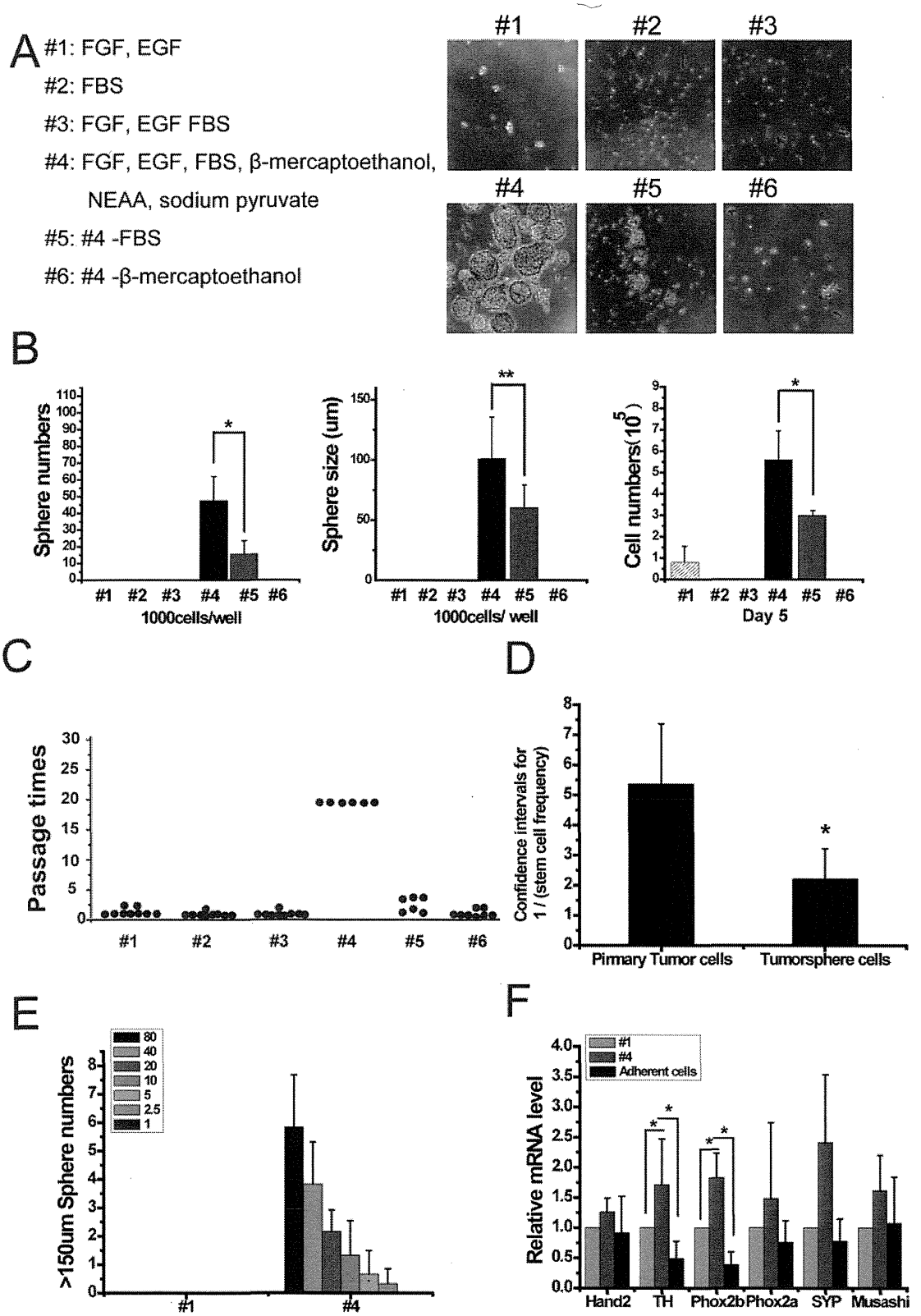

Figure 2. A suitable condition for tumorspheres from primary tumors. A, Important ingredients and a representative photo are shown for each culture condition. Photos were taken at day 5 after culturing in a 24-well plate at 10^3 cells/well. B, Quantitative results of tumorsphere formation. For proliferation assay, 10^5 cells were cultured under the indicated conditions in 6-cm petri dishes for 5 days, and the cells were then counted with trypan blue. Representative results of three independent experiments are shown here. *P<0.05, **P<0.001. C, Passageable number of tumorspheres. Culture was stopped after 20 passages. D, Self-renewal capacity of tumor cells and tumorsphere cells after several passages was evaluated by ELDA

method described under Materials and Methods. *P<0.05. E, Sphere-forming efficiency of #4 medium. We did a limited dilution adjusting cell density to 1 to 80 per well and incubated the cells for 7 days, and compared sphere (with a diameter more than 150 μm)-forming efficiency under different culture conditions, i.e., #1 and #4. F, Real-time PCR results of sphere cells cultured in #1 and #4 condition and adherent cells. For adherent cell culture, cells were seeded in 10 cm-dish coated with PDL/laminin/fibronectin. Cells were maintained with medium comprising D/F, 2% B27, 1% FBS, β-mercaptoethanol. Medium was changed 24 h later to D/F, 2% B27, 20 ng/ml NGF, 10 ng/ml NT3. mRNA was collected after 3 days culture. *P<0.05.
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Together, the results allowed us to conclude that the #4 condition supported long-term culture of tumorspheres from primary NB tissues. We call this the culture condition for primary neuroblastoma-derived spheres, or PrimNeuS hereafter.

Sphere cells cultured under PrimNeuS had a differentiation property, since radial neurites grew out of these cells under a differentiation condition for 3 days (Fig. 3A). The sphere cells also gave rise to tumors after subcutaneous injection (Fig. 3B, C). We next compared the tumor formation potential of the tumor cells and the corresponding tumorsphere cells from both the primary tumors and allograft tumors. Consistent with Fig. 3C and 3D, allograft tumor cells and their tumorsphere cells showed stronger tumor forming potentials than primary tumor cells and their tumorsphere cells (Fig. 3D). Furthermore, tumorsphere cells from primary tumors needed less cell number to give rise to tumor as compared with tumor cells from primary tumors, supporting the idea that sphere cells are more tumorigenic (Fig. 3D). A set of genes are differentially expressed in primary tumors, tumorspheres from primary tumors, allograft tumors and tumorspheres from allograft tumors (Fig. 3E). Among these genes, it was characteristic that TH, a differentiation marker for sympathetic neurons, was more expressed, and that the stem cell markers Pax6 and Sox2 were less expressed in primary tumors and their spheres (Fig. 3E). These expression profiles were maintained in tumors derived from tumorspheres of allograft or primary tumors (Fig. 3F).

We next asked whether or not sphere cells from primary tumors of *MYCN* Tg mice could metastasize. To answer this, we used spheres from primary tumors after three passages and infected them with lentivirus carrying an EF-promoter-Venus expression vector. After two more passages, we sorted Venus-expressing sphere cells followed by another two passages (Fig. 4A, B). Cells were then subcutaneously injected into syngenic 129/SvJ wild-type mice. Bone marrow cells were analyzed 6 weeks later, after subcutaneous tumors were observed. We detected a few clusters of Venus- and TH-positive cells in the femoral bone section by immunohistochemistry (data not shown). Next, we tried to determine the percentage of the positive cells on the bone marrow cells by FACS, but it was below the detectable level. However, in PrimNeuS medium, Venus-positive cells grew and formed spheres (Fig. 4C, D). These spheres could be passaged (Fig. 4E, F). In contrast, PrimNeuS did not support sphere formation of normal bone marrow cells (data not shown). We concluded that sphere cells from primary tumors metastasize to the bone marrow. Consistent with this, we found that PrimNeuS supported sphere formation from the bone marrow of *MYCN* transgenic mice harboring sympathetic ganglia-derived primary tumors, but not from that of wild-type mice (Fig. 4G, H). These spheres could be passaged under PrimNeuS (Fig. 4H). TH and Snail showed significantly higher expression in both tumorspheres from primary tumor (Primary TS) and tumorspheres from bone marrow (Bone marrow TS) as compared with primary tumors, suggesting that primary TS and bone marrow TS have similar properties (Fig. 4I).

Discussion

Primary tumors in *MYCN* transgenic mice could be serially transplanted without exhaustion (we called these serially transplanted tumors allograft tumors), suggesting that these primary tumors contain cells with a high self-renewal potential. Nevertheless, primary tumors of *MYCN* transgenic mice did not give rise to tumorspheres in a serum-free neurosphere culture condition, which is commonly used for tumorsphere cultures for neural tumors. In contrast, allograft tumors gave rise to tumorspheres in this condition. These phenomena are reminiscent of those of clinical samples; tumorspheres are rarely obtained from primary or low-grade neural tumors, in contrast to metastatic or high-grade tumors [1,2,14]. In this study, we established the new culture condition PrimNeuS, by which tumorspheres could be formed from a primary tumor and passaged indefinitely. Thus, this study has shown the self-renewal capacity of primary NB cells *in vitro*.

Barrett et al. recently reported that self-renewal does not predict tumor growth potential in mouse models of high-grade glioma [15]. Thus, while $Id1^{low}$ cells generate tumors more rapidly than $Id1^{high}$ cells, $Id1^{low}$ cells show non-self-renewal capacity in a neurosphere culture condition. However, there is still a possibility that $Id1^{low}$ cells might show self-renewal *in vitro* if an appropriate culture condition were provided. PrimNeuS has enabled us to estimate self-renewal capacity *in vitro*. Furthermore, PrimNeuS revealed for the first time that tumorspheres from primary tumors (Fig. 4A–F) as well as primary tumors themselves (Fig. 4G–I) have the potential for metastasis to the bone marrow. This is unexpected, since bone marrow metastasis has not been reported in the model of *MYCN* transgenic mice. PrimNeuS could be applicable as a sensitive tool to detect tiny bone marrow metastases or minimal residual disease of NB.

Personalized therapy has become a realistic concept for cancer treatment. For example, anti-GD2 antibody-treated NB patients lacking HLA class I ligands for their inhibitory killer cell immunoglobulin-like receptors have significantly higher survival rates than those with HLA class I ligands. Unlicensed NK cells are thought to be responsible for this phenomenon [16]. If tumorspheres are obtained from every patient regardless of tumor status, i.e., primary or metastatic, high risk or low risk and high grade or low grade, those are precious resources for evaluating the efficacy of the treatment prior to its clinical use. Although PrimNeuS medium tended to allow a longer culture of tumorspheres from human NB as compared with the classical serum-free medium (#1 medium) (data not shown), a limited number of samples did not allow us to statistically validate the usefulness of PrimNeuS for human NB. Further evaluation or improvement of the culture condition for human NB will facilitate studies of stem cells of NB.

To successfully eradicate tumors, it may be necessary to eliminate both the TICs and their more differentiated progeny [17,18]. In glioma, the capacities for self-renewal and tumor initiation are not necessarily restricted to a uniform population of stem like cells, but can be shared by a lineage of self-renewing cell types expressing a range of markers of forebrain lineage [1]. In this context, it was interesting to find in our study that tumorspheres from primary tumors and allograft tumors express distinct markers, but both show tumorigenecity. The success of culturing long-term tumorspheres from primary NB tumors may open new avenues to identify novel stem cell markers for diagnostic and therapeutic NB.

We found that FBS is essential for the formation and indefinite passaging of tumorspheres. In the case of embryonic stem cells,

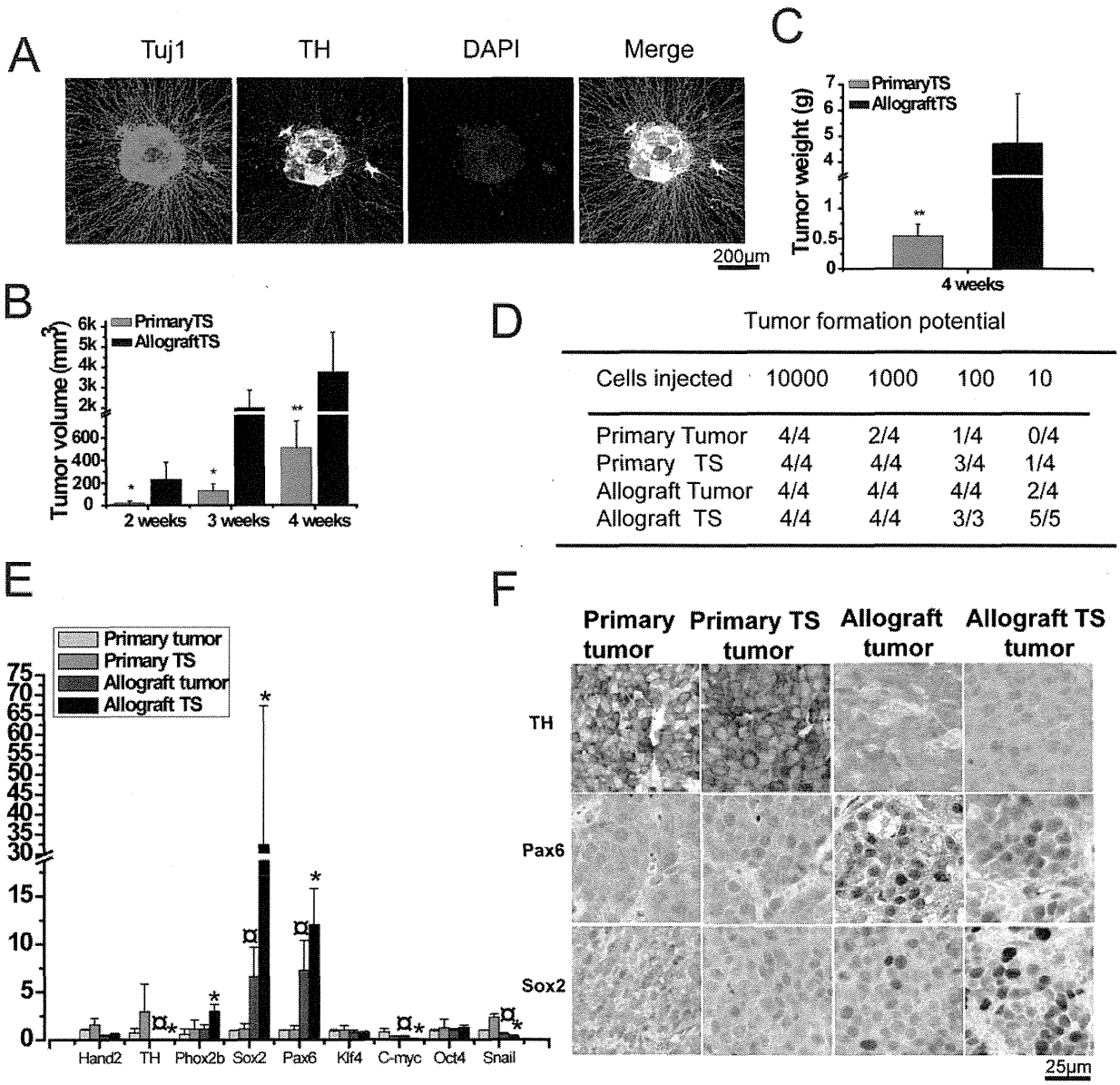

Figure 3. Tumorsphere cells have a differentiation capacity. A, Long radial neurites grew out of tumorspheres under differentiation condition. Representative photos are shown. B, C, Tumorspheres were maintained under PrimNeuS. Totally 10^4 tumorsphere cells together with 30% matrigel were subcutaneously inoculated into wild-type syngenic mice, tumor volumes and weights were monitored. *P<0.05, **P<0.005. D, The primary tumor cells, the corresponding primary tumor sphere cells, allograft tumor cells and the corresponding allograft tumor sphere cells were injected subcutaneously into 4 to 5 week-old wild-type mice at different cell numbers, and investigated for 1.5 months to evaluate the potential. E, mRNA expression profile of primary tumors, tumorspheres from corresponding primary tumors, allograft tumors and tumorspheres derived from corresponding allograft tumors. The allograft tumorshperes were maintained under the traditional serum-free condition (#1 medium), and the tumorspheres from primary tumors were maintained under PrimNeuS (#4 medium). ¤ P<0.05, primary tumor vs. allograft tumor. *P<0.05, primary TS vs. allograft TS. F, Immunohistochemistry of tumors. Representative photos are shown.
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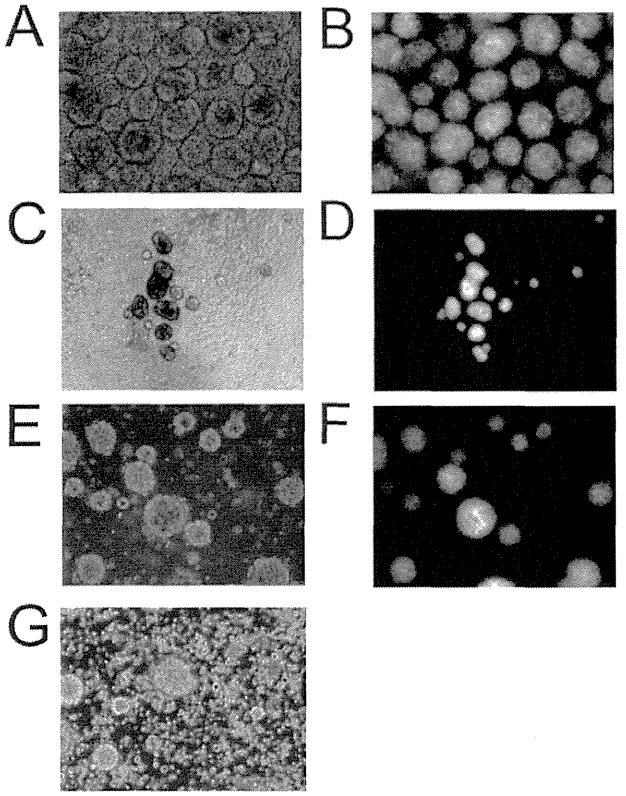
they differentiate into neurons if FBS is removed from the medium. In our case, the decreased concentration of FBS facilitated the outgrowth of neurites of primary tumor spheres, supporting the idea that FBS strongly inhibits neural differentiation of these cells [7,19,20]. Our results suggest that FBS helps to keep NB cells undifferentiated. In addition, we found that β-mercaptoethanol was critical for tumorsphere formation. If β-mercaptoethanol was removed after several passages, the tumorspheres were no longer passaged. This suggests that β-mercaptoethanol is also essential for indefinite passaging. The mechanisms underlying β-mercaptoethanol's functions remain to be verified.

PrimNeus supported the sphere formation from primary tumors and bone marrow in a neuroblastoma model. In contrast, it did not support the sphere formation from normal bone marrow cells. Therefore, PrimNeus may provide an appropriate culture condition for a subset of tumor cells in neuroblastoma (e.g., tumor initiating cells), but may not be generally applicable to stem cells such as normal bone marrow stem cells. This suggests that an



H

Bone marrow metastasis

genotype	age (weeks)	sphere formation	passage
WT	12-13	0/2	0
hemizygote	12-13	5/5	>10

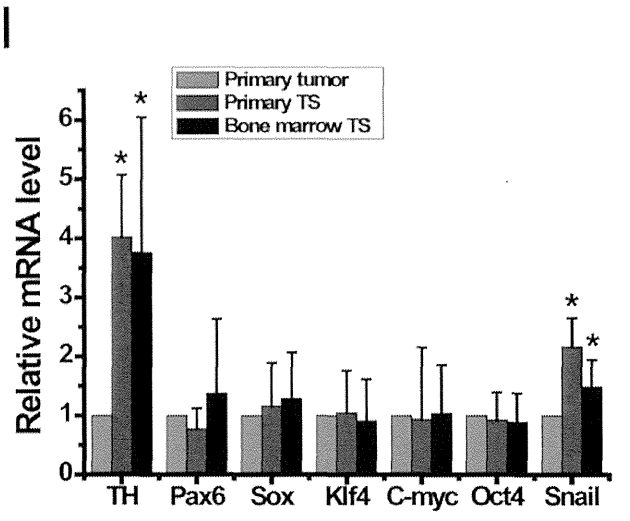


Figure 4. PrimNeuS can support the survival of metastasized cells of neuroblastomas. A–F, Primary tumorspheres were labeled with EF-promoter-Venus by lentivirus, and Venus-positive cells were purified by FACS sorting after 2 passages (A, B). Cells were then subcutaneously inoculated into wild-type mice. One and a half month after inoculation, the bone marrow cells from both femoral bones were cultured under PrimNeuS (C, D). These formed spheres were further expanded under PrimNeuS (E, F). G, The bone marrow of MYCN transgenic mice can form spheres in PrimNeuS medium. H, Bone marrow metastases presented in MYCN transgenic, but not wild-type mice. I, Real-time PCR results of the gene transcripts listed. *$P<0.05$, primary tumors vs. primary tumorsphere or bone marrow tumorspheres.
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appropriate culture condition may depend on cell types. Indeed, hematopoietic stem cells or progenitor cells require several growth factors, such as insulin, IL-3, IL-6, G-CSF and GM-CSF, but these are dispensable for the culture of tumor initiating cells [21,22,23].

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Author Contributions

Conceived and designed the experiments: DC SD KK. Performed the experiments: DC PH PM. Analyzed the data: DC PM. Contributed reagents/materials/analysis tools: ST MM. Wrote the paper: KK.

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TSPAN12 is a critical factor for cancer–fibroblast cell contact-mediated cancer invasion

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Communication between cancer cells and their microenvironment controls cancer progression. Although the tumor suppressor p53 functions in a cell-autonomous manner, it has also recently been shown to function in a non–cell-autonomous fashion. Although functional defects have been reported in p53 in stromal cells surrounding cancer, including mutations in the p53 gene and decreased p53 expression, the role of p53 in stromal cells during cancer progression remains unclear. We herein show that the expression of α-smooth muscle actin (α-SMA), a marker of cancer-associated fibroblasts (CAFs), was increased by the ablation of p53 in lung fibroblasts. CAFs enhanced the invasion and proliferation of lung cancer cells when cocultured with p53-depleted fibroblasts and required contact between cancer and stromal cells. A comprehensive analysis using a DNA chip revealed that tetraspanin 12 (TSPAN12), which belongs to the tetraspanin protein family, was derepressed by p53 knockdown. TSPAN12 knockdown in p53-depleted fibroblasts inhibited cancer cell proliferation and invasion elicited by coculturing with p53-depleted fibroblasts in vitro, and inhibited tumor growth in vivo. It also decreased CXC chemokine ligand 6 (CXCL6) secretion through the β-catenin signaling pathway, suggesting that cancer cell contact with TSPAN12 in fibroblasts transduced β-catenin signaling into fibroblasts, leading to the secretion of CXCL6 to efficiently promote invasion. These results suggest that stroma-derived p53 plays a pivotal role in epithelial cancer progression and that TSPAN12 and CXCL6 are potential targets for lung cancer therapy.

cancer-associated fibroblasts | cancer invasion | cancer–stromal cell interaction | p53 | tetraspanin family

The interaction between cancer and stroma plays a key role in tumor progression. Cancer cells alter adjacent stroma to orchestrate a supportive microenvironment. Normal stroma maintains tissue homeostasis, whereas activated stroma promotes tumor growth, angiogenesis, and metastasis, mainly by secreting growth factors, extracellular matrix (ECM) components, and matrix metalloproteinases (1–3). Cancer-associated fibroblasts (CAFs) are representative cells in the cancer microenvironment, and their activity promotes cancer cell proliferation, migration, and invasion (4–6). Tumor growth by prostatic epithelial cells transformed with an SV40-T antigen was greater than that by normal fibroblasts when nude mice were inoculated with CAFs (7).

p53 is a representative tumor suppressor inactivated by mutations or deletions in approximately half of human cancers. It is known as a transcription factor that regulates the expression of genes associated with cell-cycle arrest, apoptosis, and senescence to prevent tumorigenesis (8, 9). However, recent studies suggested that p53 also possessed non–cell-autonomous functions in the interaction between cancer and stromal cells (10, 11). The growth of inoculated cancer cells was more pronounced with the ablation of p53 in host mice (p53 knockout mice) than in p53 intact mice (wild-type mice) (12). A previous study also found that the growth-promoting activity of cancer cells was greater in SCID mice inoculated with cancer cells with p53-deficient fibroblasts than with wild-type fibroblasts (13). Genetic analyses of stromal tissues from cancer patients revealed p53 somatic mutations and the loss of heterozygosity (LOH) in these stromal cells (14–16). p53 expression was also lower in CAFs than in normal fibroblasts (17). Conditioned culture media from cancer cells also inhibited p53 expression in adjacent fibroblasts (18).

Tetraspanin 12 (TSPAN12) belongs to the tetraspanin family, characterized by four transmembrane domains and two extracellular loops (19). Tetraspanin family proteins act as signaling platforms by forming tetraspanin-enriched microdomains, and tetraspanins are involved in the suppression of metastasis (e.g., CD82 and CD9) and tumor progression (e.g., CD151 and TSPAN8) (20–23). Most studies on TSPAN12 have been performed on familial exudative vitreoretinopathy (24, 25), and in vivo functional analyses using TSPAN12-deficient mice revealed that TSAPN12 contributed to retinal vascular development by cooperating with FZD4 and LRP5, and regulated Norrin-induced

Significance

Cancer-associated fibroblasts (CAFs) are abundant and promote cancer proliferation, invasion, and metastasis. Mutations in the p53 gene and decreased p53 expression are often detected in CAFs, and a dysfunction in p53 in CAFs contributes to cancer progression. However, how host-derived p53 influences cancer cells remains unclear. We herein established coculture systems to monitor enhancements in invasiveness and proliferation elicited by p53-depleted fibroblasts and demonstrated that tetraspanin 12 (TSPAN12), identified as a p53-regulated gene, was required for these processes through the contact of cancer cells with stromal fibroblasts and β-catenin–mediated CXC chemokine ligand 6 (CXCL6) secretion. These results suggest that antibodies against TSPAN12 and CXCL6 may be effective therapeutic agents for cancer.

Author contributions: R.O., C.O., and M.E. designed research; R.O., C.O., Y.M.-H., and M.M. performed research; R.O., C.O., H.I., T.K., and M.E. analyzed data; R.O., F.T., T.O., J.Y., H.N., Y.T., and M.E. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE58753).

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β-catenin signaling (26). Furthermore, TSPAN12 has been shown to promote the maturation of tumor-facilitating sheddase ADAM10 (27) and supports human breast cancer growth (28). Thus, TSPAN12 has been suggested to play a role in cancer progression (29).

We herein constructed several coculture assays to elucidate the effects of fibroblasts on cancer cell invasion and proliferation and found that the inactivation of p53 in fibroblasts contributed to cancer cell invasion and proliferation by up-regulating TSPAN12 in fibroblasts. Enhancements in cancer cell invasion and proliferation depended on direct cancer–stromal cell contact and the up-regulation of CXC chemokine ligand 6 (CXCL6) through the TSPAN12–β-catenin pathway.

Results

p53-Depleted Fibroblasts Had Characteristics of Cancer-Associated Fibroblasts. Previous studies using laser-capture microdissected tissue showed that cancer-adjacent stroma had mutations in the p53 gene and LOH in the p53 gene locus (14–16). Therefore, we examined the expression levels of p53 in cancer-associated stromal cells using the Oncomine database (www.oncomine.org). A microarray dataset from the Oncomine database (30) showed that p53 expression was significantly lower in cancer-associated stromal cells than in normal stromal cells (Fig. S1 *A* and *B*). This was consistent with p53 protein levels being lower in cultured CAFs than in normal fibroblasts (17). To address the effects of conditioned media including various factors secreted from lung cancer cells on p53 expression in stromal cells in vitro, human lung fibroblast TIG-7 cells were cultured in conditioned media from lung cancer cells: H1299, A549, and H460 cells, and normal cells: TIG-7 cells and immortalized small airway epithelial cells (SAECs) (Fig. 1*A*). p53 expression in TIG-7 cells was lower when cultured in conditioned media from lung cancer cells than in control media from normal cells (Fig. 1*B* and Fig. S1*C*), which is consistent with the recent finding that the expression of p53 in human lung fibroblast WI-38 cells was decreased by a treatment with conditioned medium derived from cancer cells (18). We then investigated the relationship between p53 and α-smooth muscle actin (α-SMA), a marker of CAFs. The same dataset (30) from the Oncomine database revealed that p53 expression negatively correlated with ACTA2 (α-SMA) expression (Fig. 1*C*). p53 knockdown in fibroblasts increased α-SMA expression, suggesting that the decreased expression of p53 was altered to exhibit CAF-like characteristics (Fig. 1 *D* and *E*).

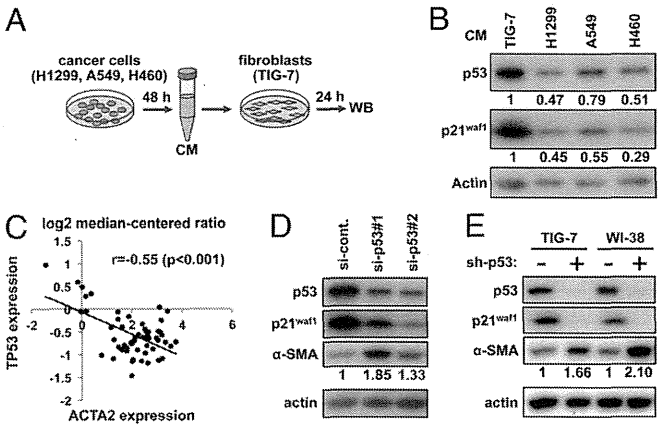

Fig. 1. Down-regulation of p53 in fibroblasts exhibited CAF-like properties. (*A*) Scheme of the experimental design to evaluate p53 expression in TIG-7 cells treated with conditioned media collected from cancer cells. (*B*) p53 expression in TIG-7 cells was down-regulated by conditioned media from cancer cells. TIG-7 cells were treated with conditioned media for 24 h, and expression levels of the indicated proteins in these cell lysates were determined by immunoblotting and quantified using ImageJ version 1.47c software. (*C*) The expression level of α-SMA (ACTA2) negatively correlated with that of p53 (TP53). Expression data were obtained from the Oncomine dataset and plotted to calculate Pearson's product-moment correlation coefficient. $r = -0.55$, $P < 0.001$. (*D* and *E*) The expression level of α-SMA in fibroblasts was up-regulated by p53 knockdown. (*D*) TIG-7 cells were transfected with the indicated siRNAs and, 72 h after transfection, the expression levels of the indicated proteins in these cell lysates were determined by immunoblotting. (*E*) Fibroblasts were infected with either control lentiviruses or lentiviruses for the expression of shRNA against p53. Expression levels of the indicated proteins in these cell lysates were determined by immunoblotting.

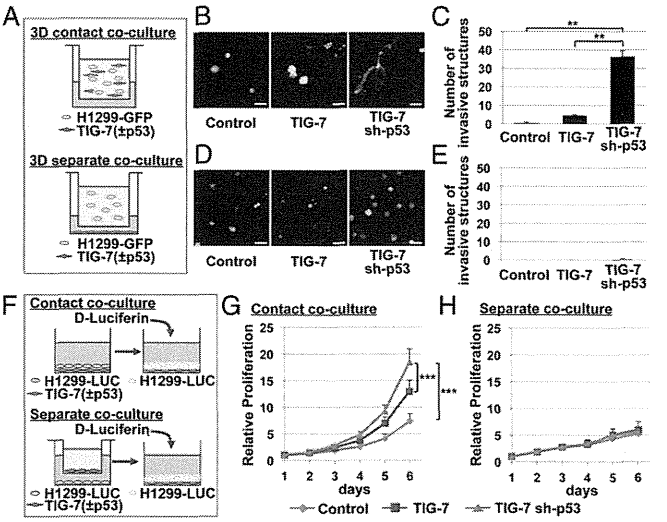

Fig. 2. p53-depleted fibroblasts enhanced invasion and proliferation of cancer cells through direct cell-to-cell contact. (*A*) Scheme of the contact (*Top*) and noncontact (*Bottom*) coculture system using a three-dimensional invasion assay. (*B* and *C*) p53-depleted TIG-7 cells enhanced invasiveness in GFP-labeled H1299 (H1299-GFP) cells using a contact coculture system. H1299-GFP cells were cocultured with parental TIG-7 cells or p53-depleted TIG-7 cells, or cultured alone in Matrigel. After 4–5 d, H1299-GFP cells were observed under a confocal microscope. (Scale bar, 100 μm.) (*B*). Quantification of invasive phenotypic cells in H1299-GFP cells (*C*). (*D* and *E*) p53-depleted TIG-7 cells did not enhance invasiveness in H1299-GFP cells in a noncontact coculture system. H1299-GFP cells were cultured in Matrigel in a Transwell insert, and parental TIG-7 cells or p53-depleted TIG-7 cells were cultured in the bottom well. After 4 or 5 d, H1299-GFP cells were observed under a confocal microscope (*D*). (Scale bar, 100 μm.). Quantification of invasive phenotypic cells in H1299-GFP cells (*E*). (*F*) Coculture systems for cell proliferation assay. Scheme of the contact coculture system using a cell proliferation assay (*Top*). Scheme of the noncontact coculture system using a Transwell insert for cell proliferation assay (*Bottom*). (*G*) p53-depleted TIG-7 cells enhanced proliferation in H1299 cells expressing luciferase (H1299-LUC cells) in a contact coculture system. H1299-LUC cells were cocultured with parental TIG-7 cells or p53-depleted TIG-7 cells, or cultured alone. Luciferase activity was measured every day up to day 6. (*H*) p53-depleted TIG-7 cells did not enhance cell proliferation in H1299-LUC cells using a noncontact coculture system. H1299-LUC cells were cultured in the bottom well, and parental or p53-depleted fibroblasts were cultured in a Transwell insert. Luciferase activity was measured every day until day 6. Data are the mean ± SD of three or more independent experiments. Statistical analyses were performed using the Student *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$.

Cancer Cell Invasion and Proliferation Were Enhanced Through Contact Between Cancer Cells and Fibroblasts. Although the contribution of p53 in fibroblasts to cancer progression has been implied, the underlying molecular mechanism remains unclear. To elucidate the mechanism by which stromal p53 regulates the invasiveness and proliferation of cancer cells, we developed three-dimensional coculture assays using Matrigel. Green fluorescent protein (GFP)-labeled H1299 (H1299-GFP) cells were

mixed with fibroblasts and cocultured in Matrigel, which enabled H1299-GFP cells to directly contact fibroblasts (Fig. 2*A*, *Top*). H1299-GFP cells were then cultured in Matrigel separately from fibroblasts, which enabled them to communicate only through secretions from fibroblasts (Fig. 2*A*, *Bottom*). With direct contact, coculturing H1299-GFP cells with p53-depleted fibroblast TIG-7 cells markedly enhanced the invasiveness of H1299-GFP cells over that of H1299-GFP cells with normal TIG-7 cells (Fig. 2 *B* and *C*). With separation, little or no enhancement was observed in invasiveness in H1299-GFP cells by culturing with fibroblasts, regardless of p53 expression in fibroblasts (Fig. 2 *D* and *E*). Increases in cancer cell numbers also had a negligible effect on cancer invasiveness (Fig. S2 *A–C*), indicating that enhancements in invasiveness were not due to increases in cancer cell numbers in the top chamber. The migration assay showed that a contact coculture with p53-depleted fibroblasts moderately enhanced cancer cell migration over that with controls (Fig. S2 *D–F*), and no significant difference was noted in cell motility under separate coculture conditions (Fig. S2 *G–I*). These results implied that direct contact between cancer cells and stromal fibroblasts was important for cancer cell invasion elicited by stromal fibroblasts when p53 expression levels decreased. We established a coculture system using H1299 cells expressing firefly luciferase (H1299-LUC cells) to assess the effects of fibroblasts on the proliferation of cancer cells. In the direct contact coculture assay, H1299-LUC cells were seeded on TIG-7 monolayer cells and luciferase activity was measured as an indicator of cell proliferation (Fig. 2*F*, *Top*). The rate of proliferation of H1299-LUC cells was greater in a coculture with p53-depleted TIG-7 cells than with H1299-LUC cells alone or with parental TIG-7 cells (Fig. 2*G*). We examined two different approaches as noncontact coculture assays using cell culture inserts to separate each cell (Fig. 2*F*, *Bottom*) and conditioned media from TIG-7 cells (Fig. S3*A*). No significant differences were observed in the rate of proliferation of H1299-LUC cells when cultured with p53-depleted TIG-7 cells or with parental fibroblasts (Fig. 2*H*). The treatment of H1299-LUC cells with conditioned media from p53-depleted TIG-7 cells resulted in a similar rate of cell proliferation to that of H1299-LUC cells with parental TIG-7 cells (Fig. S3*B*). These results suggested that cancer-fibroblast cell contact was required to increase the rate of cancer cell proliferation by coculturing with p53-depleted fibroblasts.

TSPAN12 Was a Derepressive Gene with p53 Ablation. To elucidate the mechanisms responsible for cancer cell invasion and proliferation due to the down-regulation of p53 in fibroblasts, we performed a comprehensive analysis using mRNAs from p53-depleted fibroblasts and control fibroblasts. Microarray experiments showed that 51 genes were up-regulated (fold change, >3) and 9 genes were down-regulated (fold change, <0.33) in p53-depleted fibroblasts (Fig. S4*A*). Of the up-regulated genes, we extracted those that encoded cell-surface proteins because antibodies against such proteins were expected to be candidates for cancer therapy (Fig. S4*B* and Table S1). We also selected and focused on the TSPAN12 gene encoding the tetraspanin family protein that contributes to cancer progression as a less characterized gene from those encoding cell-surface proteins because the induction of TSPAN12 expression in TIG-7 fibroblasts by p53 knockdown was highly reproducible and confirmed that the expression level of TSPAN12 was derepressed in p53-depleted TIG-7 fibroblasts using qRT-PCR and immunoblotting (Fig. 3 *A* and *B*). TSPAN12 was also derepressed by transient p53 knockdown, suggesting that p53 directly regulated TSPAN12 expression (Fig. S4*C*). p53 knockdown in WI-38 fibroblasts also derepressed TSPAN12 expression (Fig. 3 *A* and *B*). We subsequently analyzed TSPAN12 expression in cancer-associated stromal tissues from cancer patients using the Oncomine database (30) and found that TSPAN12 expression was higher in cancer-associated stromal tissues than in stromal tissues from noncancerous regions (Fig. S4 *D* and *E*).

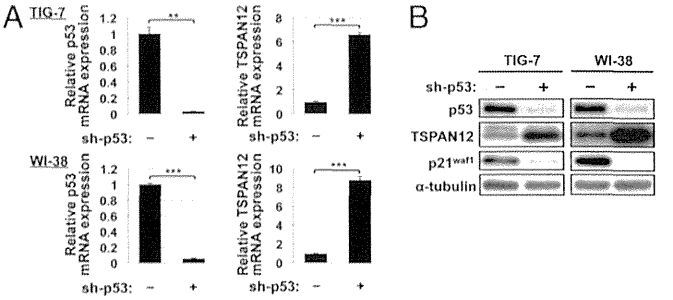

Fig. 3. TSPAN12 was derepressed by p53 knockdown. (*A* and *B*) p53 knockdown derepressed TSPAN12 expression. Total RNAs were prepared from the indicated fibroblasts and expression levels of the indicated genes were subjected to qRT-PCR (*A*). Cell lysates were prepared from the indicated fibroblasts and expression levels of indicated genes were determined by immunoblotting (*B*). Data are the mean ± SD of three or more independent experiments. Statistical analyses were performed using the Student *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$.

Cancer Cell Invasiveness and Proliferation Elicited by p53-Depleted Fibroblasts Were Inhibited by TSPAN12 Knockdown in p53-Depleted Fibroblasts. We determined whether TSPAN12 expression in fibroblasts enhanced cancer cell invasiveness and proliferation. The expression of p53 and TSPAN12 in p53-depleted TIG-7 cells transfected with control siRNAs or siRNAs targeted against TSPAN12 was confirmed by qRT-PCR (Fig. S5*A*) and immunoblotting (Fig. S5*B*), and TIG-7 cells transfected with these siRNAs were cocultured with H1299-GFP cells in Matrigel (Fig. 4*A*). An invasion assay using Matrigel showed that cancer invasiveness elicited by p53-depleted fibroblasts was inhibited by TSPAN12 knockdown in p53-depleted fibroblasts (Fig. 4*B*). These results suggested that the derepression of TSPAN12 by p53 knockdown in fibroblasts was a critical step for enhancing cancer invasiveness. We also investigated whether TSPAN12 knockdown in p53-depleted fibroblasts affected the proliferation of cancer cells using a cell-to-cell contact coculture proliferation assay (Fig. 4*C*). The transfection of si-TSPAN12 in p53-depleted TIG-7 cells decreased the proliferation rate of H1299-LUC cells to that when cocultured with parental TIG-7 cells (Fig. 4*D*). However, the effects of TSPAN12 knockdown in p53-depleted fibroblasts on cancer cell migratory activity were weak even though cancer cell migration was moderately inhibited (Fig. S5*C*). TSPAN12 knockdown in normal TIG-7 cells did not affect basal levels of cancer cell invasiveness and proliferation (Fig. S6 *A–C*). In contrast, the ectopic expression of TSPAN12 in normal TIG-7 cells increased cancer cell invasiveness and proliferation (Fig. S6 *D–F*), suggesting that derepressed TSPAN12 was crucial for enhancing the invasiveness elicited by the down-regulation of p53. We then examined the effects of the extracellular loop of TSPAN12 on invasiveness in p53-depleted cells. The large extracellular loop (LEL) of TSPAN12 could inhibit invasiveness up to basal levels (Fig. S6 *G–I*), implying that TSPAN12-LEL may compete with some factors transducing signals for invasion into cells.

Derepression of TSPAN12 in p53-Depleted Fibroblasts Accelerated Tumor Progression. We tested the effects of stroma-derived p53 on tumor growth in vivo. H1299-LUC cells were mixed with TIG-7 cells in Matrigel, inoculated s.c. into the backs of balb/c-nu/nu mice, and tumor growth was measured using an IVIS bioluminescence imaging system (Fig. 5*A*). TIG-7 cells stably expressed shRNAs targeting p53 and TSAPN12, and reductions in the expression levels of these proteins were confirmed by

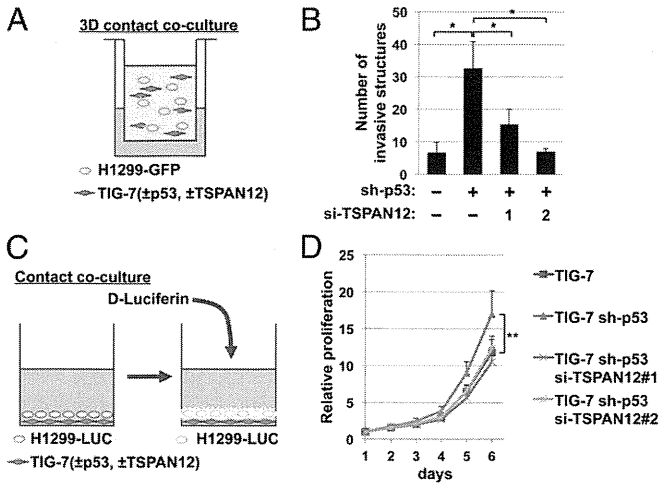

Fig. 4. TSPAN12 regulated cancer cell invasiveness and proliferation enhanced by p53-depleted fibroblasts. (*A*) Scheme of the contact coculture system using a three-dimensional invasion assay. (*B*) TSPAN12 knockdown in p53-depleted TIG-7 cells inhibited invasiveness in H1299-GFP cells. H1299-GFP cells were cocultured with either parental TIG-7 cells, p53-depleted TIG-7 cells, or TIG-7 cells depleted of both p53 and TSPAN12 in Matrigel. After 4 or 5 d, invaded H1299-GFP cells were observed and quantified. (*C*) Scheme of the contact coculture system for a cell proliferation assay. (*D*) TSPAN12 knockdown in p53-depleted TIG-7 cells inhibited proliferation in H1299-LUC cells. H1299-LUC cells were cocultured with either parental TIG-7 cells, p53-depleted TIG-7 cells, or TIG-7 cells depleted of both p53 and TSPAN12. Luciferase activity was measured every day until day 6. Data are the mean ± SD of three or more independent experiments. Statistical analyses were performed using the Student *t* test. $*P < 0.05$, $**P < 0.01$.

Western blotting (Fig. 5*B*). Using these cells, we determined whether p53-depleted fibroblasts enhanced tumor growth in mice. H1299-LUC cells mixed with parental TIG-7 cells were injected into the left back, and H1299-LUC cells mixed with p53-depleted TIG-7 cells were injected into the right back. Tumor growth was greater by H1299-LUC cells with p53-depleted TIG-7 cells than by H1299-LUC cells with parental TIG-7 cells (Fig. 5*C* and Fig. S7*A*). We then examined whether TSPAN12 derepression was required for enhanced tumor growth. TSPAN12 knockdown in p53-depleted fibroblasts suppressed tumor growth in H1299-LUC cells (Fig. 5*D* and Fig. S7*B*). These results demonstrated that stroma-derived TSPAN12 was a critical factor for enhancing tumor growth by p53-depleted fibroblasts.

TSPAN12 in Fibroblasts Promoted CXCL6 Secretion Through the β-Catenin Signaling Pathway to Increase Cancer Cell Invasion. TSPAN12 regulates the Norrin/β-catenin signaling pathway by binding to Frizzled-4, a WNT/Norrin receptor. Therefore, we evaluated the effects of β-catenin knockdown in fibroblasts on cancer cell invasiveness. The knockdown efficiency of siRNAs targeting β-catenin (si–β-catenin) was confirmed by qRT-PCR (Fig. S8*A*) and immunoblotting (Fig. S8*B*). Coculturing H1299-GFP cells with p53-depleted TIG-7 cells transfected with si–β-catenin in Matrigel had less ability to elicit the invasion of H1299-GFP cells than control p53-depleted TIG-7 cells (Fig. 6*A*). To further elucidate the mechanism by which TSPAN12 in fibroblasts enhanced cell invasion, we extracted genes regulated by both p53 and TSPAN12, which may function in cancer cell proliferation, invasion, and metastasis, using the microarray dataset in Fig. S4*A*. The expression levels of these genes were analyzed by semi-quantitative RT-PCR. CXCL6 expression was down-regulated by TSPAN12 knockdown (Fig. S8*C*) and was confirmed by qRT-PCR (Fig. 6*B*). The production of CXCL6 secreted from fibroblasts was suppressed by TSPAN12 knockdown, as determined by ELISA (Fig. 6*C*). The knockdown of β-catenin also decreased the expression (Fig. 6*D*) and secretion of CXCL6 (Fig. 6*E*). We next examined whether CXCL6 produced from fibroblasts influenced cancer cell invasiveness. The decreased expression of CXCL6 in TIG-7 cells by si-RNAs was confirmed by qRT-PCR (Fig. S8*D*) and ELISA (Fig. S8*E*), and siRNA-treated cells were cocultured with H1299-GFP cells in Matrigel. Similar to the knockdown of TSPAN12 and β-catenin, that of CXCL6 in p53-depleted TIG-7 cells inhibited the invasiveness of H1299-GFP cells (Fig. 6*F*). The treatment of TIG-7 cells with nutlin-3, a p53 activator, decreased the expression of TSPAN12 (Fig. S8*F*) and CXCL6 (Fig. S8*G*). Although CXCL6 expression was moderately up-regulated by ectopic TSPAN12 expression (Fig. S8 *H* and *I*), its level did not reach that achieved by p53 knockdown, suggesting that further factors are required for the complete up-regulation of CXCL6. Neutralizing antibodies against CXCL6 also inhibited H1299-GFP invasiveness enhanced by coculturing with p53-depleted TIG-7 cells (Fig. 6*G*). The microarray dataset from the Oncomine database (30) revealed that the expression levels of β-catenin (Fig. S9 *A* and *B*) and CXCL6 (Fig. S9 *C* and *D*) were significantly higher in cancer-associated stromal cells than in normal stromal cells. These results suggested that TSPAN12 was required to increase cancer cell invasiveness caused by fibroblasts and orchestrated the transduction of not only cell-to-cell contact-dependent signaling, but also paracrine signaling.

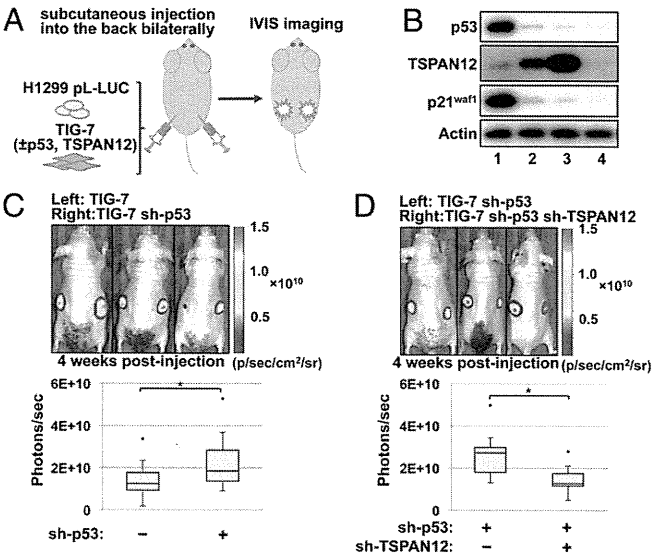

Fig. 5. Knockdown of TSPAN12 derepressed in p53-depleted fibroblasts suppressed cancer cell growth enhanced by coculturing with p53-depleted fibroblasts. (*A*) Scheme of the experimental design to evaluate cancer cell growth coinjected with fibroblasts. H1299-LUC cells mixed with parental TIG-7 cells, p53-depleted TIG-7 cells, or TIG-7 cells depleted of both p53 and TSPAN12 were s.c. injected into the backs of BALB/c-nu/nu mice. Cancer cell growth was quantified using the IVIS imaging system. (*B*) Efficiency of p53 and TSPAN12 knockdown in TIG-7 cells. TIG-7 cells were infected with the indicated viruses and the expression levels of proteins were determined by immunoblotting. Lane 1, parental TIG-7 cells; lane 2, TIG-7 cells expressing sh-p53; lane 3, control of TIG-7 cells expressing sh-p53; lane 4, TIG-7 cells expressing sh-p53 and sh-TSPAN12. (*C*) p53-depleted TIG-7 cells promoted cancer cell growth. Four weeks after the inoculation, cancer cell growth was measured using the IVIS imaging system. (*Left* back) Coinjection with parental TIG-7 cells. (*Right* back) Coinjection with p53-depleted TIG-7 cells ($n = 8$ per group, paired *t* test $*P < 0.05$). (*D*) The depletion of both p53 and TSPAN12 in TIG-7 cells inhibited cancer cell growth increased by p53 depletion in TIG-7 cells. Four weeks after the inoculation, cancer cell growth was measured using the IVIS imaging system. (*Left* back) Coinjection with p53-depleted TIG-7 cells. (*Right* back) Coinjection with TIG-7 cells depleted of both p53 and TSPAN12 ($n = 9$ per group, paired *t* test $*P < 0.05$).

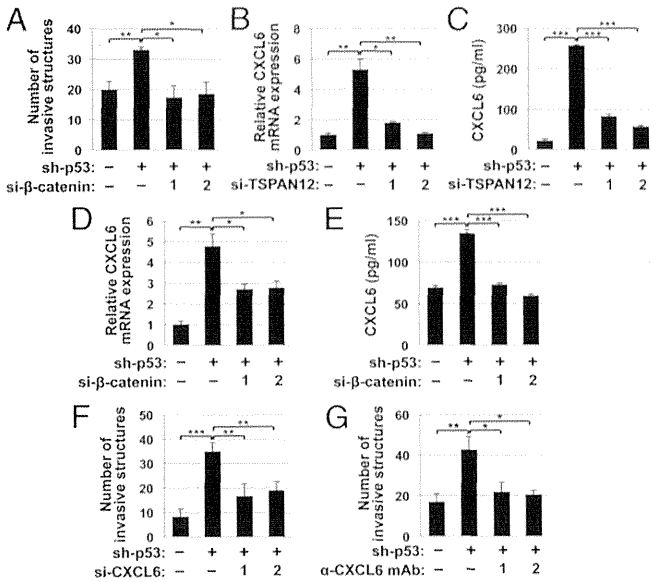

Fig. 6. TSPAN12 promoted CXCL6 expression through the β-catenin signaling pathway. (*A*) β-Catenin knockdown in p53-depleted TIG-7 cells inhibited invasiveness in H1299-GFP cells. H1299-GFP cells were cocultured with parental TIG-7 cells, p53-depleted TIG-7 cells, or TIG-7 cells depleted of both p53 and β-catenin in Matrigel. After 4 or 5 d, invaded H1299-GFP cells were observed under a fluorescent microscope and quantified by counting GFP-positive cells. (*B*–*E*) TSPAN12 knockdown decreased CXCL6 expression through the β-catenin–mediated pathway. CXCL6 expression was decreased by the knockdown of TSPAN12 and β-catenin. p53-depleted TIG-7 cells were transfected with control siRNAs, si-TSPAN12, or si–β-catenin. CXCL6 expression in cells depleted of TSPAN12 (*B*) or β-catenin (*D*) was determined by qRT-PCR. The production of CXCL6 secreted from cells depleted of TSPAN12 (*C*) or β-catenin (*E*) was quantified by ELISA. (*F*) CXCL6 knockdown in p53-depleted TIG-7 cells canceled fibroblast-elicited invasiveness in H1299-GFP cells. H1299-GFP cells were cocultured with parental TIG-7 cells, p53-depleted TIG-7 cells, or TIG-7 cells depleted of both p53 and CXCL6 in Matrigel. After 4 or 5 d, invaded H1299-GFP cells were observed under a fluorescent microscope and quantified by counting GFP-positive cells. (*G*) Neutralizing antibodies against CXCL6 inhibited invasiveness in H1299-GFP cells. H1299-GFP cells were cocultured with either parental TIG-7 cells or p53-depleted TIG-7 cells, and control IgG or an anti-CXCL6 antibody was added. Four to 5 d after the treatment with these antibodies, invaded H1299-GFP cells were observed under a fluorescent microscope and quantified by counting GFP-positive cells. Data are the mean ± SD of three or more independent experiments. Statistical analyses were performed using the Student *t* test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Discussion

Fibroblasts are the principal components of connective tissue and function to maintain the homeostasis of ECM and adjacent epithelia (5). CAFs include several mesenchymal cells, including myofibroblast-like cells and normal fibroblasts altered by factors secreted from cancer cells (5, 6). Previous studies reported that mutations in the p53 gene and decreased p53 expression in CAFs, implying functional defects in p53, contributed to cancer progression (14–18). We herein found that culturing fibroblasts with conditioned medium derived from cancer cells suppressed p53 expression in fibroblasts, consistent with the previous finding that epithelial cancer cells suppressed the induction of p53 in neighboring fibroblasts (18). Communication between cancer and stromal cells may be mediated by secreted proteins, including growth factors and cytokines (1–3). However, the mechanism by which p53 expression in stromal cells is regulated by proteins secreted from cancer cells currently remains unknown. One possibility is that TGF-β contributes to the down-regulation of p53 because it activates normal fibroblasts to support cancer and repress p53 expression through the induction of MDM2 (31, 32). Alternatively, cancer-derived exosomes may also be involved in down-regulating p53 expression in stromal cells because cancer cells release exosomes expressing specific proteins and RNAs to influence the expression of various proteins (33, 34). We here demonstrate that α-SMA expression was derepressed by the down-regulation of p53 and negatively correlated with p53 expression levels in stromal tissues from cancer patients. α-SMA is a well-known marker of CAFs (6) and our results suggest that the down-regulation of p53 is, at least in part, involved in the acquisition of a CAF-like phenotype. Genetic studies reported various genetic alterations, including LOH and mutations, in CAFs (2), and our results supported not only p53 mutations and LOH, but also alterations in p53 expression levels contributing to the transition of fibroblasts possessing CAF-like properties from normal fibroblasts.

We focused on the mechanism by which stromal fibroblasts enhanced cancer progression and found that p53-depleted fibroblasts possessing CAF-like properties enhanced cancer cell proliferation and invasion more efficiently than normal fibroblasts. Furthermore, TSPAN12 was identified as a critical factor derepressed by the down-regulation of p53, and TSPAN12 in fibroblasts promoted cancer cell proliferation and invasion through direct cancer-to-stromal cell contact. It still remains unclear how TSPAN12 in fibroblasts promotes cancer cell invasion and proliferation; however, it may bind to other membrane proteins in the transmembrane of neighboring cancer cells and activate a signaling cascade in both fibroblasts and cancer cells because tetraspanin family proteins function as scaffold factors to assemble cell-surface proteins transducing various signals. Although it was not straightforward to elucidate this mechanism, recent studies found that TSPAN12 functioned in the regulation of the Norrin/β-catenin signaling pathway (26). TSPAN12 in fibroblasts regulated CXCL6 expression through the β-catenin–mediated pathway. Therefore, we speculated that TSPAN12 may activate the β-catenin signaling pathway upon binding to a certain membrane protein on cancer cells to promote CXCL6 expression, although certain factors, including SDF-1 (CXCL12), a key tumorigenic factor secreted from p53-depleted

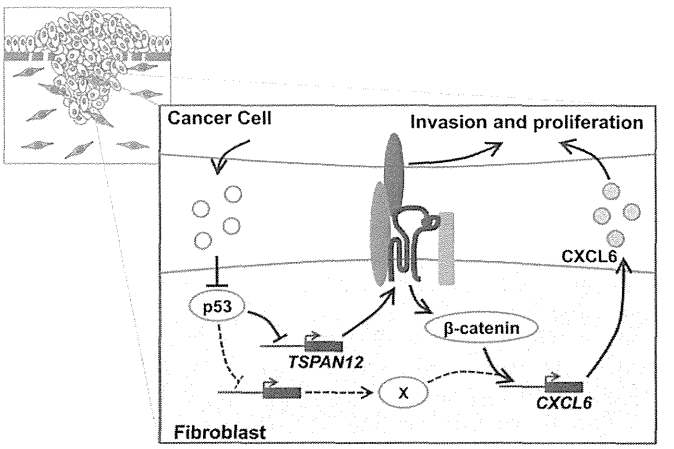

Fig. 7. A model of enhanced cancer invasiveness and proliferation elicited by adjacent fibroblasts. Cancer cells produce various secreted factors, including growth factors, cytokines, and chemokines, and these soluble factors presumably suppress p53 expression in fibroblasts, leading to the up-regulated expression of α-SMA, a marker of CAF-like characteristics. The down-regulation of p53 in fibroblasts derepresses TSPAN12 expression, and TSPAN12 is required to enhance cancer invasiveness and proliferation elicited by p53 down-regulated fibroblasts through contact between cancer cells and fibroblasts. The up-regulated expression of TSPAN12 promotes CXCL6 expression through the β-catenin–mediated pathway, which leads to enhanced cancer progression.

fibroblasts (13, 35), and transmembrane proteins that interact with cancer cells, may also contribute to fibroblast-elicited cancer progression (Fig. 7). We cannot exclude the possibility that TSPAN12 in fibroblasts may enhance the creation of tunnels by destroying ECM, enabling cancer cells to follow through their contact with fibroblasts (36). Further investigations of these issues will be required to elucidate the role of TSPAN12 in cancer progression.

Genes that are derepressed by the down-regulation of p53, such as MDR1 (37), CD44 (38), TCTP (39), and TSPAN2 (40), have been identified, and we found that TSPAN12 was derepressed by the down-regulation of p53 in fibroblasts. The mechanism underlying the transcriptional derepression of TSPAN12 through the down-regulation of p53 is complex, and even though a reporter assay using the TSPAN12 promoter region (−1,000 to −1) and ChIP analysis of the proximal TSPAN12 promoter region (−230 to −1) using various specific primers were conducted, we could not confirm that TSPAN12 was a direct p53-target gene. One reason is that p53 may block the recruitment of some coactivators to the TSPAN12 promoter by binding p53 to these coactivators (41). Alternatively, TSPAN12 expression may be regulated by p53 through the inhibition of p53-distal enhancer activity because p53 suppresses the expression of many genes in embryonic stem (ES) cells and influences the *cis* element far from promoter regions (42). However, further investigations will be required to elucidate how p53 regulates TSPAN12 expression. Cancer-associated stromal cells were recently recognized as an effective target for cancer therapy (43–45), and the recombinant soluble extracellular region of TSPAN12, antibodies against TSPAN12 and CXCL6, may become effective therapeutic agents.

Materials and Methods

Cell cultures and the immunoblotting analysis were performed as described in Endo et al. (46). *SI Materials and Methods* include detailed additional information on cell cultures, viral infection, plasmid construction, antibodies, siRNA transfection, and immunoblot analysis, as well as descriptions on quantitative and semiquantitative RT-PCR, the ELISA, invasion assay, proliferation assay, Transwell migration assay in coculture, microarray analysis, production and purification of large extracellular loops of TSPAN12, tumor growth assay, and statistical analysis.

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