

Involvement of c-Src in Carcinoma Cell Motility and Metastasis

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Carcinoma cells exhibit dysfunction/dysregulation of cell adhesion systems that correlates with their abilities to migrate, invade, and metastasize. Here we show that the tyrosine kinase c-Src is required for motility and metastasis of two carcinoma cell lines. Adherent KYN-2 cells having a high level of c-Src kinase activity become scattered, extend lamellipodia, and exhibit high motility. Expression of a dominant-negative mutant form of c-Src caused formation of stress fibers and focal adhesions, and markedly reduced motility. HCT15 cells extended lamellipodia and became scattered in response to lysophosphatidic acid stimulation in parallel with transient activation of c-Src, which was inhibited by expression of a dominant-negative mutant form of c-Src or treatment with a specific Src kinase inhibitor. Furthermore, implantation of dominant-negative c-Src transfectants into the peritoneal cavity of SCID mice resulted in reduced peritoneal dissemination compared with control transfectants. These findings indicate that c-Src activation is critically involved in carcinoma cell migration and metastasis.

Key words: c-Src — Rho — Cell motility — Metastasis

Cell-cell and cell-substratum adhesion systems connected to the actin cytoskeleton play important roles in normal development, differentiation and regeneration. Dysfunction/dysregulation of these cell adhesion systems and actin cytoskeletal organization has been correlated with tumor development and progression, particularly with invasion and metastasis.^{1–3} Cadherins and integrins have been found to be major players regulating cell adhesiveness to other cells or to the substratum, and the interaction of these cell adhesion molecules regulates carcinoma cell motility and invasiveness.³ Both cadherins and integrins interact with several different cytoskeletal proteins and signaling molecules, forming multimolecular complexes, but the regulation of these molecules in the process of cell migration is poorly understood, especially in epithelial cells.^{4–6} We recently reported that cell motility, which is mediated by the small guanosine triphosphatase Rho and its downstream effector, Rho-associated kinase, through actin reorganization, is critically involved in intrahepatic metastasis of hepatocellular carcinoma cells.⁷ One highly metastatic and motile hepatocellular carcinoma cell line, KYN-2, formed trabecular structures mediated by tight cell-cell adhesion in suspension, but dissociated and migrated when attached to collagen.³ We found that c-Src was activated by cell adhesion to the substratum. c-Src belongs to a family of cytoplasmic tyrosine kinases, and the role of its oncogenic form, v-Src, in cellular transformation has been well characterized. Despite the potent action of v-Src, the normal cellular function of the proto-

oncogene *c-Src* is poorly understood.⁸ Several studies have reported potential roles of c-Src in cell proliferation, the functions of osteoclasts, and cell adhesion, spreading and migration.^{8–10} Studies of several types of human carcinoma tissue have shown a strong association between c-Src activity and advancing tumor stage and metastasis.^{11, 12} To assess directly the role of endogenous c-Src in carcinoma cell migration and metastasis, we introduced a dominant-negative mutant form of c-Src into highly motile KYN-2 cells and also into HCT15 cells, which become motile in response to lysophosphatidic acid (LPA) stimulation. The results indicated that c-Src is critically involved in migration of both types of carcinoma cell, and also in peritoneal dissemination.

MATERIALS AND METHODS

Cell culture The human HCC cell line KYN-2¹³ was kindly provided by Dr. M. Kojiro (Kurume University, Kurume). The human colon cancer cell line HCT15 was obtained from the American Type Culture Collection. The cells were cultured on collagen-coated dishes in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Before stimulation with LPA (2 µM) (Sigma Chemical, St. Louis, MO), HCT15 cells were starved overnight by replacing the serum-containing culture media with serum-free media. Src kinase inhibitor PP1 (Calbiochem-Novabiochem Corp., Nottingham, UK) was added 30 min prior to LPA stimulation and was present during stimulation.

Cell motility was analyzed by recording time-lapse phase-contrast images from 3 to 9 h after plating, as

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reported previously.⁷⁾ Motile cells were defined as those that had moved their whole body completely out of the initial area within 6 h.

cDNA transfection For stable transfection of dominant-negative c-Src, 5 μ g of pUSE-plasmid containing the complete mouse cDNA of dominant-negative c-Src¹⁴⁾ (two mutations Lys296Arg and Tyr528Phe, provided by UBI, Lake Placid, NY) or empty vector as a control were transfected using Superfect Transfection Reagent (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Geneticin-resistant clones were isolated and screened for enhanced c-Src protein expression and neomycin phosphotransferase II expression in immunoblots.

Immunocytochemistry Cells were cultured on glass coverslips coated with 30 μ g/ml collagen type I, fixed with 4% paraformaldehyde and 2% sucrose in phosphate-buffered saline and permeabilized with 0.1% Triton X-100. Then the cells were treated with 2% normal swine serum and incubated with mouse monoclonal anti-vinculin antibody (Sigma Chemical) overnight at 4°C, followed by incubation with FITC-labeled secondary antibody (Vector Laboratories, Burlingame, CA). Actin filaments were visualized with TRITC (tetramethyl rhodamine iso thiocyanate)-labeled phalloidin (Sigma Chemical). Labeled cells were mounted in Perma Fluor (Lipshow Immunon, Pittsburgh, PA) and examined with a Zeiss LSM410 confocal microscope (Carl Zeiss, Thornwood, NY).

Immunoblotting For immunoblotting, cells were lysed for 20 min at 4°C with a buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin and 2 μ g/ml pepstatin A. Lysates were cleared by centrifugation and protein concentration was determined with the Lowry assay (Bio-Rad Laboratories, Hercules, CA). Cell lysates were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilon membranes (Millipore, Bedford, MA). After blocking, the filters were reacted with primary antibody followed by horseradish peroxidase-conjugated secondary antibodies. Peroxidase-labeled bands were visualized with an enhanced chemiluminescence detection system (Amersham International, Buckinghamshire, UK). Antibodies used were mouse monoclonal anti-c-Src and rabbit polyclonal anti-neomycin phosphotransferase II (UBI).

In vitro kinase assay Cells were lysed and immunoprecipitated with anti-c-Src antibodies as described previously.³⁾ The tyrosine kinase activity of immune complexes containing c-Src was determined with a TaKaRa Tyrosine Kinase Assay kit (TaKaRa Shuzo, Shiga) according to the manufacturer's instructions.

Animal experiments SCID mice were obtained from Clea Japan Inc. (Tokyo) and maintained in a specific pathogen-free environment. The animals received humane

care, and the studies were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Five- to 6-week-old male mice were used in this experiment. Single cell suspensions of HCT15 cells (2×10^6 cells/0.2 ml) were injected into the peritoneal cavity, and 3 weeks later, autopsies were performed and the number of tumors were counted macroscopically; all tumors were then processed for histological examination.

RESULTS

Involvement of c-Src in constitutive motility of KYN-2 cells

We recently showed that KYN-2 cells formed trabecular structures in suspension, but lost E-cadherin-mediated cell-cell adhesion, became scattered and showed motility even without serum when attached to collagen.³⁾ These changes were suggested to be mediated by c-Src activation, because tyrosine dephosphorylation of c-Src was observed in adherent cells and the tyrosine kinase activity of c-Src was approximately two-fold higher in adherent cells than in suspended cells.³⁾ To examine whether c-Src is required for this constitutive motility of adherent KYN-2 cells, we stably transfected KYN-2 cells

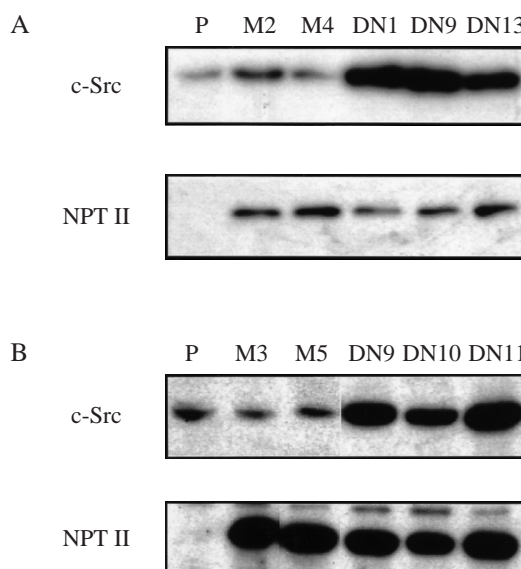


Fig. 1. Establishment of dominant-negative c-Src transfectants and mock transfectants. A, KYN-2 cells and B, HCT15 cells. Immunoblot analysis of c-Src and neomycin phosphotransferase II (NPT II) shows enhanced c-Src protein expression in dominant-negative c-Src transfectants (DN1, DN9 and DN13 of KYN-2 cells and DN9, DN10 and DN11 of HCT15 cells) and neomycin phosphotransferase II expression in dominant-negative c-Src transfectants and mock transfectants (M2 and M4 of KYN-2 cells and M3 and M5 of HCT15 cells) compared with each parent cell line (P).

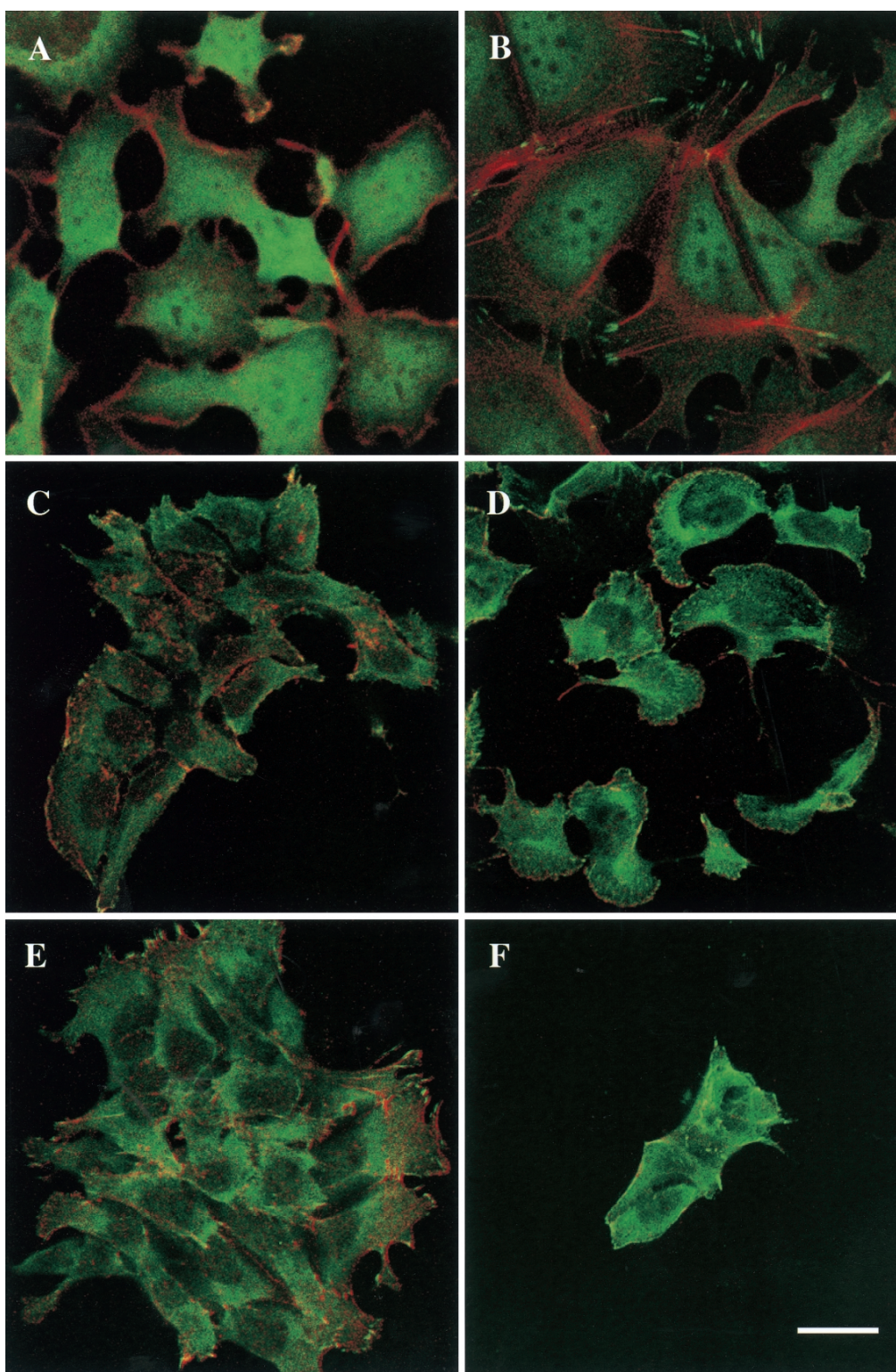


Fig. 2. Confocal fluorescence microscopy showing the localization of vinculin (green) and actin filaments (red) in KYN-2 (A and B) and HCT15 (C to F) cells. A, Mock transfectants and B, dominant-negative c-Src transfectants of KYN-2 cells. C, Mock transfectants of HCT15 cells and D, at 60 min after LPA stimulation. E, Dominant-negative c-Src transfectants of HCT15 cells at 60 min after LPA stimulation. F, Parent HCT15 cells at 60 min after LPA stimulation pretreated with 20 μ M PP1. Bar, 25 μ m.

with a dominant-negative form of c-Src, in which the ATP binding site is inactivated (K296R mutation) and the intramolecular interaction of the SH2 domain is prevented (Y528F mutation).¹⁴⁾

Three dominant negative c-Src transfectants and two mock transfectants were established and analyzed (Fig. 1A). After plating on collagen, all dominant-negative and mock clones attached in 5 to 10 min and spread almost completely within 1 h, in a similar way to parent cells, indicating that c-Src had little effect on cell adhesion and spreading in this cell line (data not shown). Conversely, the morphology of the dominant-negative clones was quite different from that of the mock clones and parent cells (Fig. 2). Mock clones extended lamellipodia with ruffling and had relatively rounded margins. Confocal microscope imaging showed accumulation of actin in the cell periphery, no stress fibers and no obvious focal adhesions (Fig. 2A). In contrast, dominant-negative clones were polygonal and showed no membrane ruffling. Confocal microscope imaging showed formation of stress fibers and distinct focal adhesions, and no accumulation of actin in the cell periphery (Fig. 2B).

The phenotype of the mock clones was characteristic of motile cells and similar to that of the parent cells described previously, and that of dominant-negative clones appeared to be characteristic of stable cells. Therefore, cell motility was analyzed by recording time-lapse phase-contrast images. Mock clones migrated randomly and 73% of the cells were motile, whereas only 20% of dominant-negative clones were motile.

The effect of the Src family kinase inhibitor PP1 was analyzed using KYN-2 parent cells. At the concentration of 10 μ M, PP1 induced rounding of KYN-2 cells, probably because of nonspecific effects, and the involvement of c-Src in cell motility was not evaluable by this approach.

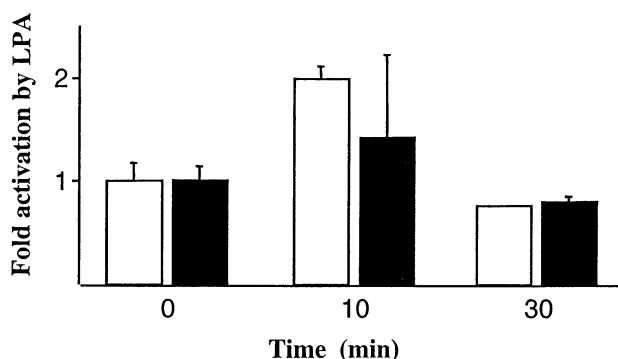


Fig. 3. Stimulation of c-Src kinase activity by LPA in HCT15 cells. Tyrosine kinase activity of immunoprecipitates collected with anti-c-Src antibody was analyzed as described in "Materials and Methods." Open bars and solid bars represent independent experiments carried out in triplicate.

Involvement of c-Src in LPA-mediated motility of HCT15 cells Unlike the KYN-2 cells described above, several types of carcinoma cells form cohesive colonies even under adherent conditions, and become scattered in response to several soluble factors.⁷⁾ In the case of HCT15 cells, LPA induces extension of lamellipodia within 10 min and scattering after 30 min. The involvement of c-Src in this LPA-mediated motility was examined.

In vitro kinase assay revealed that the tyrosine kinase activity of c-Src is very low in unstimulated control cells (approximately 10-fold less than that of adherent KYN-2 cells when the same amount of each cell lysate was analyzed), became approximately two-fold higher at 10 min after LPA stimulation and returned to the control level in 30 min (Fig. 3). To examine whether this transient activation of c-Src is involved in LPA-mediated motility, three dominant-negative c-Src transfectants and two mock transfectants were established and analyzed (Fig. 1B). Under standard culture conditions, all clones formed cohesive colonies with faint stress fibers and lamellipodia focally in the periphery of the colony (Fig. 2C), and no significant difference was observed between transfectants, probably because of the low level of c-Src activity in unstimulated cells. LPA stimulation induced extension of lamellipodia and scattering of mock transfectants (Fig. 2D), in a similar way to parent cells, but not of dominant-negative transfectants, which remained unchanged morphologically (Fig. 2E).

The Src family kinase inhibitor PP1 at concentrations up to 50 μ M had little effect on cell morphology, growth and viability in HCT15 cells. Addition of 20 to 50 μ M PP1 inhibited LPA-mediated motility almost completely (Fig. 2F), and the inhibitory effect was reversible. Taken together, this evidence indicates that transient activation of c-Src is involved in LPA-mediated motility of HCT15 cells.

Reduced peritoneal dissemination of HCT15 transfectants with a dominant-negative mutant form of c-Src

We previously showed that HCT15 cells have a high potential for peritoneal dissemination after intraperitoneal injection into SCID mice.¹⁵⁾ Using this model we analyzed the metastatic potential of dominant-negative transfectants. As shown in Table I, the numbers of disseminated peritoneal tumors were markedly reduced after injection of dominant-negative transfectants compared with mock transfectants. The histology of the disseminated tumors (Fig. 4) revealed that those from mock transfectants showed invasive growth into the surrounding tissues, whereas those from dominant-negative transfectants showed less invasive growth and frequent fibrosis in the border between the tumor and the host tissue. Fibrosis was observed in 21% and 74% of tumors caused by mock transfectants and dominant-negative transfectants, respectively. Invasion into the muscular tissues (Fig. 4B) or pancreas was observed in 5 of 10 mice and 1 of 15 mice

Table I. Peritoneal Dissemination of Mock and Dominant-negative Transfectants of HCT15 Cells

Transfectants ^{a)}	No. of mice	No. of peritoneal tumors per mouse (mean±SD)
M3	5	8.0±4.6
M5	5	8.2±7.9
DN9	5	0.8±0.8
DN10	5	2.8±1.1
DN11	5	1.0±0

a) Two mock transfectants (M3 and M5) and three dominant-negative transfectants (DN9, DN10 and DN11) were analyzed.

injected with mock transfectants and dominant-negative transfectants, respectively. These data indicate that c-Src is involved in peritoneal dissemination of HCT15 cells.

DISCUSSION

In the present study we showed that endogenous c-Src is involved in two types of carcinoma cell motility: constitutive motility of KYN-2 cells and LPA-mediated motility of HCT15 cells. In KYN-2 cells, c-Src was activated under adherent conditions compared with suspended conditions, suggesting that c-Src was activated by integrin-mediated cell-substratum adhesion, as reported previously.^{3, 9, 16)} Motile KYN-2 cells with high c-Src activity formed lamellipodia, and expression of dominant-negative c-Src caused formation of focal adhesions and stress fibers, and inhibition of lamellipodial extension and motility. The mechanism of regulation of integrin-cytoskeletal interactions by c-Src is not fully understood,^{5, 6, 8)} but our present observation suggests that inhibition of c-Src activation stabilizes focal adhesions, which were not obvious in motile parent or mock transfectant cells, probably due to the high turnover of the focal adhesion complexes. It has been reported that migratory defects in cells lacking Src family kinase or focal adhesion kinase reflect an inhibition of focal adhesion turnover, because in each case focal adhesion formation is not impaired.⁶⁾

In HCT15 cells, LPA stimulation induced cell scattering and transient activation of c-Src, which was blocked by expression of dominant-negative c-Src or by addition of the c-Src inhibitor PP1. Several reports have indicated that Rho is involved in the motility of several types of cells,⁷⁾ and c-Src and Rho are believed to cooperate in the regulation of actin cytoskeleton reorganization.^{17, 18)} Inhibition of Rho activity by addition of C3 exoenzyme to HCT15 cells (unpublished observation) and to KYN-2 cells⁷⁾ inhibited the motility of both cell types, indicating that both c-Src and Rho activity are required for their motility.

Cell motility plays an important role in metastasis of carcinoma, and our unpublished observations have shown

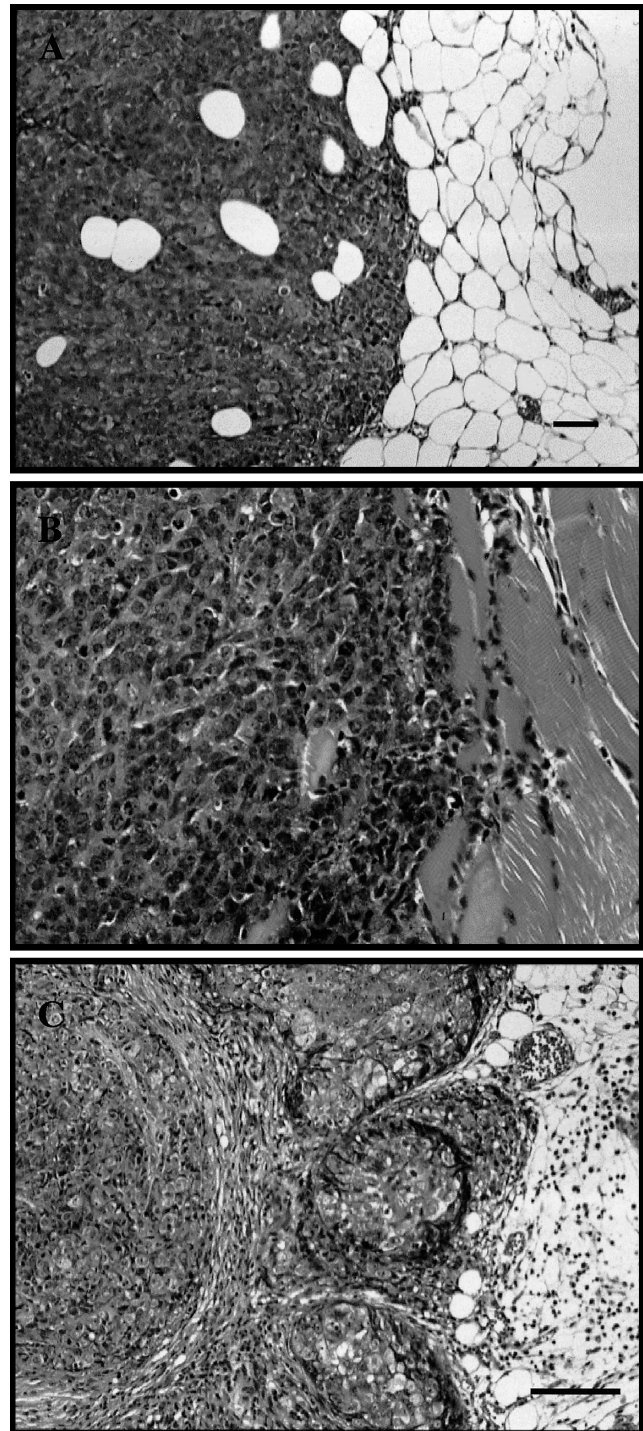


Fig. 4. Histology of disseminated peritoneal tumors caused by mock transfectants and dominant-negative c-Src transfectants of HCT15 cells. A, Mock transfectants, showing invasive growth into the surrounding adipose tissue (note many adipocytes seen within the tumor) and B, muscular tissues. C, Dominant-negative c-Src transfectants showing less invasiveness and surrounded by fibrous tissues at the tumor boundary. Bars, 100 μm.

a good correlation between LPA-mediated motility and peritoneal metastatic potential. In the present study we could reduce peritoneal dissemination of HCT15 cells by expression of dominant-negative c-Src. Histological study of the resulting tumors indicated the less invasive nature of tumors caused by dominant-negative transfectants compared with mock transfectants. Invasiveness *in vivo* is believed to reflect motility *in vitro*,⁷⁾ and inhibition of LPA-mediated motility by dominant negative c-Src should therefore suppress invasiveness *in vivo*. In dominant-negative transfectants, inhibition of c-Src activity did not affect proliferative activity, and therefore it is unlikely that the observed reduction of peritoneal dissemination was mediated by modulation of cell proliferation. Studies of several types of human carcinoma tissue have shown a strong association between c-Src activity and advancing tumor stage and metastasis.^{11,12)} We speculate that this is because

c-Src activity modulates the invasiveness and metastasis of carcinoma cells. In HCT15 cells we could inhibit LPA-mediated motility with the c-Src inhibitor PP1. This suggests the possibility of *in vivo* application of c-Src inhibitors to prevent peritoneal dissemination and also other types of metastasis.

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