g)	No. metastatic colonies*		
			Liver	Lymph node	Bone
SBC-3	<0.25	3.8 (0.7-4.1)	6 (4-21)	2 (0-3)	1 (0-2)
SBC-3/mock †	<0.25	2.9 (2.9-3.4)	22 (12-23)	2 (1-2)	1 (0-1)
SBC-3/FST10	10.8	1.4 (1.1-1.5)	All 0 ‡,§	All 0	All 0
SBC-3/FST12	37.2	2.4 (1.8-2.5)	4 (0-7) ¶	2 (0-12)	All 0

NOTE: SBC-3 cells (1×10^6) with or without FST gene were inoculated i.v. into NK cell-deleted SCID mice on day 0 (five mice per group). The mice were killed on day 49, and the formation of metastases was evaluated.

Data shown are representative of two independents with similar results.

The statistical significance of difference was analyzed by one-way ANOVA, and post-hoc pairwise comparisons were done by Newman-Keuls multiple comparison test.

*Values are the median (minimum - maximum).

† Two mice died by day 49.

‡ Statistically significant difference compared with SBC-3 ($P < 0.05$).

§ Statistically significant difference compared with SBC-3/mock ($P < 0.001$).

|| One mouse died by day 49.

¶ Statistically significant difference compared with SBC-3/mock ($P < 0.01$).

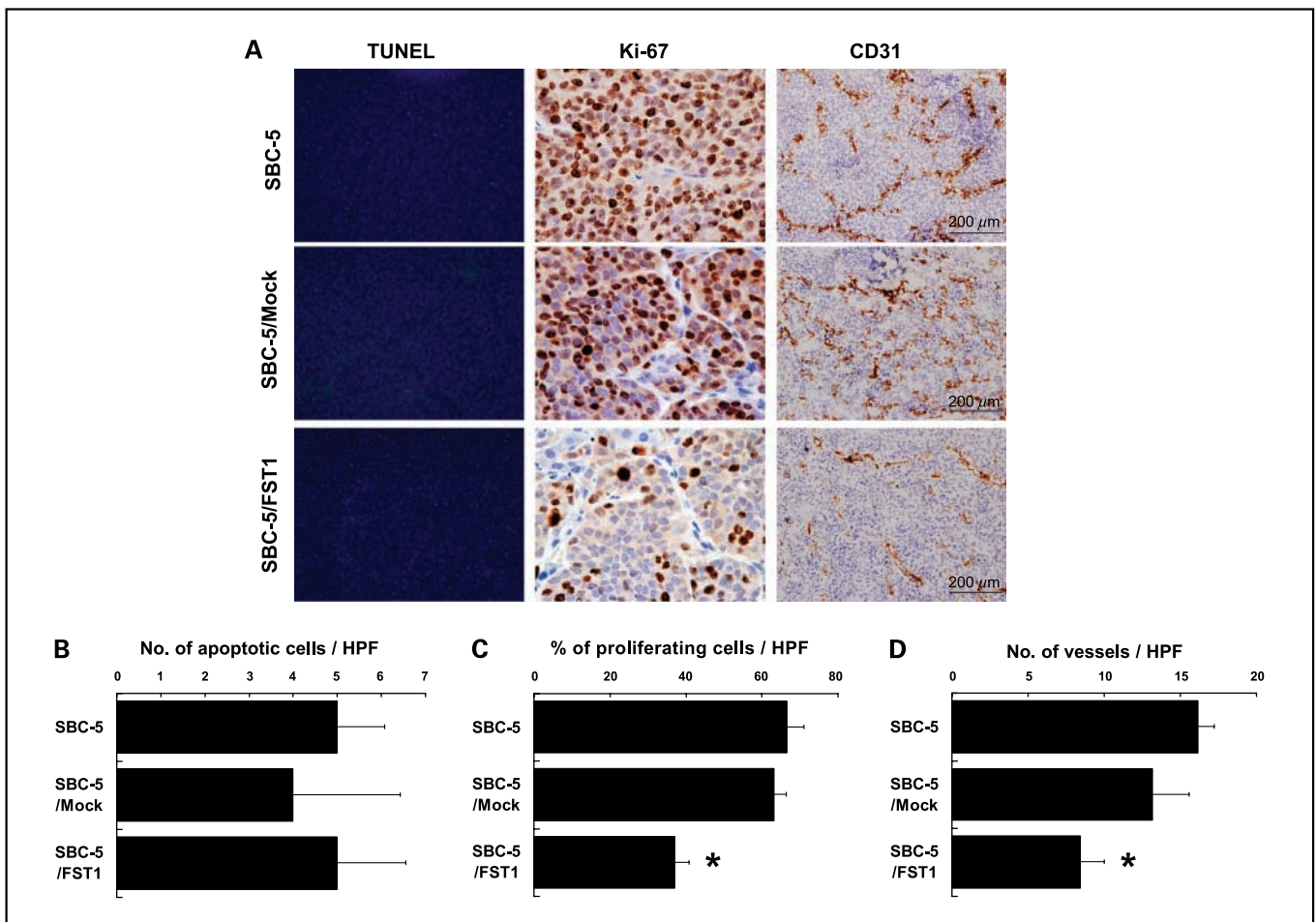


Fig. 4. The image and quantitative analyses of histological examinations of the liver metastatic tumors produced by SBC-5 cells transfected with or without the FST gene. *A* and *B*, no discernible difference was found in the number of apoptotic tumor cells (terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling, *TUNEL*) between the tumors produced by SBC-5/FST1 cells and those produced by SBC-5 of SBC-5/mock cells. However, both the number of proliferating tumor cells determined by Ki-67 staining in SBC-5/FST1 tumors shown in *A* and *C* and the vascularization determined by CD31 staining shown in *A* and *D* were significantly less than that in SBC-5 tumors of SBC-5/mock tumors. *, $P < 0.01$ compared with SBC-5-tumors and SBC-5/mock tumors (one-way ANOVA).

which will grow only on fertile soils (21). Until now, under this theory, various molecules, such as adhesion molecules, cytokines, chemokines, hormones, and their receptors, have been reported to play important roles in preferential metastasis (11, 17, 22, 23). The present study showed that FST over-expression by gene transfection resulted in the suppression of the experimental multiple organ metastasis (both of macro-metastasis and micrometastasis) in NK cell-depleted SCID mice by SCLC cells. Whereas FST gene transfection did not affect the proliferation, migration, or invasion *in vitro* and s.c. tumor growth *in vivo* of SCLC cells, it suppressed the neovascularization resulting in the suppression of *in vivo* proliferation of tumor cells in the metastatic lesions. Therefore, FST may be one of the determinants which affect the properties of organ microenvironments to inhibit metastasis. This is the first report to show the role of FST in the progression of multiple organ metastases.

In the previous report, FST was screened as a 1 of 11 genes up-regulated in derivative highly bone metastatic subpopulations in breast cancer cells (11). However, whereas the authors showed the functional roles of the four genes in forming bone metastasis, the function of FST and whether FST promote the

bone metastasis are not clarified. Moreover, it is possible that the role of FST in producing bone metastasis is different between breast cancer and SCLC. We previously established a multiple-organ metastasis model of human SCLC cells in NK cell-depleted SCID mice (9, 10) and analyzed the gene expression profiles of metastatic lesions in the lung, liver, and bone produced by a human SCLC cell line, SBC-5, using a cDNA microarray consisting of 23,040 genes (24). In the process, FST was screened as a candidate gene that may promote the cancer metastasis especially to bone, because the expression of FST was up-regulated only in the bone metastatic lesions (24). However, unexpectedly, the FST gene transfection resulted in the suppression of tumor metastasis not only to the bone but also to the visceral organs. We have reported that SBC-5 cells secreted parathyroid hormone-related peptide (PTHrP) as the primary stimulator of the bone resorption (10). The process of bone resorption releases TGF- β , which is abundantly stored in the bone matrix (25), and TGF- β is reported to up-regulate the FST expression (26); therefore, FST might be up-regulated as a consequence of TGF- β release from the osteolytic bone metastatic lesions. We considered that FST might be up-regulated in the bone lesions not to promote but

to suppress metastasis during the interaction between tumor cells and the host microenvironment as one of the defending reactions.

Activin A, a homodimer of two inhibin β A subunit, is a member of the TGF- β superfamily, which consists of TGF- β , activin, inhibin, bone morphogenic proteins, and others (1). Activin has multiple physiologic activities, including stimulation of follicle-stimulating hormone release from the anterior pituitary (27, 28), erythroid differentiation (29), and bone growth promotion (30). However, the role of activin on cancer progression is still controversial. Activin inhibits the growth of several types of tumors, such as prostate cancer (31), breast cancer (32), and others. On the other hand, the overexpression of activin A is associated with a poor prognosis of esophageal (33), pancreatic (34), ovarian (35), and colon carcinomas (36). Moreover, the circulating levels of activin A are significantly higher in the patients with bone metastasis than the patients without bone metastasis in breast or prostate cancer (37). These results suggest that activin A may be involved in the carcinogenesis, progression, and metastasis of several types of cancer in humans. According to these findings, SBC-3 and SBC-5 cells expressed mRNA of β A subunit (data not shown). Whereas FST gene transfection did not affect the expression of β A subunit (data not shown), it is possible that the inhibition of activin activity from host stromal cells might be one of the mechanisms suppressing metastasis. For example, the macrophages express activin A and it stimulates matrix metalloproteinase-2, which is one of the key regulators of cell invasion, in an autocrine manner (38). Moreover, activin A produced by vascular endothelial cells markedly enhance vascular endothelial growth factor-induced angiogenesis, and it was blocked by the addition of FST (39). These findings suggested that the inhibition of activin from the organ microenvironments, such as stromal cells and endothelial cells, might modulate metastatic features of cancer cells.

Angiogenesis, neovascularization to supply oxygen and nutrition, has been shown to play a critical role in the progression of tumors (40). Angiogenesis is regulated by the balance of proangiogenic molecules, such as vascular endothe-

lial growth factor, fibroblast growth factor-2, interleukin-8, and antiangiogenic molecules, such as thrombospondin-1, angiostatin, and endostatin (41). Whereas the roles of FST and activin in angiogenesis still remain controversial (42), several reports showed that activin induces the expression of vascular endothelial growth factor and that it controls tubulogenesis of endothelial cells (39, 43, 44). The present study showed that the tumor microvessel densities were significantly less in the liver metastatic lesions produced by FST transfectants when compared with the parental cells or vector control clone. These results suggest that FST might suppress angiogenesis in the metastatic lesions through the interaction between tumor cells and organ microenvironments. To clarify the mechanisms by which FST gene transfection resulted in the suppression of angiogenesis, the gene expression levels of various angiogenesis-related molecules, such as vascular endothelial growth factor, fibroblast growth factor-2, and interleukin-8, were analyzed in the liver metastatic colonies produced by parental cells, vector control clone, or FST transfectants. However, no discernible difference on the expression levels of these molecules was observed in the metastatic lesions (data not shown). Other mechanisms may exist by which FST suppresses angiogenesis, and therefore, additional experiments to reveal the underlying mechanisms will be needed in the future.

In conclusion, these experiments showed that FST gene transfection resulted in the suppression of the experimental multiple organ metastases, via inhibition of angiogenesis, in NK cell-depleted SCID mice by SCLC cells (SBC-3 and SBC-5). The novel strategy with FST to modify organ microenvironments may be a unique and effective strategy to inhibit multiple organ metastasis of SCLC in humans. Further experiments to clarify the mechanism by which FST inhibited angiogenesis are now under way in our laboratory.

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