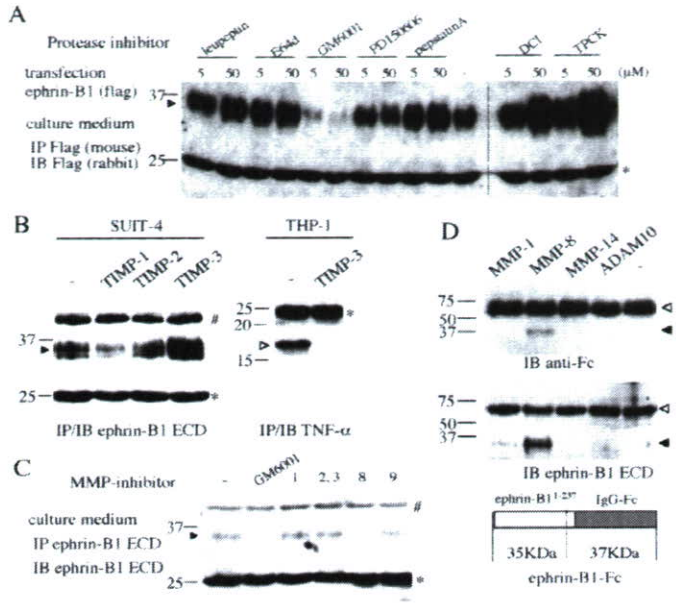

Fig. 3. Screening of a protease that cleaves the extracellular domain of ephrin-B1. (A) Panc-1 cells transiently expressing Flag-tagged ephrin-B1 were incubated with various protease inhibitors for 4 hours in medium containing 0.5% FBS: leupeptin and E64d (Loxistatin), a cystein protease inhibitor; GM6001, a pan-MMP inhibitor; PD150606, a calpain inhibitor; pepstatin A, an aspartic protease inhibitor; DCI (3,4-dichloroisocoumarin), a serine protease inhibitor; TPCK (N^{α}-tosyl-phe chloromethyl ketone), a chymotrypsin inhibitor. The cleavage of ephrin-B1 ectodomain was examined as described in Fig. 2A. (B) Left: SUIT-4 cells were incubated with EphB2-Fc (2 μg/ml) together with or without TIMPs (100 nM for each) as indicated for 2 hours. The processed ephrin-B1 fragment was detected in the culture medium. Right: THP-1 cells were treated with PMA (10 ng/ml) together with or without TIMP-3 (100 nM) for 6 hours. Open arrowhead indicates the processed fragment of TNF-α in the medium detected by immunoprecipitation with anti TNF-α antibody. (C) SUIT-4 cells were treated with various MMP inhibitors as indicated (1 μM for MMP-8 inhibitor and 5 μM for others) for 4 hours. Ephrin-B1 fragment was detected in the medium. (D) Purified ephrin-B1-Fc protein was incubated with activated MMP in vitro for 1 hour at 37°C, separated by SDS-PAGE, and immunoblotted with anti-Fc mouse IgG or anti-ephrin-B1. Bottom: a schematic representation of ephrin-B1-Fc with the MMP-8 cleavage site indicated by a dotted line. Open and filled arrowheads indicate uncleaved ephrin-B1-Fc and the processed fragments, respectively.

MMP-8 was reduced in SUIT-4 cells by treatment of cells with siRNA, the amount of processed ephrin-B1 ectodomain in the culture medium was decreased (Fig. 4B). By contrast, overexpression of activated MMP-8 cDNA, in which the propeptide domain was removed, in SUIT-4 cells evidently increased the cleavage of ephrin-B1 (Fig. 4C).

As some MMPs and their substrates physically associate directly or indirectly and make a protein complex, interaction between MMP-8 and ephrin-B1 was examined (Sawicki et al., 2005; Yu et al., 2007). Ephrin-B1 was coprecipitated with MMP-8 from extracts of L ephrin-B1 cells by MMP-8-specific antibodies, but not by control normal rabbit IgG1, indicating that MMP-8 forms a complex with ephrin-B1 (Fig. 4D). However, association of MMP-8 with the receptor EphB2 was not detected (data not shown). These results also suggest that MMP-8 is the key metalloproteinase that cleaves the ephrin-B1 ectodomain.

Stimulation of ephrin-B1 activates secretion of MMP-8, which is regulated by the C-terminus of ephrin-B1

To understand the mechanism of activation of ephrin-B1 cleavage by EphB2, we next examined whether it is accompanied by an increase in MMP-8 expression. The level of intracellular expression of MMP-8 mRNA was not affected by treatment with EphB2-Fc or contact of ephrin-B1-expressing cells with EphB2-expressing cells for 4 hours or longer (Fig. 5A). Moreover, the amount of processed ephrin-B1 ectodomain produced in the medium was not altered by the addition of cyclohexamide or actinomysin D, inhibitors of de novo synthesis of mRNA and proteins, respectively (Fig. 5B). These results indicate that activation of ephrin-B1 cleavage by EphB2 does not depend on the increased amount of MMP-8. In addition, when the de novo synthesis of MMP-8 was blocked, the amount of intracellular MMP-8 protein decreased slightly after 4 hours or longer treatment of ephrin-B1-expressing cells with EphB2-Fc (Fig. 5C), which suggests that stimulation of ephrin-B1 activates extracellular release of MMP-8 protein from the cytoplasm. Actually, the amount of MMP-8 protein in the culture medium was remarkably elevated after incubation of the cells with EphB2-Fc as detected by immunoprecipitation (Fig. 5D).

In order to show directly that stimulation of ephrin-B1 increased exocytosis of MMP-8, which was already synthesized and present in the cytoplasm, SUIT-4 cells were pulse-labeled with [^{35}S]methionine. When pulse-labeled cells were treated with EphB2-Fc, a higher amount of labeled MMP-8 protein was detected in the medium compared to treatment of cells with Fc (Fig. 5E, left). However, secretion of labeled MMP-7, which was examined as a control, was not altered by EphB2-Fc treatment (Fig. 5E, right). In addition, the secretion of [^{35}S]methionine-labeled MMP-8 was also increased by EphB2-Fc treatment of Capan-1 cells expressing wild-type ephrin-B1, but it was not found in the media of cells expressing ephrin-B1 lacking the C-terminus (ΔC4 and ΔC19) (Fig. 5F, left). Consistently, the total amount of MMP-8 protein in the culture medium from cells expressing wild-type ephrin-B1 was higher than in the medium from ΔC4 ephrin-B1-expressing cells (Fig. 5F, right). These results suggest that stimulation of ephrin-B1 by EphB2 upregulates the process of MMP-8 exocytosis, and the C-terminus of ephrin-B1 regulates this event.

Stimulation of ephrin-B1 by EphB2 induces activation of Arf1 GTPase

To further confirm that activation of MMP-8 secretion is involved in the elevated ephrin-B1 cleavage in response to stimulation with EphB2, the cells were treated with brefeldin A, an inhibitor of membrane trafficking through the Golgi, which blocks the secretion of proteins (Tamaki and Yamashina, 2002). EphB2-stimulated cleavage of ephrin-B1 was apparently reduced by brefeldin A treatment (Fig. 6A). As a mode of action of brefeldin A is to inhibit activation of ADP ribosylation factor 1 (Arf1), a ras family GTPase, by blocking of the exchange reaction from Arf1-GDP to Arf1-GTP (Niu et al., 2005; Zeeh et al., 2006), we further examined the activity of Arf1 in ephrin-

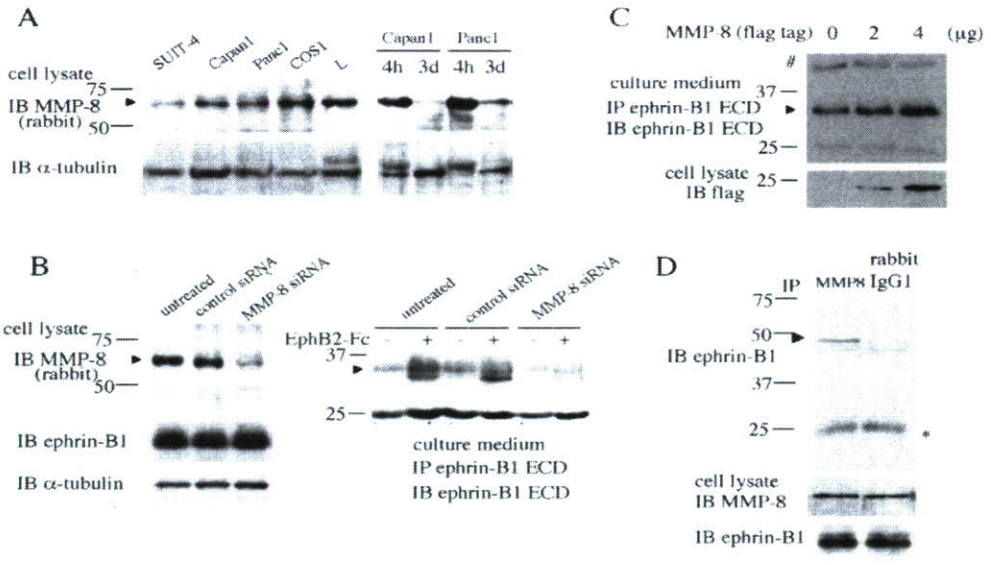

Fig. 4. MMP-8 is the key protease of ephrin-B1 cleavage. (A) Expression of MMP-8 in cell lysates. Left: The indicated cells were seeded on plates not to reach confluence. Cell lysates were prepared on the day after plating. Right: cell lysates were prepared 4 hours (4h) or 3 days (d3) after being plated on dishes. The cells were confluent on day 3 after plating. (B) SUIT-4 cells treated with either MMP-8 siRNA or control scrambled siRNA (control), or left untreated. The cells were detached 48 hours later, replated on new plates and further incubated for 24 hours. Left: Cellular levels of MMP-8 were analyzed 72 hours after treatment of SUIT-4 cells with siRNAs. Right: the culture medium was replaced with one fresh medium or medium containing EphB2-Fc (2 g/ml) and incubated for 2 hours to detect ephrin-B1 ectodomain in the medium. (C) SUIT-4 cells were transiently transfected with the indicated volume of a plasmid encoding Flag-tagged activated MMP-8 cDNA. After 48 hours of transfection, the medium was replaced and the cells were further incubated for 6 hours to detect processed ephrin-B1 ectodomain in the medium. The expression of transfected MMP-8 in each cell lysate was confirmed by immunoblotting with anti-Flag antibody (bottom). (D) The lysate of L ephrin-B1 cells was immunoprecipitated with anti-MMP-8 polyclonal antibody or control rabbit IgG1, and subjected to immunoblotting with anti-ephrin-B1 C18 antibody. HRP-conjugated anti-rabbit IgG (TrueBlot) was used as the secondary antibody for immunoblotting to avoid cross reaction with denatured rabbit IgG heavy chain of the antibody used for immunoprecipitation. The arrowhead indicates coprecipitated ephrin-B1. The asterisk indicates the IgG light chain.

B1-expressing cancer cells. To examine the amount of activated, GTP-bound Arf1, a pull-down approach was performed with an adaptor protein GGA3, which specifically binds to GTP-bound Arfs (Dell'Angelica et al., 2000). As a control experiment, we confirmed that constitutively activated Arf1 (Q71L), but not a dominant negative mutant of Arf1 (T31N) was coprecipitated with GST-tagged GGA3 (Fig. 6B, right panel). Incubation of SUIT-4 cells with EphB2-Fc increased the amount of Arf1-GTP coprecipitated with GST-tagged GGA3 (Fig. 6B). Similar results were also observed in PANC-1 cells expressing ephrin-B1, whereas treatment with EphB2-Fc did not affect activation of Arf1 in parent PANC-1 cells, which express ephrin-B1 in trace amounts (Fig. 6B). The amount of Arf1-GTP was also increased by EphB2-Fc treatment of Capan-1 cells expressing wild-type ephrin-B1 or ephrin-B1 4YF mutant, but not in cells expressing ΔC4 ephrin-B1 (Fig. 6C). These results suggest that stimulation of ephrin-B1 by EphB2 activates Arf1, and the signaling mediated by the C-terminus of ephrin-B1 is involved in this pathway. Furthermore, treatment of cells with brefeldin A, or overexpression of dominant negative mutant of Arf1, Arf1 T31N, decreased the secretion of MMP-8 in Capan-1 cells, indicating that Arf1 regulates the secretion of MMP-8 (Fig. 6D).

The C-terminus of ephrin-B1 is involved in the invasion by cancer cells

To investigate the biological implication of metalloproteinase activation caused by ephrin-B1-mediated signaling, we examined changes in the invasiveness of ephrin-B1-expressing cells with or without stimulation by EphB2 using an in vitro cell invasion assay. The invasion of collagen by Capan-1 cells was promoted by expression of ephrin-B1 and treatment of the cells with EphB2-Fc, and this invasion was inhibited by the addition of an MMP-8 inhibitor (Fig. 7A). On the other hand, the invasion by Capan-1 cells expressing ΔC4 ephrin-B1, or by parent Capan-1 cells was not significantly promoted by EphB2-Fc treatment (Fig. 7A).

We further examined whether expression of ephrin-B1 actually promotes cancer cell invasion in vivo using PANC-1 cells as a model system to study peritoneal dissemination. PANC-1 cells stably expressing wild-type ephrin-B1 (PANC-1 ephrin-B1) or ΔC4 ephrin-B1 (PANC-1 ΔC4) were established to compare their invasiveness with that of parent PANC-1 cells. Expression of wild-type or mutated ephrin-B1 did not affect BrdU incorporation into cells grown under normal two-dimensional cell culture conditions (data not shown). When these cells were injected intraperitoneally into nude mice, PANC-1 ephrin-B1 cells formed many tumor nodules in the mesenteric sheets and also in the peritoneal cavity, including the rectouterine region. By contrast, in mice injected with parent PANC-1 cells or PANC-1 ΔC4 cells, such tumors in mesentery sheets were fewer and smaller, and the total tumor volume involving the rectouterine region was much less (Fig. 7B,C, Table 1). The cells composing the mesenteric sheets express cognate receptors for ephrin-B1, EphB2 and

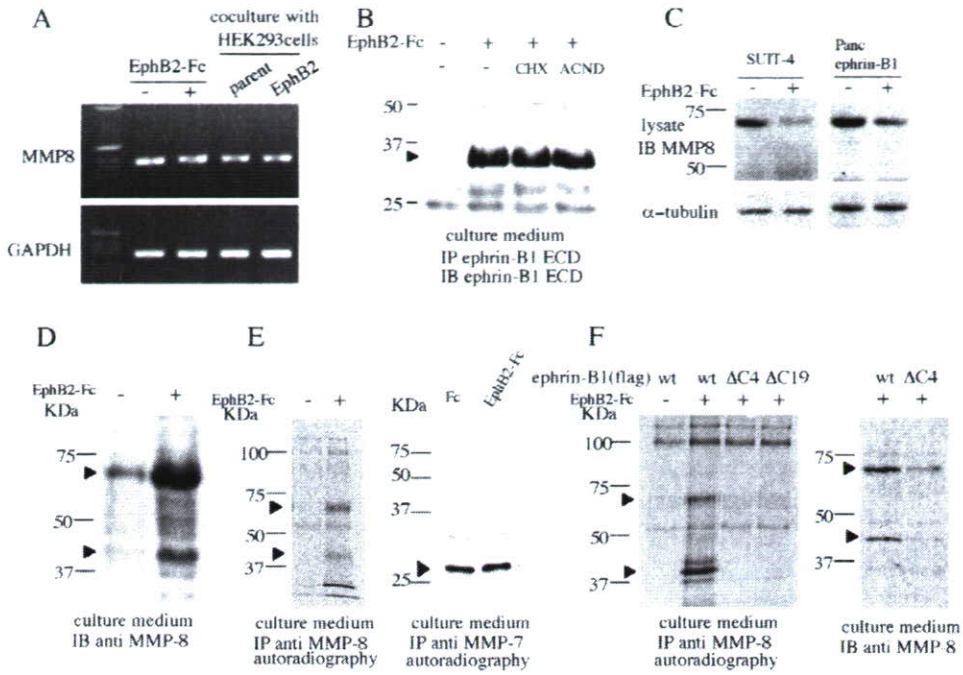

Fig. 5. Stimulation of ephrin-B1 with EphB2 increases MMP-8 secretion. (A) SUIT-4 cells were left untreated or were treated with EphB2-Fc or co-cultured with either parent or EphB2-expressing HEK293 cells for 4 hours. Cellular levels of MMP-8 were analyzed by RT-PCR using GAPDH as a control. (B) SUIT-4 cells were incubated with or without EphB2-Fc for 2 hours in the presence or absence of cyclohexamide (CHX, 100 μg/ml) or actinomycin D (ACND, 5 μg/ml) to detect the ephrin-B1 ectodomain in the medium. (C) SUIT-4 cells or PANC-1 cells stably expressing ephrin-B1 were treated with actinomycin D (5 μg/ml) together with or without EphB2-Fc (2 μg/ml) for 4 hours. Cell lysates were prepared and intracellular expression levels of MMP-8 were analyzed by western blotting using α-tubulin as a loading control. (D) Conditioned medium of SUIT-4 cells were collected after the cells were treated with EphB2-Fc or left untreated for 4 hours in serum-free medium. Proteins secreted in the medium were precipitated with trichloroacetic acid (10%), resuspended in sample buffer, and subjected to immunoblotting with anti MMP-8 polyclonal antibody. Arrowheads indicate MMP-8 protein (proenzyme and activated). (E) SUIT-4 cells were metabolically labeled with [^{35}S]methionine, then treated with EphB2-Fc or control Fc for 2 hours. The amount of labeled MMP-8 (left panel) or MMP-7 (right panel) in the medium was evaluated through immunoprecipitation from the conditioned medium followed by SDS-PAGE and autoradiography. Arrowhead indicates MMP-8 (proenzyme and activated; left) or MMP-7 (right) in the medium. (F) Wild-type and mutants of ephrin-B1 were expressed in Capan-1 cells as in Fig. 2A, and [^{35}S]methionine-labeled MMP-8 was detected in the medium (left panel). Right: The total amount of MMP-8 in the conditioned medium of Capan-1 cells was evaluated using the trichloroacetic acid (TCA) precipitation procedure, followed by immunoblotting with anti MMP-8 antibody as in D.

EphB4 (see Fig. S1 in supplementary material). These results indicate that ephrin-B1 actually promotes cancer cell invasion, which requires the C-terminus of ephrin-B1. EphB2 and EphB4 expressed in cells of the mesenteric sheet might act as interaction partners for ephrin-B1 present on PANC-1 cells.

Discussion

The interaction of Eph family receptor protein tyrosine kinases with their ligands, ephrin family proteins, induces bi-directional signaling. In this study, we showed for the first time that ephrin-B1 regulates the activation and release of a metalloproteinase. We observed that binding of EphB2 to ephrin-B1 promotes secretion of MMP-8 without increasing the expression level of MMP-8. Activation of several molecules, such as Erk, p38 and PI 3-kinase or Akt cause transcriptional activation of metalloproteinases (Chinni et al., 2006; Raymond et al., 2006; Reuben and Cheung, 2006). In our study, however, activation of ephrin-B1 by EphB2 binding did not alter the phosphorylation levels of Erk-1, 2, p38 or Akt (data not shown), as also reported by others (Huynh-Do et al., 2002). Our observation that ephrin-B1-induced secretion of MMP-8 was sensitive to brefeldin A, which blocks the membrane trafficking of coated vesicles at the Glogi/trans-Golgi network suggests that ephrin-B1 signaling resulted in increased transport of MMP-8 from the cytoplasm to the cell surface (Tamaki and Yamashina, 2002).

Regulation of the secretion of MMP-8 enables ephrin-B1 signaling to play an important role in regulating MMP-8 activity. Like other soluble MMPs, MMP-8 is proteolytically activated extracellularly by removal of its propeptide domain, and physiologically relevant level of MMP protease activity requires efficient release of the protease to the cellular surface (Sternlicht and Werb, 2001). Although the molecular mechanism of the MMP-8 secretory pathway is not well understood, our data indicate that signaling mediated by the carboxyl-terminal region of ephrin-B1 is involved. Notably, removal of the C-terminus of ephrin-B1 resulted in significant reduction of MMP-8 secretion and cleavage of the extracellular domain of ephrin-B1. In addition, the C-terminus of ephrin-B1 regulates the signal leading to activation of Arf1, a critical regulator of membrane traffic in the secretory pathway and one target of brefeldin A (Tamaki and Yamashina, 2002; Donaldson

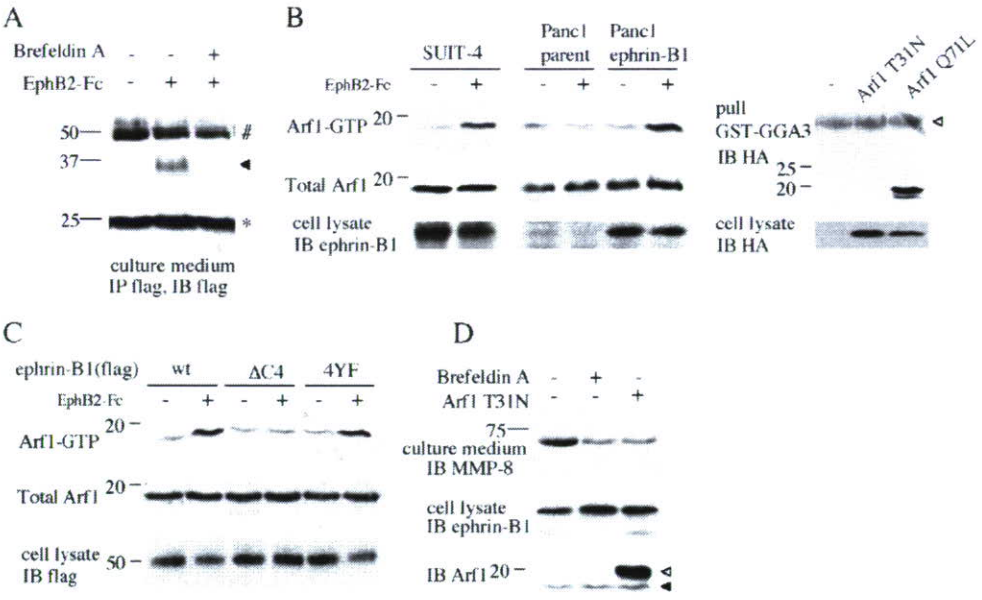

Fig. 6. Stimulation of ephrin-B1 with EphB2 activates Arf1. (A) Flag-tagged ephrin-B1 was expressed in Capan-1 cells. The cells were treated with or without EphB2-Fc (2 μg/ml) and brefeldin A (10 μg/ml) as indicated for 1.5 hours, and conditioned medium was assayed for the 35 kDa ectodomain of ephrin-B1. (B,C) The activity of Arf1 was analyzed in the indicated cells. (C) Wild-type or mutant ephrin-B1 was expressed in Capan-1 cells as in Fig. 2A. The cells were incubated with EphB2-Fc (4 μg/ml) for 20 minutes before being lysed. Arf1-GTP was pulled down with GST-GGA3 bound to glutathione-Sepharose. As controls, lysates of COS-1 cells transiently transfected with plasmids encoding Arf1 T31N or Arf1 Q71L, HA tagged at the C-terminus were analyzed (B, right panel). Open arrowhead indicates cross-reacted GST-GGA3 used for the pull-down assay. (D) Suppression of Arf1 activation decreased the MMP-8 secretion. Capan-1 cells stably expressing ephrin-B1 were used. In the right lane, Arf1 T31N was also transiently expressed in the cells by retrovirus mediated gene transfer. All cells were treated with EphB2-Fc together with (middle lane) or without brefeldin A for 4 hours, and the conditioned medium was subjected to TCA precipitation to detect MMP-8 through immunoblotting. The filled arrowhead indicates endogenous Arf1, and the open arrowhead indicates HA-tagged Arf1 T31N (bottom).

et al., 2005). Arf1 GTPase regulates the membrane association of coat proteins involved in intracellular membrane trafficking, which is critical for the vesicle transport of secretory proteins at the Golgi (Donaldson et al., 2005). Actually we observed that treatment of cells with brefeldin A, or expression of Arf1 T31N inhibited the secretion of MMP-8. Therefore, as one possibility, secretion of MMP-8 is upregulated by activation of Arf1 GTPase through ephrin-B1 signaling, although the molecular mechanisms connecting ephrin-B1 and Arf1 are still not well understood. As ephrin-B1 has a docking site for the PDZ domain at the C-terminus, some protein containing PDZ domains may be involved in this pathway. As a consequence, the increase of secreted MMP-8 may trigger the degradation of extracellular matrix and the cleavage of ephrin-B1 as a possible feedback mechanism (Fig. 8). In our preliminary observations, Arf1 activation occurs as fast as 10 minutes after stimulation with EphB2-Fc, and some increase of MMP-8 in the culture medium was detected from 0.5 hours after addition of EphB2-Fc. By contrast, the cleavage of ephrin-B1 was observed at 1 hour, but not at 10 minutes after stimulation with EphB2-Fc, by immunoblotting of a cleaved C-terminal fragment of ephrin-B1, p17, in cell lysates (data not shown). Although we cannot determine the precise time point of MMP-8 secretion because of the limitation of the antibody's sensitivity, these observations are compatible with the model that ephrin-B1 reverse signaling induces Arf1 activation, which leads to MMP-8 release and ephrin-B1 cleavage. As extracellular activation of MMPs can be triggered by activation of other MMPs, there is the possibility that ephrin-B1-mediated signaling may synergistically promote activity of several MMPs. In addition, Arf1 GTPase may involve the intracellular transport of not only MMP-8, but also several other MMPs. Whether metalloproteinases other than MMP-8 are also upregulated by EphB2 stimulation of ephrin-B1 should also be investigated.

We show that the 35 kDa ephrin-B1 fragment in the culture medium of SUIT-4 cells was generated by cleavage at aa 218. From the experiments using natural inhibitors of metalloproteinases, TIMP-1 but not TIMP-2 and TIMP-3 effectively blocked the cleavage of ephrin-B1. Among these TIMPs, TIMP-3 most effectively inhibits the function of ADAM family metalloproteinase, including ADAM-17 (TACE), ADAM-10 and ADAM-TS4 (Amour et al., 2000; Brew et al., 2000; Hashimoto et al., 2001). In addition, membrane-type metalloproteinases (MT-MMPs) are preferentially inhibited by TIMP-2 and TIMP-3, but not by TIMP-1 (Brew et al., 2000). Our observation that cleavage of ephrin-B1 was most effectively inhibited by TIMP-1, but not by TIMP-2 or TIMP-3 suggests that it is unlikely that those members of the ADAMs family are critically involved. However, we cannot exclude that TIMP-3-independent ADAM metalloproteinases may contribute to the processing of ephrin-B1. Together with the observation that ephrin-B1 cleavage was at least partially inhibited by RNA interference of MMP-8, and it was increased by overexpression of MMP-8, our results suggest that MMP-8 is the key metalloproteinase that cleaves ephrin-B1 ectodomain.

In addition to an extracellular 35 kDa peptide derived from the N-terminal of ephrin-B1, stimulation of ephrin-B1-expressing cells with EphB2-Fc resulted in the production of a 17 kDa intracellular fragment (p17) derived from the C-terminus of ephrin-B1. As detection of p17 was abolished in L cells expressing the ephrin-B1 4A mutant (data not shown), p17 was generated by cleavage of ephrin-B1 within the extracellular domain at aa 218. Ephrin-B1 and ephrin-B2 are cleaved within the transmembrane region by presenilin-dependent γ-secretase, which releases an approximately 12

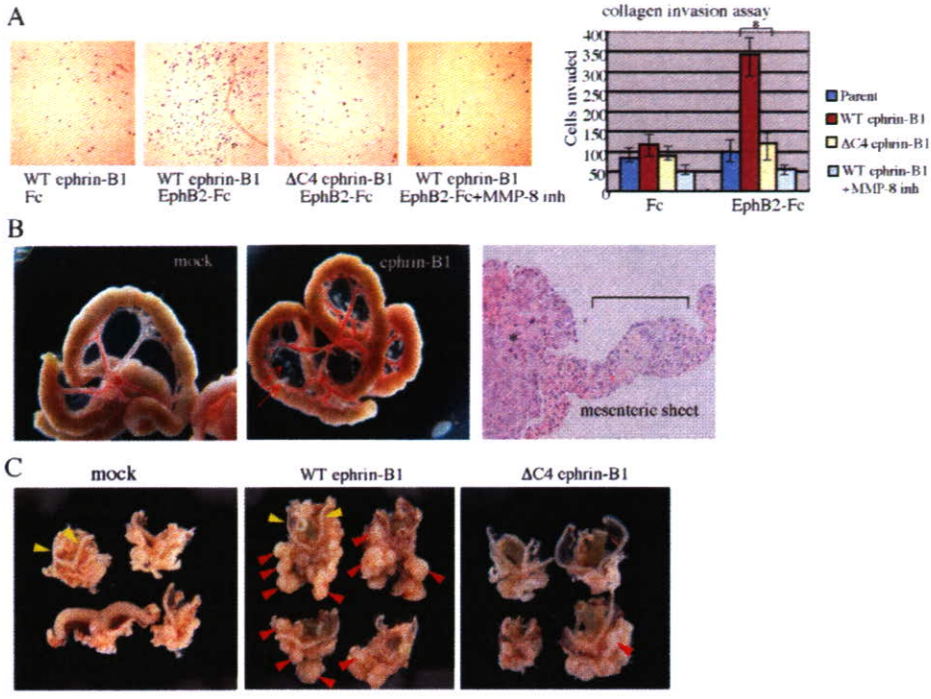

Fig. 7. The C-terminus of ephrin-B1 regulates the invasion of cancer cells. (A) Wild-type (WT) ephrin-B1 or ΔC4 ephrin-B1 mutant was expressed in Capan-1 cells. The cells were seeded onto a Transwell membrane coated with a collagen matrix (25 μg/ cm^2) in serum-free medium containing control Fc or EphB2-Fc (4 μg/ml) with or without addition of the MMP-8 inhibitor (1 μM). In the lower chamber, medium containing 5% FBS was added as a chemoattractant. After 8 hours incubation, the wells were harvested and cells that had invaded the collagen were counted. Representative fields are shown. (Right) The results from three independent experiments, each in duplicate, are shown as the mean ± s.d. *P<0.01. (B) PANC-1 ephrin-B1 cells or PANC-1 cells transfected with a mock vector (mock) were injected intraperitoneally into nude mice. The representative appearance of intestinal loops 8 weeks after injection is shown. Arrows indicate disseminated tumor nodules in the mesentery. The right panel shows the histology of the tumors in the mesentery (×100). The asterisk indicates a tumor nodule. Microscopic invasion of cancer cells was observed in the mesenteric sheet (blanket). (C) Representative appearance of the tumors of panc1 cells expressing either mock vector, wild-type or ΔC4 ephrin-B1 in the rectouterine region was compared. Yellow and red arrowheads indicate uterine horns and tumor nodules, respectively.

kDa intracellular fragment (Georgakopoulos et al., 2006; Tomita et al., 2006). Although p17 may be further processed by γ-secretase and produce a small intracellular peptide, we did not detect such a product, possibly because of its rapid degradation or cell type-dependent differences in protease activity.

One possible function of ephrin-B1-mediated MMP-8 secretion is processing of ephrin-B1 and downregulation of EhpB2-stimulated ephrin-B1 intracellular signaling. Unlike wild-type ephrin-B1 protein, ΔC ephrin-B1 protein was not reduced after EphB2-Fc treatment, which seems to suggest that cleavage of ephrin-B1 contributes to the down regulation of ephrin-B1 after stimulation. However, recent reports show the trans-endocytosis of ephrin-B1 after engagement with EphB receptors, which regulates ephrin-B-mediated cell repulsion (Zimmer et al., 2003; Marston et al., 2003; Parker et al., 2004). We also observed that ephrin-B1 4A, which also triggers Arf1 activation and release of MMP-8, but is resistant to cleavage, was degraded and decreased in amount after stimulation with EphB2-Fc almost to the same degree with wild-type ephrin-B1 (data not shown). Because the C-terminus of ephrin-B1 may also modify its endocytosis, we cannot conclude at present that ephrin-B1 cleavage greatly affects the stability and turnover of the EphB-ephrin-B1 complex on the cell surface. Rather, the biological significance of ephrin-B1-mediated MMP-8 secretion is considered to be the promotion of the invasion potential of cancer cells via degradation of surrounding extracellular matrix. However, addition of the 35 kDa ephrin-B1 ectodomain prepared from conditioned medium of ephrin-B1 expressing COS-1 cells inhibited the motility of EphB2-expressing cells, similar to the effect of unclustered ephrin-B1-Fc, indicating the possible biological effect of secreted fragments on cell movement, whereas it did not affect cell proliferation (see Fig. S2 in supplementary material).

The potential significance of ephrin-B1 in cancer cell invasion is supported by our finding that ephrin-B1-mediated collagen invasion by Capan-1 cells was related to MMP-8. The inability of ΔC-ephrin-B1 to promote collagen invasion in the same assay revealed that signaling through C-terminus of ephrin-B1 affects the invasion ability of cancer cells. In addition, expression of ephrin-B1 in the pancreas cancer cells, PANC-1, promoted the dissemination of intraperitoneally injected cells into the mesentery and peritoneal cavity where they formed tumor nodules, demonstrating for the first time that ephrin-B1 actually promotes cancer cell invasion in vivo. Overexpression of B-type ephrins in cancer cells would affect cell-cell adhesion by their interaction with cell adhesion proteins such as claudin (Tanaka et al., 2005). Ephrin-B1-mediated intracellular signaling also results in aberrant

Table 1. Mesenterial dissemination after intraperitoneal inoculation of cancer cells

	Number of nodules*		
Cell line	0-10	10-30	30+
Mock	16	2	0
WT ephrin-B1	0	4	17
ΔC4 ephrin-B1	12	4	3

Data are shown as the number of mice with tumors in the mesentery.
*Number of tumor nodules larger than 2 mm in the mesentery per body.

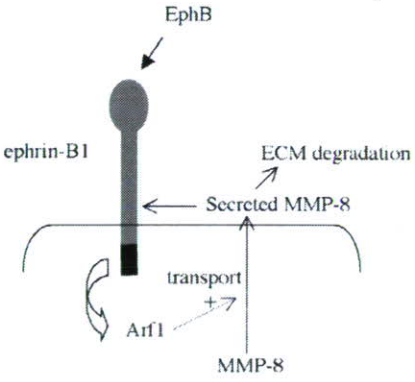

Fig. 8. Diagram showing the possible mechanism of ephrin-B1-mediated stimulation of MMP-8 secretion and cell invasion. When ephrin-B1 is stimulated by EphB receptors, Arf1 GTPase is activated through signaling mediated by the C-terminus ephrin-B1, which may stimulate the transport of MMP-8 for extracellular release. The increase of secreted MMP-8 triggers the degradation of extracellular matrix (ECM) and cleavage of ephrin-B1.

activation of RhoA and Rac1 (Tanaka et al., 2003; Tanaka et al., 2004; Lee et al., 2005). Together, events such as these would result in increased cell motility. These findings in conjunction with the ephrin-B1 induction of MMP-8 secretion, indicate that ephrin-B1 overexpression would result in an enhanced potential for invasion of surrounding tissues. For example, both MMP-8 and ephrin-B1 are frequently expressed in ovarian cancers, and their expression correlates with tumor grade and a poor prognosis (Castellvi et al., 2006; Varelias et al., 2002). Ephrin-B1 could also be involved in invasion of cancer cells circulating in the blood into sheets of endothelial cells which express EphB receptors and play a role in extravasation and metastasis. The inhibition of a specific cellular signal originating in ephrin-B1 stimulation may be a good candidate for regulating tumor invasion.

Materials and Methods

Plasmids, antibodies and reagents

Plasmids encoding full-length cDNAs of human ephrin-B1 and the Fc fusion protein construct of EphB2 and ephrin-B1 have been described previously (Tanaka et al., 2004). Fc fusion proteins were purified from the culture medium of COS-1 cells transfected with plasmids encoding EphB2-Fc or ephrin-B1-Fc using a protein A Sepharose column as described previously (Tanaka et al., 2004). Mutants of ephrin-B1 lacking the cytoplasmic tail (ΔC4 and ΔC19, truncation of four or 19 aa residues at the C-terminus, respectively) were generated using PCR-based techniques. Alanine substitution of four aa in the extracellular domain (aa 216-219) of ephrin-B1, ephrin-B1 4A, was performed using the Altered Sites Mutagenesis System (Promega). Generation of Ephrin-B1 with mutations of four tyrosine residues in the cytoplasmic domain (Y313, 317, 324, 329) and ephrin-B1 4YF, have been described previously (Tanaka et al., 2005). For making flag-tagged ephrin-B1, a DNA fragment encoding the Flag tag was inserted 3′ to the signal peptide of ephrin-B1 (aa 1-24). To generate a plasmid encoding human activated MMP-8, cDNA corresponding to nucleotides 336-824 of the reported sequence (GenBank accession number BC074988) was amplified with RT-PCR from a cDNA template derived from U937 cells. The amplified MMP-8 cDNA was tagged with the signal peptide and Flag at the 5′ terminus, and cloned into pcDNA3. GST-GGA3 was generated by cloning of PCR-amplified cDNA corresponding to aa 1-313 of human GGA3 short isoform (GenBank accession number AF219139) into pGEX4T2 (Amersham Pharmacia). The plasmids encoding wild-type Arf1 and Arf1 T31N bearing the HA epitope at the C-terminus were donated from J. S. Bonifacino (National Institute of Child Health and Human Development, NIH, Bethesda, MA). To generate the recombinant retrovirus, cDNAs were subcloned into pDON-AI vector (Takara). Monoclonal and polyclonal antibodies that recognize the Flag tag were purchased from Sigma and Affinity Bioreagents (Affinity Bioreagents, Golden, CO), respectively. Antibodies that recognize the HA tag were from InvivoGen (InvivoGen, San Diego, CA; monoclonal antibody) and Santa Cruz Biotechnology Inc. (polyclonal antibody). Rabbit polyclonal antibody that recognizes ephrin-B1 (C18) was purchased from Santa Cruz Biotechnology, Inc. The goat polyclonal antibody against ephrin-B1, which reacts with the entire extracellular domain, was purchased from R&D Systems. The polyclonal antibody against tyrosine-phosphorylated ephrin-B1 (ephrin-B1 pY317, aa residues 314-321) was raised in rabbits and affinity-purified as described previously (Tanaka et al., 2005). EphB2 and EphB4 polyclonal antibodies were from R&D Systems. Polyclonal and mouse monoclonal antibodies for MMP-8 were purchased from Chemicon and Daiichi Fine Chemical (Takaoka, Japan), respectively. The monoclonal antibody for phosphotyrosine (4G10) and Arf1 was from Upstate Biotechnology and Affinity Bioreagents, respectively. TrueBlot anti-rabbit IgG secondary antibody was purchased from eBioscience (San Diego, CA). Cyclohexamide and actinomycin D were purchased from Sigma. Purified TIMP-1, and 2 were purchased from Calbiochem, and TIMP-3 was from Sigma. The protease inhibitors shown in Fig. 3 and purified MMP-1 (proenzyme), MMP-8 (proenzyme), MT1-MMP (catalytic domain, aa 89-265) and ADAM10 (mature active ectodomain, aa 19-673) were purchased from Calbiochem. The MMP-8 inhibitor is (3R)-(+)-[2-(4-methoxybenzenesulfonyl)-1,2,3,4-tetrahydroisoquinoline-3-hydroxamate]. Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and the structural analog 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3) were purchased from Calbiochem.

Cell culture, transfection and retrovirus infection

SUIT-4 (Kawano et al., 2004) and the other pancreas carcinoma cell lines were cultured in RPMI1640 supplemented with 10% fetal bovine serum. Mice fibroblast L cells and COS-1 cells were cultured in DMEM with 10% fetal bovine serum. For transient expression assays, COS-1 cells and SUIT-4 cells were transfected with plasmid DNA using Lipofectamine 2000 reagent (Invitrogen). Recombinant retroviral plasmid, pDON-AI was cotransfected with pCL-10A1 retrovirus packaging vector (IMGENEX) into 293gp cells to allow the production of retroviral particles. Capan-1 cells were infected with retroviruses for transient expression of ephrin-B1 or Arf1 mutants, and used for experiments 48 hours after infection. For some experiments, Capan-1 cells stably expressing wild-type ephrin-B1 were established after retrovirus infection through the selection in medium containing G418 (600 μg/ml). L cells stably expressing ephrin-B1 or EphB2 were established as described and cultured in medium containing hygromycin B at a concentration of 400 μg/ml (Tanaka et al., 2005). PANC-1 cells stably expressing ephrin-B1 were established through selection in medium containing puromycin at a concentration of 2 μg/ml for 2-3 weeks. Well isolated colonies were characterized further.

In vitro siRNA treatment

Stealth siRNA (Invitrogen) of MMP-8 was synthesized as follows. Sense: 5′-AAGGCAUGAGCAAGGAUUCCAUUGG-3′; antisense: 5′-CCAAUGGAAUCC-UUGCUCAUGCCUU-3′. The control siRNA (scramble II duplex: 5′-GCG-CGCUUUGUAGGAUUCGdTdT-3′) was purchased from Dharmacon. SiRNAs were incorporated into cells using Lipofectamine™ 2000 according to the manufacturer's instructions (Invitrogen). Assays were performed 72 hours post treatment.

Peptidomic analysis of secretory proteins

SUIT-4 cells were cultured in serum-free RPMI1640 medium and the conditioned medium was collected. Cleared supernatant was loaded onto a SepPak C18 cartridge (Waters) for peptide extraction. Peptides bound to the cartridge were eluted with 0.1% trifluoroacetic acid (TFA)/60% acetonitrile (ACN) and lyophilized. The resultant sample was reconstituted with the same solvent and applied to an HPLC gel filtration column (Pharmacia). Fractions containing peptides with a molecular mass below 8,000 Da were subjected to reductive alkylation as described previously (Sasaki et al., 2002) and desalted with an Empore disk cartridge (3M). The desalted material was separated with a 75 mm × 100 mm C18 column (LC Packings, Sunnyvale, CA) before matrix assisted laser desorption ionisation (MALDI)-MS/MS analysis using an Ultimate HPLC pump and gradient programmer (LC Packings). The solvent system was 5% acetonitrile (ACN) (solvent A) and 95% ACN (solvent B); both contained 0.1% TFA. A linear gradient from 5% B to 60% B over 50 minutes was used. Eluates were spotted at 20-second intervals using Probot (LC Packings) on a MALDI target plate. Mass spectra were obtained in reflector mode on a (MALDI-TOF/TOF 4700 mass spectrometer (Applied Biosystems). Ion signals above S/N 25 observed in the MSMS spectra were selected for MSMS ion search against human entries in the NCBI nr database using the Mascot (Matrix Science) search algorithm with no enzyme specification, with the mass tolerance of precursor ions and product ions set at 100 ppm and 0.25 Da, respectively.

Immunoprecipitation and immunoblotting

Cell lysates were prepared with protease inhibitors in PLC buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 100 mM NaF, 1 mM Na_3VO_4 and 1% Triton X-100]. To precipitate the proteins, 1 μg of

monoclonal or affinity purified polyclonal antibody was incubated with 500 μg of cell lysate for 2 hours at 4°C, and then precipitated with protein G agarose for 1 hr at 4°C. Immunoprecipitates were extensively washed with PLC buffer, separated by SDS-PAGE, and immunoblotted. In some experiments, TrueBlot anti-rabbit IgG (eBioscience), which does not react to the denatured rabbit IgG, was used as the secondary antibody of the immunoblotting. For detection of MMP-8, rabbit polyclonal antibody was basically used in this study, however, almost the same results were obtained by using mouse monoclonal antibody.

RT-PCR

Total RNA was prepared from cultured cells by Isogen (Nippon Gene) according to the manufacturer's instructions and treated with DNase I. cDNA was synthesized from 2 μg of total RNA, and polymerase chain reactions (PCR) were performed in a 25 μl reaction volume at an annealing temperature of 55°C. The linear area of the PCR for each reaction was defined; 15 cycles for GAPDH and 25 cycles for MMP-8. Specific primers for MMP-8 and GAPDH have been described previously (Wahlgren et al., 2001; Woo et al., 2003); the expected PCR products were 352 bp and 300 bp, respectively. PCR products were subjected to electrophoresis on 2% agarose gels, and DNA was visualized by ethidium bromide staining.

Metabolic labeling

Cells cultured in 60 mm diameter dishes were preincubated in methionine-free DMEM (Sigma) for 1 hour, then cultured in 1.5 ml of methionine-free medium containing 0.15 mCi of [^{35}S]methionine (Amersham) for a further 4 hours. The cells were rinsed extensively and incubated in medium supplemented with EphB2-Fc or control Fc at 4 μg/ml for 2 hours. MMP-8 in the conditioned medium was purified by immunoprecipitation using an anti-MMP-8 polyclonal antibody and separated on SDS-PAGE. The gel was dried and subjected to autoradiography. The results were visualized with a Bio Imaging Analyzer (BAS1000; Fuji).

In vitro cleavage of ephrin-B1

Purified MMP-1 and MMP-8 were activated prior to use by treatment with 2 mM *p*-aminophenylmercuric acetate for 90 minutes at 37°C and dialyzed against 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM $CaCl_2$ and 50 μM $ZnCl_2$. Activated enzymes (10 nM, each) were incubated with ephrin-B1-Fc (2 μM) in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$ and 0.05% SDS at 37°C for 1 hour, separated by SDS-PAGE, and immunoblotted with anti-Fc of mouse IgG.

Arf1-GTP pull-down assay

To assess the amount of activated Arf1-GTP in cells, we performed a pull-down assay by using a GST-GGA3 construct (Dell's Angelica et al., 2000). Briefly, cells were left untreated or treated with EphB2-Fc (4 μg/ml) for 20 minutes. Cell lysates were prepared in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 100 mM NaF, 1 mM Na_3VO_4 and 1% Triton X-100], and then incubated with gluthathione-Sepharose beads containing a GST-GGA3 fusion protein for 45 minutes at 4°C. Precipitates were washed four times in the same buffer, and the precipitated Arf1 was detected by immunoblotting.

In vivo tumor invasion assay

The animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the guidelines for Animal Experiments of the National Cancer Center. Peritoneal dissemination of tumors was tested by intraperitoneal injection of 1×10^7 PANC-1 cells suspended in 0.3 ml of RPMI1640 medium into 6-week-old BALB/c nude mice (CLEA Japan, Inc.). The mice were sacrificed 8 weeks after injection, and peritoneal dissemination was evaluated. To examine expression of Eph receptors in mesentery-derived cells, the mesenteric sheets were cut along the streak of arteries as described previously (Akedo et al., 1986). The cells were collected from dissected sheets by incubating at 37°C for about 20 minutes in 0.25% trypsin in PBS.

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Growth Factors, Cytokines, Cell Cycle Molecules

Phosphorylation of Ephrin-B1 Regulates Dissemination of Gastric Scirrhous Carcinoma

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Interaction of the Eph family of receptor protein tyrosine kinase and its ligand ephrin family induces bidirectional signaling via cell-cell contacts. High expression of B-type ephrin is frequently found in various cancer cells, and their expression levels are associated with high invasion of tumors and poor prognosis. However, whether ephrin-B1 actually promotes invasion of cancer cells *in vivo* has not been shown. We investigated the involvement of ephrin-B1 in regulating the invasiveness of scirrhous gastric cancer, which is a diffusely infiltrative carcinoma with high invasion potential. Reduction of ephrin-B1 expression by short interfering RNA or overexpression of phosphorylation-defective mutant suppressed migration and invasion of scirrhous gastric cancer cells *in vitro* without affecting tumor cell proliferation and apoptosis. Blocking of tyrosine phosphorylation of ephrin-B1 attenuates not only dissemination of cancer cells injected intraperitoneally but also local invasion and dissemination of orthotopically implanted cancer cells in the gastric wall of nude mice. Furthermore, blocking of ephrin-B1 phosphorylation attenuated the activation of Rac1 GTPase in these invasive gastric cancer cells. Our results suggest that tyrosine phosphorylation of ephrin-B1 promotes invasion of cancer cells *in vivo* and is a potential therapeutic target in some types of gastrointestinal cancers. *(Am J Pathol 2007, 171:68–78; DOI: 10.2353/ajpath.2007.070033)*

Members of the Eph receptor family can be classified into two groups based on their sequence similarity and their preferential binding to the subset of ligands tethered to the cell surface either by a glycosylphosphatidyl inositol-anchor (ephrin-A) or a transmembrane domain (ephrin-B).[1–3] Interaction of the EphB family of receptor protein tyrosine kinases and its ligand ephrin-B family induces bidirectional signaling via cell-cell contacts.

The biological functions of Ephs and ephrins in epithelial cells and tumors have recently been highlighted.[4–9] For example, EphB receptors and ephrin-B ligands are expressed in normal intestinal epithelium, which contributes for the restriction of cell migration and positioning along the crypt-villus axis.[4] Overexpression of B-type ephrin in cancer cells correlates with poor prognosis characterized by high invasion and high vascularity of the tumors.[10–15] Expression of ephrin-B2 has been reported in invasive tumor cells and is often highly expressed in the peripheral region of the tumors especially at the front of the invasion.[11] Ephrin-B1 is frequently overexpressed in gastrointestinal tumors, especially in poorly differentiated invasive tumor cells.[15] Although an accumulating number of reports have suggested that expression of B-type ephrin is closely associated with tumor cell invasion, whether ephrin-B modifies tumor invasion *in vivo* has not been well established.

Ephrin-Bs are tyrosine phosphorylated via Src family kinases in response to the interaction with EphB receptors, which serves as a docking site for Src homology 2 domain of adaptor protein Grb4, and transduce intracellular signaling.[16,17] We have also demonstrated that ephrin-B1 is phosphorylated independently of Eph receptors through association with an intercellular adhesion molecule, which leads to attenuation of cell-cell adhesion.[7] In our recent observations, signaling mediated by ephrin-B1 promoted the process of intracellular transport and secretion of matrix metalloproteinase (M. Tanaka, K. Sasaki, R. Kamata, and R. Sakai, unpublished data), which led us to examine in this study whether disruption of ephrin-B1-mediated signaling, especially through the tyrosine phosphorylation of ephrin-B1, could suppress tumor cell invasion.

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Scirrhous gastric carcinoma diffusely infiltrates a broad region of the stomach and frequently associates with metastasis to lymph nodes and peritoneal dissemination and, therefore, has the worst prognosis among various types of gastric cancers. We previously established two cell lines of human gastric scirrhous carcinoma possessing high infiltrative potential by repeating cycles of orthotopic transplantation in nude mice and collecting cancer cells from the ascitic fluid formed as a result of cancerous peritonitis.[18,19] In this study, we show that reduction of ephrin-B1 expression or blocking of tyrosine phosphorylation of ephrin-B1 inhibits tumor invasion of these highly invasive gastric cancer cells. Our results suggest that ephrin-B1 represents a rational therapeutic target and that suppression of its phosphorylation is a strategy for modulating the invasion of some types of cancers.

Materials and Methods

Plasmids, Antibodies, and Reagents

Plasmids encoding full-length cDNAs of human ephrin-B1 and ephrin-B1 with mutations of four tyrosine residues in the cytoplasmic domain (Y313, 317, 324, and 329) ephrin-B1 4YF have already been described.[7] To generate the recombinant retrovirus, cDNAs were subcloned into the vector pDON-AI (Takara, Kyoto, Japan). The rabbit polyclonal antibodies for ephrin-B1 (C18) and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody against tyrosine-phosphorylated ephrin-B1 (ephrin-B1 pY317, amino acids 314 to 321, which is identical to corresponding region of ephrin-B2, 301 to 308, and ephrin-B3, 308 to 315) was raised in rabbits and affinity-purified as described previously.[7] The monoclonal antibodies for phosphotyrosine (4G10) and Rac1 were from Upstate Biotechnology (Lake Placid, NY). The polyclonal antibodies for EphB2 and EphB4 were from R&D Systems (Minneapolis, MN). Fibronectin (bovine), collagen type I, and Matrigel basement membrane matrices were purchased from Sigma (St. Louis, MO), Nitta Gelatin, Inc. (Osaka, Japan), and BD Biosciences (San Jose, CA), respectively.

Cell Culture and Transfection

Gastric carcinoma cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Mouse fibroblast L cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. L cells stably expressing EphB2 (L EphB2) were established through transfection of a plasmid encoding human ephrin-B1 in parent L cells, which do not express cognate receptors for ephrin-B1 as previously described,[7] and selection in medium containing hygromycin B at a concentration of 400 μg/ml. Recombinant retroviral plasmid pDON-AI was cotransfected with pCL-10A1 retrovirus packaging vector (Imgenex, San Diego, CA) into 293gp cells to allow the production of retroviral particles. Gastric cancer cells stably expressing ephrin-B1 4YF were established by infecting cancer cells with retroviruses and selected in the medium containing G418 at a concentration of 500 μg/ml for 3 weeks. The mixture of selected cells was used for the experiments.

In Vitro *Short Interfering RNA (siRNA) Treatment*

Two sets of Stealth siRNAs of ephrin-B1 were synthesized as follows (Invitrogen, Carlsbad, CA): ephrin-B1 sense 1, 5′-UAAGGGAAUGAUGAUGUCGCUGGGC-3′; ephrin-B1 antisense 1, 5′-GCCCAGCGACAUCAUCAUUCCCUUA-3′; ephrin-B1 sense 2, UAGUCCGUAAGGGAAUGAUGAUGUC-3′; and ephrin-B1 antisense 2, GACAUCAUCAUUCCCUUACGGACUA-3′. The control siRNA (scramble II duplex, 5′-GCGCGCUUUGUAGGAUUCGdTdT-3′) was purchased from Dharmacon (Lafayette, CO). siRNAs were incorporated into cells using Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). Assays were performed 72 hours after treatment.

Immunoprecipitation

Cell lysates were prepared with protease inhibitors in PLC buffer [50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 10% glycerol, 100 mmol/L NaF, 1 mmol/L Na_3VO_4, and 1% Triton X-100]. To precipitate the proteins, 1 μg of affinity-purified polyclonal antibody was incubated with 500 μg of cell lysate for 2 hours at 4°C and then precipitated with protein G agarose for 1 hour at 4°C. Immunoprecipitates were extensively washed with PLC buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted.

Affinity Precipitation

Affinity precipitation with GST-PBD (p21-binding domain of p21-activated kinase 1) was performed as described previously.[20] In brief, cells were lysed in the lysis buffer [50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 150 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 10% glycerol, 100 mmol/L NaF, 1 mmol/L Na_3VO_4, and 1% Triton X-100] and incubated with GST-PBD on Sepharose for 1 hour at 4°C. Precipitants were washed three times in the same buffer, and endogenous Rac1 was detected by immunoblotting with anti-Rac1 antibody.

5-Bromo-2′-Deoxyuridine Incorporation

Cell proliferation was assessed by measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation into the DNA with Cell Proliferation enzyme-linked immunosorbent assay, BrdU (colorimetric) kit (Roche, Basel, Switzerland). In brief, gastric cancer cells were plated onto 96-well plates (1×10^4 cells/well) 48 hours after treatment of siRNAs and further incubated for 24 hours before the addition of BrdU. Cells were reincubated for 6 hours,

and incorporated BrdU was detected with peroxidase-labeled anti-BrdU antibody and developed with tetramethyl-benzidine as a chromogenic substrate according to the manufacturer's instructions (Roche). The absorbance of the samples was measured at the wavelength of 450 nm using a microplate reader (model 550; Bio-Rad, Hercules, CA).

Apoptosis Assays

Gastric cancer cells were plated in triplicate onto 96-well plates (1×10^4 cells) 48 hours after treatment of siRNAs and incubated for 24 hours. Cells were lysed to detect apoptosis by measurement of nucleosomes in the cytoplasm of apoptotic cells using a Cell Death enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Roche Molecular Biochemicals). In brief, nucleosomes in cell lysates were detected with peroxidase-labeled anti-DNA antibody and developed with 2,2′-azino-di[3-ethylbenzthiazolin-sulfonate] as a chromogenic substrate. The absorbance of the samples was measured at the wavelength of 405 nm using a microplate reader (model 550; Bio-Rad).

Cell Attachment Assay

Cancer cells were detached by phosphate-buffered saline(−) [$PBS^{(-)}$] containing ethylenediamine tetraacetic acid (2 mmol/L) and replated on the chamber slides coated with either collagen type I (100 μg/ml; Nitta Gelatin, Inc.), fibronectin (50 μg/ml; Sigma), or Matrigel (85 μg/ml; Asahi Techno Glass Co., Tokyo, Japan). After incubation for 30 minutes, unattached cells were removed by washing the slides in $PBS^{(-)}$ several times, and the remaining cells were stained with Giemsa's solution. The number of attached cells on each substrate was counted.

Cell Staining

Cells were fixed for 5 minutes at room temperature with 4% paraformaldehyde in PBS and permeabilized for 10 minutes with 0.2% Triton X-100. The cells were preincubated in 2% bovine serum albumin for 0.5 hour and incubated with Alexa546-conjugated phalloidin (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Photos were taken with a Radiance 2100 confocal microscope (Bio-Rad).

Overlay Tumor Invasion Assay

Invasion of tumor cells into the monolayer of stromal cells was monitored basically as described previously.[20] Gastric cancer cells treated with ephrin-B1 siRNA or control siRNA were labeled with 2 mmol/L lipophilic tracer DiO (Molecular Probes) and then detached with Hanks' balanced salt solution$^-$ containing 2 mmol/L ethylenediamine tetraacetic acid and seeded on the confluent monolayer of parent L cells or L EphB2 cells. After being cultured in the medium with 10% fetal bovine serum for 15 hours, the cells were fixed with 4% paraformaldehyde in PBS, and the number of invasion foci of cancer cells was counted using fluorescence microscopy.

In Vivo *Tumor Cell Invasion Assay*

The animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the guidelines for Animal Experiments in the National Cancer Center. Peritoneal dissemination of tumors was examined by intraperitoneal injection of 5×10^6 gastric cancer cells suspended in 0.3 ml of RPMI 1640 medium into 6-week-old BALB/c nude mice (CLEA Japan, Inc., Tokyo, Japan). The mice were sacrificed 2 weeks after injection, and peritoneal dissemination was evaluated. Orthotopic implantation of gastric cancer cells into BALB/c nude mice has been described previously.[18,19] In brief, 1×10^6 cells were inoculated into the middle wall of the greater curvature of the glandular stomach by using a 30-gauge needle. The mice were sacrificed at different time points after the orthotopic transplantation of the cancer cells and subjected to macroscopic and histopathological examination of the tumors.

Results

Reduction of Ephrin-B1 Expression Attenuates Tumor Invasion of Gastric Cancer Cells

To examine the involvement of ephrin-B1 for invasion of tumors, we analyzed cell lines of scirrhous gastric carcinoma, which is characterized as reduced cell-cell adhesion with high invasion potential. HSC-44PE and HSC-58 were originally established from the patients of scirrhous gastric carcinoma, and highly invasive sublines were further selected from these parent cells (44As3 from HSC-44PE; 58As1 and 58As9 from HSC-58[18,19]). Both expression and phosphorylation levels of ephrin-B1 were higher in cells of invasive sublines than in corresponding parent cell lines, whereas the expression level of control α-tubulin was not altered in these cell lines (Figure 1a). In addition, EphB2 was expressed in all of these cell lines, and HSC-44PE and 44As3 cells also expressed EphB4, showing the existence of cognate receptors (Figure 1a).

We next examined whether reduction of ephrin-B1 expression affects cell motility and proliferation of these gastric cancer cells. The treatment of cells with two independent siRNA of ephrin-B1 effectively reduced ephrin-B1 expression level in 58As9 cells and 44As3 cells (Figure 1b). In addition, phosphorylation of B class ephrin was greatly reduced by knocking down ephrin-B1, as judged by the antibody recognizing phosphorylation of all three members of ephrin-Bs at the tyrosine in the cytoplasmic region (Figure 1b). From the analysis of *in vitro* Transwell assay, reducing the amount of ephrin-B1 in 44As3 cells inhibited migration and invasion through the extracellular matrix (Figure 2a). Similar results were also observed in 58As9 cells (Figure 2a). On the other

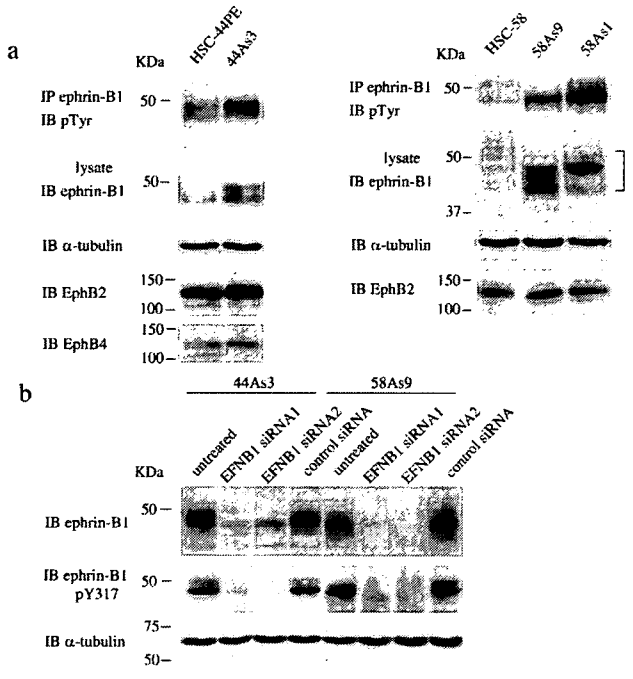

Figure 1. Tyrosine phosphorylation of ephrin-B1 is higher in invasive gastric cancer cell lines. **a:** As indicated, lysates from cells were subjected to immunoprecipitation (IP) with anti-ephrin-B1 antibody and immunoblotting (IB) with anti-phosphotyrosine antibody. The expression levels of ephrin-B1, α-tubulin, EphB2, and EphB4 in each cell lysate were confirmed by immunoblotting (bottom). When cell lysates were blotted with ephrin-B1 antibody, several ephrin-B1-reactive bands (blanket) were detected because of the glycosylation and difference in tyrosine phosphorylation as reported.[31] **b:** Cellular levels of ephrin-B1 were analyzed 72 hours after treatment with siRNAs by Western blotting using α-tubulin as a loading control. Expression of ephrin-B1 was reduced in cells treated with ephrin-B1 siRNA (EFNB1siRNA1, 2). The phosphorylation level of ephrin-Bs was assessed by the antibody recognizing phosphorylation of Tyr317 of ephrin-B1, which also detects phosphorylation of corresponding tyrosine of ephrin-B2 (Tyr304) and ephrin-B3 (Tyr311) as described in Materials and Methods.

hand, proliferation and apoptosis of these cells were not significantly affected (Figure 2b). In addition, reduction of ephrin-B1 expression did not cause remarkable change in the adhesion of 44As3 and 58As9 cells on different extracellular matrices, including type I collagen, fibronectin, and Matrigel (Figure 2c). We further examined whether overexpression of ephrin-B1 is sufficient to promote the migration and invasion of cancer cells by stably expressing ephrin-B1 in one parental cell line HSC-58. The migration and invasion through extracellular matrix was apparently increased by the overexpression of ephrin-B1 (Figure 2d).

Phosphorylation of Ephrin-B1 Promotes Migration and Invasion of Gastric Cancer Cells

Because the level of tyrosine phosphorylation of ephrin-B1 was higher in invasive sublines of gastric cancer cells, we next examined whether blocking of ephrin-B1 phosphorylation in these cells attenuates their migration and invasion. The stable expression of ephrin-B1 with mutations of four tyrosine residues in the cytoplasmic domain (Y313, 317, 324, and 329) ephrin-B1 4YF reduced the tyrosine phosphorylation level of ephrin-B1 in 44As3 and 58As9 cells, because overexpression of ephrin-B1 4YF prevents endogenous ephrin-B1 from association with EphB receptors expressed in these cells (Figure 3a). From the analysis of *in vitro* Transwell assay, migration and invasion of cancer cells stably expressing ephrin-B1 4YF mutant (44As3 4YF and 58As9 4YF cells) were decreased compared with the control cells expressing mock vector (44As3 mock and 58As9 mock cells) (Figure 3b). On the other hand, expression of ephrin-B1 4YF did not affect cell proliferation under usual two-dimensional cell culture condition (Figure 3c). When these cancer cells expressing mock vector or ephrin-B1 4YF were implanted subcutaneously in nude mice, the mean size and weight of the tumors were not significantly different (Figure 3d). To understand the mechanism by which ephrin-B1 4YF attenuates the cell migration, we examined activity of Rac1 GTPase, which is a critical molecule controlling the organization of actin cytoskeleton. The activation of Rac1 was examined by affinity precipitation of GTP-bound Rac1 with the GST-fusion protein of the p21-binding domain of p21-activated kinase 1.[21] The activated Rac1 was apparently reduced in 44As3 or 58s9 cells expressing ephrin-B1 4YF compared with the cells expressing mock vector (Figure 3e). When the appearance of cytoskeleton of these cancer cells was examined, formation of large lamellipodia was observed in most of the mock-containing 44As3 and 58As9 cells, whereas it was less frequently observed in 44As3 4YF and 58As9 4YF cells (Figure 3f), which may be consistent with the reduced Rac1 activity in cancer cells expressing ephrin-B1 4YF.

Gastric scirrhous carcinoma frequently associates with peritoneal dissemination through the process that cancer cells perforate gastric serosa and become exfoliated and free and then attached on the surface of the peritoneum and start to invade there. The effect of ephrin-B1 expression on tumor invasion was further monitored *in vitro* by overlay tumor cell invasion assay as a model system for stromal invasion of cancer cells. When 44As3 cells were plated onto the monolayer of fibroblasts, L cells, the formation of tumor cell islands was observed as tumor cells invaded and grew between the fibroblasts (Figure 4a). The formation of such tumor islands is the sign of penetration of tumor cells into the sheet of stromal cells, as used for the evaluation of cancer cell invasion through endothelial cells or mesothelial cells.[20,22,23] The invasion of 44As3 cells into the fibroblasts monolayer was more evident when the cancer cells were plated onto L cells stably expressing EphB2 (L EphB2) than on the parent L cells, which do not express receptors for ephrin-B1[7] (Figure 4b). The invasion foci of 44As3 in monolayer of L EphB2 cells were decreased in number and size when expression of ephrin-B1 in cancer cells was reduced (Figure 4a). In addition, the invasion of 44As3 4YF cells into the monolayer of L EphB2 cells was also decreased compared with 44As3 mock cells (Figure 4, a and b). These results indicate that activation of the signaling mediated by ephrin-B1 phosphorylation in cancer cells induced by the interaction with EphB2 receptor expressed in stromal cells enhanced the tumor invasion.

The effect of ephrin-B1 phosphorylation on tumor invasion was further examined *in vivo* using 44As3 and 58As9

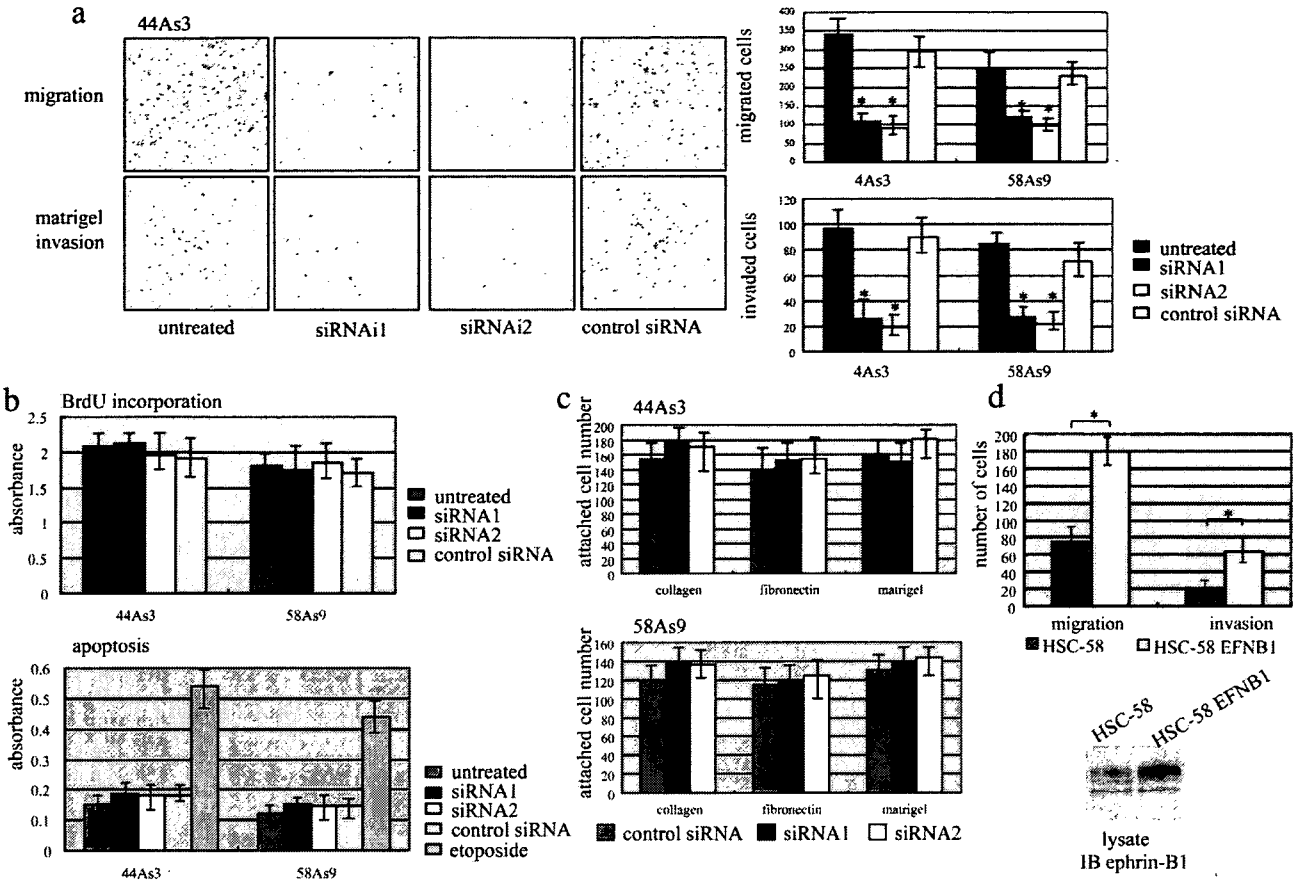

Figure 2. Reduction of ephrin-B1 expression suppressed cell motility and invasion in 44As3 and 58As9 cells. **a:** 44As3 and 58As9 cells treated with ephrin-B1 siRNA (siRNA1, 2) or control siRNA or left untreated were plated onto a Transwell membrane coated with Matrigel (bottom; 85 $\mu g/cm^2$) or uncoated (top) in serum-free medium. In the lower chamber, medium containing 5% fetal bovine serum was added as a chemoattractant. After 10 hours of incubation, the wells were harvested, and cells that migrated to the lower surface of the membrane were counted. Representative fields of 44As3 cells are shown. The results from three independent experiments, each in duplicate, are shown at the right as mean ± SD. The **asterisks** indicate differences from the cells treated with control siRNA. $^*P < 0.01$. **b:** Proliferation and apoptosis of cells were evaluated 72 hours after treatment of siRNAs. Top: Proliferation of the cells was evaluated by measurement of DNA synthesis through detection of BrdU incorporation by enzyme-linked immunosorbent assay (Roche). Bottom: Apoptosis of the cells was examined by measurement of nucleosomes through Cell death detection enzyme-linked immunosorbent assay kit (Roche). As a control, cells were treated with 50 μmol/L etoposide for 12 hours. The results from three independent experiments, each in duplicate, are shown as the mean ± SD. **c:** Reduction of ephrin-B1 expression did not significantly affect the cell adhesion to the extracellular matrix. 44As3 and 58As9 cells treated with ephrin-B1 siRNA (siRNA1, 2) or control siRNA were detached by ethylenediamine tetraacetic acid and replated on the chamber slides coated with collagen type I (100 μg/ml), fibronectin (50 μg/ml), or Matrigel (85 μg/ml). After incubation for 30 minutes, unattached cells were removed by washing the slides in PBS(−) several times, and the remaining cells were stained with Giemsa's solution. The number of attached cells on each substrate was counted, and the results from three independent experiments, each in duplicate, are shown as the mean ± SD. **d:** Expression of wild-type ephrin-B1 promotes migration and invasion of HSC-58 cells. Wild-type ephrin-B1 was stably expressed in HSC-58 cells by retrovirus-mediated gene transfer (HSC-58 EFNB1). The cells indicated were plated onto a Transwell membrane coated with Matrigel (invasion) or uncoated (migration) in serum-free medium and assayed as described in **a**. The results from three independent experiments, each in duplicate, are shown as mean ± SD. $^*P < 0.01$.

cells as a model system for peritoneal dissemination. When 58As9 or 44As3 cells expressing mock vector were injected intraperitoneally into nude mice, severe carcinomatous peritonitis was observed, as previously described (Figure 5a, top).[19] Innumerable whitish nodules were observed in the mesentery of almost all mice injected with 58As9 mock cells (Figure 5a, top left) and 44As3 mock cells (data not shown). In addition, many tumor nodules of 44As3 mock cells were observed in the peritoneal cavity, including the rectouterine region with invasion into the retroperitoneum (Figure 5a, top right). On the other hand, dissemination of cancer cells expressing ephrin-B1 4YF was apparently modest. Tumor nodules of 58As9 4YF cells in the mesentery were small in size and number compared with those of control 58As9 mock cells (Figure 5b; Table 1). Such reduction of tumor nodules in the mesentery was also observed in 44As3 cells expressing ephrin-B1 4YF (Table 1). In addition, the tumor volume involving the rectouterine region was reduced in the mice injected with 44As3 4YF cells (Figure 5c). These results suggest that ephrin-B1 promotes peritoneal dissemination of 58As9 and 44As3 gastric cancer cells through signaling mediated by its tyrosine phosphorylation.

Blocking of Tyrosine Phosphorylation of Ephrin-B1 Attenuates Tumor Invasion of Orthotopic Implanted Gastric Carcinoma

To further evaluate the effect of ephrin-B1 on the process of tumor invasion, we implanted gastric cancer cells orthotopically in the gastric submucosa of nude mice. At 15 days after implantation, 70% of 44As3 tumors expressing

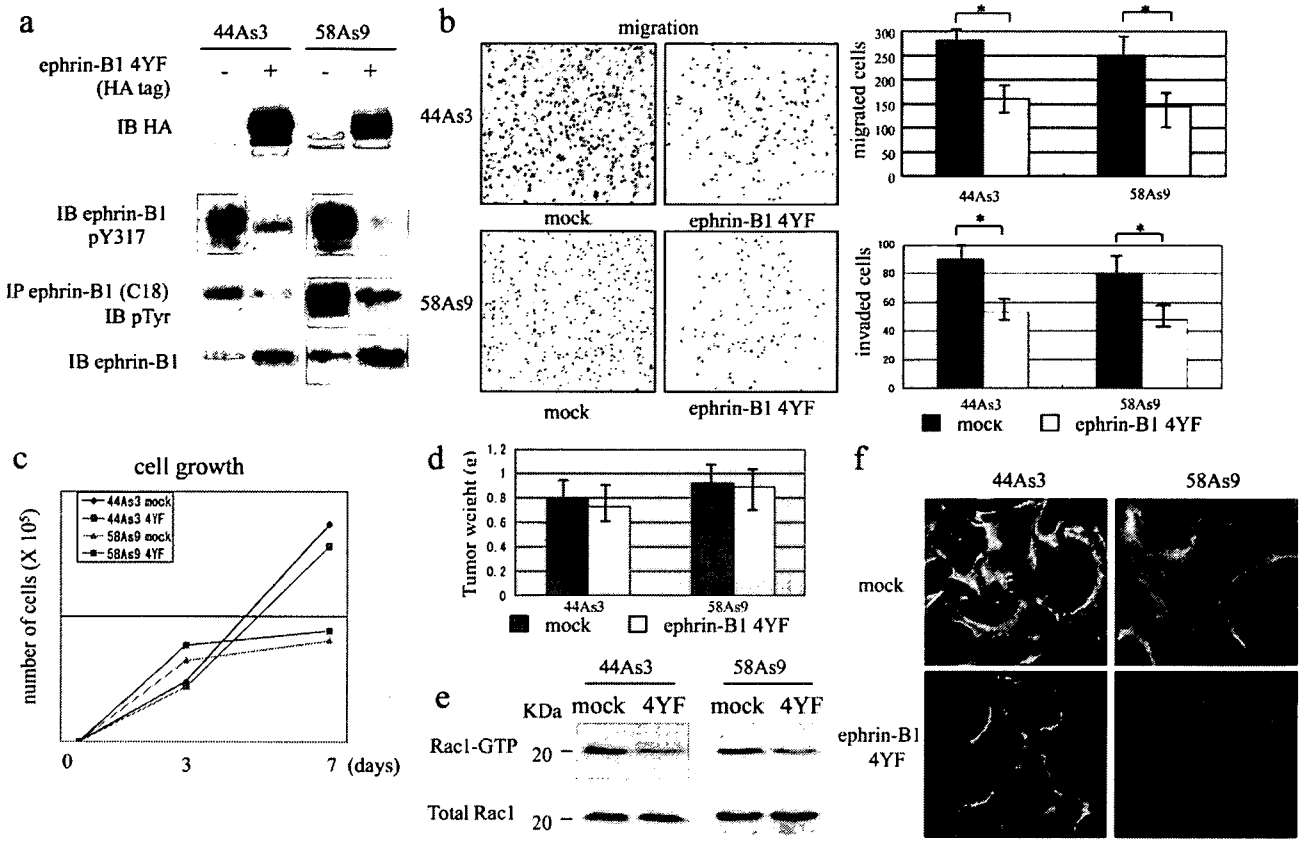

Figure 3. Blocking of ephrin-B1 phosphorylation suppressed cell migration *in vitro*. **a:** Lysates of cells stably expressing ephrin-B1 4YF tagged with HA at carboxyl terminus (+) or control mock vector (−) were subjected to immunoblotting with anti-HA antibody or tyrosine-phosphorylated ephrin-Bs (ephrin-B1 pY317) or immunoprecipitation of total ephrin-B1 and immunoblotting with anti-phosphotyrosine antibody (pTyr). The amount of immunoprecipitated ephrin-B1, which includes both endogenous ephrin-B1 and transfected ephrin-B1 4YF, is shown at the bottom. **b:** As indicated, the cells were seeded onto a Transwell membrane coated with Matrigel (invasion) or uncoated (migration) in serum-free medium as in Figure 2a. In the lower chamber, medium containing 5% fetal bovine serum was added. After 10 hours of incubation, the wells were harvested, and cells that migrated to the lower surface of the membrane were counted. Representative fields are shown. The results from three independent experiments, each in duplicate, are shown at the bottom as mean ± SD. $^*P < 0.01$. **c:** Proliferation of the cells under the culture in the medium containing 10% serum was evaluated by counting the cell number at different time points after being plated onto dishes. **d:** 44As3 and 58As9 cells (1×10^7 cells/mice) expressing either mock or ephrn-B1 4YF were implanted subcutaneously in the flank of nude mice, and the mice were sacrificed 5 weeks after implantation. The mean weight ± SD of eight subcutaneous tumors is shown. **e:** Activation of Rac1 was examined by affinity precipitation of GTP-bound Rac1 (Rac1-GTP) with GST-PBD as described in Materials and Methods. The precipitated Rac1-GTP and total Rac1 in each cell lysate were detected by immunoblotting with anti-Rac1 antibody. **f:** Morphology of gastric cancer cells was monitored. The cells were fixed around 12 hours after plating and stained with phalloidin as described in Materials and Methods for detection of F-actin.

the mock vector formed a large tumor mass involving the greater omentum because of the disruption of gastric serosa and invasion into the surrounding fat tissue (Figure 6a). On the other hand, such local invasion into the omentum was less frequently observed (18%) in mice implanted with 44As3 4YF cells, and most tumors remained within the gastric wall at day 15 (Figure 6a; Table 2). The dissemination of 44As3 mock cells was also observed in several tissues, including the liver surface and mesenteric sheets (Figure 6, b and c). However, we rarely observed cancer dissemination in the peritoneal cavity in mice implanted with 44As3 4YF cells (Figure 6, b and c; Table 2).

Histological examination revealed that expression of ephrin-B1 4YF did not greatly change the morphology of the tumor in the gastric wall (Figure 7, c, d, l, and m). However, invasion of control 44As3 mock cells into lymphatic vessels in subserosa and metastasis to the regional lymph nodes was more frequently observed than 44As3 4YF cells at day 10 after tumor implantation (70 and 18%, respectively; Figure 7, e–g; Table 3). Histological analysis also revealed that the tumor had already reached the outside of the serosal surface and invaded into the surrounding fat tissue in 80% of mice implanted with 44As3 mock cells, whereas it was of low frequency in mice implanted with 44As3 4YF cells (27%) (Figure 7, h and i; Table 3). These phenotypes, including lymphatic vessel invasion, lymph node metastasis, and perforation of gastric serosa, were developed in the same mice, and all three phenotypes are overlapped in 7 of 10 mice in the mock group and in 2 of 10 mice in the 4YF group, suggesting that these are sequential or correlated events. These results indicate that the disruption of signaling mediated by phosphorylation of ephrin-B1 suppressed the invasion and peritoneal dissemination of scirrhous gastric carcinomas.

Discussion

Ephrin-B1 plays pivotal roles in the migration and invasion of cancer cells. High invasion potential is one of the

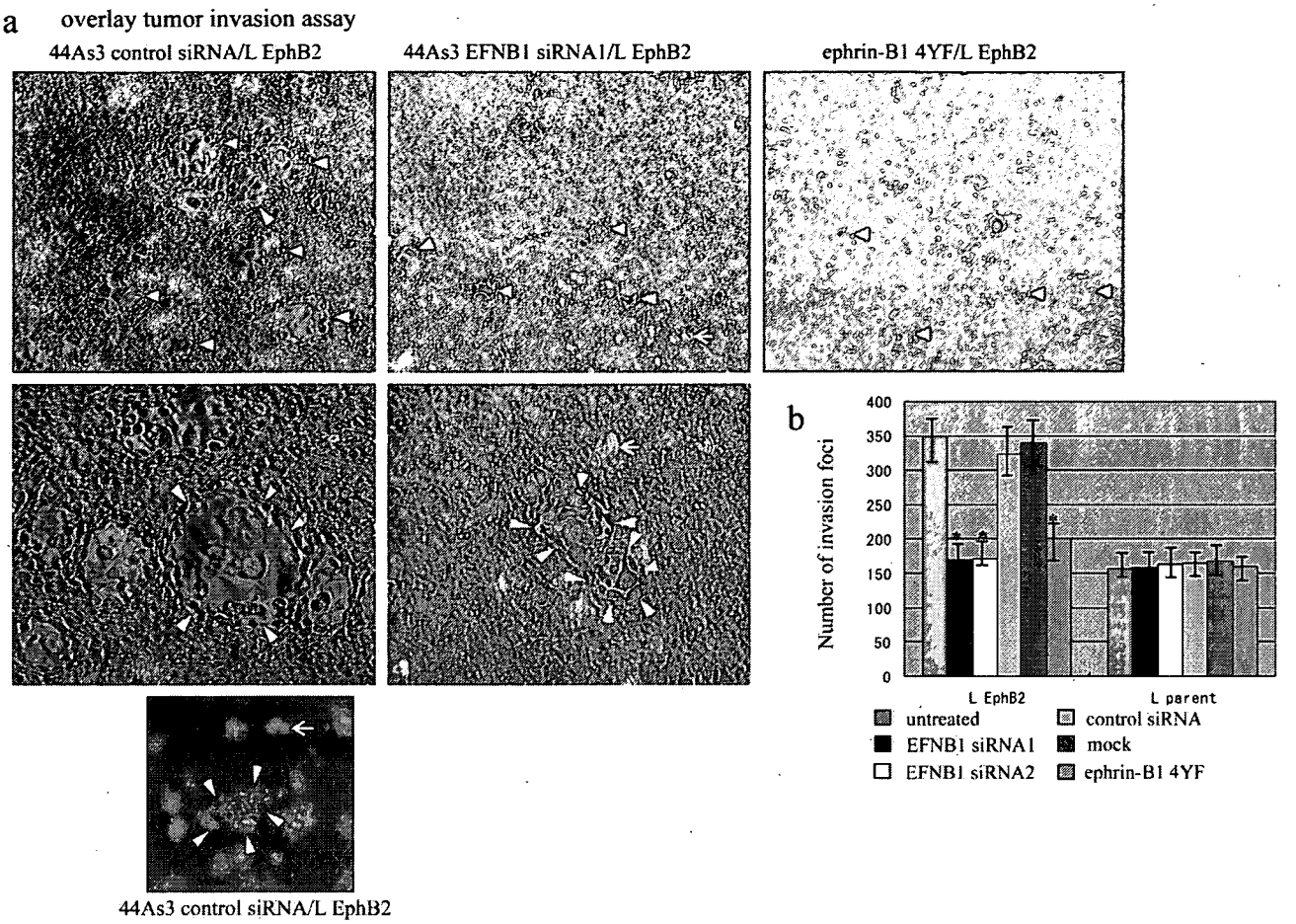

Figure 4. Invasion of ephrin-B1-expressing cells was promoted by interaction with EphB2 receptor. **a:** 44As3 cells were highly invasive into the monolayer of fibroblasts expressing EphB2, which was attenuated by reduction of ephrin-B1 expression. When L cells or L EphB2 cells grew to confluent state in 24-well plates, DiO-labeled 44As3 cells treated either with control or ephrin-B1 siRNA or with 44As3 mock or 44As3 4YF cells (1×10^4, each) were seeded onto the monolayer and incubated in a medium containing 10% fetal bovine serum for 15 hours. Representative fields of the co-culture are shown (top, ×100; bottom, ×200). **Arrowheads** indicate typical invasion foci of tumor cells. Bottom: Typical invasion focus of tumor cells is shown in the fluorescence field (×200). The nest of invaded tumor cells is surrounded by **arrowheads**. **b:** The number of invasion foci was counted as described in Materials and Methods, and the results from three independent experiments, each in duplicate, are shown as mean ± SD. The **asterisks** indicate differences from the cells treated with control siRNA or control cells expressing mock vector. $^*P < 0.01$. The **arrows** indicate unfocused Dio-labeled tumor cells, which did not invade but grew on the monolayer.

major characteristics of scirrhous carcinoma, which determines the poor prognosis of this type of cancer. Using two scirrhous gastric cancer cell lines with high invasion potential, we show for the first time that ephrin-B1 modifies cancer invasion *in vivo*. Blocking of the signaling mediated by tyrosine phosphorylation of ephrin-B1 suppressed the invasion and peritoneal dissemination of these scirrhous cancer cells. Notably, attenuation of phosphorylation of ephrin-B1 suppressed orthotopically implanted scirrhous cancer cells invading through the gastric wall and into the lymphatic vessels. The significance of ephrin-B1 in the invasive phenotype of cancer cells was further obtained from the result that stable expression of wild-type ephrin-B1 in HSC-58, the parental cell line of 58As9 cells, actually promoted the migration and invasion of this cell line *in vitro* (Figure 2d).

Association with EphB receptors triggers tyrosine phosphorylation of ephrin-B1, including Tyr317 (corresponding to Tyr298 of *Xenopus* ephrin-B1), by Src family kinases, which is a critical requirement for interaction with an Src homology 2/Src homology 3 adaptor, Grb4, to transduce signaling.[16] In the physiological conditions, phosphorylation of ephrin-B1 is induced by the contact of ephrin-B1-expressing cells with heterologous cells expressing EphB receptors. On the other hand, ephrin-B1 also promotes migration of cancer cells in a cell-autonomous mechanism as observed in Transwell assay (Figures 2 and 4b). Because EphB2 receptor was expressed in 44As3 and 58As9 cells and expression of EphB4 was also detected in 44As3 cells (Figure 1a), there is a possibility that ephrin-B1 in these cancer cells may be constitutively stimulated by EphB receptors coexpressed in the same cell surface or contacting neighboring cancer cells. This conclusion may be consistent with the observation that the basal phosphorylation level of ephrin-B1 is elevated in 44As3 and 58As9 cells under the usual two-dimensional culture condition *in vitro* (Figure 1a). Overexpression of ephrin-B1 4YF may suppress tumor invasion *in vivo* by blocking cell autonomous phosphorylation of ephrin-B1 in cancer cells and the induction of ephrin-B1 phosphorylation through the interaction with stromal cells. There is still the possibility that expression of eph-

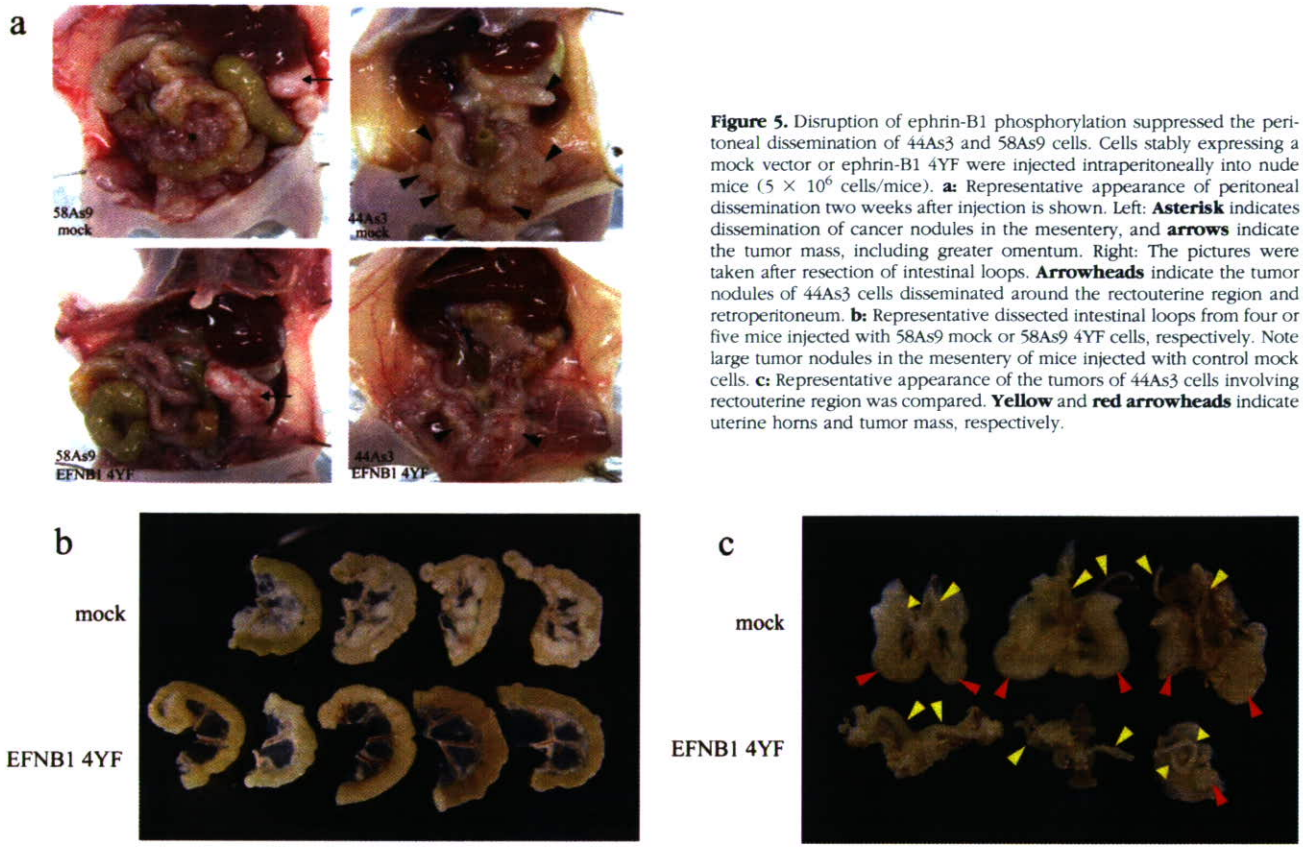

Figure 5. Disruption of ephrin-B1 phosphorylation suppressed the peritoneal dissemination of 44As3 and 58As9 cells. Cells stably expressing a mock vector or ephrin-B1 4YF were injected intraperitoneally into nude mice (5×10^6 cells/mice). **a:** Representative appearance of peritoneal dissemination two weeks after injection is shown. Left: **Asterisk** indicates dissemination of cancer nodules in the mesentery, and **arrows** indicate the tumor mass, including greater omentum. Right: The pictures were taken after resection of intestinal loops. **Arrowheads** indicate the tumor nodules of 44As3 cells disseminated around the rectouterine region and retroperitoneum. **b:** Representative dissected intestinal loops from four or five mice injected with 58As9 mock or 58As9 4YF cells, respectively. Note large tumor nodules in the mesentery of mice injected with control mock cells. **c:** Representative appearance of the tumors of 44As3 cells involving rectouterine region was compared. **Yellow** and **red arrowheads** indicate uterine horns and tumor mass, respectively.

rin-B1 4YF may also block the signaling mediated by other members of ephrin-Bs that also bind the similar group of EphB receptors. However, knocking down of ephrin-B1 by siRNA greatly reduced the phosphorylation of total ephrin-Bs (Figure 1b), suggesting that ephrin-B1, but not ephrin-B2 or ephrin-B3, is the major member of B-class ephrin, which is phosphorylated in these cells. Consistent with this conclusion, at least treatment of 44As3 and 58As9 cells with ephrin-B2 siRNA did not affect the phosphorylation level of the corresponding tyrosine residue of ephrin-Bs examined by anti-ephrin-B1 pY317 antibody (data not shown).

The ephrin/Eph interaction provides both ephrin-B1-mediated reverse signaling and EphB-mediated forward signaling. The expression of ephrin-B1 4YF may suppress the invasion of gastric cancer cells by stimulation of EphB receptor-mediated forward signaling in cancer cells through acting as a stimulator of EphB receptors, because EphB receptor-mediated forward signaling inhibited migration of colorectal tumors in a recent report.[24] However, cancer cell invasion was also inhibited by treatment with ephrin-B1 siRNA, which should cause reduction of forward signaling. Therefore, such inhibitory effect of forward signaling caused by the overexpression of the ligand, even if it exists, does not seem to be strong. In addition, we previously observed that overexpression of ephrin-B1 in Panc1 and Capan1 pancreas cancer cells, in which EphB2 is endogenously expressed, promoted the peritoneal dissemination of these cells by the similar experiment in this study (data not shown), which may also support this conclusion.

We observed that ephrin-B1-expressing cancer cells invaded more frequently into the monolayer of fibroblasts expressing EphB2 receptor than into the parent fibroblasts in overlay invasion assay. This result suggests that cancer cells expressing ephrin-B1 may actively invade into the stromal tissues that express EphB receptors, and such mechanism may also be involved in the process of peritoneal dissemination of ephrin-B1-expressing cancer cells *in vivo*. For example, stromal cells composed of mouse mesentery sheets actually expressed cognate receptors for ephrin-B1 (Supplemental Figure 1 at *http://ajp.amjpathol.org*). Again, there is a possibility that EphB2-mediated forward signaling in L EphB2 fibroblasts also contributes to the formation of invasion foci of cancer cells in this overlay assay. However, expression of ephrin-B1 4YF prevented the invasion of 44As3 cells into the monolayer of L EphB2 cells, suggesting that this invasion

Table 1. Mesenterial Dissemination after Intraperitoneal Inoculation of Gastric Cancer Cells

Cell	Number of nodules*		
	0 to 10	10 to 30	>30
44As3 mock	0	5	15
44As3 4YF	14	5	1
58As9 mock	0	2	18
58As9 4YF	16	3	1

Mice were sacrificed at 14 days after inoculation. Data are shown as the number of mice bearing tumors in the mesentery.
*Number of tumor nodules larger than 2 mm in the mesentery.

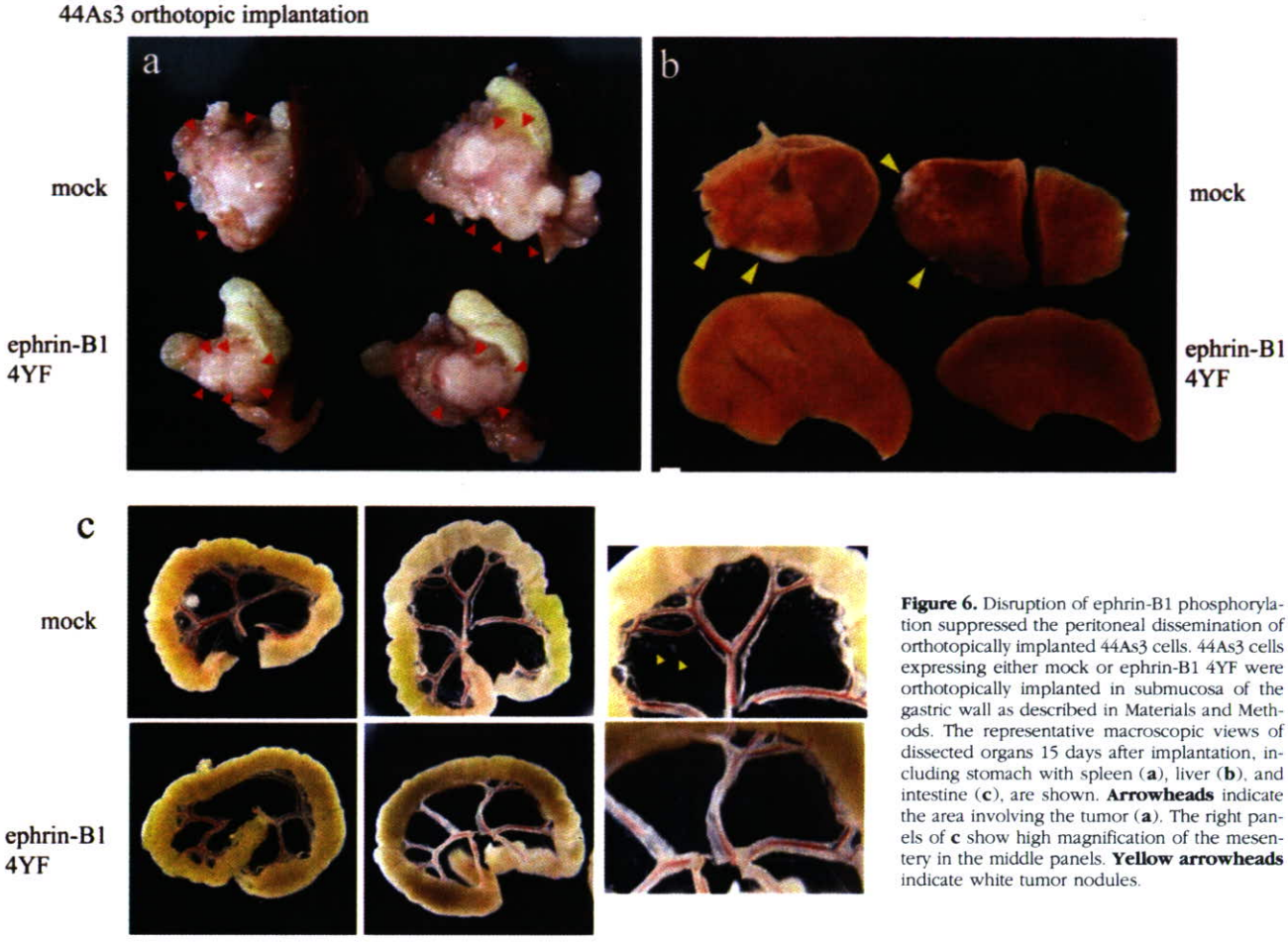

Figure 6. Disruption of ephrin-B1 phosphorylation suppressed the peritoneal dissemination of orthotopically implanted 44As3 cells. 44As3 cells expressing either mock or ephrin-B1 4YF were orthotopically implanted in submucosa of the gastric wall as described in Materials and Methods. The representative macroscopic views of dissected organs 15 days after implantation, including stomach with spleen (**a**), liver (**b**), and intestine (**c**), are shown. **Arrowheads** indicate the area involving the tumor (**a**). The right panels of **c** show high magnification of the mesentery in the middle panels. **Yellow arrowheads** indicate white tumor nodules.

is considered to depend predominantly on the ephrin-B1-mediated reverse signaling in cancer cells rather than the forward signaling in fibroblasts.

Tumor cells expressing ephrin-B1 may gain invasiveness in multiple steps of the cell invasion process. Phosphorylation of ephrin-B1 leads to the recruitment of scaffolding protein disheveled via Grb4, which results in aberrant activation of RhoA.[25,26] Ephrin-B1 makes a complex with Tiam1 to induce Rac1 activation,[20] and we showed blocking of tyrosine phosphorylation of ephrin-B1 decreased the Rac1 activity in this study. Ephrin-B1 is also phosphorylated on tyrosine residues through physical association with cell adhesion proteins such as claudin, which attenuates the cell-cell adhesion.[7] In addition, EphB2-induced phosphorylation of ephrin-B1 has been reported to modify the cell-to-substrate adhesion,[17] although reduction of ephrin-B1 with siRNA did not significantly affect cell adhesion to several substrates *in vitro* under the condition that ephrin-B1 was not stimulated with exogenous EphB2 receptor. Therefore, phosphorylation of ephrin-B1 may regulate cell motility also by affecting the cell adhesion to substrates in the process of stromal invasion of tumors *in vivo*. Blocking of ephrin-B1 phosphorylation by the ephrin-B1 4YF mutant may inhibit tumor invasion through blocking such multiple events.

Table 2. Dissemination at 15 Days after Orthotopic Implantation of 44As3 Gastric Cancer Cells

Cell	Omentum	Liver	Mesenterium
Mock	7/10 (70)	5/10 (50)	7/10 (70)
4YF	2/11 (18)	1/11 (9)	2/11 (18)

Number of mice bearing tumor at the site per total number of mice bearing tumor (%).

We cannot exclude a possibility that phosphorylation-independent signaling of ephrin-B1 is also involved in the regulation of cancer cell invasion. For example, proteins containing PDZ domains make a stable complex with ephrin-B1 via PDZ domain-binding motif -YXV located at the carboxyl terminus of ephrin-B1.[27] It may be important to examine the phosphorylation state of ephrin-B1 *in vivo* in a wide range of cancers to estimate what types of cancers will be sensitive for blocking ephrin-B1 phosphorylation on tumor suppression. For example, phosphorylation of B-type ephrins in invading glioblastoma has been reported recently.[14]

We performed intraperitoneal inoculation of cancer cells into nude mice as a model of peritoneal dissemination of the tumor, as also reported by others.[28,29] Although many gastric cancer cell lines do not show typical histological appearance of human scirrhous carcinoma when inoculated into nude mice, our system of orthotopic transplantation of 44As3 cells is a highly reproducible animal model of peritoneal dissemination of human scir-

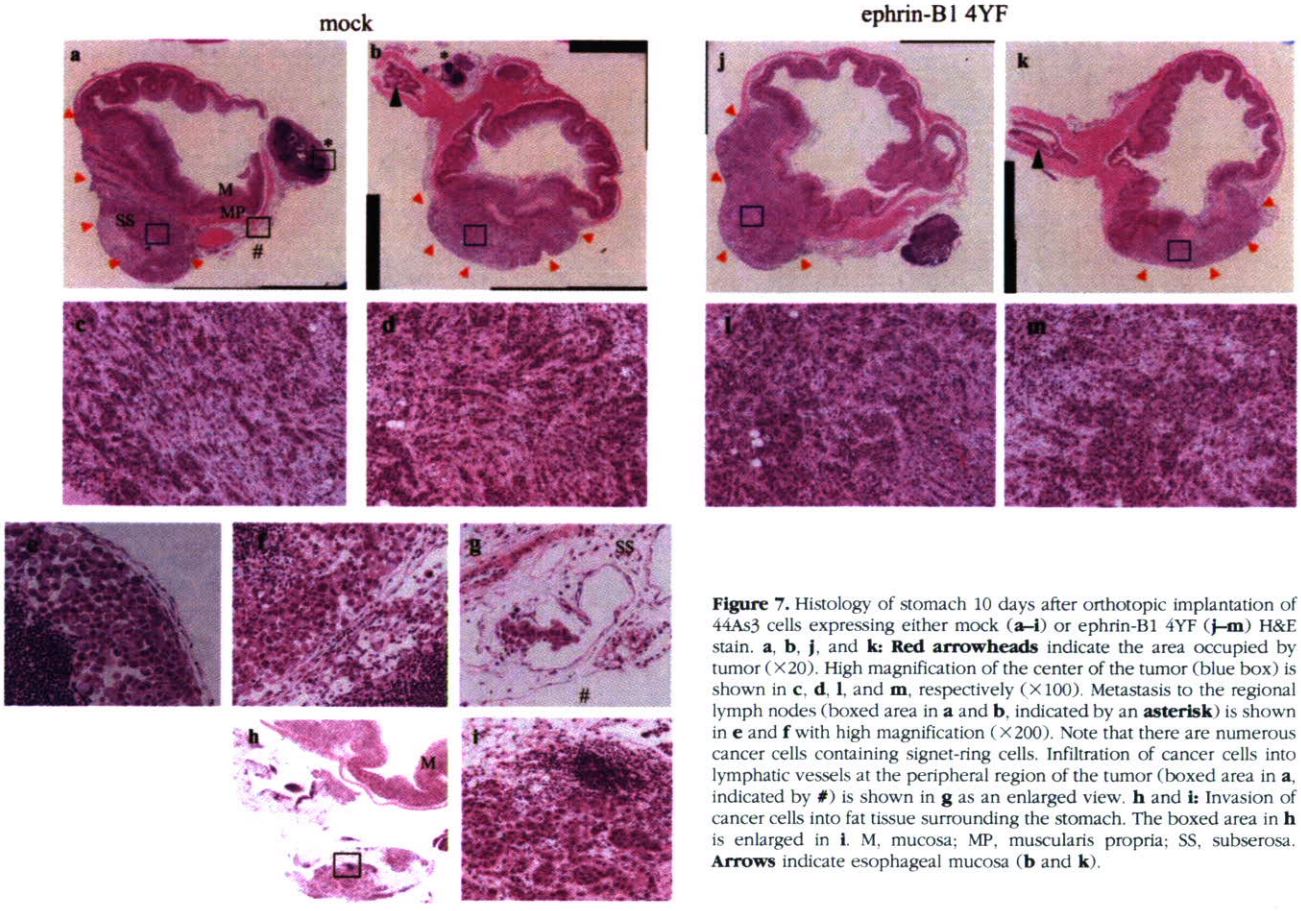

Figure 7. Histology of stomach 10 days after orthotopic implantation of 44As3 cells expressing either mock (**a–i**) or ephrin-B1 4YF (**j–m**) H&E stain. **a**, **b**, **j**, and **k: Red arrowheads** indicate the area occupied by tumor (×20). High magnification of the center of the tumor (blue box) is shown in **c**, **d**, **l**, and **m**, respectively (×100). Metastasis to the regional lymph nodes (boxed area in **a** and **b**, indicated by an **asterisk**) is shown in **e** and **f** with high magnification (×200). Note that there are numerous cancer cells containing signet-ring cells. Infiltration of cancer cells into lymphatic vessels at the peripheral region of the tumor (boxed area in **a**, indicated by **#**) is shown in **g** as an enlarged view. **h** and **i:** Invasion of cancer cells into fat tissue surrounding the stomach. The boxed area in **h** is enlarged in **i**. M, mucosa; MP, muscularis propria; SS, subserosa. **Arrows** indicate esophageal mucosa (**b** and **k**).

rhous gastric carcinoma.[18,19,30] Because early clinical diagnosis of scirrhous gastric carcinoma is difficult, peritoneal dissemination or metastasis to lymph nodes has frequently occurred by the time the diagnosis is made. We observed that expression of ephrin-B1 4YF suppressed not only local invasion of 44As3 cells in the gastric wall but also the infiltration of cancer cells into lymphatic vessels and lymph node metastasis. These results suggest that ephrin-B1 phosphorylation-mediated signaling may modulate the process of the invasion of cancer cells to lymphatic vessels. It may be useful to examine the expression and phosphorylation level of ephrin-B1 in a surgical specimen of scirrhous gastric carcinomas to predict lymphatic metastasis of tumors. Dissemination is a frequent form of the recurrence of scirrhous gastric carcinoma, which serves as a major factor determining the prognosis. Ephrin-B1 is considered to be a prognostic factor of gastric scirrhous carcinoma, and the inhibition of a specific cellular signal originating in ephrin-B1 phosphorylation may be a good candidate for regulating its invasion and dissemination.

Table 3. Histological Analysis of the Stomach 10 Days after Orthotopic Implantation of 44As3 Cells

Cell	ly ves	Lymph node	Perforation
Mock	7/10 (70)	7/10 (70)	8/10 (80)
4YF	2/11 (18)	2/11 (18)	3/11 (27)

Data are shown as the number of mice bearing tumor with lymphatic vessel invasion (ly ves), metastasis to regional lymph nodes (lymph node), or perforation of the gastric serosa and infiltrating to the surrounding fat tissue (perforation) per total number of mice bearing tumor (%).

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CUB Domain-Containing Protein 1 Is a Novel Regulator of Anoikis Resistance in Lung Adenocarcinoma▽†

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Malignant tumor cells frequently achieve resistance to anoikis, a form of apoptosis induced by detachment from the basement membrane, which results in the anchorage-independent growth of these cells. Although the involvement of Src family kinases (SFKs) in this alteration has been reported, little is known about the signaling pathways involved in the regulation of anoikis under the control of SFKs. In this study, we identified a membrane protein, CUB-domain-containing protein 1 (CDCP1), as an SFK-binding phosphoprotein associated with the anchorage independence of human lung adenocarcinoma. Using RNA interference suppression and overexpression of CDCP1 mutants in lung cancer cells, we found that tyrosine-phosphorylated CDCP1 is required to overcome anoikis in lung cancer cells. An apoptosis-related molecule, protein kinase Cδ, was found to be phosphorylated by the CDCP1-SFK complex and was essential for anoikis resistance downstream of CDCP1. Loss of CDCP1 also inhibited the metastatic potential of the A549 cells in vivo. Our findings indicate that CDCP1 is a novel target for treating cancer-specific disorders, such as metastasis, by regulating anoikis in lung adenocarcinoma.

Src family kinases (SFKs) play important roles in various cell functions, including cell proliferation, cell adhesion, and cell migration, under the control of extracellular stimuli (26). Many studies have shown elevated activity of SFKs or increased protein expression in a variety of human cancers (31). The activities of SFKs often correlate with the malignant potential of cancer and poor prognosis (36). SFKs may contribute to various aspects of tumor progression, including uncontrolled proliferation and migration, disruption of cell-cell contacts, invasiveness, angiogenesis, and resistance to apoptosis.

Anoikis is a form of apoptosis triggered by the loss of cell survival signals generated from interaction with the extracellular matrix (10). Anoikis is considered to be physiologically important in the maintenance of homeostasis and tissue architecture (24). On the other hand, the resistance to anoikis acquired during carcinogenesis has been described as a core aspect of cancer cells for tumor progression and metastasis (12). This property indicates the existence of survival signals in tumor cells, which compensate for similar signals supported by cell-matrix interactions. Since they were originally described by Frisch and Francis (9), several previous reports have shown the crucial role of SFKs in the anoikis resistance of tumor cells. Viral Src oncoprotein abrogates anoikis in epithelial cells (13). Src activation is also important for resistance to anoikis in various cancers, such as colon tumor and lung adenocarcinoma cells (33, 35). However, the exact mechanism that is responsible for the anoikis resistance mediated by SFKs in human cancer cells has not been clearly elucidated.

The purpose of this study, therefore, was to identify the key molecules of anoikis resistance, which mediate signals from activated SFKs in human cancer cells. For that purpose, we analyzed proteins binding to SFKs with and without cell attachment in a number of human lung cancer cell lines. We found that tyrosine phosphorylation of a 135-kDa SFK-binding protein is associated with elevated anchorage independence in a group of lung cancer cell lines, especially in a cell suspension condition. This 135-kDa phosphoprotein was purified and identified as CUB-domain-containing protein 1 (CDCP1) by mass spectrometry. The protein CDCP1 is a type I transmembrane protein that has possible roles in cell-cell and cell-matrix adhesion (3, 5). The molecule has been reported to be highly expressed in lung, breast, and colon cancers (6, 28). Using an RNA interference (RNAi) technique, it was determined that CDCP1 is required for the survival of lung cancer cells both in suspension culture and in soft agar. This study identifies a novel modulator that sustains anoikis resistance under the control of SFKs in lung cancer cells.

MATERIALS AND METHODS

Plasmids, antibodies, and reagents. Full-length cDNA of human CDCP1 with a FLAG tag at the C terminus (wild type [WT]) was obtained by reverse transcription-PCR amplification from the mRNA of A549 human lung adenocarcinoma cells and cloned into pcDNA3.1 (Invitrogen). The cytoplasmic-domain mutants of CDCP1, Y734F (Tyr734 to Phe), Y762F (Tyr762 to Phe), and Y2F (Y734 and Y762 double mutant), were generated by PCR using the overlap extension method of Ho et al. (14). The C2 domain of protein kinase Cδ (PKCδ) corresponding to amino acids (1 to 160) with a hemagglutinin (HA) tag at the C terminus was obtained by PCR and cloned into pcDNA3.1 (Invitrogen). To express the Fyn Src homology 2 (SH2) domain fused with glutathione *S*-transferase (GST) protein (GST-FynSH2), a cDNA fragment of the Fyn SH2 domain corresponding to nucleotides 1018 to 1299 of the reported sequence (GenBank accession number NM 002037) was amplified by PCR and cloned into pGEX4T2 (Amersham Pharmacia).

Antiphosphotyrosine antibody (4G10) and anti-c-Src antibody (clone GD11) were purchased from Upstate Biotechnology. Anti-Akt antibody, anti-phospho-Akt (Ser473) antibody, anti-ERK1/2 antibody (p44/p42 mitogen-activated protein kinase [MAPK] antibody), anti-phospho-ERK1/2 antibody (phospho-p44/p42 MAPK [Thr202/Tyr204] antibody), anti-p38MAPK antibody, anti-phospho-

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