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癌幹細胞を標的とする人工ウイルスを用いた癌幹細胞特異的 Drug delivery activation system(DDAS)の確立

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研究代表者　大内田　研宙

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厚生労働科学研究費補助金（医療機器開発推進研究事業）
総合研究報告書

癌幹細胞を標的とする人工ウイルスを用いた癌幹細胞特異的新規 Drug delivery activation system(DDAS)の確立

研究代表者　大内田　研宙

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研究要旨

現在、癌治療における主たる課題は治療抵抗性と転移浸潤であるが、これまでの本研究で、膵癌を用いて、治療抵抗性の根幹となっている癌幹細胞と転移浸潤に関わる細胞集団における特異的な分子の機能解析を行い有望な標的分子を複数同定した。これらに加え、癌の悪性度に深く関わる癌間質細胞の標的分子を同様にして同定した。それらの分子を標的にした新規分子治療薬を内包した人工ウイルスを作成し、癌幹細胞及び癌間質細胞特異的新規 DDAS を構築した。

分担研究者

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（九州大学大学院医学研究院　災害・救急医学分野・特任准教授）

A. 研究目的

固形癌の多くは、依然として根治切除ができなければその後の再発再燃の制御が困難で、その治療法の開発は、社会的要請度が高い。最近、癌組織中の特定の微量細胞集団だけが腫瘍形成能を持つとする癌幹細胞の概念が注目されている。この癌幹細胞は薬剤耐性を有し、再発に関与する。また、微小環境を形成している間質細胞にも癌の悪性度に深く関わる集団がある。本研究では、異なる蛋白を認識する人工ウイルスを作成することによって、癌幹細胞や癌の悪性度に深く関わる間質細胞に薬剤や分子標的薬を選択的に輸送することにより、細胞内特異的な薬剤濃度上昇や治療薬の活性化/効果増強を実現し、重篤な副作用を抑えることにより、治療抵抗性が高く再発、転移の原因となる標的細胞を制御する新規 Drug delivery activation system(DDAS)を開発することが目的である。

B. 研究方法

本研究は、以下の１）～ 3）の課題に対する研究を分担して行った。

１） 癌幹細胞及び癌の悪性度に深く関わる癌間質細胞の標的分子の機能解析（大内田担当）

膵癌、大腸癌の切除組織を対象に、癌幹細胞マーカーCD133 および CD44, c-kit, CD24, c-Met について、セルソーターを用いて prospective isolation を行い、その細胞集団の存在と臨床病理学的な所見との付き合わせをおこない、該当細胞集団の生物学的な意義を検討した。また、切除組織からソートした癌幹細胞様細胞や治療抵抗性株を対象にマイクロアレイ解析を行い有望な標的分子や治療抵抗性に関わる分子を同定した。更に、癌間質相互作用に着目し、大腸癌及び膵癌の手術切除サンプルより作成した fibroblast に対しセルソーターを用いて prospective isolation を行い、機能解析を行った。

２） 抗癌剤含有幹細胞特異的認識人工ウイルスの作成（村田担当）

我々が独自に開発した人工ウイルスに１）で同定した癌細胞及び癌幹細胞膜表面上に特異的に発現する標的マーカーを認識し結合するアンテナ分子を作成・付加することにより、標的細胞に特異的に集積するように人工ウイルスを改変した。さらに人工ウイルスを高い細胞膜透過性を持ち、標的細胞内で刺激応答的に崩壊するように機能化した。

３） 癌細胞及び癌幹細胞を標的とする新規人工ウイルスの細胞選択性及び治療効果の検証（大内田担当）

in vitro にておいて２）で作製した新規人工ウイルスの細胞特異的な治療効果を検証した。さらに、in vivo において、腫瘍モデルを作成し、MRI を用いて新規人工ウイルスの細胞特異的な集積性及び治療効果を検証した。

（倫理面への配慮）

本研究は、癌に含まれ後天的に出現する特定の細胞集団を対象としており、マイクロアレイや RT-PCR を用いた発現解析も同様に後天的な特定の分子の発現異常を解析するものであり、ゲノム解析は行わず、平成 13 年の三省の「ヒトゲノム・遺伝子解析研究に関する倫理指針」の対象になる研究ではない。しかし、臨床検体を使用した解析を含む研究であるので、平成 15 年 7 月の厚生労働省「臨床研究に関する倫理指針」に従い、九州大学倫理委員会で承認済みである。本研究でのマウスの飼育・管理・実験は、動物愛護、生命倫理の観点に十分に配慮し、「研究機関等における動物実験等の実施に関する基本指針」および九州大学の学内規定に基づいて適切に行う。

実験用各種ウイルス・plasmid の取扱いは、九州大学の学内規定に基づき厳正に行う。すでに P2 レベルの動物実験施設、培養実験施設を専用に確保しており、承認された計画調書に従い、安全性の確保に最大限の注意を払って研究を遂行する。

C. 研究結果

1） 癌幹細胞及び癌の悪性度に深く関わる癌間質細胞の標的分子の機能解析

癌間質細胞特異的分子の機能解析

癌幹細胞マーカーCD133およびCD44, c-kit, CD24, c-Metについて、セルソーターを用いてprospective isolationを行い、その細胞集団の存在と臨床病理学的な所見との付き合わせをおこない、該当細胞集団の生物学的な意義を検討した。その結果、CD133 陽性細胞は、腫瘍増大とin vitroでの細胞浸潤が有意に促進したものの、明らかな腫瘍形成性の差を見出すには至らなかった。またCD44は癌細胞だけでなく腫瘍関連間質細胞でも発現があり、CD24は同一細胞集団内における経時的な発現変化が大きく、恒久的な標的マーカーとしては適さないものであった。そこで、切除組織からソートした癌幹細胞様細胞や治療抵抗性株を対象にマイクロアレイ解析をした結果、有望な標的分子や治療抵抗性に関わる分子として、CXCR4 および c-Metが同定された。また、腸癌及び膵癌の手術切除サンプルより作成したfibroblast に対しセルソーターを用いてprospective isolationを行い、機能解析を行った結果、CD10を発現しているfibroblastが、特異的に膵癌細胞及び大腸癌細胞の浸潤能を亢進させることが明らかになった。

2） 抗癌剤含有癌細胞特異的認識人工ウイルスの作成

1）の結果をもとに、人工ウイルスHsp16.5にPolyethylene Glycol (PEG)で被包化したPEG鎖末端にCXCR4、c-Metに特異的に結合するペプチドアンテナ分子を付加することに成功した。PEG化Hsp16.5-P4を、動的光散乱法（DLC）によって評価したところ、期待通り約24nmの球状構造体であることが確認された。同手法を用いて、膵癌細胞特異的に高発現しているMUC1をターゲットとして、そのIgG抗体を人工ウイルスに付加することに成功した。

3）癌細胞及び癌幹細胞を標的とする新規人工ウイルスの治療効果の検証

2）で作成した新規人工ウイルスの細胞選択性について、in vitroにて検証したところ、CXCR4、c-Met、MUC1陽性細胞特異的に新規人工ウイルスが集積することが確認された。また、in vivoにおける腫瘍特異性を確認するためにMRI造影剤を人工ウイルスに内包し、マウスモデルにおける人工ウイルスの集積を評価したところ、標的細胞に選択的に人工ウイルスが集積することが確認された。さらに、癌治療の第一選択薬である塩酸ゲムシタビン(GEM)を内包し、その治療効果をin vitro、in vivoにおいて検証した。その結果、新規人工ウイルスの癌細胞選択性は得られ、一定の治療効果をえることができた。

D. 考察

本研究において、我々は癌幹細胞や治療抵抗性株に着目し、その標的分子としてCXCR4、c-Metを同定した。また、癌間質相互作用に着目した研究において、CD10を発現しているfibroblastが、特異的に膵癌細胞及び大腸癌細胞の浸潤能

を亢進させることが明らかになった。特に膵癌では，この CD10 陽性細胞は，膵星細胞とよばれる細胞集団と部分的にオーバーラップした細胞集団で，膵癌の豊富な間質の制御に深く関わっていくもので、癌間質相互作用あるいは物理的な薬剤拡散制限などと直接的に関与していると考えられる。さらに、本研究においては、標的細胞に特異的に集積し、治療効果をもたらす新規人工ウイルスの作成にも成功した。これらの結果より、正常細胞への影響を抑え、標的細胞のみを抑制するという、理想的な DDAS の実現への礎となるものと考えられる。今後は癌幹細胞の根幹となる腫瘍形成性に関わる細胞集団だけでなく悪性度に関わる癌細胞あるいは周囲の間質細胞を表面マーカーにより絞り込んでいき、同時に，今までの成果をふまえて、総合的にあるいは個別に人工ウイルスに付加することで多機能化人工ウイルスを作成し、さらに治療薬放出システムを強化し、患者予後と直結する転移、浸潤、治療抵抗性に関わる細胞集団を制御するための DDAS を開発する。

E. 結論

最新の分子生物学的手法を用いて、癌治療抵抗性の根幹をなす癌幹細胞に特異的な分子の機能解析を行うことにより、有望な標的分子を同定し、標的細胞に特異的に作用する人工ウイルスの作成に成功した。本研究の結果は、癌幹細胞、癌細胞、癌間質細胞に特異的に作用する DDAS の確立への基盤となる。本研究の成果により、効率的かつ副作用の少ない、新たな治療戦略の開発が期待できる。

F. 健康危険情報

該当なし

G. 研究発表

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H. 知的財産権の出願・登録状況

該当なし

別添 4

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Original Article

Enhanced Cell Migration and Invasion of CD133$^+$ Pancreatic Cancer Cells Cocultured With Pancreatic Stromal Cells

Taiki Moriyama, MD[1]; Kenoki Ohuchida, MD, PhD[1,2]; Kazuhiro Mizumoto, MD, PhD[1,3]; Lin Cui, MD[1]; Naoki Ikenaga, MD[1]; Norihiro Sato, MD, PhD[1]; and Masao Tanaka, MD, PhD[1]

BACKGROUND: Recently, cancer stem cells have been reported as a new therapeutic target in pancreatic cancer as well as other cancers, but the specific role of these cells is unknown. **METHODS:** The authors investigated the functional roles of CD133$^+$ cells, 1 of the putative cancer stem cell candidates in pancreatic cancer. CD133 expression was assessed in human pancreatic cancer and cancer cell lines by quantitative real-time reverse transcriptase polymerase chain reaction and flow cytometry. Next, they compared the ability of CD133$^+$ and CD133$^-$ cells to proliferate, migrate, and invade using 2 pancreatic cancer cell lines. In particular, they evaluated the relationship between CD133$^+$ cells and primary pancreatic stromal cells. **RESULTS:** CD133 was expressed in primary human pancreatic cancer tissues and some cancer cell lines, whereas there was little expression in primary normal pancreatic epithelial cells and primary pancreatic stromal cells. CD133$^+$ cells, isolated by flow cytometry, showed increased cell proliferation under anchorage-independent conditions ($P < .01$), and enhanced migration and invasion, particularly when cocultured with primary pancreatic stromal cells ($P < .001$). Chemokine-related receptor-4 (CXCR4), markedly overexpressed in CD133$^+$ cells, may be responsible for the increased invasive ability of the cells cocultured with pancreatic stromal cells, which express stromal derived factor-1, the ligand to CXCR4. **CONCLUSIONS:** These data suggest that CD133$^+$ cells exhibit more aggressive behavior, such as increased cell proliferation, migration, and invasion, especially in the presence of pancreatic stromal cells. The targeting therapy for the interaction between CD133$^+$ cancer cells and stromal cells may be a new approach for the treatment of pancreatic cancer. ***Cancer*** **2010;116:3357–68.** *© 2010 American Cancer Society.*

KEYWORDS: CD133, CXCR4, cell invasion, cancer-stromal interaction, pancreatic cancer.

Pancreatic cancer is the fifth leading cause of cancer-related death in Japan[1] and the fourth leading cause in the United States,[2] with annual deaths reaching >17,000 in Japan[1] and almost 30,000 in the United States.[2] The prognosis for pancreatic cancer is the worst of all cancers, because of the lack of improvement in early detection, diagnosis, and treatment strategies. Therefore, novel diagnostic modalities for early diagnosis and new therapeutic strategies are urgently needed.

Recently, cancer stem cells have been defined as a very small subset of cells within the tumor population that have the capacity to initiate and sustain tumor growth. It is most notable that cancer stem cells possess the ability to self-renew and undergo multilineage differentiation.[3] The study of cancer stem cells has developed through 2 important techniques, fluorescence-activated cell sorting (FACS) and tumor xenograft models in immunocompromised mice. Bonnet and Dick[4] isolated cancer stem cells in human acute myeloid leukemia for the first time by using cell surface markers (CD34$^+$ CD38$^-$) and a xenograft model in nonobese diabetic/severe combined immunodeficiency mice.

CD133 was reported as a marker of cancer stem cells in the brain,[5-7] colon,[8,9] liver,[10,11] and prostate.[12] In pancreatic cancer, Li and colleagues[13] have determined that pancreatic cancer is hierarchically organized and originates from a primitive stem/progenitor group of cells for which CD44$^+$ CD24$^+$ ESA$^+$ precursors constitute 1 of the most immature stages. However, Hermann and colleagues[14] have reported that a distinct subpopulation of CD133$^+$ cancer stem cells

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determined the metastatic phenotype of individual tumors. Therefore, there are 2 possible sources for cancer stem cells in pancreatic cancer; 1 is $CD44^+$ $CD24^+$ ESA^+ cells, and the other is $CD133^+$ cells. Hermann et al.[14] reported that these 2 populations overlap but are not identical. In this study, we analyzed the 2 kinds of cancer stem cell markers and found that CD44 was indeed expressed in almost 100% of pancreatic cancer cell lines. Therefore, CD44 seemed to be an inappropriate marker to isolate CD44-positive and -negative cells. Therefore, we used CD133 as a candidate marker of cancer stem cells in pancreatic cancer, and investigated the biological function of $CD133^+$ cells.

Previous studies have attributed the high expression levels of specific adenosine triphosphate-binding cassette drug transporters to the increased resistance of $CD133^+$ cancer stem cells to chemotherapeutic agents in hepatocellular carcinomas[15] and brain tumors.[16] Others have also demonstrated that cancer stem cells promoted radioresistance in glioblastoma[17] and breast cancer.[18] In pancreatic cancer, however, there are few reports regarding the biological functions of $CD133^+$ cancer cells. In the present study, we investigated the biological function of $CD133^+$ cells isolated by FACS in pancreatic cancer cell lines. Our data showed that $CD133^+$ cells might play important roles in cell proliferation, migration, and invasion in pancreatic cancer.

Invasive ductal adenocarcinoma of the pancreas is often characterized by abundant desmoplastic stroma. Recently, the stromal reaction has been a focus of attention as a characteristic feature of the majority of pancreatic cancers.[19] Therefore, we also investigated the involvement of $CD133^+$ cells in tumor-stromal interactions and found that $CD133^+$ cells significantly increased cell migration and invasion when cocultured with primary pancreatic stromal cells compared with $CD133^-$ cells. These data suggest that $CD133^+$ cells contribute to cancer-stromal interaction more than $CD133^-$ cells.

MATERIALS AND METHODS

Human Pancreatic Tissue Samples

The tissue samples analyzed in this study were obtained from patients who underwent a surgical procedure to resect a portion of the pancreas in Kyushu University Hospital, Fukuoka, Japan. Normal pancreatic tissues were also taken from areas of peripheral tissue away from the tumor or from the non-neoplastic pancreas resected because of biliary disease. Tissues were removed as soon as possible after resection and used for the experiments in the present study. The samples were suspended in Hank solution with collagenase, and mechanically dissociated using scissors and then minced with a sterile scalpel blade over ice to yield 1 × 1-mm pieces. The pieces were washed with Hank solution 3× before analysis by flow cytometry. The primary cultures of pancreatic stromal cells were established and maintained as described previously.[20] Written informed consent was obtained from all patients, and the study was approved by the ethics committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Cell Lines and Cultures

The following 14 pancreatic cancer cell lines were used: AsPC-1, KP-1N, KP-2, KP-3, PANC-1, and SUIT-2 (Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan); MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan); CAPAN-1, CAPAN-2, CFPAC-1, H48N, HS766T, and SW1990 (American Type Culture Collection, Manassas, Va); and NOR-P1 (established by Dr. N. Sato in our laboratory). A human pancreatic ductal epithelial cell line (HPDE6-E6E7 clone 6) immortalized by transduction with the E6/E7 genes of human papillomavirus 16 was kindly provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada), and maintained as described previously.[20] Primary cultures of human normal pancreatic epithelial cells were obtained from Cell Systems (Kirkland, Wash), and maintained in Cell Systems Corporation (CS-C) medium containing 10% fetal bovine serum (FBS), according to the supplier's instructions. Total cell numbers were quantified using a particle distribution counter (CDA500; Sysmex, Kobe, Japan).

Flow Cytometry and Cell Sorting

Cells were incubated in phosphate-buffered saline containing 1% FBS with phycoerythrin (PE)-conjugated antihuman CD133/2 antibody (Miltenyi Biotec, Aubum, Calif). Isotype-matched mouse immunoglobulins served as controls. For flow cytometry, samples were analyzed using an EPICS ALTRA flow cytometer (Beckman Coulter, Fullerton, Calif) and a FACS Calibur (Becton Dickinson, Bedford, Mass). For cell sorting by flow cytometry, samples were analyzed and sorted on the EPICS ALTRA. For the positive and negative population, only the top 10% most brightly stained cells or the bottom 10% most dimly stained cells were selected, respectively. Aliquots of $CD133^+$ and $CD133^-$ sorted cells were evaluated for

purity with the same machine, using the CD133/2 antibody. We also analyzed expression of cell surface markers using the antibodies of chemokine-related receptor-4 (CXCR4)-PE (R & D systems, Minneapolis, Minn).

Transfections

Transfections were performed by electroporation using a Nucleofector system (Amaxa Biosystems, Cologne, Germany). All studies were performed in triplicate. Cells (1-2 × 10^6) were centrifuged at 1200 rpm for 5 minutes, and the medium was removed. The cells were resuspended in 98 μL of Nucleofector solution (Amaxa Biosystems) at room temperature, followed by the addition of 2 μL of 50 μmol CXCR4-siRNA or a control-siRNA (both obtained from B-Bridge International, Mountain View, Calif). The transfected cells were resuspended and cultured in regular culture medium containing 10% serum for 24 hours before analysis.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction Assay for CD133 mRNA Expression

Total RNA was isolated from cell lines using the High Pure RNA isolation kit (Roche Diagnostics, Indianapolis, Ind), and RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del) at 260 and 280 nm (A260/280). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif). Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assays were performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Tokyo, Japan) with a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer's instructions. PCR was performed for 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, followed by a thermal denaturation protocol. The expression levels of CD133 mRNA in all cell lines were normalized to the expression level of 18S rRNA. The following primer pairs were used for PCR amplification: CD133-forward, 5′-GCCACCGCTCTAGATACTGC-3′; CD133-reverse, 5′-GCTTTTTCCTATGCCAAACCA-3′; CXCR4-forward, 5′-GAAGCTGTTGGCTGAAAGG-3′; CXCR4-reverse, 5′-CTCACTGACGTTGGCAAAGA-3′; stromal cell-derived factor-1 (SDF-1) (CXCL12)-forward, 5′-GATTGTAGCCCGGCTGAAGA-3′; SDF-1 (CXCL12)-reverse, 5′-TTCGGGTCAATGCACACTTGT-3′; 18S rRNA-forward, 5′-GATATGCTCATGTGGTGTTG-3′; and 18S rRNA-reverse, 5′-AATCTTCTTCAGTCGCTCCA-3′.

Cell Proliferation Assay

Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide (PI) as described previously.[21,22] $CD133^+$ and $CD133^-$ cells in pancreatic cancer were seeded at 2 × 10^4 cells/well in 24-well tissue culture plates (Becton Dickinson). In anchorage-independent proliferation experiments, cells were plated at 1 × 10^5 cells/well in 24-well plates coated with Ultra Low Attachment Surface (Corning Inc., Corning, NY). Cell proliferation was evaluated after culture for a further 72 or 120 hours after the initial cell number determination by the PI assay. PI (30 μM) and digitonin (600 μM) were added to each well to label all nuclei with PI. The fluorescence intensity, corresponding to the total cell number, was measured using a CytoFluor II multiwell plate reader (PerSeptive Biosystems, Framingham, Mass) with 530-nm excitation and 645-nm emission filters. A separate well, which possessed the same medium but no cells, was used for a baseline PI signal as a control. We evaluated the difference between each sample well and the control well. Cell proliferation was defined relative to the cell number measured at the beginning of the experiment. All experiments were performed in triplicate wells and repeated at least 3×.

Migration Assay and Invasion Assay

Migration of pancreatic cancer cells was measured by counting the number of cells that migrated from transwell chambers 24 hours after seeding cells into the upper chamber. Invasive ability of pancreatic cancer cells was measured by the number of cells invading Matrigel-coated transwell chambers. We also assessed cancer-stromal interaction by coculture with pancreatic cancer-related stromal cells.

In the migration assay, a total of 5 × 10^4 isolated cells were resuspended in 250 μL of Dulbecco modified Eagle medium (DMEM) containing 10% FBS and placed in the upper transwell chamber (8 μm pore size, Becton Dickinson, Franklin Lakes, NJ). The upper chamber was placed in a 24-well culture dish containing 750 μL of the medium described above supplemented with 5 × 10^4 primary pancreatic stromal cells. After 24 hours of incubation at 37°C, the number of migrated cells was counted. In the invasion assay, 5 × 10^4 isolated cells were plated in the upper Matrigel-coated transwell chambers (filled with 20 μg of Matrigel/well) and reconstituted with 10% FBS-containing medium for 2 hours before the experiment.

Cells were seeded into the upper chambers in 250 μL of DMEM supplemented with 10% FBS. The same medium (750 μL) was placed in the lower wells. After 72 hours of incubation at 37°C, the number of invading cells was counted.

Cells that had degraded the Matrigel and invaded the lower surface of the Matrigel-coated membranes were fixed with 70% ethanol, stained with H & E, and counted in 5 random fields at ×200 magnification under a light microscope. The results were expressed as the average number of invading cells per field.

Statistical Analysis

All data were expressed as the mean ± standard deviation and evaluated using an analysis of variance on Microsoft Office Excel software (Redmond, Wash). Values of $P < .05$ were accepted as statistically significant in any analysis.

RESULTS

CD133 Is Markedly Overexpressed in Pancreatic Cancer Tissues Compared With Normal Pancreatic Tissues

We measured the expression of CD133 in human pancreatic cancer tissues and normal pancreatic tissues by flow cytometry, and found that the expression of CD133 in cancer tissues was much higher than in nonmalignant tissues (Fig. 1A, $P < .001$).

We performed quantitative RT-PCR and flow cytometry to measure the expression of CD133 in 14 pancreatic cancer cell lines, HPDE cells, and primary cultures of normal pancreatic epithelial cells and pancreatic fibroblasts (Fig. 1B and C). There was a wide range of relative CD133 expression levels among the pancreatic cancer cell lines. The primary normal pancreatic epithelial cells and fibroblasts did not express detectable levels of CD133. We selected SUIT-2 and KP-2 cells in which >10% of cells were determined to be isolated $CD133^+$ cells and $CD133^-$ cells by FACS for the following experiments.

More $CD133^+$ Cells Survive Than $CD133^-$ Cells Under Anchorage-Independent Conditions

To investigate cell proliferation and cell survival, we performed PI assays using SUIT-2 and KP-2 cells. In anchorage-dependent conditions, there was no significant difference in cell proliferation between $CD133^+$ and $CD133^-$ cells (Fig. 2A). However, in anchorage-independent conditions, cell survival of $CD133^+$ cells was significantly increased compared with $CD133^-$ cells in both SUIT-2 and KP-2 cells (Fig. 2B; $P = .004$).

$CD133^+$ Cells Show High Migration and Invasion Ability, Especially When Cocultured With Primary Pancreatic Stromal Cells

Next, we compared the migration and invasion abilities of $CD133^+$ and $CD133^-$ cells using SUIT-2 and KP-2 cells. The $CD133^+$ SUIT-2 and KP-2 cells exhibited slightly increased cell migration compared with $CD133^-$ cells 24 hours after seeding in monoculture conditions (Fig. 3A, $P = .0028$; Fig. 3B, $P = .035$, respectively). When cocultured with pancreatic stromal cells, $CD133^+$ cells exhibited markedly increased invasive potential compared with $CD133^-$ cells (Fig. 3A, $P = .0002$; Fig. 3B, $P < .0001$, respectively). Although we found no significant difference in cell invasion between $CD133^+$ and $CD133^-$ cells at 72 hours after seeding under monoculture conditions (Fig. 3C; $P = .12$), $CD133^+$ cells exhibited markedly increased invasive potential compared with $CD133^-$ cells when cocultured with pancreatic stromal cells (Fig. 3C; $P < .0001$). Similar results were again found in KP-2 cells (Fig. 3D, $P = .023$ and $P < .0001$, respectively).

$CD133^+$ Cells Expressed CXCR4 at Much Higher Level Than $CD133^-$ Cells

To assess the mechanism underlying the increase in the migration and invasion ability of $CD133^+$ cells in cocultures with pancreatic stromal cells, we focused on 2 major tumor-stromal cell interactions in pancreatic cancer, CXCR4/SDF-1[14] and c-Met/hepatocyte growth factor (HGF).[20] SUIT-2 and KP-2 $CD133^+$ cells expressed a significantly higher level of CXCR4 mRNA than $CD133^-$ cells (Fig. 4A; $P = .0003$ and .001, respectively). However, there was no significant difference in c-Met mRNA expression (Fig. 4A). These results suggest that CXCR4 may be responsible for the increased migration and invasion ability of $CD133^+$ cells when cocultured with pancreatic stromal cells secreting SDF-1 (Fig. 4B, CAF-3).

Down-Regulation of CXCR4 Decreases Cell Migration and Invasion Only in $CD133^+$ Cells

To assess whether the CXCR4 /SDF-1 axis plays an important role in the migration and invasion of $CD133^+$ cells in pancreatic cancer, we down-regulated CXCR4 using RNA interference. At 24 hours (Day 1) after transfection with 100 pmol of CXCR4-siRNA or control-siRNA, $CD133^+$ and $CD133^-$ cells transfected with CXCR4-siRNA showed 0.3-fold lower levels of CXCR4 mRNA than the cells transfected with control-siRNA (Fig. 5A). At 120 hours (Day 5) after transfection, CXCR4-siRNA was

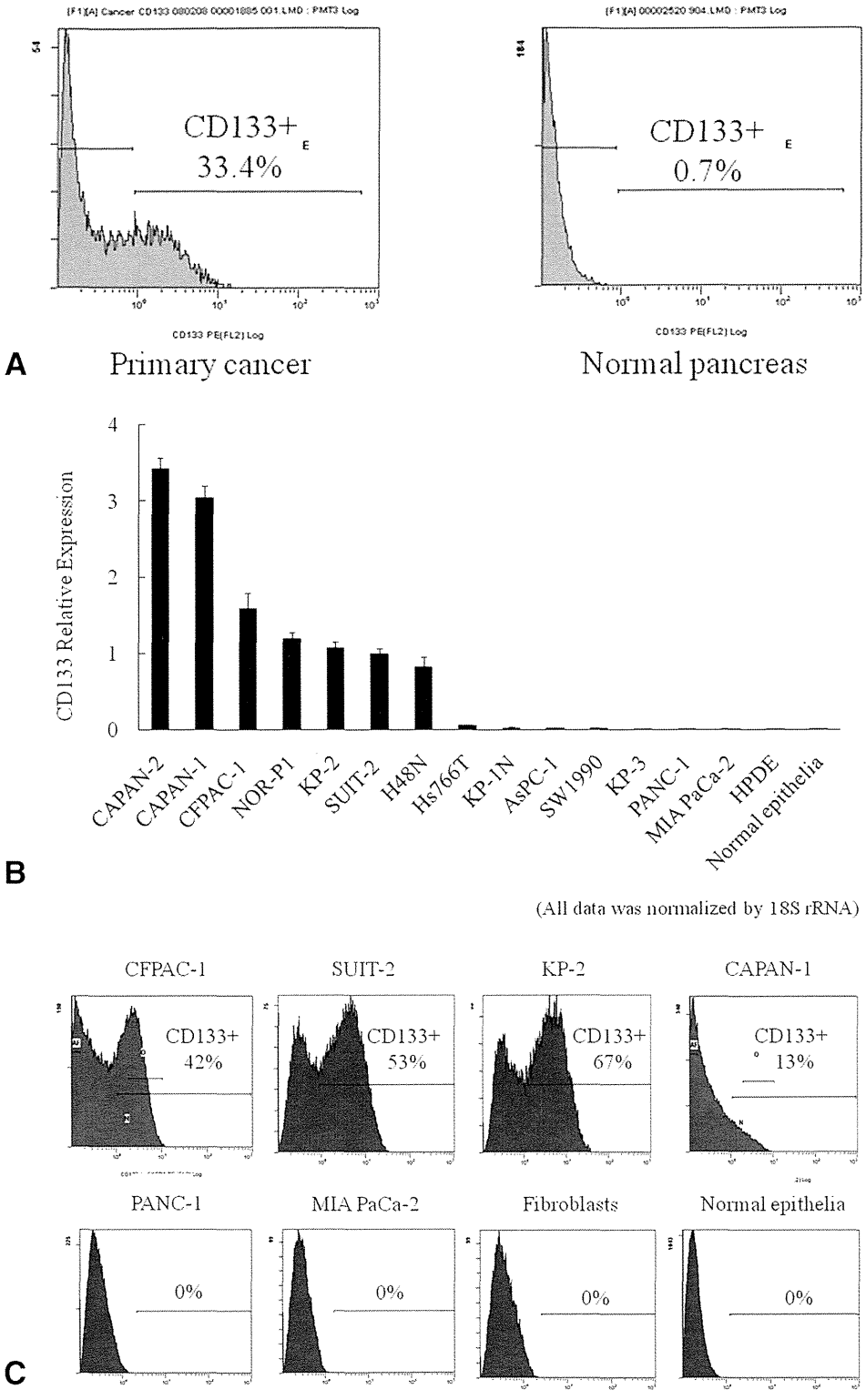


Figure 1. Levels of CD133 expression in human pancreatic tissues and cell lines are shown. (A) Levels of CD133 expression in pancreatic cancer tissues and normal pancreatic tissues as determined by flow cytometry are shown. (B) Levels of CD133 mRNA expression were assessed by quantitative reverse transcriptase polymerase chain reaction and normalized to the level of 18S rRNA in each sample. Data represent the mean ± standard deviation of triplicate measurements. (C) Levels of CD133 expression in pancreatic cancer cell lines were assessed by flow cytometry. Data represent triplicate measurements.

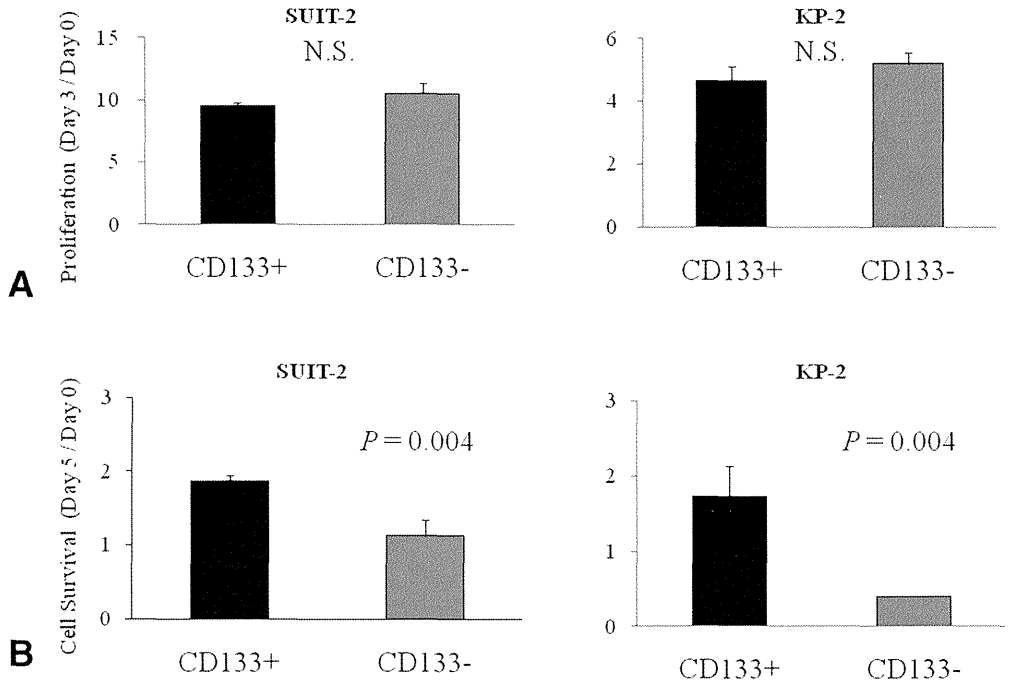


Figure 2. Cell proliferation and cell survival of pancreatic cancer cells are shown. The propidium iodide assay was used to compare cell proliferation and cell survival between $CD133^+$ and $CD133^-$ cells. Two kinds of pancreatic cancer cell lines, SUIT-2 and KP-2, were used and sorted by CD133 expression. (A) In anchorage-dependent conditions, there was no significant difference in proliferation between $CD133^+$ and $CD133^-$ cells. (B) In anchorage-independent conditions (floating culture), cell survival of $CD133^+$ cells was significantly increased compared with $CD133^-$ cells in both SUIT-2 and KP-2 cells (P=.004). Each value represents the mean ± standard deviation of triplicate measurements. N.S. indicates not significant.

effective (Fig. 5A), and no morphological differences between these transfected cells were observed.

Down-regulation of CXCR4 significantly decreased cell migration and invasion only in $CD133^+$ cells cocultured with pancreatic stromal cells (CAF-3) (Fig. 5B-E). These data suggest that the CXCR4/SDF-1 pathway strongly affects the migration and invasion ability of $CD133^+$ cells. We also evaluated several invasion-related genes, and found that $CD133^+$ cells expressed slightly increased levels of FOXM1, MMP-9, and VEGF-A mRNA (data not shown). These results suggest that $CD133^+$ cells may have several pathways influencing cell invasion in addition to the CXCR4/SDF-1 axis.

DISCUSSION

In the present study, we have evaluated the biological function of $CD133^+$ cells in pancreatic cancer, and obtained the first data regarding the specific relationship between $CD133^+$ cells and primary pancreatic stromal cells. We found that CD133 expression was increased in pancreatic cancer tissue compared with normal pancreatic tissues as well as in cancer cell lines compared with normal pancreatic epithelial cells, and that $CD133^+$ was a marker of high proliferative potential in floating cultures, migration, and invasion. In particular, migration and invasion of $CD133^+$ cells cocultured with primary pancreatic stromal cells was greatly enhanced.

Recently, CD133 expression has been shown to be a prognostic marker for poor survival in patients with colon cancer,[23,24] and brain tumors.[25,26] Maeda et al.[27] also reported that CD133 expression is correlated with lymph node metastasis, vascular endothelial growth factor-C expression, and poor prognosis in pancreatic cancer. As well as these previous studies, our data also suggest that $CD133^+$ cells are involved in pancreatic tumor progression, through processes such as invasion and migration.

In a further step, we evaluated the relationship between $CD133^+$ cancer cells and stromal cells, focusing on the CXCR4/SDF-1 axis. Previously, some studies reported that CXCR4/SDF-1 contributed to tumor progression. The CXCR4/SDF-1 axis promotes migration and invasion in breast cancer.[28] We have also previously reported that DNA methylation influenced CXCR4 expression in pancreatic cancer.[29] It is possible that these findings provide new insights into the role of CXCR4/

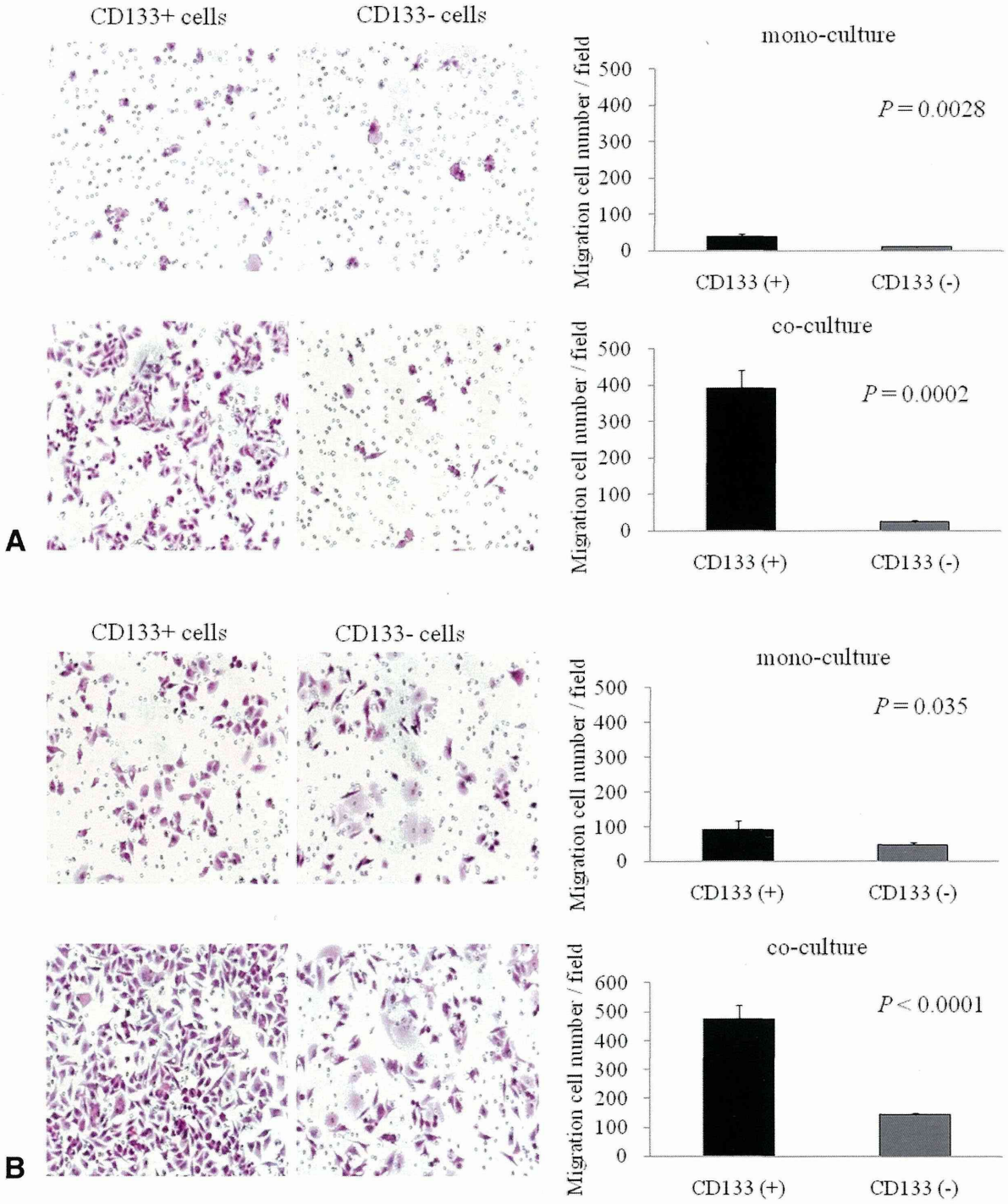
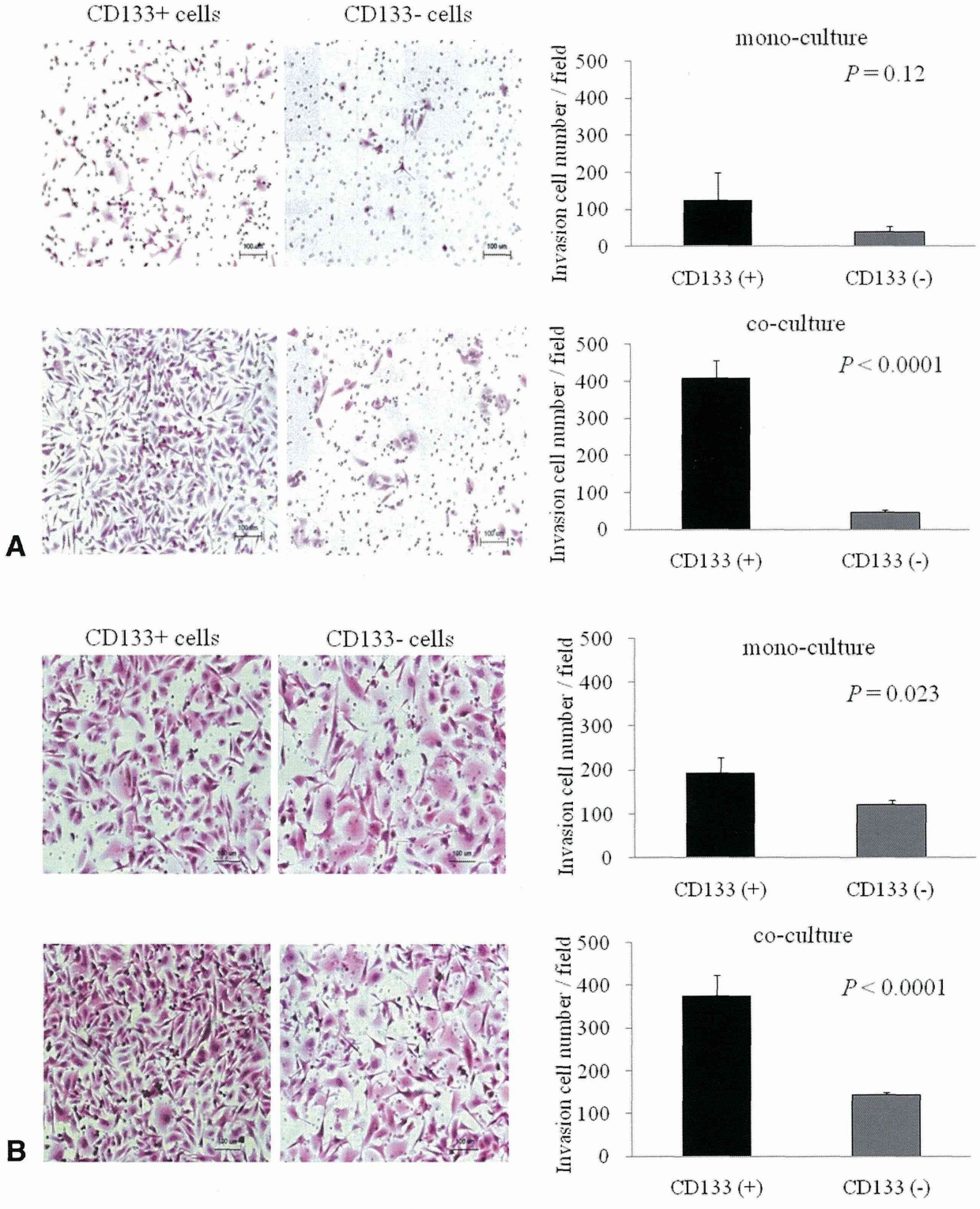


Figure 3. Cell migration and invasion of CD133$^+$ and CD133$^-$ cells in pancreatic cancer are shown. (A) Migration of SUIT-2 CD133$^+$ cells increased slightly compared with CD133$^-$ cells in monoculture conditions (P=.0028), and increased markedly when cocultured with pancreatic stromal cells (P=.0002). (B) Migration of KP-2 CD133$^+$ cells increased slightly compared with CD133$^-$ cells in monoculture conditions (P=.035), and increased markedly when cocultured with pancreatic stromal cells ($P < .0001$). (C, D) A comparison of invasive ability of CD133$^+$ and CD133$^-$ cells is shown. (C) The invasive ability of SUIT-2 CD133$^+$ cells increased slightly compared with CD133$^-$ cells in monoculture conditions (P=.12), and increased markedly when cocultured with pancreatic stromal cells ($P < .0001$). (D) The invasive ability of KP-2 CD133$^+$ cells increased slightly compared with CD133$^-$ cells in monoculture conditions (P=.035), and increased markedly when cocultured with pancreatic stromal cells ($P < .0001$).



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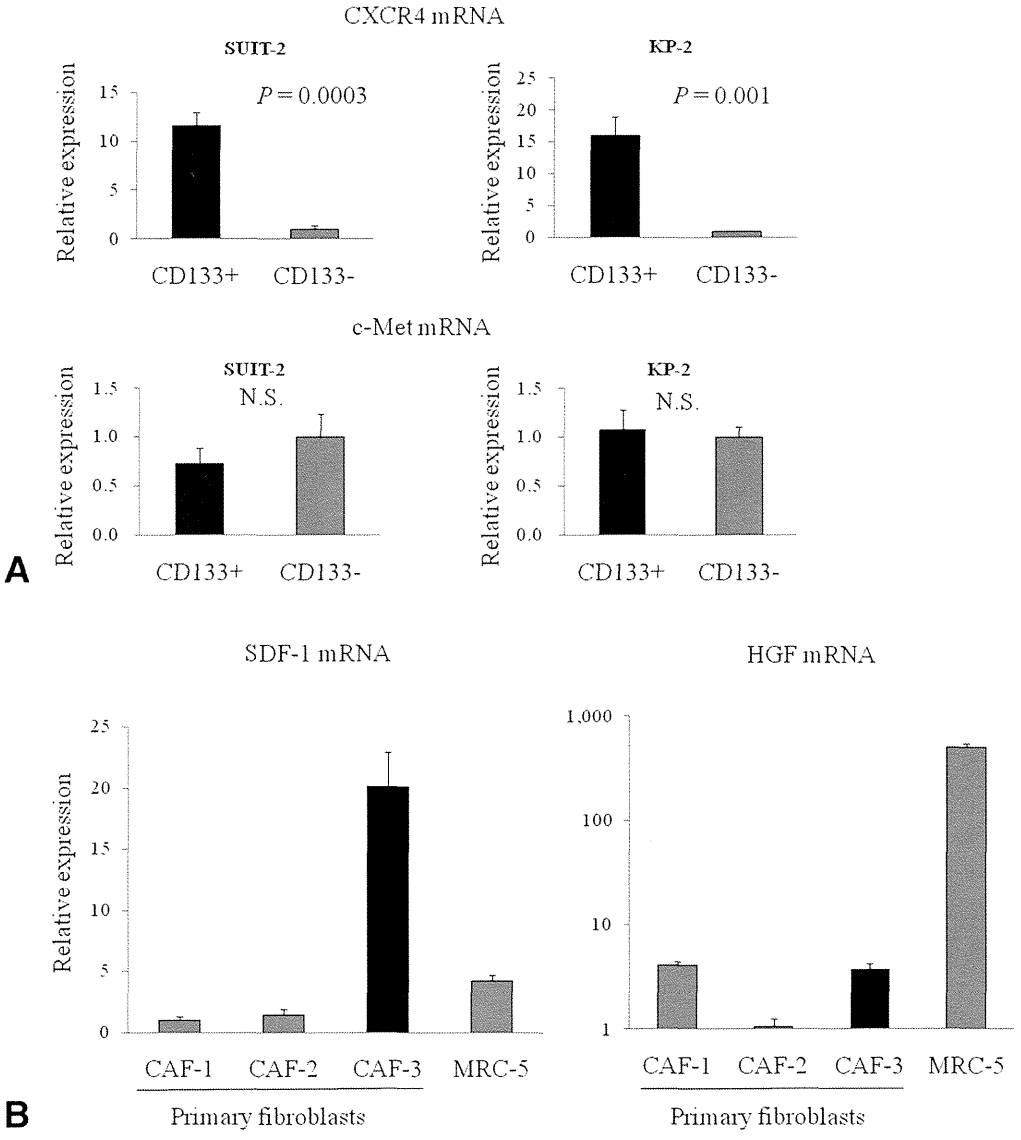

Figure 4. Levels of chemokine-related receptor-4 (CXCR4) and c-Met mRNA in pancreatic cancer are shown. (A) CXCR4 mRNA was assessed, and a significant difference between $CD133^+$ and $CD133^-$ cells in both SUIT-2 and KP-2 cells was found ($P = .0003$ and .001, respectively). However, there was no significant difference in c-Met mRNA expression. (B) Stromal cell-derived factor-1 (SDF-1) and hepatocyte growth factor (HGF) mRNA were expressed in the pancreatic stromal cells used in this study. N.S. indicates not significant.

SDF-1 interaction in tumor progression. Other studies have demonstrated that the CXCR4/SDF-1 axis is involved in tumor progression, influencing cell invasion, metastasis, and neoangiogenesis, in acute myeloid leukemia,[30] glioma,[31] breast cancer,[32] ovarian cancer,[33] and others. CXCR4 expression is associated with poor survival in colon cancer,[34] malignant melanoma,[35] and sarcoma.[36,37] In this study, we found that CD133 expression correlated with CXCR4 expression, and that $CD133^+$ cells exhibited markedly increased cell invasiveness compared with $CD133^-$ cells when cocultured with pancreatic stromal cells secreting SDF-1. The data suggest that $CD133^+$ cells increase tumor progression via the CXCR4/SDF-1 axis through tumor/stromal cell interaction in pancreatic cancer. There were no differences in the expression of c-Met mRNA between $CD133^+$ cells and $CD133^-$ cells (Fig. 4A), suggesting that the differences in stromal cell-enhanced invasion between $CD133^+$ cells and $CD133^-$ cells is not dependent on the differences in activation of c-Met/HGF pathway. However, there may be contribution of other signaling molecules, which were not examined here.

Although some studies have demonstrated that high expression levels of specific adenosine triphosphate-binding cassette drug transporters increase resistance of

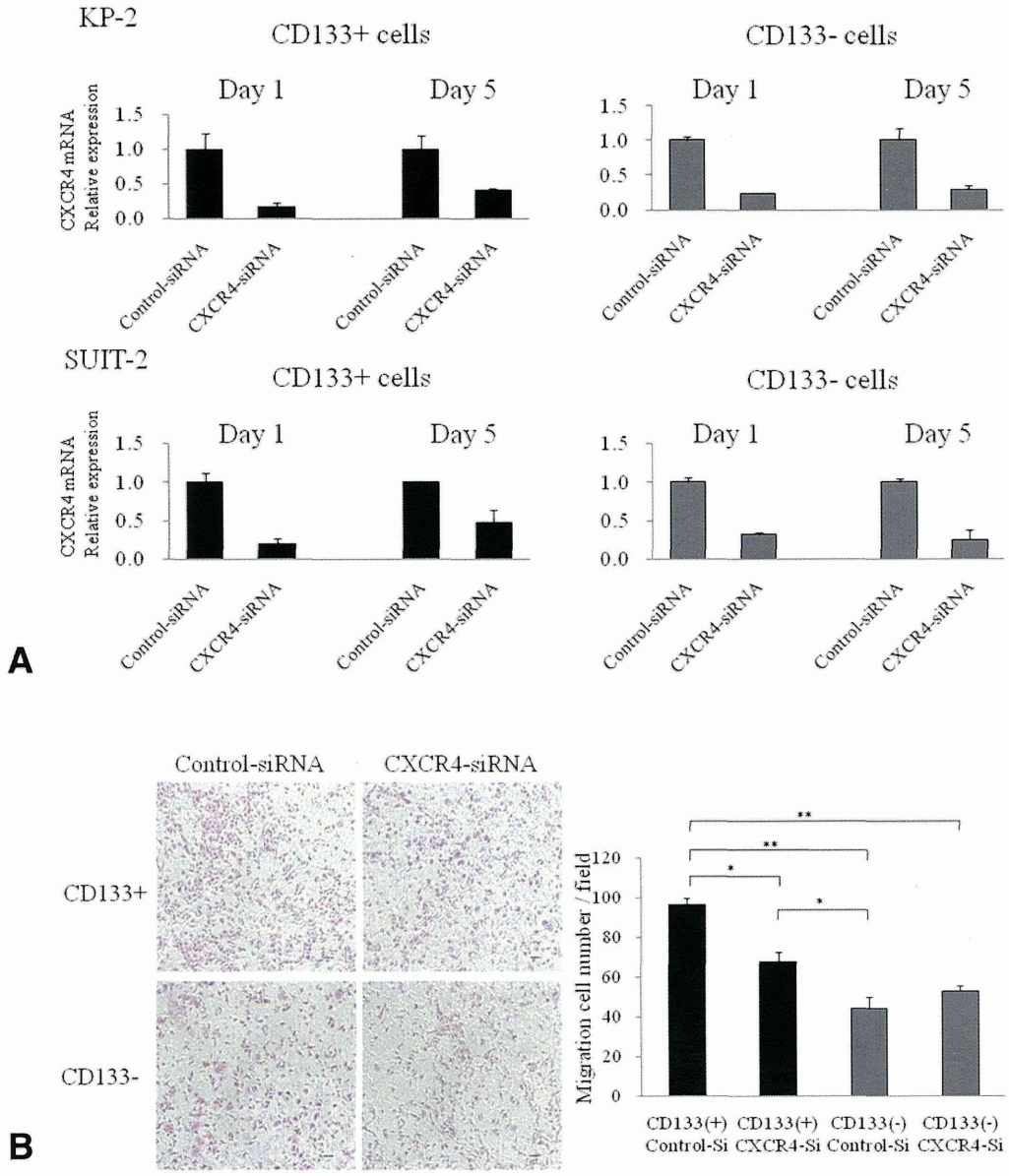

Figure 5. The effect of chemokine-related receptor-4 (CXCR4) on cell migration and Matrigel invasion in $CD133^+$ cells cocultured with pancreatic stromal cells is shown. (A) Levels of CXCR4 mRNA in $CD133^+$ and $CD133^-$ cells transfected with CXCR4-siRNA or control-siRNA at 24 hours (Day 1) and 120 hours (Day 5) in KP-2 and SUIT-2 cells are shown. Transfection with CXCR4-siRNA led to 0.3-fold lower levels of CXCR4 mRNA than the cells transfected with control-siRNA in both $CD133^+$ and $CD133^-$ cells. (B, C) Down-regulation of CXCR4 significantly decreased cell migration in $CD133^+$ cells cocultured with pancreatic stromal cells in KP-2 cells and SUIT-2 cells (*$P < .05$, **$P < .01$). (D, E) CXCR4 down-regulation decreased Matrigel invasion, especially in $CD133^+$ cells cocultured with pancreatic stromal cells in KP-2 cells and SUIT-2 cells(*$P < .05$, **$P < .01$, ***$P < .001$).

CD133 cancer stem cells to chemotherapeutic agents in hepatocellular carcinoma[15] and brain tumors,[16] we found no difference in chemoresistance between $CD133^+$ cells and $CD133^-$ cells (data not shown). Collectively, our data suggest that $CD133^+$ cells possess more aggressive behavior, such as increased cell proliferation, migration, and invasion, especially when cocultured with pancreatic stromal cells. The targeting therapy for the interaction between $CD133^+$ cancer cells and stromal cells may be a new approach to the treatment of pancreatic cancer.

CONFLICT OF INTEREST DISCLOSURES

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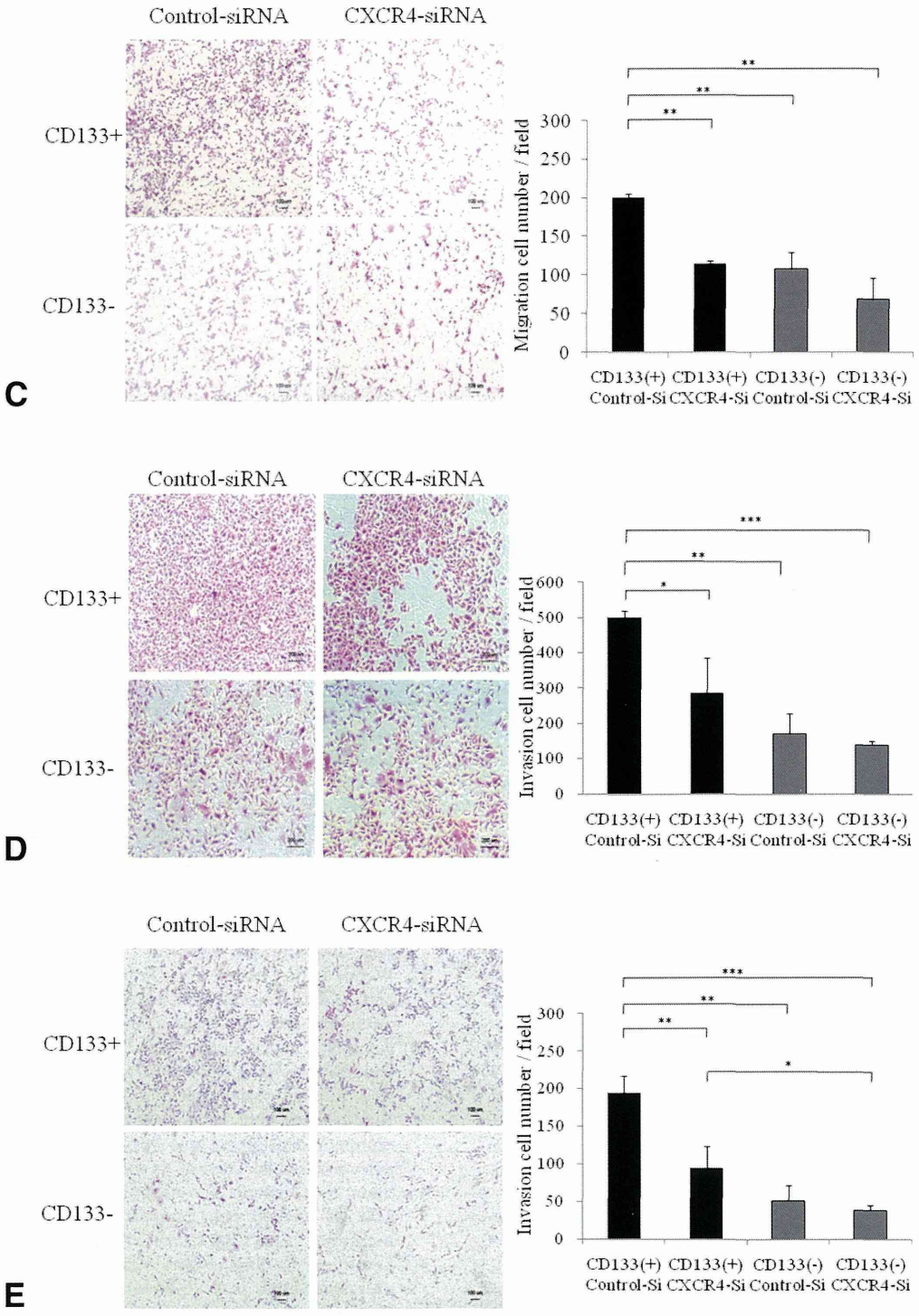

Figure 5. (*Continued*).

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