The Effects of the Stromal Cell-Derived Cyclooxygenase-2 Metabolite Prostaglandin E₂ on the Proliferation of Colon Cancer Cells

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ABSTRACT

It is well known that tumor-surrounding stromal tissues support tumor development through secreting soluble factors such as various cytokines, chemokines, and growth factors. It has also been suggested that tumor-associated fibroblast and immune cells have a high expression of cyclooxygenase-2 (COX-2) and produce and secrete several prostaglandins (PGs) to adjacent cancer tissues. From these findings, we assumed that COX-2 inhibition might have an anticancer effect on cancer cells even without COX-2 expression in COX-2-dependent mechanisms through blocking the effect of stroma-derived PGs. Here, because of the complex involvement of various factors in vivo, we investigated this possibility with an in vivo-mimicking model using a Transwell system. To test our hypothesis, we used COX-2-transfected cell lines as stromal cells in our model. When we cocultured cancer cells (LS174T cells without COX-2 expression) with COX-2-high stromal cells in the Transwell membrane system, we observed that the proliferation of cancer cells was promoted and vascular endothelial growth factor synthesis was up-regulated significantly. These effects were blocked completely by COX-2 inhibitors and phosphoinositide-3-kinase inhibitors and partially by the PG E₂ receptor 4 antagonist. Even if some cancer cells did not express COX-2, they were found to have expression of PG receptors and PG-related downstream signaling molecules associated with cell viability. Our observation suggests that these cells can be influenced by PGs derived from stromal tissues. These findings also suggest that COX-2 inhibitors can be used to control the interaction between cancer and surrounding stromal tissues and suppress the proliferation of cancer cells regardless of the expression of COX-2 in cancer cells.

INTRODUCTION

Cyclooxygenase-2 (COX-2) has been described as modulating cell viability and invasiveness (such as cell proliferation, apoptosis, angiogenesis, migration, and metastasis) mainly in solid tumors and more recently in hematological malignancies (de Souza Pereira, 2009; Jang et al., 2009; Ozuysal et al., 2009). A good deal of available clinical data has established the protective effect of COX-2 inhibition on human cancer progression (Li et al., 2007; Kurihara et al., 2009; Lanas and Ferrandez, 2009). However, despite these encouraging outcomes, the various actions of COX-2 in many different cancers remain a major hurdle for the general application of COX-2 inhibitors as effective cancer drugs (Borer, 2006; Abdel-Tawab et al., 2009). Hence, a better understanding of the molecular signals downstream of COX-2 is needed for the elucidation of drug targets that may improve cancer therapy.

ABBREVIATIONS: COX-2, cyclooxygenase-2; PG, prostaglandin; VEGF, vascular endothelial growth factor; PI3K, phosphoinositide-3-kinase; 2',5'-dA, 2',5'-dideoxyadenosine; EP1, prostaglandin E₁ receptor 1; EP2, prostaglandin E₂ receptor 2; EP3, prostaglandin E₃ receptor 3; EP4, prostaglandin E₄ receptor 4; DP1, prostaglandin D₁ receptor 1; DP2, prostaglandin D₂ receptor 2; FP, prostaglandin F₂α receptor; TP, thromboxane A₂ receptor; PKA, cAMP-dependent protein kinase; PDK1, phosphoinositide-dependent kinase-1; EIA, enzyme immunoassay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; AH23848, [1α,2β,3β,5α]-7-[5-[[1,1'-biphenyl]-4-yl][methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid; L-161982, N-[[4'-[3-butyl-1,5-dihydro-5-oxo-1-[2-( trifluoromethyl)phenyl]-4H-1,2,4-triazol-4-yl][methyl][1,1'-biphenyl]-2-yl][sulfonyl]-3-methyl-2-thiophencarboxamid; MDL-12330A, cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide; Dup-697, 5-bromo-2-(4-fluorophenyl)-3-(4- methysulfonlphenyl)thiophene.
At present, there is growing evidence that the interaction between cancer cells and surrounding stroma (notably fibroblasts) plays a critical role in cancer growth, invasion, metastasis, angiogenesis, and chemoresistance (Liao et al., 2009; Pietras and Ostman, 2010; Sharma et al., 2010). Many mechanisms have been reported to explain the cancer cells’ ability to survive the attack of immune cells (Grimm et al., 2010; Yigit et al., 2010). It has also been found that several immune cells can promote the development of some cancers by changing their characteristics from cancer-killing to cancer-promoting by means of unknown soluble factors released from cancer cells (Kataki et al., 2002; Lamagna et al., 2006). Several observations have attracted our attention to stromal cells such as cancer-associated fibroblasts, which seem to interact with cancer cells in various levels of cancer development (De Wever et al., 2008). Despite the importance of cancer-stromal interactions in cancer progression, their underlying molecular mechanisms have not been well characterized, partly because of their diversity and complexity. The identification and characterization of genes/pathways involved in cancer-stromal interactions can identify targets for novel therapeutic strategies.

Several researchers have reported the key role of COX-2 in the cancer microenvironment using the cancer-stromal interaction in various cancer models and found that the cancer growth and progression-promoting effects of stroma are caused at least in part by increased COX-2 expression in cancer, provoked by their interaction with stromal cells such as fibroblasts (Hu et al., 2009). In addition to the importance of COX-2 up-regulation in cancer cells themselves from the interactions of cancer-stromal cells, researchers have observed that stromal cells such as cancer-associated fibroblasts express COX-2 endogenously and play carcinogenic roles by providing their prostaglandins (PGs) to cancer tissues in a paracrine route (Vandoros et al., 2006; Konstantinopoulos et al., 2007).

Previously, using a mouse syngenic model, we suggested the possible residual tumor-promoting mechanism of inflammatory environments from wound healing after clinical surgery (Roh et al., 2004). We assessed that COX-2 inhibition retarded wound-induced tumor growth by blocking the inflammatory condition of the wound, which would otherwise be able to indirectly promote tumor development. In addition, we assumed the possibility of direct promotion of cancer cell viability by PGs from wound-related inflammatory condition. However, we could not perform further study on the concise mechanism because of the complex involvement of various factors in vivo.

From these findings, we developed an interest in the idea that COX-2 inhibition could be a promising cancer therapy even in cancer cells with no endogenous PGs because of genetically silenced COXs (by promoter methylation and other epigenetic modifications), if the viability of cancer cells was promoted by PGs provided by the stroma. In particular, in cancer cells whose PG-related machineries exist and may mediate normal PG signaling, the cancer cells’ viability may be affected and maintained by stromal PGs, although their COXs are not expressed in themselves and cannot be induced by exogenous inducers. In that case, COX-2 inhibition may be useful in the treatment and prevention of cancers, regardless of their status in terms of COX-2 expression.

This study is an attempt to identify this possibility through an in vivo mimicking model at the molecular level. First, we found cancer cells with little PG but good responses to exogenous PGs and confirmed normal expression of several PG receptors in our cell model. Then, through coculture experiments using a Transwell system, we demonstrated the possibility that COX-2 inhibition may show anticancer effects even in cancers without COX-2 activity by blocking only PGs derived from stromal cells.

### Materials and Methods

#### Cell Culture.
Human colon cancer cell lines (LS174T, HCT15, HT29, HCT116, DLD1, SW480, and LOVO) were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). 293T is a cell line from human embryonic kidney. Cells were maintained at 37°C in a humidified, 5% CO₂, 95% air atmosphere and routinely subcultured using trypsin–EDTA (0.25% w/v). Unless otherwise stated, all cell culture reagents were obtained from Gibco BRL (Grand Island, NY).

#### Chemicals.
COX-2-specific inhibitors (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) and 5-bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)phenyl)-thiophene (Dup-697), 1α(Z),2β,5α-17β-7-5-[(1,1′-biphenyl)-4-yl]methoxy]-2-(4-morpholino)-3-oxo-cyclopropylbenzoic acid (AH23848) (an EP4 antagonist), arachidonic acid, and PGE₂ were obtained from Cayman Chemical (Ann Arbor, MI). H929, H11032 (2′5′-dideoxyadenosine (2′,5′-ddA; an adenylyl cyclase inhibitor), cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine (MDL-12330A) (an adenylyl cyclase inhibitor), wortmannin (a PI3 kinase inhibitor), forskolin (an adenylyl cyclase activator), and dibutyryl cAMP (cell-permeable analog of cAMP) were obtained from Calbiochem (La Jolla, CA). N-[4′-(3-butyl-1,5-dihydro-6-oxo-1-[2-(trifluoromethyl)phenyl]-4H-1,2,4-triazol-4-yl-methyl][1,1′-biphenyl]-2-yl]sulfonamido)-3-methyl-2-thiophenecarboxamide (L-161982) (an EP4 antagonist) and L-161983 (an inactive analog of L-161982) were generous gifts from Dr. R. Young and Dr. K. McCusker (Merek Frost Canada and Co, Kirkland, Quebec, Canada). All chemicals were used according to the suggestions provided (IC₅₀ in manufacturer’s information).

#### Quantification of PGE₂ and VEGF Production.
The amount of PGE₂ and VEGF released by the cells was determined using enzyme immunoassay (ELISA) kits for PGE₂ (Cayman Chemical) and VEGF (R&D Systems, Minneapolis, MN) according to the manufacturers’ instructions.

#### Cell Proliferation Assay.
Cells were seeded in 96-well plates, incubated for 24 h at 37°C, and treated with specific drugs for the indicated time at 37°C. After the drug treatment, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma-Aldrich, St. Louis, MO) and cell counting kit-8 (Dojindo, Tokyo, Japan) were used for the investigation of cell proliferation according to the manufacturers’ instructions.

#### Western Blot Analysis.
Denatured protein lysates were resolved by 4 to 12% NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (Whatman Schleicher and Schuell, Dachen, Germany). The membranes were incubated with anti-EP1, EP2, EP3, EP4, DP1, DP2, FP, and TP (Cayman Chemical); anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-PDK1 (Ser241), anti-p-Akt (Ser473), and anti-pan-Akt (Cell Signaling, Danvers, MA); or monoclonal anti-α-tubulin (Sigma-Aldrich) for 2 h at room temperature or overnight at 4°C. Membranes were then washed (four times) with Tris-buffered saline/Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibody (Fierce Chemical, Rockford, IL) for 1 h. Immuneactive proteins were visualized by developing them with Lumi-light Western blotting substrate (Roche Diagnostics, Mannheim, Germany), followed by exposure in a LAS-3000 imager (Fuji Film Co., Tokyo, Japan) according to the manufacturer’s instructions.
Transcription Factor Activation Assay for PKA Activity. The fusion transactivator plasmid (pFA-cAMP response element-binding protein), consisting of the DNA-binding domain of Gal4 (residues 1–147) and the transactivation domains of cAMP response element-binding protein, was purchased from Stratagene (La Jolla, CA). pFA2-db/d, which contains only the DNA-binding domain of Gal4 (residues 1–147), was used as the negative control. Experiments were performed according to the manufacturer’s instructions. Cells were cotransfected with 0.05 μg of fusion transactivator plasmid, 0.5 μg of pPR-Luc plasmid, and 0.3 μg of pSV-β-galactosidase control vector by electroporation, seeded in culture plates, and then incubated for 24 h. Subsequently, cells were treated for an additional 24 h with the indicated doses of PGE2. After a total of 48 h, cells were analyzed using a transcription factor activation assay. Luciferase activity was measured using a TR717 Microplate Luminometer with a Bioluminescent Reporter Gene Assay kit according to the manufacturer’s instructions (Tropix, Bedford, MA). Luciferase activity was normalized in relation to cotransfection with pSV-β-galactosidase control vector.

Transfection. The cells were transfected with the indicated plasmids by electroporation using a Microporator MP-100 (NanoEnTek Inc., Seoul, South Korea), following the protocol provided by the manufacturer.

COX-2-Expressing Plasmids. We used COX-2 cDNA (a gift from Dr. William L. Smith, Department of Biological Chemistry, University of Michigan, Ann Arbor, MI) to establish the pcDNA3.1-expressing vectors (pcDNA3.1-COX-2). Cells were transfected with 0.5 to 1.5 μg of plasmid and seeded in culture plates.

Cell Coculture with Transwell System. 293T cells (used as stromal cells providing PGs) were transfected with 1 μg of pcDNA3.1-COX-2 or LacZ and placed at once in the upper side of a Transwell chamber (NUNC A/S, Roskilde, Denmark) partitioned by a polycarbonate membrane (0.2- to 8.0-μm pore size; Corning Life Sciences, Lowell, MA). Then 293T cells were cocultured with LS174T cells (cancer cells) seeded in the lower part of the Transwell chamber in Dulbecco’s modified Eagle’s medium for 24 h. Subsequently, cells were treated with chemicals as indicated. LS174T cells were applied to the cell proliferation assay (for a total of 96 h).

Analysis of VEGF Promoter Activity in LS174T Cells after Coculture. The VEGF luciferase reporter gene construct was a kind gift of Dr. Su-Hyeong Kim (Seoul National University) (Kim et al., 2006). 293T cells were transfected with 1 μg of pcDNA3.1-COX-2 and seeded in the upper side of a Transwell plate. LS174T cells were transfected with 0.5 μg of VEGF luciferase reporter gene construct and 0.3 μg of pSV-β-galactosidase control vector by electroporation and seeded in the lower parts of the Transwell plate. Then two cells were coincubated for 24 h. Subsequently, cells were treated for an additional 48 h with chemicals as indicated. After a total of 72 h, lysates of LS174T cells were applied to analyze the VEGF promoter activities. Luciferase activity was measured with a TR717 Microplate Luminometer with a Bioluminescent Reporter Gene Assay kit according to the manufacturer’s instructions (Tropix, Bedford, MA). Luciferase activity was normalized in relation to cotransfection with a pSV-β-galactosidase control vector.

Statistical Analysis. The data are presented as the mean ± S.D. of triplicates as representative of three separate experiments. Levels of significance between treated and untreated groups were determined by two-sided Student’s t test. P values < 0.05 were considered statistically significant.

Results

The Promoting Effect of PGE2 on Cell Proliferation and VEGF Synthesis in Colon Cancer Cells. We found cells suitable for our study among colon cancer cell lines whose cell viability was known to be well stimulated by PGs (Sheng et al., 2001). We found that most of the tested cells, except DLD1 and LOVO cells, had low levels of PGE2 (Fig. 1). We observed no COX-1/COX-2 expression in LS174T and HCT15 cells. In addition, PGE2 was not detected in LS174T and HCT15 cells, which showed good response to treatment of PGE2. Treatment of exogenous PGE2 up-regulated VEGF synthesis (by 50–350%) as well as cell proliferation (by 30–250%) in most of the tested cells. In particular, LS174T cells showed the best response to PGE2 (Fig. 2).

Analysis of the Expression of Several PG Receptors in Colon Cancer Cells. Because our study was performed to
show the possible carcinogenic action of PGs in the interaction of cancer and its stroma at the molecular level, we examined the expression of PG receptors to identify precisely the signaling pathways associated with PGs in our models. Using Western blotting analysis for major PG receptors (EP1-EP4 for PGE2, DP1-DP2 for PGD2, FP for PGF2α, and TP for thromboxane A2), we observed that some PG receptors were expressed even in cancer cells with no endogenous expression of COX-2 and COX-1, although various patterns of their expression seemed to occur among cell lines (Fig. 3A).

Activation of the cAMP-PKA Pathway by PGE2 in LS174T and HCT15 Cells. PGE2 is a major PG produced by the COX-2 pathway and is known to play the most important roles in the carcinogenic action of COX-2. EP1 and EP3 of the PGE2 receptors seem to mediate PGE2 action through activating Ca2+ signaling, whereas EP2 and EP4 mediated the physiological action of PGE2 by increasing the cAMP level (Sugimoto and Narumiya, 2007). To show the anticancer effect of blocking PGs secreted from stromal COX-2 more precisely at the molecular level, we worked to identify the PGE2-related signaling pathway in colon cancer cells. From our finding of strong expression of EP4 in the tested cells (Fig. 3A), we examined the involvement of the cAMP-PKA pathway by PGE2-induced activation of EP4 in LS174T and HCT15 cells. PKA was activated by treatment of PGE2 in a dose-dependent manner in both cell types (Fig. 3B), suggesting the possible role of cAMP accumulation in PGE2-promoted cell viability of LS174T and HCT15 cells.

Inhibition of PGE2-Induced Proliferation and VEGF Production by Adenylyl Cyclase Inhibitors. In the presence of exogenous PGE2, 2'-5'-dDA (an inhibitor of adenylyl cyclase) blocked partially PGE2-promoted cell proliferation and VEGF production in LS174T and HCT15 cells. In addition, MDL-12330A (another inhibitor of adenylyl cyclase) reversed up-regulation of cell viability by PGE2 (Fig. 4). Dibutyryl cAMP and forskolin (activators of adenylyl cyclase) promoted VEGF production as well as cell proliferation in a dose-dependent manner in LS174T and HCT15 cells.

Activation of the PI3K-Akt Pathway by PGE2 in LS174T Cells. It has been reported that the PGE2-EP4 interaction can activate PI3K in some situations and PI3K activation plays a key role in PGE2-induced cell viability (Fujino et al., 2003; Leone et al., 2007). Because PGE2-induced cAMP seemed not to be the only key regulator of PGE2-induced cell viability in our cell model (Fig. 4), we investigated whether PI3K activation might be involved in PGE2-induced cell viability in LS174T cells. We observed PGE2-induced PDK1 and Akt activation in LS174T cells (Fig. 5A). Wortmannin (an inhibitor of PI3K-Akt) perfectly blocked PGE2-induced cell proliferation (Fig. 5B) and VEGF production (Fig. 5C) of LS174T cells, suggesting a key role for PI3K activation in the cell viability of LS174T cells induced by PGE2. The inhibition of PI3K decreased the cell viability significantly.
of LS174T cells somewhat, even without treatment of PGE₂, suggesting the important role of PI3K in keeping the basic cell viability of LS174T cells. In our cell model, PGE₂-induced cAMP seemed not to be involved in the activation of the PI3K-Akt pathway by PGE₂. Wortmannin did not block dibutyryl cAMP-induced cell proliferation and VEGF production of LS174T cells (Fig. 5, B and C), and dibutyryl cAMP had no effect on the phosphorylation of PDK1 and Akt (data not shown). Even though PGE₂ activated both cAMP-PKA and PI3K-Akt each pathway seemed to mediate PGE₂-induced cell proliferation and VEGF production independently.

Promoted Cell Viability of LS174T Cells by Coculture with 293T with High Expression of COX-2. Finally, to show that COX-2 in cancer-associated stromal cells may be a promising therapeutic target at the molecular level, we performed a coculture with LS174T cells and COX-2-transfected 293T cells (as stromal cells) in a Transwell system (Fig. 6). The up-regulated expression of COX-2 and PGE₂ by transfection of plasmids expressing COX-2 was confirmed in 293T cells. In this experiment, we focused on the effect of the

COX-2, EP4, and PI3K inhibition on the stromal PGE₂-promoted cell viability of LS174T cells, because we identified the promotion of cell viability of LS174T cells by the COX-2/PGE₂/EP4/PI3K pathway. When LS174T cells were cocultured with 293T-COX-2, cell proliferation of LS174T cells was promoted by 80 to 120% (Fig. 7A) and VEGF synthesis (Fig. 7B) was promoted by 100 to 120%. This effect was blocked significantly by COX-2 inhibitors (NS-398 and DuP-697) and PI3K inhibitors (wortmannin). In addition, EP4 antagonists (L-161982 and AH23848) inhibited stromal PGE₂-induced cell growth (Fig. 8A) and VEGF production (Fig. 8B). However, the inhibition by EP4 antagonists was weaker than that by COX-2 inhibitors, suggesting the roles of other PGs other than PGE₂. The up-regulation of COX-2 expression in LS174T cells by coculture was not observed (data not shown).

Discussion

The overexpression of COX-2 in various cancers is well known (Jang et al., 2009; Ozuysal et al., 2009). The COX-2 and PGs pathways have also been considered as two of the main pathways to mediate the initiation of cancer from inflammatory situations (Giannitrapani et al., 2009; Kraus and Arber, 2009). In addition, fibroblasts and several immune cells have been suggested as cancer-associated stromal cells that promote cancer development in noninflammatory and inflammatory conditions (Kataki et al., 2002; De Wever et al., 2008; Liao et al., 2009). Previous investigations on the interaction between cancer and its stroma have suggested that expression of COX-2 in cancers may be up-regulated by unknown soluble factors derived from cancer-associated stromal cells (Tjiu et al., 2009) and that cancer-derived PGs may be related to cancer-favorable transition of stromal cells.
(Yashiro et al., 2007). From these findings, COX-2 up-regulated in cancer cells seems to be a promising molecular target for cancer treatment and prevention. Furthermore, observations on the carcinogenic role of COX-2 expressed in various cancer-associated stromal cells have increased the value of COX-2 inhibition as a cancer therapy (Adegboyega et al., 2004; Vandoros et al., 2006).

As mentioned in the Introduction, we previously assumed the possible involvement of PGs in promoting viability of cancer cells by various inflammatory factors provided from the wound (Roh et al., 2004). Their underlying mechanism remained unclear because of the complex involvement of various factors in vivo.

Since then, several researchers suggested that PGs might be one of the stroma-derived factors that are involved in cancer initiation and progression. Most articles on the role of COX-2 in tumor-stromal interaction observed that the anticancer effects of COX-2 inhibition resulted from blocking COX-2 in cancer tissues, provoked by their interaction with stromal cells (Hu et al., 2009; Tjiu et al., 2009). It has been also postulated that COX-2 seemed to be important in the viability of the stromal cell itself, as well as in transition from normal stroma to cancer-associated stromal tissues (Adegboyega et al., 2004; Konstantinopoulos et al., 2007). In this study, we assumed that COX-2 inhibition can have a significant anticancer effect through blocking stroma-derived PGs even in some cancer cells with no endogenous synthesis of PGs.

We verified our assumption experimentally at the molecular level with an in vivo-mimicking model using a Transwell system. We identified the expression of PG receptors in cancer cells without endogenous PGs synthesis. We selected LS174T cells with little PGs but good responses to exogenous PGs and established 293T cells transfected with COX-2 as stromal cells. We used them to investigate the underlying mechanisms of the stromal PG-promoted viability of cancer cells in this in vivo-mimicking model. Experimentally, through blocking COX-2 activity in stromal cells and partial PGE2 receptors/PI3K signaling in LS174T cells, we showed that stromal-derived PGs could be crucial factors in promoting the cell viability of LS174T cells. Because in our repeated experiments we did not observe the up-regulation of COX-2 expression in LS174T cells by coculture with 293T cells, cell proliferation and VEGF synthesis in LS174T cells seemed to be promoted by PGs provided from stromal 293T cells. These results clearly support our hypothesis that COX-2 inhibition as a cancer therapy would be useful even for COX-2-negative cancer cells, as well as cancer cells with high activity of COX-2, because of the important traits of COX-2 and PGs as mediators in the interaction between cancer and its stroma. Even cancer cells without COX-2 activity could be targeted by COX-2 inhibition through inhibiting the carcinogenic ac-
tion of stromal PGs. By elucidating cancer models suitable for this application, we can extend cancer targets for COX-2-inhibiting therapy and enhance anticancer effects easily by cotreating COX-2 inhibitors along with other molecular-targeted cancer therapy.

Ultimately, our suggestions may provide an additional theoretical basis for the possibility that prescription of nonsteroidal anti-inflammatory drugs for cancer patients during their treatment may help to prevent recurrence of residual cancer cells. Considering that the underlying molecular mechanisms of cancer-stromal interactions have not been well elucidated because of the diversity and complexity of genes/pathways involved, experimental suggestions such as those of our study using cell coculture models will be considerably helpful in understanding the precise mechanisms of cancer therapy in blocking the cancer-stromal relationship.

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