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SDF-1/CXCR4 signaling induces pancreatic cancer cell invasion and epithelial–mesenchymal transition *in vitro* through non-canonical activation of Hedgehog pathway

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Abstract

In our previous study, we found that blockade of SDF-1/CXCR4 signaling inhibits pancreatic cancer cell migration and invasion *in vitro*. However, the mechanism governing the downstream regulation of SDF-1/CXCR4-mediated invasion remains unclear. Here we report the role of SDF-1/CXCR4 in pancreatic cancer and the possible mechanism of SDF-1/CXCR4-mediated pancreatic cancer invasion. We show that there is a cross-talk between SDF-1/CXCR4 axis and non-canonical Hedgehog (Hh) pathway in pancreatic cancer. Furthermore, our data demonstrate that the ligand of CXCR4, SDF-1 induces CXCR4-positive pancreatic cancer invasion, epithelial–mesenchymal transition (EMT) process and activates the non-canonical Hh pathway. Moreover, we also demonstrate that the invasion of a pancreatic cancer and EMT resulting from the activation of SDF-1/CXCR4 axis is effectively inhibited by Smoothened (SMO) inhibitor cyclopamine and siRNA specific to Gli-1. Collectively, these data demonstrate that SDF-1/CXCR4 modulates the non-canonical Hh pathway by increasing the transcription of SMO in a ligand-independent manner. Taken together, SDF-1/CXCR4 axis may represent a promising therapeutic target to prevent pancreatic cancer progression.

Keywords

CXCR4; Hedgehog pathway; Pancreatic cancer invasion; Epithelial–mesenchymal transition

1. Introduction

Pancreatic cancer is one of the most aggressive malignancies in the world and its 5-year survival rate is less than 5% [1]. Due to the lack of early symptoms and reliable diagnostic markers for early detection, 80% of patients with pancreatic cancers are diagnosed at a locally advanced or metastatic stage. The exact molecular mechanisms which are responsible for this dismal clinical course remain largely unknown. Recently, multiple genes have been identified to be involved in the process of pancreatic cancer invasion and metastasis [2,3].

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.canlet.2012.02.035>.

The stromal-derived factor-1 (SDF-1)/CXCR4 axis is deregulated in multiple human cancers, including pancreatic cancer [4,5]. Elevated levels of SDF-1 in patients with pancreatic cancer have been linked to a poor outcome [6]. CXCR4 has also been reported to be highly expressed in pancreatic cancer cells [7] and its elevated level is correlated with a poor clinical outcome in pancreatic carcinoma patients [8]. In our previous study [9], we found that blockade of SDF-1/CXCR4 signaling inhibits pancreatic cancer cell migration and invasion *in vitro*. However, the mechanism governing the downstream regulation of SDF-1/CXCR4-mediated invasion is still the focus of investigators.

The Hedgehog (Hh) pathway, which is known to control cell proliferation and differentiation in developing embryos [10], is over-expressed in many extracutaneous cancers, including pancreatic cancers [11,12]. The Hh signaling protein functions by binding to a 12-pass-transmembrane receptor called Patched 1 (Ptch1). The binding of Hh to Ptch1 results in the release of the inhibitory effects of Ptch1 on the Smoothened (SMO), a 7-transmembrane spanning protein. The activated SMO then re-localizes to the primary cilia and initiates an intracellular signaling cascade that eventually leads to the activation of the Gli-1 transcription factor and the up-regulation of the downstream target genes, including Ptch1 [13]. Another study has examined the expression of Ptch and Gli-1 in 54 pancreatic cancer surgical specimens and seven available pancreatic cancer cell lines. This study demonstrated that Hh signaling activation is a very common event in pancreatic cancer [14]. The activation of the Hh pathway in pancreatic cancer could induce an epithelial–mesenchymal transition (EMT), which results in invasion and metastasis through up-regulating the expression of E-cadherin and down-regulating the expression of vimentin [2,15].

In the present study, we aimed to study the role of SDF-1/CXCR4 in pancreatic cancer and the possible mechanism of SDF-1/CXCR4-mediated pancreatic cancer invasion. We found that CXCR4 is expressed in pancreatic cancer cells and demonstrated that activation of CXCR4 by its ligand SDF-1 leads to increased expression of SMO, resulting in Hh pathway activation and cancer cell invasion.

2. Materials and methods

2.1. Cell culture and reagents

The human pancreatic cancer cell lines BxPc-3, MiaPaCa-2, and Panc-1 were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) (HyClone, Logan, USA) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml ampicillin, and 100 µg/ml streptomycin. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Recombinant human SDF-1 was purchased from PeproTech (Rocky Hill, USA). The pharmacological reagent cyclopamine was provided by Selleck Chemicals (Houston, USA) and AMD3100 was purchased from Sigma (St. Louis, USA). Before drug treatment, pancreatic cancer cells in log phase growth were cultured in six-well plates in the media containing only 1% FBS for 24 h. The drugs (or solvent only) were then administered at given concentrations in medium containing 1% FBS, and the plates were incubated for another 72 h before a matrigel invasion assay. Antibodies were purchased from different resources: anti-CXCR4 antibody (Santa Cruz Biotechnology, Santa Cruz, USA), anti-SMO antibody (Bioworld, Minneapolis, USA), anti-Gli-1 antibody (Santa Cruz Biotechnology), anti-E-Cadherin antibody (Santa Cruz Biotechnology), anti-Vimentin (Bioworld), and anti-β-actin antibody (Santa Cruz Biotechnology).

2.2. Matrigel invasion assay

Pancreatic cancer cell invasion was assessed by a chamber-based invasion assay. In brief, the upper surface of a filter (pore size, 8.0 μ m; Millipore, Billerica, USA) was coated with basement membrane matrigel (BD Biosciences, Franklin Lakes, USA). The cells were suspended in DMEM containing 1% FBS. Then the cell suspensions (100 μ l containing 10,000 cells) were added to the upper chambers. Simultaneously, 500 μ l of DMEM containing 10% FBS was placed in the lower chambers. The cells were allowed to migrate for 24 h at 37 °C. The non-migrated cells were removed from the upper surface by scraping with a cotton swab. After incubation, the filter was fixed and stained with crystal violet. All of the cells that had migrated from the upper to the lower side of the filter were counted under a light microscope by counting 10 random fields at a magnification of 100 $\times$. The tumor cell invasion assay was performed in triplicate.

2.3. Western blotting analysis

Cells were lysed using cell lysis buffer containing 40 mM Tris-HCl (pH 7.4), 10% glycerol, 50 mM β -glycerophosphate, 5 mM ethyleneglycol-bis (aminoethylether)-tetraacetic acid, 2 mM ethylenediaminetetraacetic acid, 0.35 mM vanadate, 10 mM NaF, 0.3% Triton X-100, and protease inhibitors (Roche, Penzberg, Germany). Equivalent amounts of protein were subject to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to immobilon membranes (Millipore). The membranes were incubated with primary antibody overnight at 4 °C followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) for 2 h. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Millipore).

2.4. Real-time PCR assay

Real-time RT-PCR was performed to determine the messenger RNA (mRNA) levels of CXCR4, SMO, Gli-1, E-cadherin, vimentin, and GAPDH. Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA), and reverse transcription was performed using a PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The real-time experiments were conducted on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using a SYBR Green Real-time PCR Master Mix (TaKaRa). The PCR reactions consisted of 30 s at 95 °C followed by 40 cycles of at 95 °C for 5 s, at 60 °C for 30 s and at 72 °C for 30 s. The PCR primer sequences for CXCR4, SMO, Gli-1, E-cadherin, vimentin, and GAPDH are shown in Supplementary Table S1. The comparative C (T) method was used to quantitate the expression of each target gene using GAPDH as the normalization control [16].

2.5. RNAi transfections

siRNA against Gli-1 (Gli-1-Homo-2758: 5'-GGCUCAGCUUGUGUGUAAUTT-3', 5'-AUUACACACAAGCUGAGCCTT-3') and a negative control siRNA (NC: 5'-UUC UCC GAA CGU GUC ACG UTT-3', 5'-ACG UGA CAC GUU CGG AGA ATT-3') were purchased from GenePharm (Shanghai, China). Cells (0.2×10^6 wells) seeded in six-well plates were transfected with 100 nM siRNA using Lipofectamine RNAi MAX Reagent (Invitrogen, CA, USA) according to the manufacture's instructions. The cells were used for further experiments at 24 h after transfection.

2.6. Statistical analyses

The results are presented as the mean $\pm$ standard error. The analysis of difference was performed using one-way ANOVA with the LSD *post hoc* test for multiple comparisons with SPSS version 13.0 software. $P < 0.05$ was considered as significant difference.

3. Results

3.1. Expression of CXCR4 and SMO in pancreatic cancer cells

To explore the possible role of CXCR4 signaling in activation of Hh pathway in pancreatic cancer cells, we first explored the expression of CXCR4 and SMO in three human pancreatic cancer cell lines. According to the cell characteristics, MiaPaCa-2 is undifferentiated. Panc-1 is poorly differentiated while BxPc-3 is moderately differentiated. As shown in Fig. 1, the CXCR4 protein was detected in MiaPaCa-2 and Panc-1 cells, but not in BxPc-3. Similar results were observed while detecting the CXCR4 mRNA levels in the three cell lines by real-time RT-PCR. Additionally, SMO, a member in Hh pathway, was detected in all three cell lines.

3.2. The modulation of CXCR4 influence the invasiveness of pancreatic cancer cells

To determine the effect of SDF-1/CXCR4 signaling on the cell invasiveness, pancreatic cancer cells were cultured for 24 h with or without SDF-1 and then assessed for invasion, the results show that 50 ng/ml or 100 ng/ml of SDF-1 significantly increased the invasion rates of MiaCaPa-2 and Panc-1 cells; however, SDF-1 had no effect on the rate of cell invasion of BxPc-3, a CXCR4-negative cell ($P > 0.05$, Fig. 2). These results suggest that SDF-1 modulates the cell invasion through CXCR4 receptor.

EMT, an important process for cancer progression, is characterized by the loss of cell–cell adhesion with diminished expression of epithelial marker such as E-cadherin and increased expression of mesenchymal marker such as vimentine [17]. To investigate the possible role of SDF-1/CXCR4 signaling on EMT process, we performed Western blotting and real-time RT-PCR to explore the expression of E-cadherin and vimentine at both protein and mRNA levels. The results showed that both 50 ng/ml and 100 ng/ml SDF-1 significantly decreased the expression of E-cadherin at both mRNA and protein levels ($P < 0.05$). Simultaneously, the expression levels of vimentin mRNA and protein were significantly increased in response to the SDF-1 treatment (Fig. 3).

Although SDF-1 could influence the EMT process and the cell invasive ability, the mechanism remains unclear. To further confirm whether the SDF-1-induced invasion is dependent upon the activation of its receptor CXCR4, we used a CXCR4 antagonist AMD3100 (2 μ g/ml) to block the function of CXCR4 in the pancreatic cancer cells, that in turn reduce the effect of SDF-1 on these cells. The result showed that inhibition of CXCR4 significantly decreased pancreatic cancer cell invasion ($P < 0.05$, Fig. 4A). Furthermore, the SDF-1 induced down-regulation of E-cadherin and up-regulation of vimentin in pancreatic cancer cells were abolished upon addition of AMD3100 (Fig. 4B and C). These data suggest that SDF-1 could promote the pancreatic cancer progression through CXCR4.

3.3. SDF-1 activates Hh pathway in pancreatic cancer in a CXCR4-dependent manner

Since Hh pathway has been shown to be a treatment target for pancreatic cancer [18], we next investigated the effect of SDF-1 on the activation of Hh pathway. A high dose (100 ng/ml) of SDF-1 significantly increased the transcription of Hh pathway-related genes, including SMO and Gli-1, in MiaPaCa-2 and Panc-1 cells (Fig. 5A and B).

To confirm if the increased invasion resulted by SDF-1-induced Hh pathway activation in pancreatic cancer cells is dependent upon its receptor CXCR4, we treated the cells with AMD3100 to block the function of CXCR4 prior to the addition of SDF-1. As shown in Fig. 4A, AMD3100 inhibited the invasion of CXCR4-positive pancreatic cancer cells. At the same time, inhibition of CXCR4 significantly decreased the expression of Hh pathway-related molecules SMO and Gli-1 compared to the SDF-1 alone (Fig. 5C and D). Thus, the

data indicates that SDF-1 increases the activation of Hh pathway in a CXCR4-dependent manner.

3.4. Activation of CXCR4 increases pancreatic cancer invasion and Hh pathway activation by increasing the expression of SMO

Since the activation of CXCR4 simultaneously induces tumor cell invasion and Hh pathway activation, we hypothesized that a cross-talk exists between the SDF-1/CXCR4 axis and the non-canonical Hh pathway and this cross-talk contributes to pancreatic cancer invasion. To test this hypothesis, we investigated the relationship between SDF-1/CXCR4-induced invasion and Hh pathway activation using a SMO antagonist, cyclopamine, and siRNA targeting Gli-1. 10 μ M of cyclopamine or Gli-1 siRNA did not affect the cell number measured by MTT assay for cell proliferation after 24 h of culture.

Interestingly, cyclopamine significantly decreased pancreatic cancer invasion (Fig. 6A), reversed the down-regulation of E-cadherin and up-regulation of vimentin (Fig. 6B and C), and reduced the expression of Gli-1 even in the presence of SDF-1 (Fig. 6B and D). Therefore, we conclude that inhibition of Hh pathway activation via blocking the function of SMO could abolish the invasion and EMT induced by SDF-1. These findings suggest that there is a cross-talk exists between the SDF-1/CXCR4 axis and Hh pathway activation, which is mediated by increased SMO expression.

To further confirm if SDF-1 could influence SMO expression earlier than Gli-1 in Hh pathway, we used Gli-1 siRNA to knockdown Gli-1 in pancreatic cells (Fig. 7A and B) and then tested for cell invasion. We found that the increased invasion of pancreatic cancer in the presence of SDF-1 was significantly abolished in the condition of the Gli-1 knockdown (Fig. 7C). Additionally, the levels of E-cadherin at both mRNA and protein expression were obviously increased, while the expression of vimentin at mRNA and protein levels significantly decreased, even though the expression of SMO was still up-regulated by SDF-1 in Gli-1 siRNA groups compared with siRNA control (Fig. 7D). Since the blockade of Gli-1 could not influence SMO expression, these data suggest that SDF-1 contributes to increased pancreatic cancer cell invasion and EMT in a SMO-dependent manner. The cross-talk between the SDF-1/CXCR4 axis and the Hh pathway is present and essential for pancreatic cancer invasion.

4. Discussion

SDF-1 is broadly expressed in a variety of tissue types and acts as a potent chemo-attractant for immature and mature hematopoietic cells [19,20]. Recently, there is increased evidence that SDF-1/CXCR4 signaling plays an important role in cancer [21,22]. We and others have corroborated the role of CXCR4 signaling in the local invasion and distant metastasis of pancreatic cancer [9,23]. CXCR4 expression was detected in pancreatic carcinoma cell lines and cancer tissues in earlier reports [7]; however, SDF-1 expression was rarely detected in all of the studied pancreatic cancer cell lines, but has been identified in all pancreatic cancer tissue samples. Consistent with recent published data [7], our results find that CXCR4 is expressed in undifferentiated MiaPaCa-2 cells and poorly differentiated Panc-1 cells. Furthermore, the results show that SDF-1/CXCR4 axis can stimulate tumor cell invasion. We analyzed the cross-talk between the SDF-1/CXCR4 axis and the Hh pathway and the contribution of this cross-talk to both EMT and invasion in pancreatic cancer. Such cross-talk could explain the mechanisms by which the chemoattractant cytokine SDF-1 induces tumor migration and invasion.

The Hh signaling pathway has been reported to be related to cancer cell mobility, migration, and invasion in several types of cancers [18,24], probably through direct participation in cell

migration and angiogenesis processes [25]. Recently, a paracrine requirement for Hh signaling from epithelial tumor cells to the stroma in pancreatic cancer has been reported [15,26]. Another report found that mutant KRAS induces or enhances sonic hedgehog expression and favors paracrine Hh signaling, and antagonizes autocrine Hh signal transduction [27]. However, the activation of Hh pathway in pancreatic tumor cells themselves under conditions of ligand blocking is unclear, despite the important role played by Hh paracrine signaling in activating tumor-associated stroma in a ligand-dependent manner in pancreatic cancer development. Here, we present compelling evidence that the Hh signaling pathway is activated in tumor cells via a ligand-independent manner to result in tumor cell EMT and invasion.

EMT is an important characteristic of human malignant tumors [28] and the loss of E-cadherin and gain of vimentin are known to play key roles in EMT process of various human cancers, including pancreatic cancer [29]. Interestingly, our results show that the activation of the Hh pathway and the process of EMT are took place in the presence of exogenous SDF-1. Based on previously published evidence and our findings, we thus speculate that there is a cross-talk exists between the SDF-1/CXCR4 axis and the Hh pathway, which is the critical for the SDF-1-induced cancer cell invasion and EMT. This notion is further supported by the factor that the inhibition of CXCR4 by AMD3100 prevented activation of the Hh pathway by SDF-1, suggesting that CXCR4 is the key factor in SDF-1-mediated activation of the Hh pathway.

Hh signaling could be regulated at different levels by multiple components of the pathway. Cyclopamine, a natural alkaloid derivative isolated from a plant of the lily family *verayum californicum*, is able to specifically inhibit the Hh pathway through inhibition of SMO activity by binding to its heptahelical bundle. To identify whether SMO or Gli-1 is the factor that is directly regulated by the SDF-1/CXCR4 axis, we treated two CXCR4-positive pancreatic cancer cell lines with cyclopamine or siRNA specific to Gli-1 in the presence of a high dose of SDF-1. Although both treatments caused dramatic negative effects on the tumor invasion and EMT induced by activation of CXCR4 by SDF-1, Gli-1 siRNA could not interrupt the SDF-1-mediated increase in SMO; in contrast, blocking SMO function by cyclopamine suppressed the expression of the transcription factor Gli-1. Hence, we conclude that activation of CXCR4 by SDF-1 up-regulates the Hh pathway through up-regulation of SMO expression.

In summary, we found a novel modulator of the non-canonical Hh pathway (SDF-1/CXCR4-SMO) in mutant RAS pancreatic cancer cells (MiaPaCa-2 and Panc-1), in concert with an important role for SDF-1/CXCR4-induced invasion and tumor cell EMT through increased SMO expression. Our results suggest that the link between CXCR4 and the Hh pathway plays an important role in pancreatic cancer progression. Therefore, SDF-1/CXCR4 axis may represent a promising therapeutic target to prevent pancreatic cancer progression.

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Abbreviations

SDF-1 stromal-derived factor-1

CXCR4	C-X-C, chemokine receptor type 4
SMO	smoothened
Gli-1	glioma-associated oncogene homolog-1
EMT	epithelial–mesenchymal transition

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Fig. 1.

The expression of CXCR4 and SMO in the human pancreatic cancer cells. (A) The expression of CXCR4 and SMO at protein level in BxPc-3, MiaCaPa-2, and Panc-1 cells was evaluated by Western blotting. 150 μ g of cellular protein were separated on a 10% SDS-polyacrylamide gel, and transferred onto Immobilon membrane. Immunoblots were probed with antibodies specific for CXCR4 and SMO. The blots were then re-probed with β -actin as a loading control. (B) The expression of CXCR4 and SMO at mRNA level was estimated by real-time RT-PCR. The expression of each target gene was quantified using GAPDH as a normalization control. The data represent the results of three independent experiments. Column: mean; bar: SD.

Fig. 2.

SDF-1 increases the invasion of human pancreatic cancer cells. Pancreatic cancer cells in DMEM medium containing 1% FBS were seeded in the matrigel-coated Transwell upper chambers. 10% FBS was used as chemoattractant. After 24 h, the cells on the upper surface of the filters were removed; the filters were then stained with crystal violet. (A) Representative staining. $\times 100$ magnification. (B) The number of migrated cells was

quantified by counting the cells from 10 random fields at $\times 100$ magnification. The data are representative of 3 independent experiments. Column: mean ($n = 10$); bar: SD; * $P < 0.05$; ** $P < 0.01$ compared to normal controls.

Fig. 3.

Activation of CXCR4 down-regulates E-cadherin and up-regulates vimentin. BxPc-3, MiaCaPa-2, and Panc-1 cells were treated with 50 ng/ml or 100 ng/ml SDF-1 for 48 h prior to harvest. Normal culture was used as the negative control. (A) Total RNA was extracted and the expression of E-cadherin and vimentin were measured by real-time RT-PCR. The expression of each target gene was quantified using GAPDH as a normalization control. The data represent the results from three independent experiments. (B) Whole cell protein extracts were subject to Western blotting analysis using E-cadherin or vimentin antibodies. β-actin was used as an internal loading control.

Fig. 4.

The effects of AMD3100 on SDF-1 induced pancreatic cancer cell invasion and EMT. (A) The effects of AMD3100 in pancreatic cancer cell invasion. The number of migrated cells was quantified by counting the number of cells from 10 random fields at $\times 200$ magnification. (B) The effects of AMD3100 on the expression of EMT-related molecules E-cadherin and vimentin at protein level. (C) The effects of AMD3100 on the expression of E-cadherin and vimentin at mRNA level. Column: mean; bar: SD; * $P < 0.05$ compared to normal controls.

Fig. 5. CXCR4 activation stimulates Hh pathway activation in human pancreatic cancer cells. (A) The expression of SMO and Gli-1 proteins in three pancreatic cancer cell lines was evaluated by Western blotting following treatment of the cells with the CXCR4 ligand SDF-1 for 48 h. The band densities were normalized to β -actin. (B) The expression of SMO and Gli-1 at an mRNA level was estimated by real-time RT-PCR for the same cells. (C) Treatment with AMD3100 diminishes the effects of SDF-1 on the expression of SMO and Gli-1 at protein level in MiaCaPa-2 and Panc-1 cells, as determined by Western blotting. (D) Treatment with AMD3100 diminishes the effects of SDF-1 on the expression of SMO and Gli-1 at mRNA level in MiaCaPa-2 and Panc-1 cells, as determined by real-time RT-PCR.

Fig. 6.

The effects of cyclopamine on SDF-1 induced pancreatic cancer cell invasion and EMT. (A) The effects of cyclopamine (10 μ M) in pancreatic cancer cell invasion. The number of migrated cells was quantified by counting the number of cells from 10 random fields at $\times 200$ magnification. (B) The effects of cyclopamine on the expression of EMT-related molecules E-cadherin and vimentin, and Hh pathway-related proteins SMO and Gli-1 were analyzed by Western blotting following treatment of MiaPaCa-2 and Panc-1 with SDF-1 for 48 h in the presence or absence of the SMO inhibitor cyclopamine. Normal culture was used as a negative control. (C) The EMT-related molecules E-cadherin and vimentin mRNA levels, and Hh pathway-related genes at mRNA level were analyzed by real-time RT-PCR following treatment of MiaPaCa-2 and Panc-1 with SDF-1 for 48 h in the presence or absence of the SMO inhibitor Cyclopamine. Normal culture was used as a negative control.

Fig. 7. Gli-1 siRNA abolished the effects of SDF-1-mediated invasion and EMT in pancreatic cancer cells. (A) The knockdown of Gli-1 by siRNA for 48 h was confirmed by Western blotting. (B) The knockdown of Gli-1 by siRNA for 48 h was confirmed by real-time RT-PCR. (C) The effect on cell invasion in response to Gli-1 knockdown. After transfection with siRNA for 48 h, the cells were seeded into a matrigel-coated invasion chamber with or without SDF-1 for 24 h. The number of cells was counted under a light microscope. (D) The effects of Gli-1 siRNA on the expression of SMO, E-cadherin, and vimentin. After transfection of the cells with siRNA for 48 h, SMO, E-cadherin, and vimentin expression levels were determined by Western blotting.