Mini Review

EP3/EP4 signaling regulates tumor microenvironment formation by bone marrow-derived fibroblasts

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Recently, it has been shown that bone marrow (BM)-derived hematopoietic cells have critical roles in the tumor microenvironment as a major components of tumor stroma and regulate tumor progression. In addition, hematopoietic cells need chemokine signaling for their recruitment. On the other hand, COX-2 and endogenous prostaglandins are important determinants for tumor growth and tumor-associated angiogenesis. However, their precise mechanisms in stromal formation and angiogenesis remain elusive. Our recent data suggest that COX-2 inhibition reduced CXCL12/CXCR4 expression as well as tumor stromal formation, tumor-associated angiogenesis and tumor growth. Consistently, PGE_2 enhanced stromal formation, angiogenesis and CXCL12/CXCR4 expression. Moreover, a COX-2 inhibitor suppressed expression of a fibroblast marker (S100A4) in tumor stroma. These suppressive activities were found by either EP3 or EP4 knockout among 4 PGE_2 receptors. Experiments using GFP-bone marrow chimeric mice revealed that $CXCL12^+CXCR4^+S100A4^+$ fibroblasts dominantly composed stromal cells and most of which were recruited from BM. Additionally, fibroblasts were stimulated to produce CXCL12 by either EP3 or EP4 specific agonist *in vitro*. Therefore, COX-2/PGE_2-EP3/EP4 signaling may play a crucial role in tumor stromal formation and angiogenesis via CXCL12/CXCR4 chemokine system. These results may lead to new approaches in further studies and cancer treatment.

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Introduction

Cancer is a systemic disease with the tumor affecting various systems in the host that can lead to spread of cancer cells such as invasion and metastasis. Primary tumor is composed of a lot of stromal cell types in addition to cancer cells. Recently, it has been shown that tumor stroma is an important regulator of carcinogenesis and a potentially effective therapeutic target. Among the stromal cell types that have been implicated in tumor progression are endothelial cells, pericytes, fibroblasts, and various bone marrow-derived cells (BMDCs), including macrophages, neutrophils, mast cells, myeloid cell-derived suppressor cells (MDSCs) and mesenchymal stem cells (MCSs)[1]. Macrophages and fibroblasts are the major components of the tumor stroma in addition to endothelial cells and they play pivotal roles in tumor-associated angiogenesis[2]. BMDCs are the major components of the tumor stroma and they determine the tumor microenvironment[1,3-8]. However, it remains unclear how they are recruited from BM into the blood and back as well as to different tissues, and what regulates the final differentiation and function of BMDCs at specific sites. Chemokine systems play important roles not only in leukocyte trafficking during inflammatory processes but also in cancer progression[9-12]. Experimental models suggest that several chemokine receptor antagonists can inhibit cancer growth either directly or indirectly through influence on the tumor stroma[13-18].

Cyclooxygenase (COX)-2 is one of two forms of COX, and is expressed at sites of inflammation and malignancies, which suggests that a COX-2 inhibitor may be available in the treatment or prevention of inflammatory diseases and tumors[19]. Actually, tumor-associated angiogenesis and tumor growth were suppressed by inhibition of the COX-2/VEGF-dependent pathway in mice[20,21], and COX-2-lacking mice were resistant to the development of colorectal neoplasia[22]. In clinical, non-steroidal anti-inflammatory drugs (NSAIDs), the prototypic inhibitors of COX, contributed the prevention of several types of cancer[23]. PGE_2 may be a relevant endogenous prostaglandin (PG), which is generated from arachidonic acid and other polyunsaturated fatty acids, in a reaction catalyzed by COX-2[24]. PGE_2 receptors are composed of four G protein-coupled receptors (GPCRs), designated as EP1, EP2, EP3, and EP4[25]. Either COX-2 inhibition or EP3 receptor knockout markedly inhibited the stromal formation around the tumors besides the attenuation of angiogenesis[20,21], suggesting that COX-2-derived PGE_2 has a crucial role in tumor stroma formation and tumor-associated angiogenesis.

In this minireview, we will present our recent study on the critical roles and the mechanisms of COX-2-derived PGE_2 in tumor stromal formation and PG-dependent tumor angiogenesis.

A COX-2 inhibitor suppressed CXCL12/CXCR4 expression in tumor stroma and recruitment of BM cells to the tumor stroma

We first analyzed effects of COX-2 inhibition on tumor stromal formation and host-side chemokine systems using tumor-bearing mice model subcutaneously injected with high-tumorgenic Lewis lung carcinoma (LLC) cells. Celecoxib, a COX-2 inhibitor administration (100 mg/kg/day) suppressed tumor growth, angiogenesis (as gauged by measurements of microvessel density (MVD) and microvessel area (MVA), and and the formation of stromal tissues (stromal thickness). Among the various chemokines (CCL2, 3, 4, 5, 6, 7, 8, 9, 12 and CXCL12) and chemokine receptors (CCR1, 2, 3, 5, 6, 7, 8, CXCR4 and CX3CR1), mRNA levels of CXCL12 (also known as stromal cell-derived factor-1, SDF-1) and its receptor CXCR 4 were significantly reduced in tumor stroma by Celecoxib (Figs. 1A and B). Substantial expression of the other chemokines and chemokine receptors was detected in the stromal tissues, but Celecoxib did not alter the mRNA levels of them. Consistently, CXCL12 protein expression was remarkably suppressed in tumor stroma (Fig. 1C), suggesting that the CXCL12/CXCR4 chemokine system may be involved in COX-2/PGE_2-dependent tumor stromal formation. Additionally, CXCR7 was recently deorphanized alternative receptor for CXCL12, and suggested that CXCR7 expression on cancer cells may promote tumor progression[26]. However, its expression in the tumor stroma was not significantly affected by COX-2 inhibition in our study[27]. Therefore, the precise mechanism of CXCL12 acting pathway might be different between tumor cells and stromal cells.

Moreover, CXC chemokines containing an ELR motif (glutamic acid-leucine-arginine) are thought to

promote angiogenesis, whereas CXC chemokines lacking this sequence are considered to be angiostatic (with the exception of CXCL12)[12,28]. In our study, the gene expression of angiostatic CXCR3 chemokine family was increased by COX-2 inhibition, while those of angiogenic CXCR2 chemokine family were not affected. These results may imply suppressive COX-2 activity against angiostatic CXCR3 chemokine family in host-side tumor stroma.

To evaluate the origin of tumor stromal cells, we used BM chimeric mice by transplantation of BM from GFP-positive mice to lethally irradiated (9.0 Gy) WT mice. Celecoxib treatment significantly reduced the tumor stromal fluorescence intensity and the mRNA levels of GFP[27]. These results suggest that COX-2-derived PGs regulate BM cell recruitment to the tumor stroma.

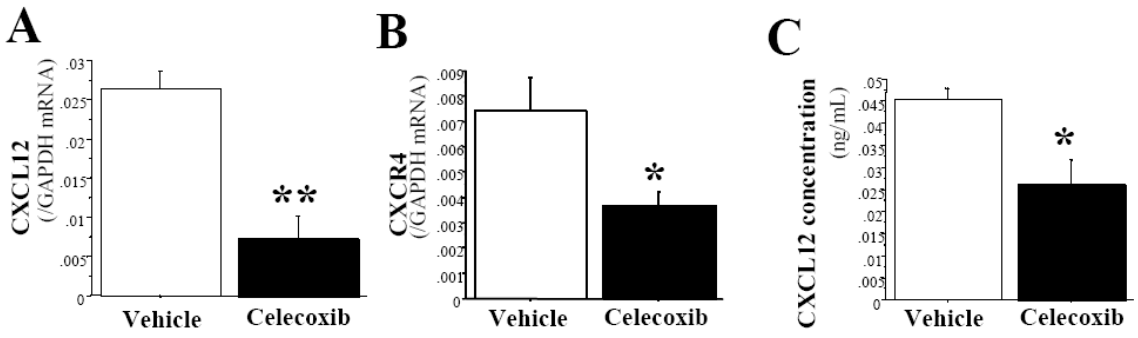

Fig. 1. Effects of COX-2 inhibitor in the tumor-bearing model. (A and B) The mRNA expression of CXCL12 and CXCR4 in tumor stroma on day 14. (C) CXCL12 protein expression in tumor stroma on day 14. n=10 per group. The results represent means ± SEM. *P<0.05; **P<0.01 by Student's t-test (compared with vehicle-treated mice on the same day). (Reprinted from Am J Pathol 2010, 176:1469-1483 with permission from the American Society for Investigative Pathology.)

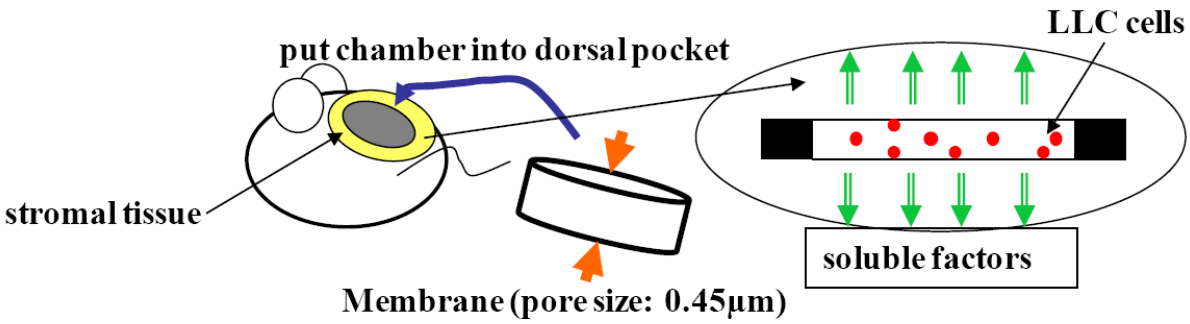

Fig. 2. Schematic representation of micropore chamber model.

In line with the above results, topical application of PGE_2 enhanced angiogenesis and stromal thickness in the Matrigel™ implantation model. This model is characterized by the formation of the granulation tissues around the gel implants[29], and is designed to mimic tumor-associated angiogenesis. PGE_2 also increased the expression of CXCL12 and CXCR4[27]. These results suggest that COX-2-derived PGE_2 actually enhances stromal formation and angiogenesis.

COX-2-dependent enhancement of stromal formation and angiogenesis around micropore chambers, and CXCL12/CXCR4 signaling involvement

To discriminate stromal cells from tumor cells, we used micropore chamber model which was filled with LLC cells and allowed the passage of only so-

luble factors through the chamber membranes as shown in Fig. 2. Similarly with tumor-bearing model, Celecoxib remarkably suppressed granulation tissue formation, which mimics the tumor stromal reaction, and angiogenesis (MVD and MVA) around the LLC-containing chambers. COX-2 inhibition also decreased the gene expression of CXCL12 and CXCR4 in the granulation tissues. CXCL12 protein level was reduced by COX-2 inhibition in ELISA assay for CXCL12. These results suggest that COX-2 facilitates not only tumor-associated angiogenesis, but also stromal formation, and that the CXCL12/CXCR4 axis may be involved in these effects.

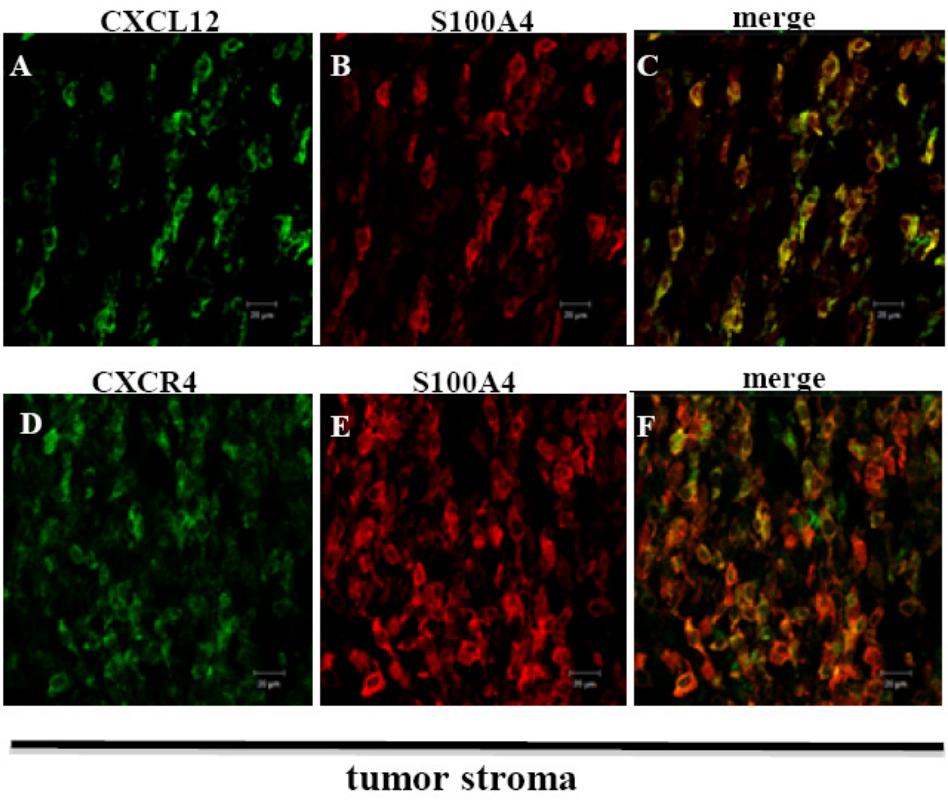

Fig. 3. Double labeling analysis of CXCL12 (A-C) or CXCR4 (D-F) with S100A4, F4/80 or CD3ε in tumor stoma 14 days after tumor implantation. Among the CXCL12-positive cells in the tumor stroma, 94.4% are S100A4 positive (A-C), 6.4% are F4/80 positive and 0% are CD3ε positive (data not shown). Among CXCR4-positive cells, 87.8% are S100A4 positive (D-F), 5.8% are F4/80 positive, and 0% are CD3ε positive (data not shown). Most $CXCL12^+$ or $CXCR4^+$ stromal cells were S100A4-positive fibroblasts. (Reprinted from Am J Pathol 2010, 176:1469-1483 with permission from the American Society for Investigative Pathology.)

To identify which type of cells have pivotal role in tumor stromal formation, major components (fibroblasts, monocytes, and lymphocytes) of stromal cells were evaluated in this micropore chamber model. Fibroblasts are thought to be the main effecter cells in cancer cell progression[30,31]. Recently, tumor stromal fibroblasts, named cancer-associated fibroblasts (CAFs), have been thought to play crucial role in cancer progression[32-34], and, moreover, may be potential initiators of certain carcinomas[35]. Elevated levels of stromal fibroblasts are also correlated with a poor prognosis for human carcinoma patients[36]. CAFs have been reported to originate partly from BM-derived progenitor cells[37-39]. α-SMA has been used as a marker of cancer-associated myofibroblasts which are more competent in enhancing tumor progression[40], however, the uncertainty in defining the myofibroblasts has existed[41]. On the contrary, S100A4 is strongly expressed in activated fibroblasts and myofibroblasts and has been used as a highly specific marker for these cells[42-44]. Moreover, S100A4 per se promotes tumor progression[45]. In

fact, S100A4 expression was detected in more fibroblasts of normal tissues than α-SMA, and most fibroblasts were S100A4$^+$α-SMA$^+$ (79.6±3.1%) in the micropore chamber model, whereas only 0.3±0.2% fibroblasts were single positive for α-SMA, and the ratio of fibroblast lineages (α-SMA$^+$/S100A4$^+$) was not altered by COX-2 inhibition despite of total fibroblasts were decreased[27]. Therefore, S100A4 may be a good marker for tumor-associated activated fibroblast in tumor stroma. Celecoxib treatment significantly reduced the gene expression of the S100A4 in the granulation tissues, while neither F4/80 nor CD3ε (markers for macrophages and lymphocytes, respectively) were affected by COX-2 inhibition. Immunohistochemistry confirmed that S100A4-positive fibroblasts in stromal tissues were declined upon COX-2 inhibition. Double labeling assay of CXCL12 and S100A4 revealed that almost all (99.7%) CXCL12-positive cells were S100A4-positive fibroblasts, and the CXCL12$^+$S100A4$^+$ stromal cells were significantly decreased by COX-2 inhibition. These results suggest that CXCL12/CXCR4 signaling recruits fibroblasts in a COX-2-dependent manner, and thereby has a role in tumor-associated stromal formation.

S100A4$^+$ fibroblasts predominantly express CXCL12/CXCR4 in tumor stroma and are recruited from BM

We also tested subcutaneous tumor-bearing model with LLC cells. Double labeling of CXCL12 and each cell marker (S100A4, F4/80 and CD3ε) indicated that S100A4$^+$ fibroblasts predominantly expressed CXCL12 and CXCR4 in the tumor stroma (94.4±1.5% of CXCL12$^+$ cells and 87.8±2.5% of CXCR4$^+$ cells) in line with the mentioned micropore chamber model (Fig. 3).

Furthermore, we analyzed whether tumor stromal fibroblasts are recruited from BM or not in tumor-bearing model using GFP-BM chimeric mice (Fig. 4A). Surprisingly, GFP$^+$S100A4$^+$ cells reached 89.6±1.0% of S100A4$^+$ stromal fibroblasts, indicating that most tumor stromal fibroblasts are recruited from BM (Fig. 4B). Collectively, most fibroblasts expressing CXCR4 are recruited from BM to tumor stroma and produce CXCL12, leading to further recruitment of stromal fibroblasts.

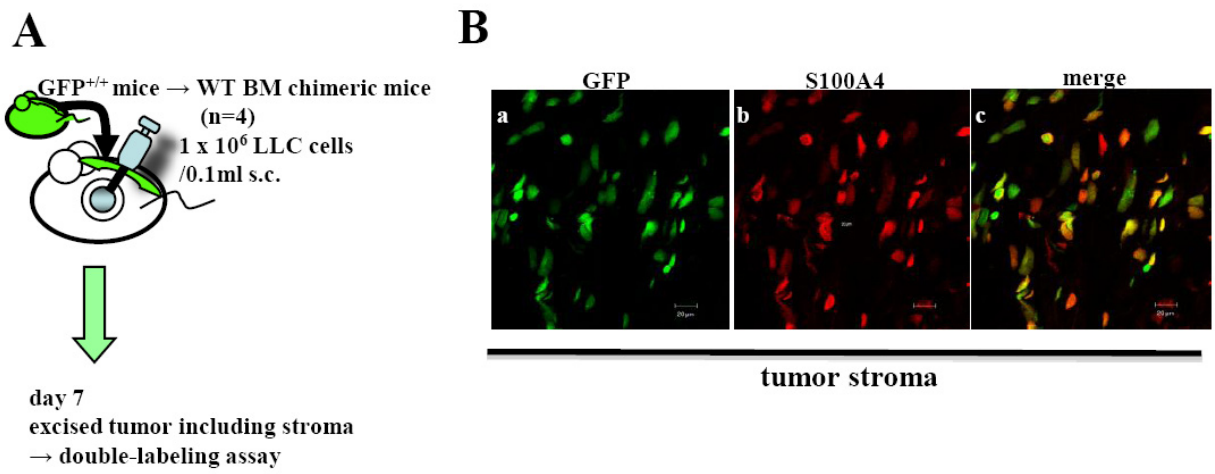

Fig. 4. (A) Schematic representation of the tumor-bearing model in GFP-BM chimeric mice. (B) Double labeling assay of GFP and S100A4 in tumor stoma of tumor implantation model in GFP-BM chimeric mice. 92.3% of GFP$^+$ cells were positive for S100A4. And GFP$^+$S100A4$^+$ cells occupied 89.6% of S100A4$^+$ stromal fibroblasts. Bars, 20 μm. (Reprinted from Am J Pathol 2010, 176:1469-1483 with permission from the American Society for Investigative Pathology.)

Effects of CXCR4 stimulation and neutralizing in stromal formation

To confirm that CXCL12 has a role in enhancement of stromal formation and angiogenesis, we exposed Matrigel^{TM} implants to topical injections of CXCL12 (SDF-1α). CXCL12 was confirmed to enhance granulation tissue formation, mimicking the stromal formation, and angiogenesis in Matrigel^{TM} model, and to upregulate VEGF-A expression[27].

On the other hand, topical injections of the neutralizing antibody against CXCR4 (2B11) around

a micropore chamber containing LLC cells markedly suppressed stromal formation and angiogenesis[27]. VEGF-A gene expression in the stroma was also attenuated by 2B11. The stromal expression of S100A4 mRNA was significantly downregulated by 2B11, whereas those of the F4/80 and CD3ε were not influenced. These results suggest that CXCR4 signaling has a crucial role in tumor-associated stromal formation and angiogenesis in this model.

Identification of the EP receptor signaling that facilitates tumor stromal formation

As previously reported, COX-2/PGE_2 promotes tumor angiogenesis and tumor growth[20,21]. We identified the PGE_2 receptor subtypes (EP1, EP2, EP3, and EP4) responsible for stromal formation in tumor-bearing model using respective EP receptor knockout mice. Tumor stromal formation was significantly suppressed in either $EP3^{-/-}$ or $EP4^{-/-}$ mice, whereas neither EP1 nor EP2 knockout did affect tumor stromal formation[27]. CXCL12 and CXCR4 gene expression and CXCL12 protein expression in stromal tissues were markedly reduced in either $EP3^{-/-}$ or $EP4^{-/-}$ mice. S100A4 gene expression was significantly suppressed in either $EP3^{-/-}$ or $EP4^{-/-}$ mice, but in neither $EP1^{-/-}$ nor $EP2^{-/-}$ mice. The populations of $S100A4^{+}$ cells were sparse in tumor stroma in $EP3^{-/-}$ mice and $EP4^{-/-}$ mice. Collectively, endogenous COX-2-derived PGE_2 may regulate tumor stromal formation through EP3/EP4 receptor signaling by fibroblast recruitment using CXCL12/CXCR4 chemokine system.

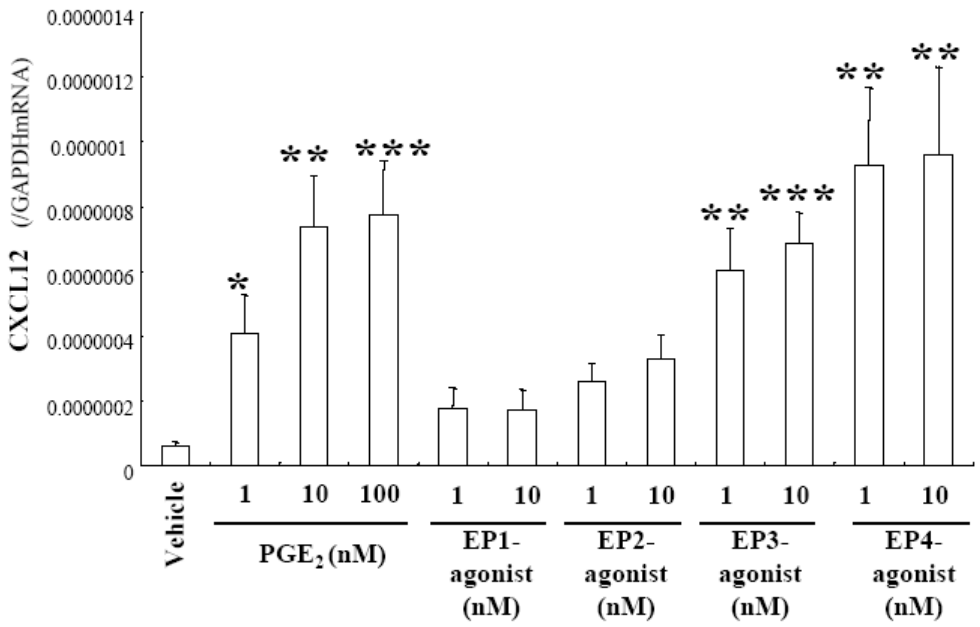

Fig. 5. Effects of EP1, EP2, EP3, and EP4 specific agonists on CXCL12 gene expression in L929 fibroblasts. L929 fibroblasts (3 x 10^5 cells/well) cultured in six-well plates were incubated for 24 hrs with the respective agonists, then CXCL12 gene expression was measured. Data were expressed as the mean ± SEM of three independent experiments. **P*<0.05; ***P*<0.01 by Student's t-test (compared with vehicle-treated cells). (Reprinted from Am J Pathol 2010, 176:1469-1483 with permission from the American Society for Investigative Pathology.)

EP3 or EP4 specific agonist stimulates CXCL12 expression by fibroblasts

To see the effects of each EP receptor specific agonist on CXCL12 production from fibroblasts, we performed *in vitro* experiments using L929 fibroblast cell line. PGE_2 at 1-100 nM markedly stimulated CXCL12 gene expression by fibroblasts (Fig. 5). Similarly, either EP3 or EP4 specific agonists remarkably promoted CXCL12 expression to the extent of PGE_2 effects, while neither EP1 nor EP2 stimulation did. These results suggest that both EP3 and EP4 receptors on fibroblast may mediate CXCL12 induction elicited by endogenous PGE_2.

And CXCL12 may act on the fibroblasts in an autocrine or paracrine fashion in tumor stroma.

Perspective

In conclusion, PGE2-EP3/EP4 signaling appears to be crucial for tumor-associated stromal formation and tumor growth (Fig. 6). EP3 and EP4 signaling on the stromal fibroblasts was correlated with the induction of a potent chemotactic and proangiogenic cytokine, CXCL12, in stromal fibroblasts. The subsequent upregulation of CXCL12/CXCR4 signaling facilitated tumor stromal formation by accelerating the recruitment of fibroblasts, which resulted in tumor growth. Highly selective EP3, EP4 and CXCR4 antagonists may therefore serve as novel therapeutic tools to treat cancer.

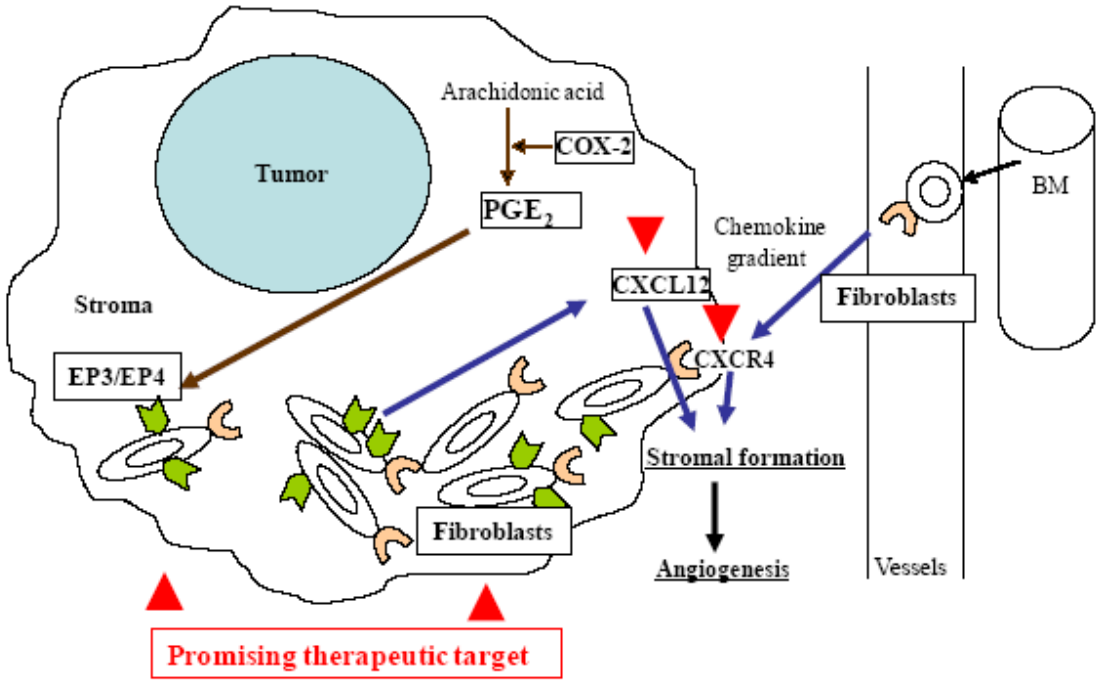

Fig. 6. Schematic representation of the mechanism in stromal formation.

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