Fibroblasts associated with cancer cells keep enhanced migration activity after separation from cancer cells: A novel character of tumor educated fibroblasts

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Received February 12, 2010; Accepted April 29, 2010

DOI: 10.3892/ijo_00000680

Abstract. It is now clear that the association between cancer cells and recruited fibroblasts (cancer-associated fibroblasts; CAFs) leads to alteration of the biological properties of both types of cells and creates a specific microenvironment. Here we report a novel biological property of CAFs and its cellular mechanism using *in vivo* and *in vitro* model. Cultured CAFs derived from human lung cancer tissue displayed significantly higher migration activity in response to PDGF-BB than that of fibroblasts from corresponding non-cancerous tissue (NCAFs). Moreover, KM104^{GFP} (GFP-labeled human fibroblast cell line) co-cultured with human cancer cell line Capan-1 showed significantly higher migration activity than KM104^{GFP} alone. No such phenomenon occurred when KM104^{GFP} and Capan-1 were cultured separately. Even after KM104^{GFP} were sorted from co-cultured Capan-1, KM104^{GFP} retained their enhanced migration activity until passage-5 of culture in the absence of cancer cells. Despite a similar level of phosphorylation of ERK1/2 after exposure to PDGF-BB, the inhibitory effect of MEK inhibitor was significantly higher on migration of KM104^{GFP} that had been sorted from co-cultured Capan-1 than of KM104^{GFP} alone. This higher dependence on ERK1/2 signaling for cell migration was also seen in CAFs obtained from cancer tissue. The results of this study indicate that by association with cancer cells, CAFs can acquire enhanced migration activity which could be kept after separation from cancer cells and suggest the possibility that higher dependence on ERK1/2 signaling for enhanced migration activity would be one of the biological properties of CAFs.

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Key words: cancer associated fibroblast, MAP kinase, migration activity

Introduction

During the process of cancer cell invasion, the cancer cells associate with several types of stromal cells that together create the specific microenvironment of the cancer tissue. It is becoming clear that the microenvironment plays an important role in allowing the tumor to express its full neoplastic phenotype (1,2). Fibroblasts, which are the major component of the newly created stroma, are recruited from neighboring tissues and remote organs (3-7). This recruitment step is called the 'desmoplastic reaction' and is a kinetic sequence of events in the invasion process. Within the cancer tissue, the fibroblasts directly communicate with the cancer cells and other types of stromal cells and acquire a specific biological phenotype (8,9). The type of fibroblast in cancer tissue that has acquired a specific biological phenotype is called cancer-associated fibroblast (CAF), and such fibroblasts have been postulated to perform supportive roles that promote tumor progression and metastasis by secreting growth factors (10,11), chemokines (12,13), and matrix metalloproteinases (MMPs) (14,15). Although an increasing number of translational studies have emphasized the prognostic significance of the altered phenotype of CAFs (16-18), the cellular mechanisms of these alterations remain unclear.

It is noteworthy that expression of specific molecules on CAFs is maintained under *in vitro* conditions, which means that alterations of gene expression in CAFs are capable of being stably maintained even in the absence of continued exposure to cancer cells. Actually, in a comparison with cultured non-cancerous fibroblasts Nakagawa *et al* found that approximately 170 of 22,000 genes were up-regulated in cultured CAFs (fold change >2, $P<0.05$), and that the upregulated genes included many genes that encode cell

adhesion molecules and growth factors (19). However, the mechanisms by which specific gene expression is induced in CAF are controversial. In some studies, the frequency of LOH in CAFs has been reported to be similar to the frequency observed in the epithelial components (20-22). Qiu *et al,* on the other hand, reported that LOH and copy number alterations were extremely rare in CAFs from breast and ovarian cancers (23). Very little is known as to how genetic or epigenetic status can modify gene expression and cellular signaling(s) in CAFs.

The desmoplastic reaction during cancer progression centers on various functions of fibroblasts (24). One of the representative phenotypes involved in this reaction is migration activity, which is thought to be related to cancer progression (25). In this study we used *in vivo* and *in vitro* models to investigate the biological characteristics of CAFs, with a special focus on their migration activity and the cellular mechanism.

Materials and methods

Human fibroblast culture. Cancer-associated fibroblasts (CAFs) and non-cancerous tissue-associated fibroblasts (NCAFs) were obtained from the surgically resected lungs of lung cancer patients as previously reported (26). Briefly, approximately 5-mm^3 carcinoma tissue and non-cancerous lung tissue specimens were cut into about ten pieces and each was placed in α-MEM (Gibco, Grand Island, NY) culture medium with 10% heat-inactivated FBS and antibiotics (penicillin and streptomycin) (Sigma, St. Louis, MO). The medium was changed every other day until the tissue was surrounded by adherent fibroblasts. The tissue was then removed and cultured for two more days. When the cells reached 80% confluence they were harvested with 0.25% trypsin and 1 mmol/l EDTA and then replated at a density of $1x10^4$ cells/cm^2. The fibroblasts were separated from contaminating epithelial and macrophages by differential trypsinization and used between passages 3 and 7. All specimens were collected after the subjects had given their written informed consent and it was approved by the Institutional Review Board of the National Cancer Center.

Animals. Six-week-old female severe combined immunodeficient (SCID) mice (C.B-17 background) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and maintained at the National Cancer Center Research Institute East (Chiba, Japan). All animals were maintained under specific-pathogen-free, temperature-controlled environmental conditions throughout this study, in accordance with the Institutional Guidelines. Written approval for all animal experiments (K03-011) was obtained from the local Animal Experiments Committee of the National Cancer Center Research Institute.

Cell lines and cell cultures. KM104 cells, SV-40 transformed human fibroblast cell line derived from bone marrow, were originally established from male patient as described previously (27). This cell line was cultured in RPMI-1640 medium (Sigma) with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). Human pancreatic cancer cell line Capan-1 was purchased from the American Type Culture Collection (Rockville, MD). The Capan-1 cells were maintained in DMEM (Sigma) medium with 20% heat-inactivated FBS and antibiotics. All cells were maintained in a 5% CO_2 incubator at 37˚C.

Green fluorescent protein (GFP) labeling and cell sorting. KM104 were transfected with pEGFP-C1 vector encoding EGFP (Clonetech, Palo Alto, CA) by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). GFP-positive KM104 ($KM104^{GFP}$) cells were sorted by using a FACSCalibur sorting system (Becton-Dickinson, San Jose, CA). After confirming that over 90% of cell line was GFP positive, they were used in the following experiment.

Intraperitoneal xenotransplantation of Capan-1 and $KM104^{GFP}$. Capan-1 cells ($5x10^6$ cells per animal) were injected into the peritoneal cavity of SCID mice as described previously, and 1 h later, $5x10^6$ $KM104^{GFP}$ were injected into each peritoneal cavity at a different injection site (28). The animals were sacrificed on day 21, and parapancreatic tumors were removed, minced, and cultured in 10% FBS RPMI-1640. Under subconfluent conditions, the adherent cells were harvested, and the GFP-positive cells were sorted with the FACSCalibur sorting system.

Co-culture system. $KM104^{GFP}$ ($1x10^6$) and Capan-1 ($2x10^5$) were co-cultured in RPMI-1640 medium with 10% FBS. After 48 h, cells were collected, and GFP-positive cells were sorted with the cell sorter, and the cultured. $KM104^{GFP}$ used in experiments were passaged by harvesting and distribution into five new plates. To obtain cell-conditioned medium, Capan-1 were cultured until semiconfluent conditions. The plate was then washed with sterile PBS, and the medium was changed to RPMI-1640+10% FBS. After incubation for 24 h, the Capan-1 culture supernatant was collected and added to the pre-seed $KM104^{GFP}$, and their chemotactic activity was assayed 48 h later.

Migration assays. In the preliminary study, we tested the ability of 10 synthetic chemoattractants and growth factors (b-FGF, CCL-21, EGF, IGF-1, IL-8, PDGF-AA, PDGF-BB, SDF-1, TGF-ß, VEGF-A), and found that PDGF-BB was the most powerful chemoattractant at the same protein concentrations (data not shown). PDGF-BB was therefore used as the chemoattractant in the subsequent experiments. The migration assay was performed by using 24-well culture chambers (Becton-Dickinson Labware, Bedford, MA) and a polycarbonate filter with an 8 μm pore size (Becton-Dickinson Labware) as previously described (28). Briefly, the lower chamber contained 0.6 ml of RPMI-1640 + recombinant PDGF-BB (final concentration; 0-100 ng/ml), or 0.6 ml of RPMI-1640 as a control. In the upper compartment, $2x10^4$ cells/well were placed in triplicate wells and incubated for 6 h at 37˚C in a humidified incubator under a 5% CO_2 atmosphere. At the end of the incubation period, the cells that had passed through the filter into the lower wells were stained with hematoxylin, and the cells in nine predetermined fields were counted under a microscope.

Western blotting. Western blot analysis was performed as follows. Cells were lysed in whole-cell extraction buffer

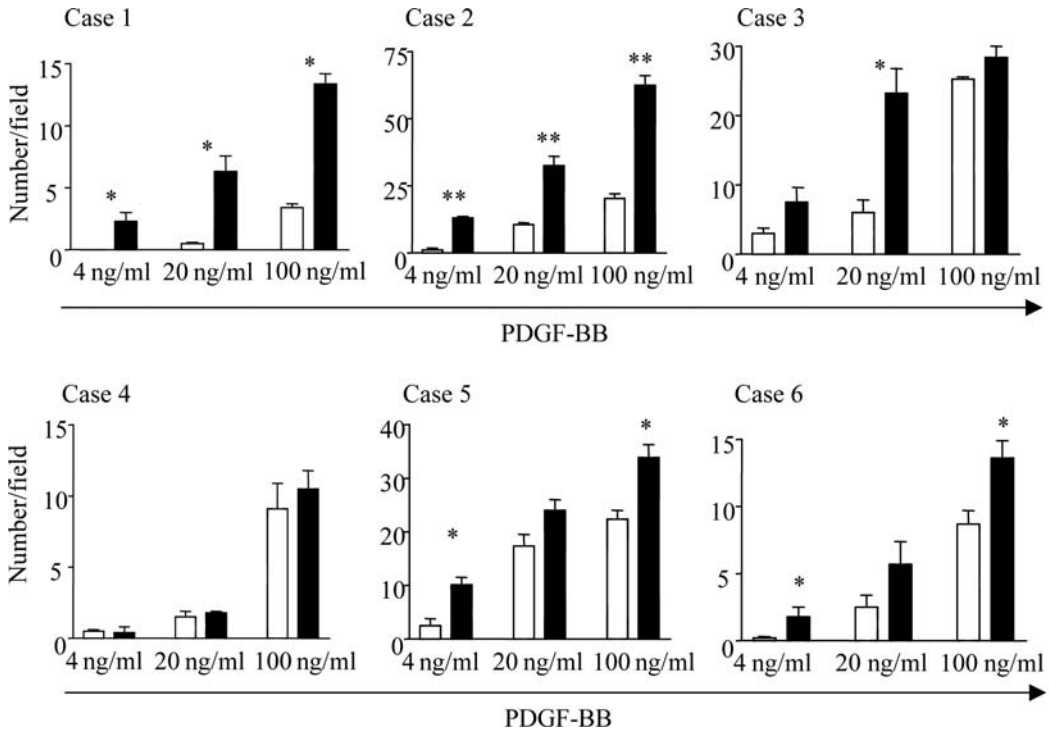

Figure 1. Cancer-associated fibroblasts (CAFs) isolated from human cancer tissue displayed enhanced migration activity in response to PDGF-BB. Fibroblasts isolated from human lung cancer tissue (cancer-associated fibroblasts; CAFs) and fibroblasts from corresponding non-cancerous lung tissues (non-cancerous-tissue-associated fibroblasts; NCAFs) were isolated and cultured, and their migration activity was compared. Except for case 4, migration activity of the CAFs in response to PDGF-BB was significantly higher than that of the NCAFs. ($^{*}P<0.05$, $^{**}P<0.01$).

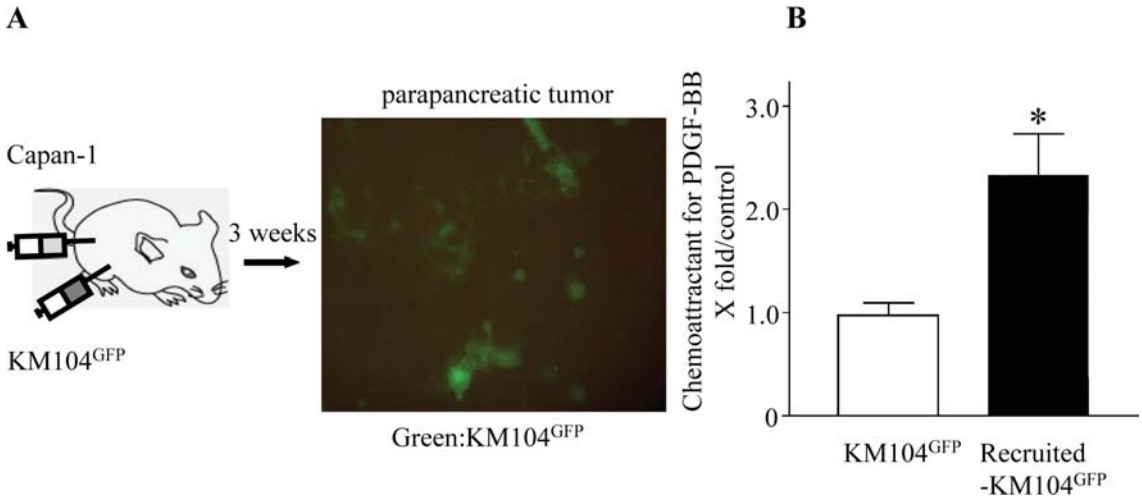

Figure 2. KM104^{GFP} recruited into cancer stroma *in vivo* display enhanced migration activity in response to PDGF-BB. (A) GFP-positive cells (KM104^{GFP}) are scattered within this abdominal tumor. (B) GFP (+) cells were sorted from the minced abdominal tumor and cultured *in vitro* (rec-KM104^{GFP}). Their migration activity in response to PDGF-BB was then compared with that of the parent cells (KM104^{GFP}). Rec-KM104^{GFP} exhibited a 2.4-fold higher migration activity than parent KM104^{GFP} (n=3, $^{*}P=0.03$).

(20 mM Hepes-NaOH, 0.5% NP-40, 15% glycerol) containing a Complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Proteins were separated on 12% SDS-polyacrylamide gels and then transferred to an Immobilon-P PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA). The blots were saturated with blocking buffer (5% skim milk in TBS-T) for 1 h at room temperature and then incubated for 1 h at 4˚C with anti-phospho-ERK1/2 antibody (mouse monoclonal IgG, E10, phospho-p44/42 MAPK antibody-Thr 202/Tyr 204) and total ERK1/2 antibody (rabbit polyclonal, p44/42 MAPK antibody) according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA, USA). After washing in TBS-T, the membranes were incubated for 1 h at room

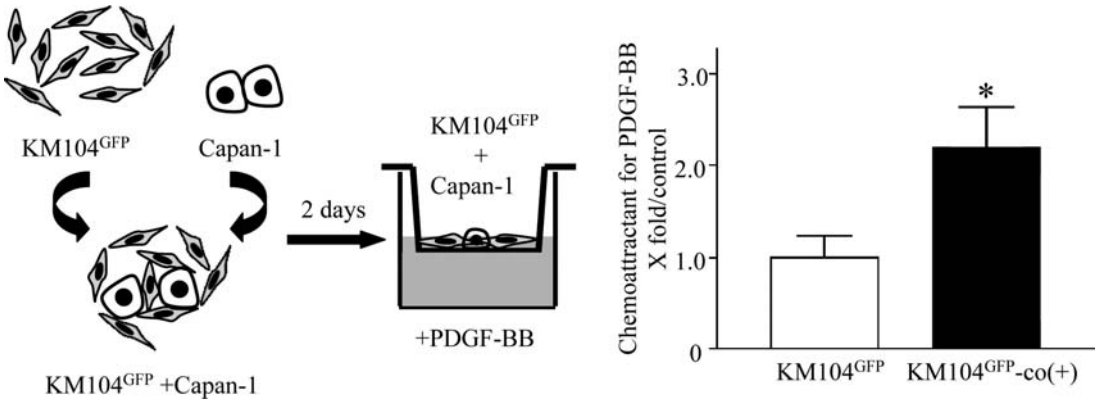

Figure 3. KM104^{GFP} co-cultured with a cancer cell line display enhanced migration activity in response to PDGF-BB. KM104^{GFP} were co-cultured with Capan-1 cells for two days [KM104^{GFP}-co(+)], and their migration activity in response to PDGF-BB was assayed. KM104^{GFP}-co(+) displayed 2.2-fold higher migration activity than KM104^{GFP} cultured in the absence of Capan-1 (n=9, *P=0.04)

temperature with HRP-Rabbit anti-mouse IgG or HRP-goat anti-rabbit IgG (Zymed, San Francisco, CA). Antibody binding was detected with an Amersham enhanced chemiluminescence system.

Methylated DNA immunoprecipitation-chip (MeDIP-chip) assay. A 5 μg sample of genomic DNA was sonicated to fragment lengths between 200 and 800 bp. After heat denaturation, the DNA was incubated with 5 μg of antibody against 5-methyl cytidine (Diagnode, Liège, Belgium) at 4˚C overnight. Immuno-complexes were collected with Dynabeads Protein A (Invitrogen Dynal AS, Oslo, Norway), treated with Proteinase K, and purified by phenol and chloroform extraction and isopropanol precipitation. The chip assay was carried out with a human CGI oligonucleotide microarray (Agilent Technologies, Santa Clara, CA). Immunoprecipitated DNA from 4.5 μg of sonicated DNA and 1 μg of input DNA were labeled with Cy-5 and Cy-3, respectively, using an Agilent labeling kit (Agilent Technologies), and then hybridized with the microarray. The microarray was scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). The methylation statuses of 8,528 promoter CGIs were evaluated by using Me values, which have been shown to have a higher correlation coefficient with the fraction of methylated DNA molecules (29,30), the methylation status of 8,528 promoter CGIs was evaluated.

MEK inhibitor treatment. Cells were pretreated with the MEK1/2 inhibitor compound UO126 (Promega, San Diego, CA) or with DMSO vehicle alone before harvesting. UO126 was used at a concentration of 25 μM, which was shown to maximally inhibit MEK in fibroblasts. In the upper compartment, $2x10^4$ cells/well were placed in medium containing 25 μM of U0126 and incubated for 6 h.

Statistical analyses. Three or four independent experiments were performed for each protocol. Results were expressed as the mean ± standard error of the mean (SEM). Statistical calculations were performed by using Prism 3.03 software. Differences in measured variables between experimental groups and control groups were assessed by using an unpaired t-test. P<0.05 was considered statistically significant.

Results

Cancer-associated fibroblasts (CAFs) isolated from human cancer tissue displayed enhanced migration activity in response to PDGF-BB. Fibroblasts isolated from human lung cancer tissue (cancer-associated fibroblasts; CAFs) and fibroblasts from corresponding non-cancerous lung tissues (non-cancerous tissue-associated fibroblasts; NCAFs) were isolated and cultured, and their migration activity was compared. Except for Case 4, migration activity of the CAFs in response to PDGF-BB was significantly higher than that of the NCAFs (Fig. 1).

Fibroblast cell line KM104^{GFP} recruited into cancer stroma displays enhanced migration activity in response to PDGF-BB. Capan-1 and KM104^{GFP} were injected intraperitoneally at a different site. Immunofluorescence examination of the parapancreatic tumor revealed the presence of KM104^{GFP} within the cancer tissue (Fig. 2A). GFP (+) cells were sorted from the minced parapancreatic tumor and cultured, and the chemoattractant response of the KM104^{GFP} to PDGF-BB was compared with that of the parent cells. KM104^{GFP} recruited into the tumor exhibited a 2.4-fold higher migration activity than parent KM104^{GFP} (n=3, Fig. 2B, P=0.03).

KM104^{GFP} co-cultured with the cancer cell line displayed enhanced migration activity in response to PDGF-BB. We co-cultured KM104^{GFP} and Capan-1 for two days and assayed the migration activity of KM104^{GFP} in response to PDGF-BB. No Capan-1 cells were able to migrate through the cell culture insert (8.0 μm pore size) under these conditions. KM104^{GFP} co-cultured with Capan-1 [KM104^{GFP}-co(+)] displayed 2.2-fold higher migration activity than KM104^{GFP} cultured in the absence of Capan-1 (n=9, P=0.04, Fig. 3).

Cell-cell contact is necessary for enhanced migration activity. To explore whether cell-cell contact is necessary for KM104^{GFP} to exhibit enhanced migration activity, we cultured KM104^{GFP} with KM104 cell conditioned medium (control) or Capan-1 cell conditioned medium for 2 days, and assayed their migration activity, but no significant difference in migration activity was found between the two groups (Fig. 4A). Next,

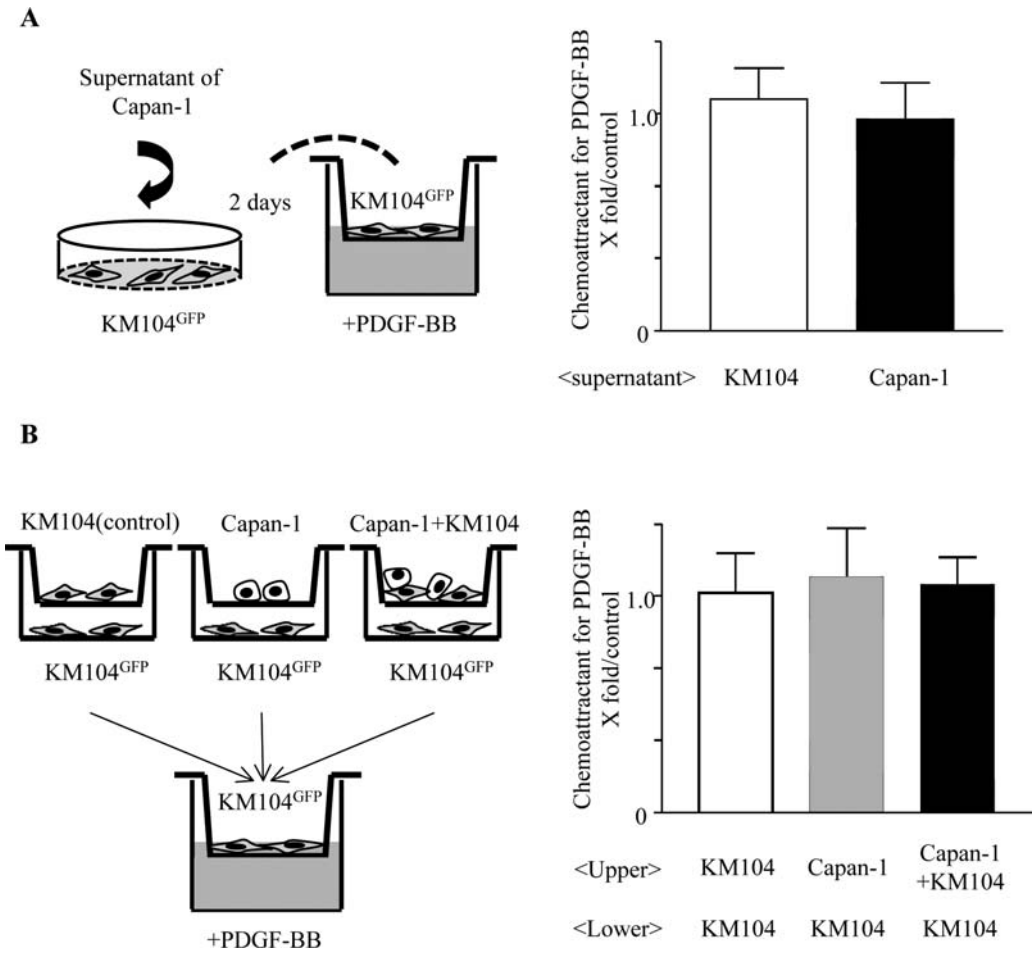

Figure 4. Cell-cell contact is necessary for enhanced migration activity. (A) KM104^{GFP} cells were cultured with conditioned medium from KM104 cell cultures (control) or Capan-1 cell cultures for 2 days, and their migration activity was assayed. (B) KM104^{GFP} were co-cultured in the 2-chamber system (upper chamber; KM104, Capan-1, or Capan-1 +KM104, lower chamber; KM104^{GFP}) for 2 days, and the migration activity of the KM104^{GFP} cultured in the lower chamber was assayed.

KM104^{GFP} were co-cultured in the 2-chamber system (upper chamber: KM104, Capan-1, or Capan-1+KM104; lower chamber: KM104^{GFP}) for 2 days, and the migration activity of the KM104^{GFP} cultured in lower chamber was assayed (Fig. 4B). Migration number of KM104^{GFP} exposed to Capan-1 cell supernatant (middle column) and KM104^{GFP} exposed to Capan-1+KM104 cells supernatant (right column) was almost the same as that of control group (left column). These findings suggested that direct KM104-Capan-1 contact is necessary for KM104^{GFP} to exhibit enhanced migration activity.

Enhanced migration activity of KM104^{GFP}-co(+) is kept after separation from cancer cells. GFP-positive KM104 were sorted (3.0x10^5) from cocultured Capan-1 and cultured (Fig. 5A). In a control experiment, KM104^{GFP} alone were cultured, and GFP-positive cells were sorted (sham-sorted) and cultured. Cells from subconfluent cultures (1.0x10^6) were then assayed for migration activity in response to PDGF-BB. The results showed that the sorted KM104^{GFP}-co(+) displayed 2.3-fold higher activity than sham-sorted KM104^{GFP} (P=0.02), indicating that the sorted KM104^{GFP}-co(+) displayed enhanced migration activity for almost 2 popu-lation doublings after separation from the cancer cells.

Sorted KM104^{GFP}-co(+) were then serially passaged by harvesting and distribution into five new plates, and migration activity was compared in each passage culture. Three independent experiments were performed, and the results are shown in Fig. 5B. The sorted KM104^{GFP}-co(+) kept their enhanced migration activity even after the passage-5 culture in every experiment. In experiments 2 and 3, the enhanced migration activity of the sorted KM104^{GFP}-co(+) returned to the levels of passaged sham-sorted KM104^{GFP} cells in the passage-6 culture. These results indicate that the sorted KM104^{GFP}-co(+) retained the enhanced migration activity for at least 13 population doublings after separation from the cancer cells *in vitro*.

MeDIP-chip analysis of CAFs. Because of the prolonged enhanced migration activity of CAFs, it was important to analyze the epigenetic changes that accompany this phenomenon. We used a methylation screening approach called MeDIP-chip to compare the DNA methylation profiles of the CAFs and corresponding NCAFs from two cases. The results of the MeDIP-chip analysis showed that 2.2% (188 promoter CGIs) (Case 1) and 1.1% (98 promoter CGIs) (Case 2) of the CGIs were hypermethylated in CAFs compared with NCAFs.

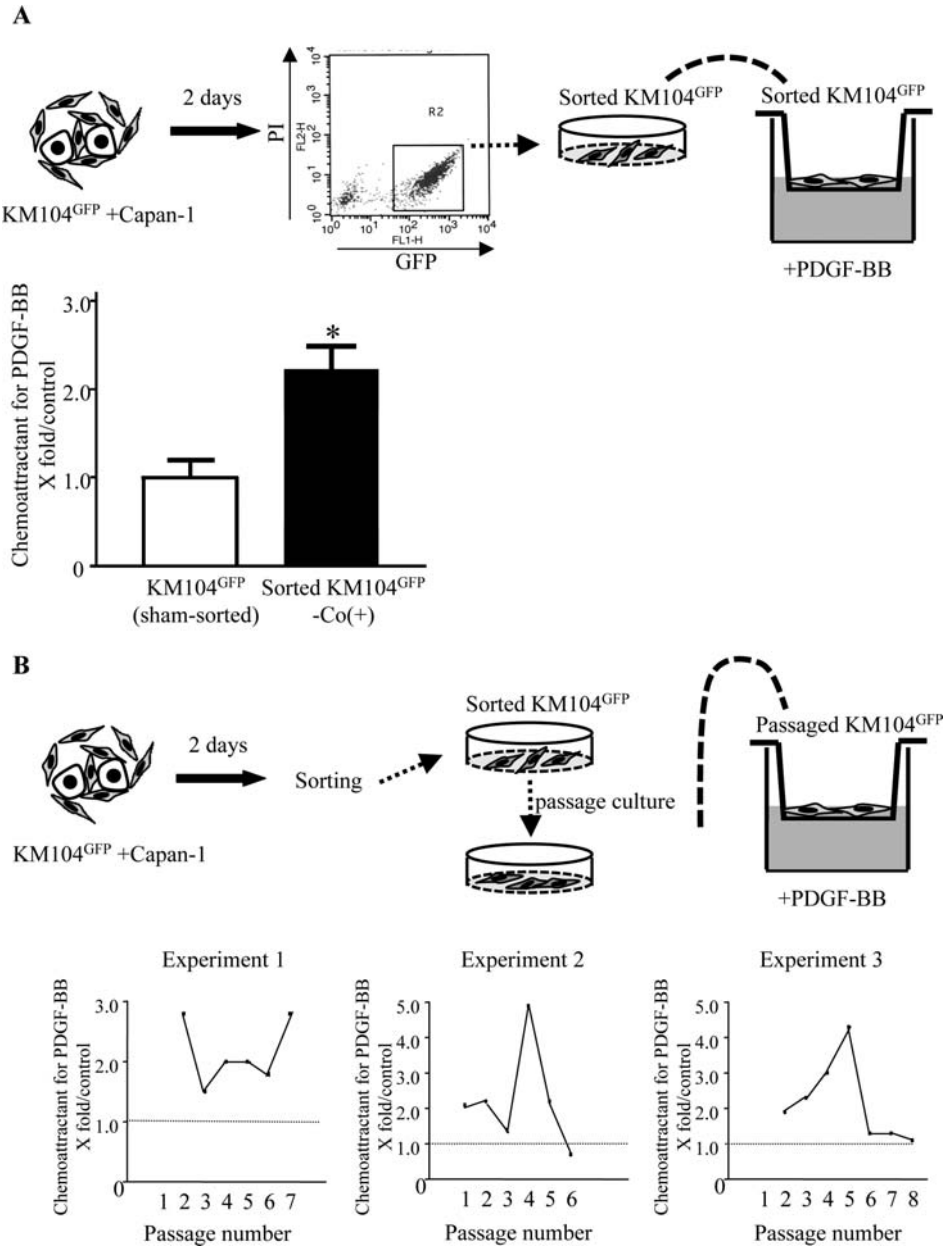

Figure 5. Sustained enhanced migration activity of KM104^{GFP}-co(+) after separation from the cancer cells. (A) After sorting from the co-cultured Capan-1, GFP-positive KM104 were sorted and cultured [Sorted KM104^{GFP}-co(+)]. In a control experiment, KM104^{GFP} alone were cultured, and the GFP-positive cells were sorted (sham-sorted) and cultured. Cells from a subconfluent culture were then examined for migration activity in response to PDGF-BB. Sorted KM104^{GFP}-co(+) displayed 2.3-fold higher activity than sham-sorted KM104^{GFP} (n=3, *P=0.02). (B) Sorted KM104^{GFP}-co(+) were serially passaged by harvesting and distribution into five new plates, and their migration activity was compared at each passage culture. Representative data of three independent experiments are shown.

0.5% (39 promoter CGIs) (Case 1) and 0.6% (50 promoter CGIs) (Case 2) were hypomethylated in CAFs compared with NCAFs (Table I). Ten promoter CGIs (*ALDH1L2*, *AP1G2*, *CENPJ*, *KBTBD6*, *MAB21L1*, *MED4*, *RCBTB2*, *SH2B3*, *TBC1D4* and *UBL3*) were hypermethylated in the CAFs from both cases (Table II), but no promoter CGIs were hypomethylated in the CAFs from either of the cases.

Role of extracellular signal-regulated kinase (ERK) 1/2 in PDGF-BB-induced migration of sorted KM104^{GFP}-co(+) and CAFs. Activation of extracellular signal-regulated kinase (ERK) 1/2 has been reported to play crucial role in PDGF-induced cell migration. We analyzed the effects of PDGF-BB

Table I. Aberrantly methylated promoter CGIs in CAF to be compared with NCAF.

	Pair	No. of genes
Hypermethylated genes		
	Case 1	188
	Case 2	98
Hypomethylated genes		
	Case 1	39
	Case 2	50

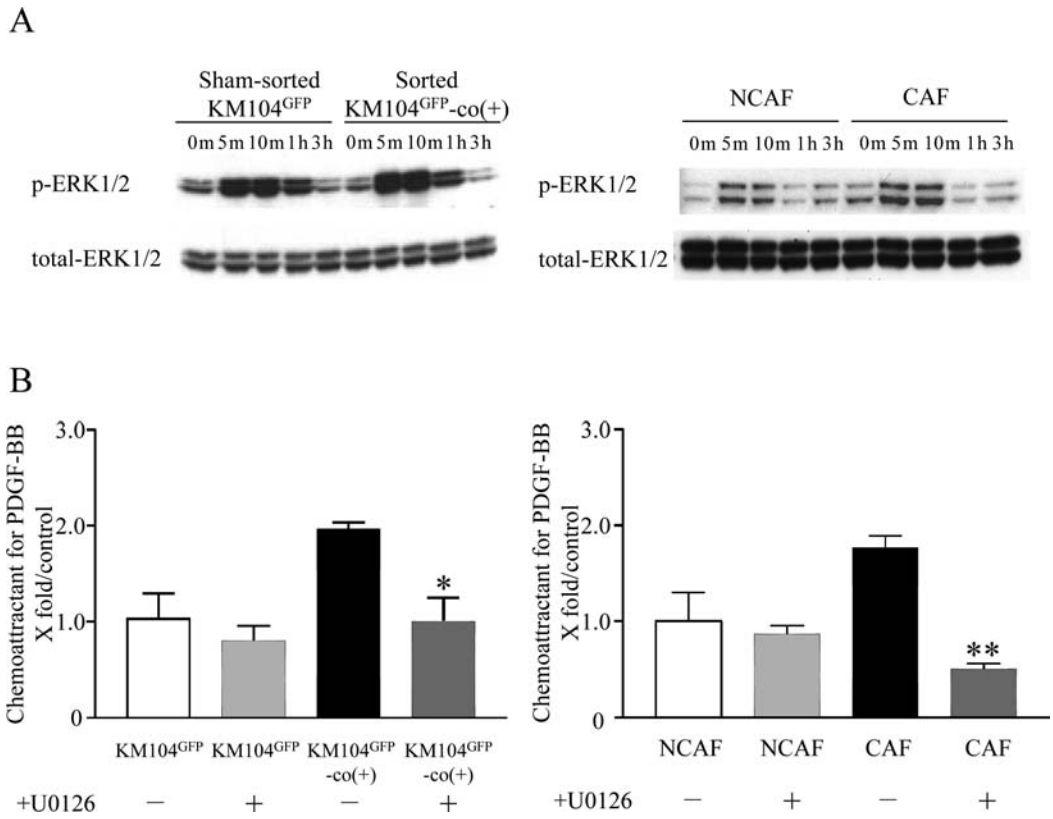

Figure 6. Role of extracellular signal-regulated kinase (ERK) 1/2 in PDGF-BB-induced migration by sorted KM104^{GFP}-co(+) cells and CAFs. (A) Western blotting of phosphorylated ERK1/2. PDGF-BB increased the level of ERK1/2 phosphorylation in sham-sorted KM104^{GFP} cells, sorted KM104^{GFP}-co(+) cells, NCAFs, and CAFs in a time-dependent manner, reaching a maximum at 300-600 sec, and the phosphorylation level decreased slightly thereafter. (B) Inhibitory effect of treatment with UO126 (25 μM) on cell migration. Treatment of the sham-sorted KM104^{GFP} and KM104^{GFP}-co(+) with UO126 decreased migration to 80.0±15.3% and 50.1±10.8% of untreated group, respectively (P=0.01). UO126 decreased the migration of NCAFs and CAFs to 86.7±8.9% and 28.1±1.6% of untreated group, respectively (P=0.001).

Table II. Genes hypermethylated in both CAFs.

Gene ID	Gene name
160428	Aldehyde dehydrogenase 1 family, member L2 (ALDH1L2)
8906	Adaptor-related protein complex 1, γ 2 subunit (AP1G2)
55835	Centromere protein J (CENPJ)
89890	Kelch repeat and BTB (POZ) domain containing 6 (KBTBD6)
4081	Mab-21-like 1 (C. elegans) (MAB21L1)
29079	Mediator complex subunit 4 (MED4)
1102	Regulator of chromosome condensation and BTB domain containing protein 2 (RCBTB2)
10019	SH2B adaptor protein 3 (SH2B3)
9882	TBC1 domain family, member 4 (TBC1D4)
5412	Ubiquitin-like 3 (UBL3)

Hypomethylated genes in both CAFs were not found.

on ERK1/2 activation of sorted KM104^{GFP}-co(+). As shown in Fig. 6A, PDGF-BB increased ERK1/2 phosphorylation in both sham-sorted KM104^{GFP} and sorted KM104^{GFP}-co(+) in a time-dependent manner up to a maximum at 300-600 sec, and the phosphorylation level decreased thereafter. The levels of phosphorylated ERK1/2 in both sham-sorted KM104^{GFP}and sorted KM104^{GFP}-co(+) was similar, and this phenomenon was also observed in CAFs and NCAFs isolated from lung cancer tissue.

Treatment of the sham-sorted KM104^{GFP} with UO126 (25 μM), a highly selective inhibitor of both MEK1 and MEK2, decreased migration to only 80.0±15.3% of untreated group, whereas it decreased migration of the sorted KM104^{GFP}-co(+) to 50.1±10.8% of untreated group (P=0.01) (Fig. 6B).

Furthermore, although U0126 decreased the PDGF-BB-induced migration of NCAFs to only 86.7±8.9% of untreated group, it decreased the migration of CAFs to 28.1±1.6% of untreated group (P=0.001).

Discussion

In the current study we demonstrated that fibroblasts that have associated with cancer cells retain their enhanced migration activity for a while after separation from the cancer cells. Furthermore, we first report the possibility that one of the biological characteristics of CAFs would be higher dependence on ERK1/2 signaling for their enhanced migration activity even in the absence of cancer cells.

It is now clear that activation of ERK1/2 is important for PDGF-induced cell migration (31-33). We examined the levels of ERK1/2 phosphorylation of sorted-KM104^{GFP}-co(+) and CAFs after PDGF-BB treatment, but there was no significant difference from the level in the control cells. However, the inhibitory effect of a selective inhibitor of both MEK1 and MEK2 on migration was significantly higher in sorted-KM104^{GFP}-co(+) and CAFs than in control cells. We also investigated the involvement of other MAPK family, including the JNK and P38, however, phosphorylation of these molecules in sorted-KM104^{GFP}-co(+)and CAFs were almost the similar level as those of control cells with or without PDGF-BB stimulation. Moreover, the inhibitory effect of their specific inhibitor SP600125 or SB203580 on cell migration in sorted-KM104^{GFP}-co(+) and CAFs and in untreated cells was almost equal (data not shown). This finding indicated that the fibroblasts that had associated with cancer cells were more dependent on the ERK1/2 pathway for cell migration. Activated ERKs regulate membrane protrusions and focal adhesion dynamics via myosin light chain kinase (MLCK), calpain, paxillin, and focal adhesion kinase (FAK) activation (32), however, the MeDIP-chip analysis did not reveal any promoter CGIs involved in these molecules. After association with cancer cells, pairs of factors that have a synthetic relationship in CAFs may differ from those in naive fibroblasts, thus increasing the dependence of cells on the downstream ERK1/2 pathway. Alternatively, the downstream ERK1/2 signaling pathway may play a more essential and qualitatively different role in a given pathway in CAFs than in naive fibroblasts, making them much more dependent on the activity of a specific signaling pathway. The direct association with cancer cells may cause 'signaling addiction' to occur in fibroblasts, which would be a similar condition explained by the concept of 'oncogene addiction' that some cancer cells are dependent on or addicted to one or a few genes for both maintenance of their malignant phenotype and cell survival (34,35).

DNA methylation is one of the molecular mechanisms that have long-term effects on gene expression. Since the enhanced migration activity of fibroblasts continues after being separated from cancer cells, we speculated that the association with cancer cells might change the methylation status of certain DNA(s) involved in cell migration. MeDIP-chip analysis identified that 10 promoter CGIs (*ALDH1L2, AP1G2, CENPJ, KBTBD6, MAB21L1, MED4, RCBTB2, SH2B3, TBC1D4* and *UBL3*) that were hypermethylated, no promoter CGIs were hypomethylated in both CAFs, and we were unable to identify any well-known methylated genes that were directly involved in cell migration. The cause of the persistent enhanced migration activity remains unknown, and additional research on chromatin modifications, such as histone deacetylation, will be necessary to understand the mechanisms underlying this phenomenon in CAFs.

Jiang *et al* detected global hypomethylation of genomic DNA by means of a methylation-sensitive SNP array analysis (MSNP), a restriction enzyme-based analysis, in the stromal myofibroblasts of gastric carcinomas (36). By contrast, when we focused on promoter CGIs in the current study, the results showed a higher number of hypermethylated genes than hypomethylated genes in CAFs (hypermethylated: 188 CGIs in Case 1 and 98 CGIs in Case 2; hypomethylated: 39 CGIs in Case 1 and 50 CGIs in Case 2). This difference might be caused by CGIs to be focused on. Our data showed for the first time that a few hundred genes were aberrantly hypermethylated in CAFs, suggesting that some regulators can be epigenetically targeted by the cancer microenvironment (37,38).

Cultured fibroblasts from idiopathic pulmonary fibrosis (IPF) were found to migrate faster than those from control fibroblasts, indicating that they also retained enhanced migration activity *in vitro* (39). Since there are many parallels between cancer and chronic active inflammation, as described in the saying, 'tumours are wounds that never heal', enhanced migration may be a common characteristic of fibroblasts recruited into fibrotic lesions.

Our current results may imply that by using this special property of CAFs, strategies that are directed to block the recruitment and tumor-associated functions of CAFs could be a possible method against tumors. Although the exact mechanisms of this phenomenon are not fully understood, cellular and molecular studies on the role of 'educated fibroblasts' should provide novel insights into the pathogenesis of a unique microenvironment of cancers.

Acknowledgements

This study was supported in part by the Grant-in-Aid for Cancer Research (19-10) from the Ministry of Health, Labour and Welfare, the Grant for Scientific Research Expenses for Health Labour and Welfare Programs, the Foundation for the Promotion of Cancer Research, 3rd-Term Comprehensive 10-year Strategy for Cancer Control, and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

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