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Dependence of fibroblast infiltration in tumor stroma on type IV collagen-initiated integrin signal through induction of platelet-derived growth factor

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ABSTRACT

Cancer-associated fibroblasts play a crucial role in accelerating tumor progression, but there is a knowledge gap regarding the chemotactic signal activated in a tumor microenvironment. In this study, the expression of type IV collagen was knocked down using a lentiviral-mediated short hairpin RNA strategy. Although there was no obvious effect on cell growth in vitro, silencing the *Col4-α1* gene decreased the tumorigenicity of B16F10 in C57BL/6 mice, which was accompanied by a reduction in the infiltration of alpha-smooth muscle actin-positive (α-SMA^{+}) fibroblasts. Silencing the *Col4-α1* gene or disrupting integrin engagement by blocking the antibody reduced the expression of platelet-derived growth factor A (PDGF-A), a potent chemotactic factor for fibroblasts. Furthermore, ectopic expression of the autoclustering integrin mutant significantly stimulated PDGF-A expression in murine B16F10 and human U118MG and Huh7 cells. PDGF-A-specific sh-RNA and neutralizing anti-PDGF-A antibody effectively inhibited the transwell migration of fibroblasts. Adding recombinant PDGF-A back to shCol cell-conditioned media restored the fibroblast-attraction ability indicating that PDGF-A is a major chemotactic factor for fibroblasts in the current study model. The integrin-associated PDGF-A production correlated with the activation of Src and ERK. High type IV collagen staining intensity colocalized with elevated PDGF-A expression was observed in tumor tissues obtained from hepatoma and glioma patients. The integrin signal pathway was activated by collagen engagement through Src and ERK, leading to enhanced PDGF-A production, which serves as a key regulator of fibroblast recruitment.

1. Introduction

Solid tumors are surrounded by distinctive basement membrane-like structural barriers that physically separate them from other tissue compartments [1,2]. Extracellular matrix (ECM) molecules constituting the basement membrane contain laminins, collagen type IV, nidogens, and heparan sulfate proteoglycans [1,3,4]. Many tumors exhibit aberrant deposition of ECM as compared with that of their normal counterparts [5,6]. In particular, a positive correlation of type IV collagen expression with tumor malignancy has been documented in several clinical studies. Type IV collagen is a major component of the complex basement membrane zone, where it has an important molecular filtration function. This microenvironmental change is considered to be important for tumor progression and metastasis. For instance, distinct organizational and distribution patterns of type IV collagen around tumors have been associated with tumor progression in melanoma and colorectal carcinoma [7,8].

Accompanied with atypical matrix composition, various types of cells, including fibroblast, vascular, glial, smooth muscle, epithelial, fat, and immune cells, infiltrate into tumor stroma, a process which further contributes to the altered tissue architecture of tumor tissue [9]. Among these cells, cancer-associated fibroblasts play a crucial role in accelerating tumor progression through multiple mechanisms for cell viability, immune evasion, and epithelial–mesenchymal transition [10–12]. Despite a positive correlation of excessive type IV collagen deposition and altered stromal infiltration, there is a knowledge gap regarding the signal transduction pathways initiated by collagen deposition as they relate to the altered stromal infiltration.

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Accumulating data show that tumor cells grown in high-density collagen matrices exhibit elevated activation of non-receptor focal adhesion kinase and small GTP binding protein, leading to increased cell proliferation and invasiveness [13]. Specifically, the signal initiated by integrin engagement with collagen confers high invasiveness of human pancreatic cells through extracellular signal-regulated kinase (ERK) activation [14]. Rac and ERK pathways have been shown to be required for fibroblast activation and migration [15,16]. These findings raise the possibility that aberrant collagen-associated signaling is involved in cancer-associated fibroblast recruitment. In this study, the molecular mechanism linking collagen deposition and fibroblast infiltration in tumors is elucidated. By knocking down the *Col4-α1* gene, integrin-associated signaling and platelet-derived growth factor A (PDGF-A) expressions in various tumor cell lines were impaired. In addition, the knockdown strategy drastically reduced the capacity of tumor cell lines to recruit myofibroblasts both in vitro and in vivo. Based on these findings, a mechanism for stromal cell infiltration initiated by the deposition of collagen is proposed.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit antibodies (Abs) for type IV collagen and PDGF-A were obtained from Novus Biologicals LLC (Littleton, CO). Rabbit Abs specific for Src, phospho-Src, ERK, and phospho-ERK were obtained from Cell Signaling Technology (Beverly, MA). Mouse Abs for GAPDH and CD29 were purchased from BD Pharmingen (San Diego, CA). Mouse anti-human integrin β1-blocking Ab was obtained from Millipore (Billerica, MA). Purified NA/LE hamster anti-rat CD29-blocking Ab was purchased from BD Pharmingen. Mouse anti-mouse and human α-smooth muscle actin (SMA) antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant PDGF-A and neutralizing anti-PDGF-A Ab were purchased from Sigma-Aldrich. Growth factor-reduced matrigel was purchased from BD Bioscience (Bedford, MA). Inhibitors for MEK (U0126) and Src family kinase (PP1) were purchased from Calbiochem (La Jolla, CA). Puromycin was purchased from Sigma-Aldrich. Short hairpin (sh) RNA interference pLKO.1-shColIV for type IV-α1 collagen and pLKO.1-shPDGF-A were obtained from the National RNAi Core Facility of Taiwan (Taipei, Taiwan). Plasmids encoding β1-integrin wild-type, constitutively active (G429N), and autoclustering (V737N) variants were kindly provided by Dr. Valerie M. Weaver (UCSF Helen Diller Family Comprehensive Cancer Center, USA).

2.2. Cell culture

Mouse melanoma cell line B16F10, mouse fibroblast cell line NIH3T3, human glioma cell line U118MG, and human fetal lung fibroblast cell line MRC-5 were obtained from the American Type Culture Collection (Manassas, VA). Human hepatocellular carcinoma cell line Huh-7 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% antibiotic–antimycotic. Viable cells were counted using a trypan blue dye exclusion assay.

Cell growth was determined using the MTT assay. About 2×10^3 cells in 100 μl of medium were seeded into wells of a 96-well plate and incubated for the indicated time. At the end of incubation, 10 μl of 5 mg/ml MTT solution was added into each well. After incubation for another 4 h, the purple crystal sediment was dissolved in 150 μl DMSO and read at 540 nm in an ELISA reader. The absorbance value was used to represent the cell number.

2.3. Lentivirus production and cell transduction

To knock down the expression of type IV-α1 collagen and PDGF-A, tumor cells were transduced with lentivirus encoding shRNA for the *Col4-α1* and *pdgf-a* genes, respectively. The packaging plasmid (psPAX2) and envelope plasmid (pMD2.G) were kindly provided by Dr. Y. L. Lin (Centre National de la Recherche Scientifique, France). To produce lentivirus, 15 μg of the transfer plasmid, 9 μg of the packaging plasmid (psPAX2), and 6 μg of the envelope plasmid (pMD2.G) were cotransfected into 293T cells using a calcium phosphate method. After 24 h of transfection, the cells were cultured in 15 ml of fresh serum-free medium for another 48 h. Culture medium with virions was collected, filtered through a 0.45-μm filter, and further cleaned using centrifugation. For transduction, tumor cells were infected with appropriate amounts of virus suspension containing polybrene (Sigma-Aldrich) for 16 h. Alternatively, plasmid DNA was directly delivered into the cells using lipofection at a ratio of 2 μg DNA/5 μl Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol.

2.4. Quantitative real-time PCR

RNA was isolated using a High Pure RNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany) and treated with DNase to eliminate genomic DNA contamination. Quantitative real-time PCR was conducted using SYBR Green PCR MasterMix according to the manufacturer's instructions. 50 ng of template cDNA in SYBR® Green master mixes in total 20 μl volume was used for PCR reaction and run on a StepOnePlus™ Real-Time PCR System in triplicate (Applied Biosystems, Foster City, CA, USA). To normalize readings, we used GAPDH as internal control for each run. The real time PCR conditions were 95 °C for 15 s, 60 °C for 60 s for 40 cycles. The primers used for amplification are listed in Table 1. Data was analyzed by StepOne Software v2.3 (Applied Biosystems).

2.5. Reverse-transcription polymerase chain reaction

Total RNA was reverse-transcribed into cDNA with Oligo(dT)10–18 primers and MMLV reverse transcriptase (Invitrogen). Supplementary Table S1 summarizes the primer sequences for the target genes used for the polymerase chain reaction (PCR) in this study. PCR products were separated by 2% TAE agarose electrophoresis and made visible by ethidium bromide staining.

2.6. Western blot analysis

Total cell proteins were extracted with a buffer containing 250 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM

Table 1
Quantitative real-time PCR oligonucleotide primers used in this study.

Gene	Primer sequences (5′ → 3′)	mRNA target sequence (NCBI)
Human		
Col4-α1	Forward primer: ACTCTTTTGTGATGCACACCA Reverse primer: AAGCTGTAAGCGTTTGCGTA	NM_001845.4
PDGF-A	Forward primer: CCCTGCCCATTCGGAGGAAGAG Reverse primer: AAGTTGGCGGACGTGGGGTCGA	XM_005249770.1
GAPDH	Forward primer: TTCCAGGAGCGAGATCCCT Reverse primer: CACCCATGACGAACATGGG	NM_001289746.1
Mouse		
Col4-α1	Forward primer: GCTCTGGCTGTGGAAAATGT Reverse primer: CTTGCATCCCGGGAAATC	XM_006508693.1
PDGF-A	Forward primer: CTCTTGGAGATAGACTCCGTAGG Reverse primer: ACTTCTCTTCCTGCGAATGG	XM_006504658.1
GAPDH	Forward primer: AAGGTCATCCCAGAGCTGAA Reverse primer: CTGCTTCACCACCTTCTTGA	NM_001289726.1

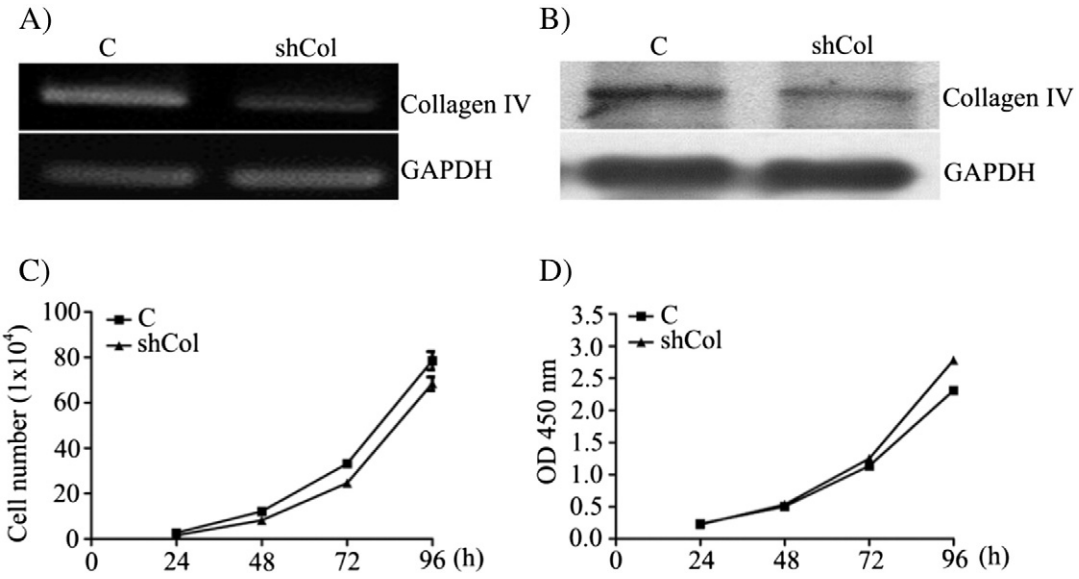


Fig. 1. Knocking down of type IV-α1 chain collagen. B16F10 cells were transduced by lentivirus carrying shRNA for the *Col4-α1* gene (denoted as shCol cells). The expression of (A) type IV-α1 collagen transcript and (B) protein levels were detected using RT-PCR and Western blot analysis, respectively. Cell growth was measured by direct cell counting using (C) hemocytometry and (D) MTT assay. Shown are the averages of three independent experiments.

Na_3VO_4, 1 mM PMSF, and 5 mM DTT, and a protease inhibitor cocktail containing 500 μM AEBSF, 1 μg/ml aprotinin, 1 μM E-64, 500 μM EDTA, and 1 μM leupeptin (Calbiochem). Proteins were separated using 8–12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. After being blocked with 5% skim milk, the membrane was probed with the primary antibody that recognized the target proteins followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. The target proteins were made visible with fluorography using an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ).

2.7. In vitro chemo-attractive invasion assay

The invasion assay was done using a chemotaxis chamber (8-μm pores, 6.5-mm diameter) with a matrigel (30 μg/well)-coated polycarbonate filter. NIH3T3 or MRC-5 cells were seeded in the upper well of each chamber compartment. Tumor-cell-conditioned media serving as the chemoattractant was loaded into the lower chamber. To visualize the transmigrated cells, the cells were fixed with 10% formaldehyde for 10 min and stained with 20% Giemsa stain for 15 min. The cells in the upper chamber were removed with a cotton swab. Transmigrated cells were counted under a light microscope.

2.8. In vivo tumor formation

To generate tumor nodules, approximately 5×10^5 B16F10 cells were subcutaneously injected into C57BL/6 mice (9–12 weeks old). The tumor volume was calculated as $3.1416 \times \text{length} \times \text{width}^2/6$. The mice were maintained in filter-top micro-isolator cages and provided with sterile water and food under regulations of the Institutional Animal Care and Use Committee of National Cheng Kung University. All manipulations of experimental animals were conducted in a laminar-flow hood using controlled procedures adhering to the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia to minimize stress or suffering [17].

2.9. Immunofluorescence staining

Glioma and hepatoma tissues were obtained from the National Core Facilities of the National Research Program for Genomic Medicine-Tumor Tissue Bank in Southern Taiwan. Tumor tissues were fixed with 3.7% formaldehyde and embedded in paraffin. Paraffin-embedded tumor samples were cut into 5-μm-thick sections. Tissue sections were deparaffinized using a standard protocol. Tissue sections were blocked with 1% BSA to prevent nonspecific antibody binding. Endogenous peroxidase was quenched with hydrogen peroxide (0.3%) in PBS for 15 min. Hybridization was done with primary antibodies at dilutions ranging from 1:200 to 1:500 to get optimal stains followed by appropriate secondary antibodies conjugated with fluorescence dyes. Tissue sections were observed under a fluorescence microscope (Olympus Cell^R microscope).

2.10. Statistical analysis

Data are expressed as the mean ± standard deviation. The differences between groups were analyzed using Student's *t*-test; $p < 0.05$ was considered significant. Data were interpolated with the software GraphPad Prism 5 (GraphPad Software).

3. Results

3.1. Impaired tumorigenesis of B16F10 cells and reduced myofibroblast infiltration by knocking down Col4-α1 gene

To reduce collagen expression, cells were transfected with lentivirus encoding shRNA specific for targeting the *Col4-α1* gene (denoted as shCol cells). The reduction of type IV collagen at both transcript and protein levels in B16F10-derived shCol cells was confirmed by RT-PCR (Fig. 1A) and Western blot analysis (Fig. 1B), respectively. Silencing the *Col4-α1* gene did not affect the growth of B16F10 cells in vitro (Fig. 1C, D). However, it significantly impaired subcutaneous tumor formation in C57BL/6 mice in terms of both the size and weight of the tumor nodules (Fig. 2A, B). In addition, histochemical staining of tissue sections with hematoxylin and eosin shows that the tumor of B16F10-derived shCol cells had a less compact fiber-like network structure with reduced stromal cell infiltration as compared to that of the control group (Fig. 2C). Most of the infiltrating cells in the tumor stroma of the control group were positive for α-SMA. Notably, a substantial reduction of infiltrating fibroblasts was found in tumors derived from B16F10-derived shCol cells (Fig. 2D).

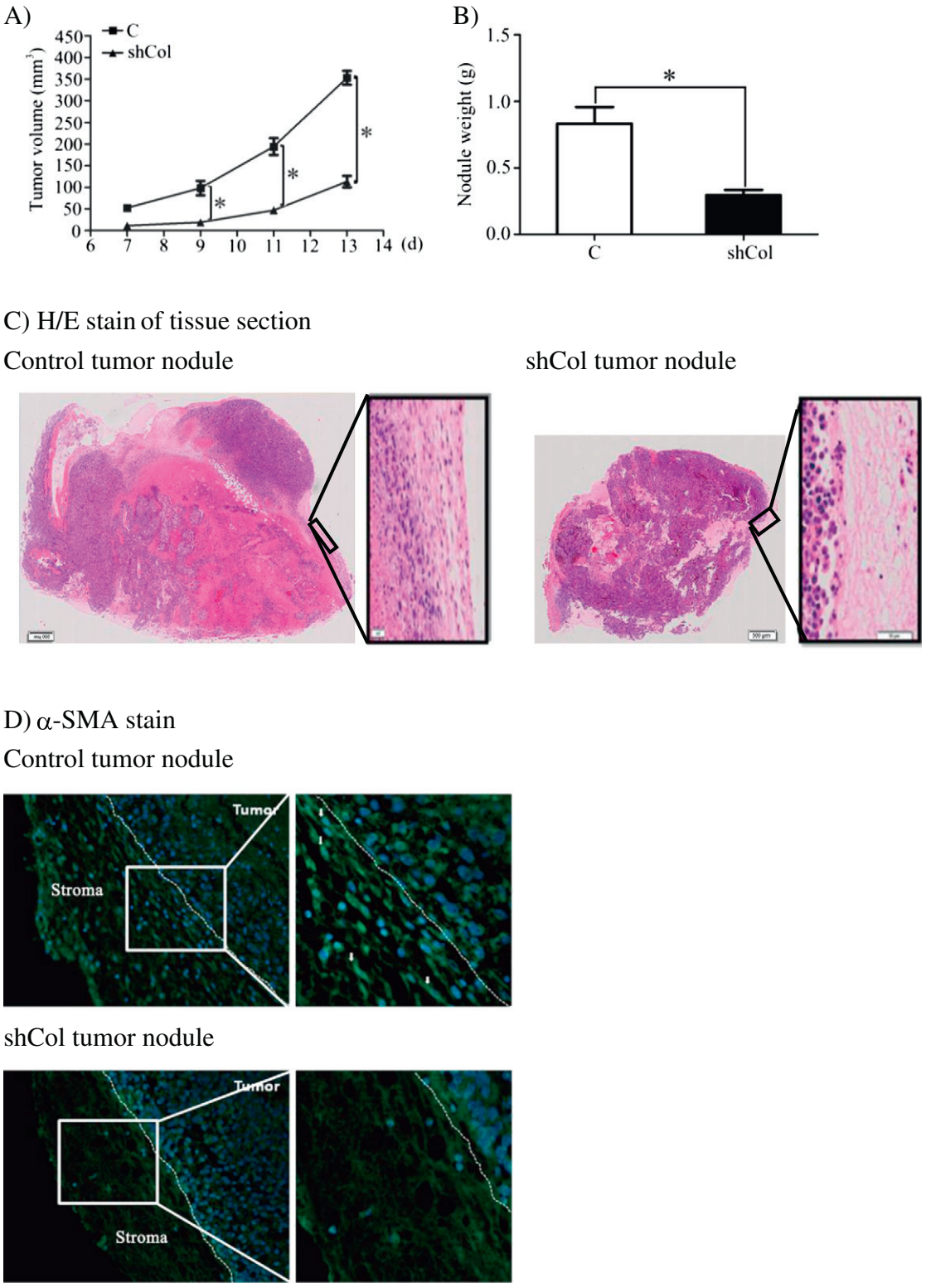


Fig. 2. Reduced tumor growth and fibroblast infiltration of B16F10-derived shCol cells. Approximately 5×10^5 cells were subcutaneously inoculated into C57BL/6 mice (n = 7/group). (A) A tumor mass appeared approximately 10 days after cell inoculation. The tumor volume was calculated at indicated intervals (*: $p < 0.05$ compared with control cells). (B) Tumor nodules were surgically obtained at 13 days post-injection and weighed. (C) Tumor samples were fixed with 3.7% formaldehyde and embedded in paraffin. Five-μm tissue sections were prepared, deparaffinized, and stained with hematoxylin and eosin (H/E). (D) Immunofluorescence staining for α-SMA. Cell nuclei were visualized by 4′,6-diamidino-2-phenylindole stain (blue). Arrows indicate some α-SMA positive cells. Shown are representative images.

3.2. Correlation of type IV collagen with PDGF-A expression

In agreement with the reduction in fibroblast infiltration in B16F10-derived shCol tumors, the conditioned medium of the shCol group was less effective in attracting NIH3T3 fibroblasts, as compared to that of the control (Fig. 3A). The B16F10 cells expressed transforming growth factor beta (TGF-β) and PDGF-A, which are potent fibroblast chemokines [18,19]. Knocking down the *Col4-α1* gene significantly reduced the

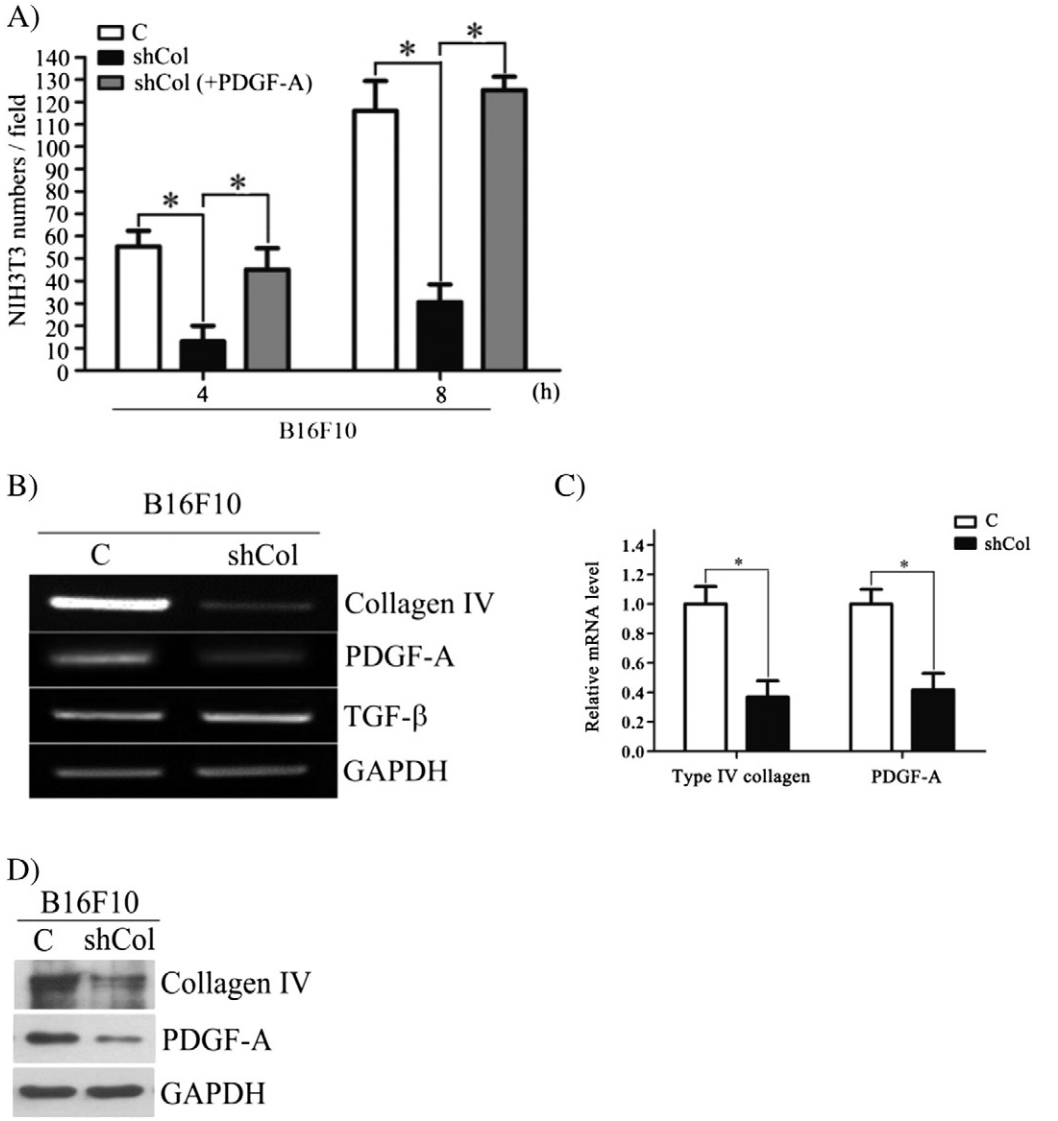


Fig. 3. Reduction of fibroblast recruitment ability and PDGF-A expression of B16F10-derived shCol cells. The B16F10-derived shCol cells were established as described in Fig. 1. (A) Fibroblast recruitment assay was done in a 6-well transmigration plate. Conditioned media collected from B16F10-derived control and shCol cells with or without 10 ng/ml PDGF-A growth factor were used as an attractant and loaded in the lower chamber. NIH3T3 fibroblasts were loaded into the upper chamber. The NIH3T3 cells migrating across the membrane in this setting at the indicated time durations were fixed, stained with 20% Giemsa stain, and counted. (B) Transcripts of PDGF-A and TGF-β in B16F10 cells were determined by RT-PCR. (C) Type IV collagen and PDGF-A mRNA expression levels were analyzed by quantitative real time PCR. (D) Type IV collagen and PDGF-A proteins were detected by Western blot analysis. GAPDH served as the loading control. Shown are the averages of three independent experiments. *: $p < 0.05$ compared with control cells.

transcription of PDGF-A, but not that of TGF-β (Fig. 3B, C). Accordingly, the B16F10-derived shCol cells produced less PDGF-A protein (Fig. 3D). To verify the role of PDGF-A in fibroblast attraction, the *pdgf-a* gene in B16F10 cells was also knocked down (Fig. 4A). B16F10 cells expressing low levels of PDGF-A had a reduced chemotactic ability to attract NIH3T3 fibroblasts as compared to the control group (Fig. 4B). Besides, neutralizing anti-PDGF-A Ab effectively inhibited the transwell migration of fibroblasts (Fig. 4C).

The expression of PDGF-A in tumor samples of human glioma and hepatoma patients was confirmed by immunofluorescence staining. Some tumor cells showed intense type IV collagen stain in the cytoplasm. Positive staining for PDGF-A was found in scattered cells of tissue areas that had high type IV collagen deposition (Fig. 5A).

The *Col4-α1* gene of human glioma cell line U118MG and hepatoma cell line Huh7 was also knocked down. In parallel with a reduced expression of type IV collagen, U118MG- and Huh7-derived shCol cells had decreased PDGF-A expression (Fig. 5B, C). Accordingly, the conditioned media of U118MG- and Huh7-derived shCol cells exhibited a lower ability to attract human MRC-5 fibroblasts. Adding PDGF-A back to shCol cell-conditioned media restored fibroblast chemotaxis (Fig. 5D). The conditioned media of U118MG and Huh7 parental cells treated with neutralizing anti-PDGF-A Ab had a lower ability to attract fibroblasts as compared to that treated with control Ab (Fig. 5E)

3.3. Contribution of integrin signaling to collagen-associated PDGF-A expression

Integrins are cell surface receptors that interact with ECM molecules. In response to ECM engagement, Src and ERK are activated, representing the main components of integrin-mediated signaling [20]. α1β1 and α2β1 integrins are the major cell binding molecules for type IV collagen [21]. Knocking down the *Col4-α1* gene severely impaired integrin signaling, as was evident in the decreased Src and ERK phosphorylation of shCol cells (Fig. 6A). To test whether integrin receptors are involved in

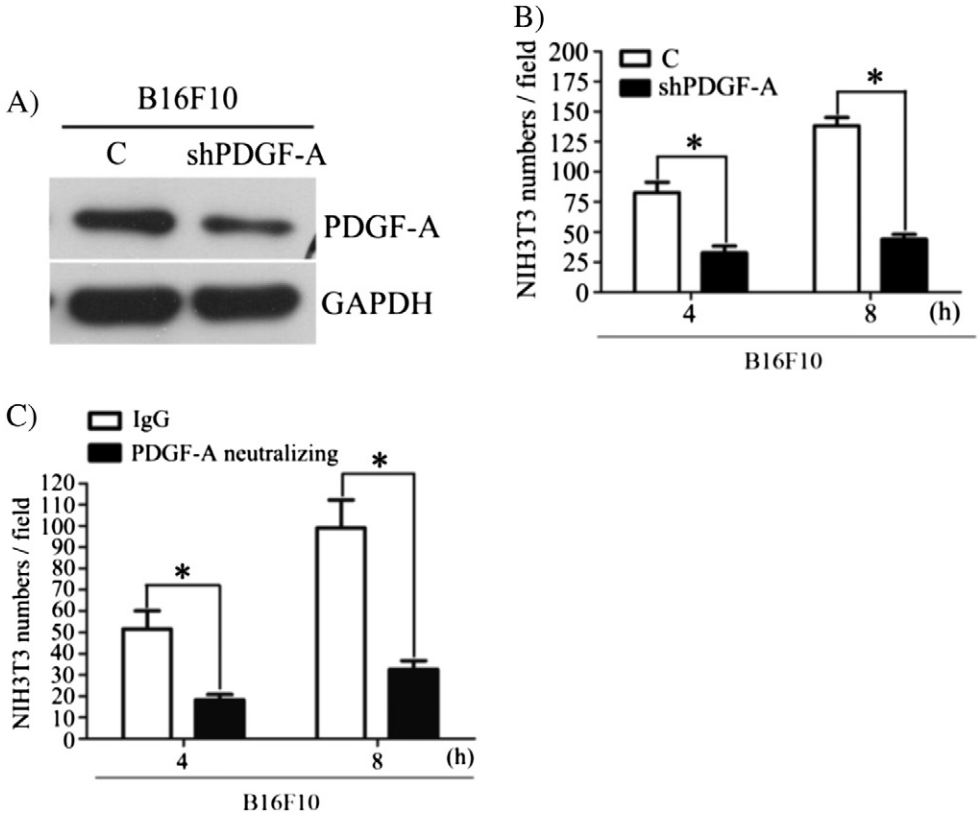


Fig. 4. Impaired fibroblast recruitment by knock-down PDGF-A expression. B16F10 cells were transduced by lentivirus encoding the shRNA for the *pdgf-a* gene. (A) The expression of PDGF-A was measured using Western blot analysis. (B) Fibroblast recruitment assay was done with the conditions described in Fig. 3. The conditioned media collected from B16F10-derived cells expressing different levels of PDGF-A were used as attractants and loaded in the lower chamber. (C) Fibroblast recruitment assay was done using the conditions as described in Fig. 3. The conditioned media collected from B16F10 parental cells treated with 20 μg/ml neutralizing anti-PDGF-A Ab were used as attractants and loaded in the lower chamber. Shown are the averages of three independent experiments (*: $p < 0.05$ compared with control group).

collagen-associated PDGF-A production, the interaction of integrin and collagen was disrupted using a β1-integrin-blocking antibody. The expressions of PDGF-A in B16F10, U118MG, and Huh7 cells were significantly inhibited by β1-integrin-blocking antibody both at RNA and protein levels. In parallel to a reduction in PDGF-A, β1-integrin blockade also suppressed the phosphorylation of Src and ERK (Fig. 6B). Distinctive signals generated by different degrees of integrin activation and clustering may have varied impacts on tumorigenesis [22,23]. To differentiate the integrin status for PDGF-A production, integrin mutants with autoactivation or autoclustering ability were ectopically expressed in B16F10 cells. Although the integrin G429N variant was less effective, wild-type and autoclustering (V737N) integrin variants triggered profound phosphorylation of ERK and Src and the expression of PDGF-A (Fig. 6C).

3.4. Effect of kinase inhibitors on PDGF-A expression

Knowing that Src and ERK were activated in the present study model, we set out to assess the causal relationship between these kinase activities and PDGF-A expression using pharmacological inhibitors. Inhibition of ERK phosphorylation by U0126 effectively suppressed the expression of PDGF-A (Fig. 7A). Treatment with PP1 for a short time period (1 h) reduced the phosphorylation of Src and ERK (Fig. 7B). However, the phosphorylation of Src and ERK rebounded quickly with prolonged exposure to PP1 (12-24 h), indicating a compensation mechanism for Src kinase. Despite the fluctuation of Src kinase, cells with a higher phospho-Src status consistently exhibited higher degrees of phospho-ERK and PDGF-A expression (Fig. 7B).

4. Discussion

Fibroblasts constitute the majority of stromal cells within the primary tumor bed in various types of human carcinomas and contribute to tumor progression [24]. Selective targeting of cancer-associated fibroblasts has recently attracted great interest and could provide novel strategies for treating cancer [25]. In the present study, we identified a collagen-associated signaling mechanism in the tumor environment for the production of PDGF, which is a major cytokine responsible for fibroblast recruitment. When the expression of type IV collagen of B16F10 cells was knocked down using the shRNA strategy, it resulted in hampered tumor growth in mice. Notably, infiltration of α-SMA-positive fibroblasts, in stromal and tumor areas, was also drastically reduced, indicating a link between collagen deposition, fibroblast recruitment, and tumor growth.

PDGF-A and TGF-β are major chemokines implicated in fibroblast recruitment in several fibrotic diseases, including tumors [26,27]. PDGF potently promotes myofibroblast proliferation and chemotaxis, while TGF-β stimulates collagen deposition in fibrogenesis by newly replicated myofibroblasts [28–30]. Although B16F10 cells expressed both PDGF-A and TGF-β, only the expression of PDGF-A was altered by knockdown of the collagen gene. A positive correlation of PDGF-A expression and the level of type IV collagen was also demonstrable in human tumor cells. In human glioma and hepatoma tissue samples, tumor fibroblasts were frequently observed in areas where there was a strong expression of type IV collagen and PDGF-A. Knockdown of the collagen gene effectively reduced the expression of PDGF-A in U118MG and Huh-7 cells and also impaired their ability to induce fibroblast migration. We have further used two strategies to establish a causal relationship between

PDGF-A expression and fibroblast recruitment in vitro. First, PDGF-A-specific sh-RNA and neutralizing anti-PDGF-A antibody effectively inhibited the transwell migration of fibroblasts. Second, adding recombinant PDGF-A back to shCol cell-conditioned media restored fibroblast chemotaxis further indicating that PDGF-A is a major chemotactic factor for fibroblasts in the current study model. This collagen-related signaling modulated the expression of PDGF-related factors, including PDGF-B, PDGF-C, and PDGF-D (Supplementary Fig. 1), which may also contribute to fibroblast recruitment [31–33]. Thus, type IV collagen deposition creates a fibroblast-favoring microenvironment by stimulating tumor cells to produce a panel of fibroblast chemoattractants.

The receptors of type IV collagen include α1β1/α2β1 integrins, DDR1, CD47, and CD44 [34–36]. Among these, β1 integrin is the major common receptor component for type IV collagen [37,38]. We noted that tumors produced from B16F10-derived shCol cells have a loose, friable texture at the stroma area (Fig. 2C), indicating altered tissue rigidity

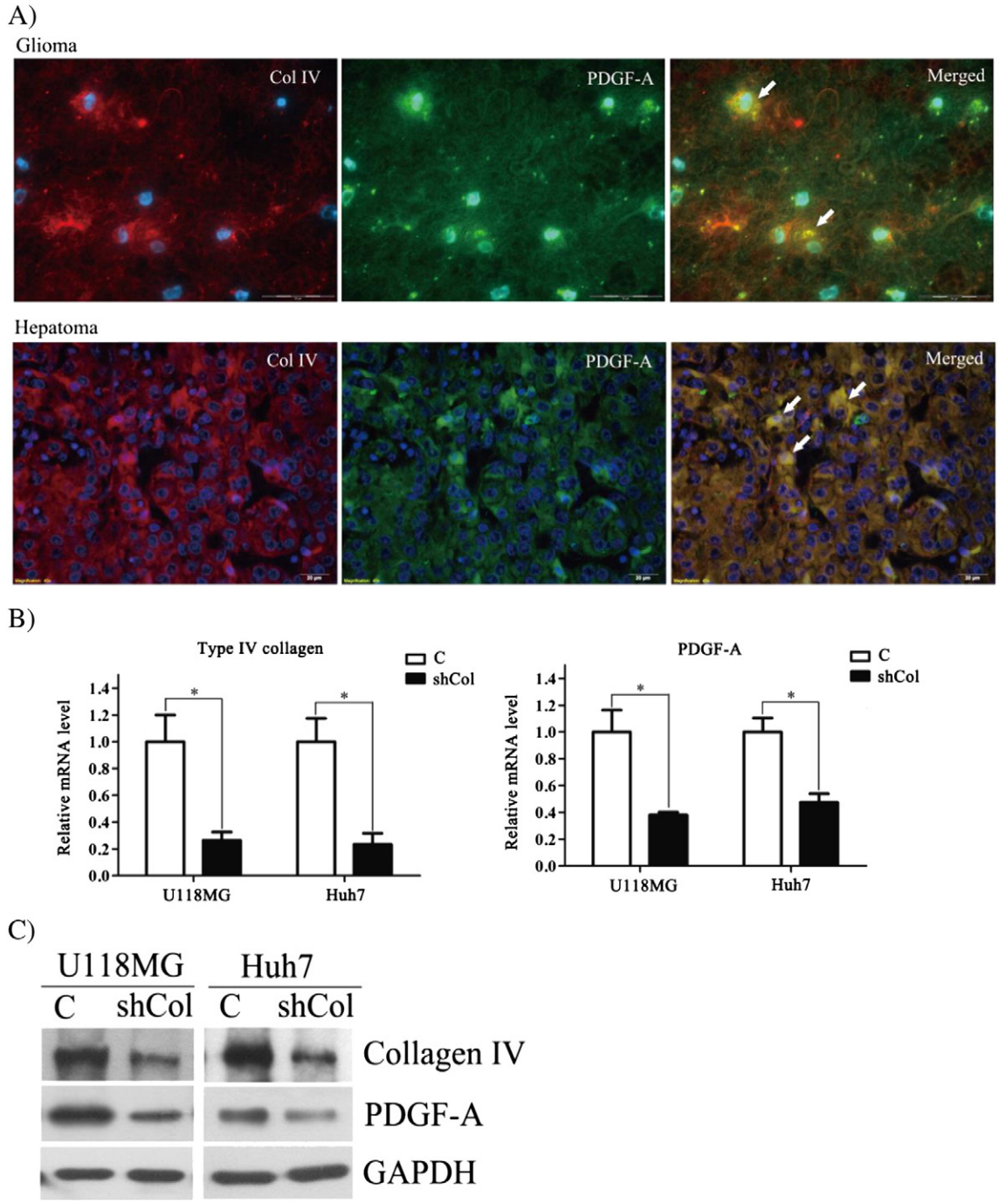


Fig. 5. Correlation of type IV-α1 collagen and PDGF-A expression in human cancer cells. (A) Five-μm paraffin-embedded tissue sections of glioma and hepatoma were prepared, deparaffinized, and dual-immunofluorescence stained for type IV collagen and PDGF-A with appropriate antibodies. Arrows indicate that type IV collagen and PDGF-A were colocalized within the tumor section. (B) The U118MG- and Huh7-derived shCol cells were established as described in Fig. 1. The transcripts of PDGF-A in U118MG- and Huh7-derived cells were determined by quantitative real time PCR. (C) Type IV collagen and PDGF-A proteins were detected by Western blot analysis. GAPDH served as the loading control. (D) Fibroblast recruitment assay was done using human fibroblast MRC-5 cells in the conditions described in Fig. 3. Conditioned media collected from U118MG- and Huh7-derived cells supplemented with or without 10 ng/ml PDGF-A growth factor were used as attractants. (E) The conditioned media collected from U118MG and Huh7 parental cells were pretreated with 20 μg/ml neutralizing anti-PDGF-A Ab or control Ab and then used as attractants and loaded in the lower chamber. Shown are the averages of three independent experiments. *: $p < 0.05$ compared with control cells.

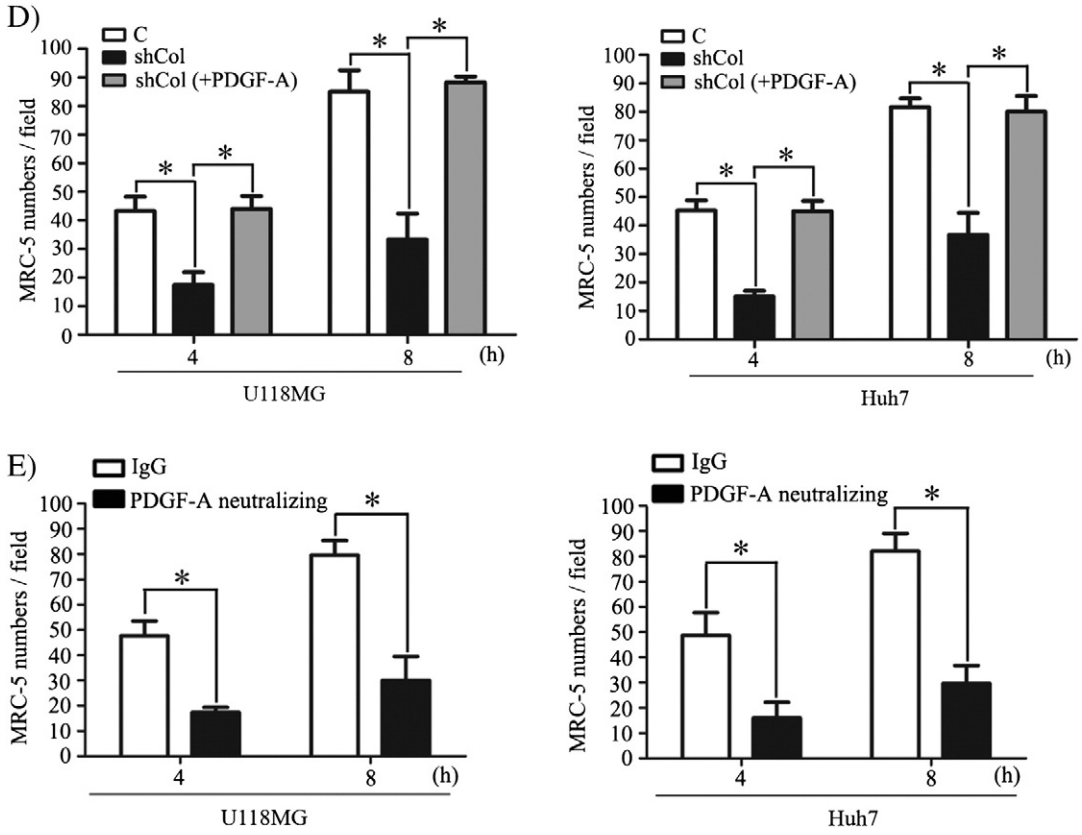


Fig. 5 (*continued*).

and receptor activation [22]. Involvement of integrin signaling in PDGF production has been reported in several cell models under physiological conditions. For example, the binding of aggretin to α2β1 integrin has been found to induce PDGF production in vascular smooth muscle cells [39]. In human platelets, binding of collagen to integrins stimulates the production of PDGF-A/B via the ERK pathway [40]. Intrinsic activation of integrin has been observed in tumors in response to high-rigidity microenvironments, which is a hallmark of solid tumors, partly due to ECM deposition [41,42]. Our findings further support that integrin receptors are candidates for PDGF indication. Indeed, preventing the engagement of collagen and integrin via a blocking antibody suppressed the phosphorylation of Src and ERK and simultaneously reduced the expression of PDGF-A. By ectopical expression of autoactivation or autoclustering integrin variants in tumor cells, we confirmed that the signal associated with integrin clustering is responsible for major Src and ERK activation and PDGF-A expression. Furthermore, pharmacological inhibition of ERK effectively reduced the expression of PDGF-A and PDGF isoforms (Supplementary Fig. 2), indicating that both PDGF-A and PDGF isoform production may be regulated by the same pathway. Thus, we propose that collagen/integrin-associated signaling is responsible for the PDGF production and the expansion of the fibroblast population in the tumor region that initiates a vicious cycle for elevated tissue stiffness and subsequent tissue abnormality (Fig. 8).

Targeting PDGF receptors in the stroma has been found to inhibit lung cancer growth and bone metastasis, and colon cancer growth and metastasis [43]. Anti-PDGF receptor treatment, however, has limitations due to its side effects, as PDGF-directed signals play important roles in development and wound repair.

5. Conclusion

The signaling pathway activated upon collagen/integrin engagement was found to be essential for the production of PDGF, which plays a prevailing role in fibroblast recruitment in tumor stroma. Targeting tumor specific integrin receptors provides an additional strategy for turning off local PDGF production that, in combination with anti-tumor cell treatment, may be of benefit in clinical applications.

Conflict of interest statement

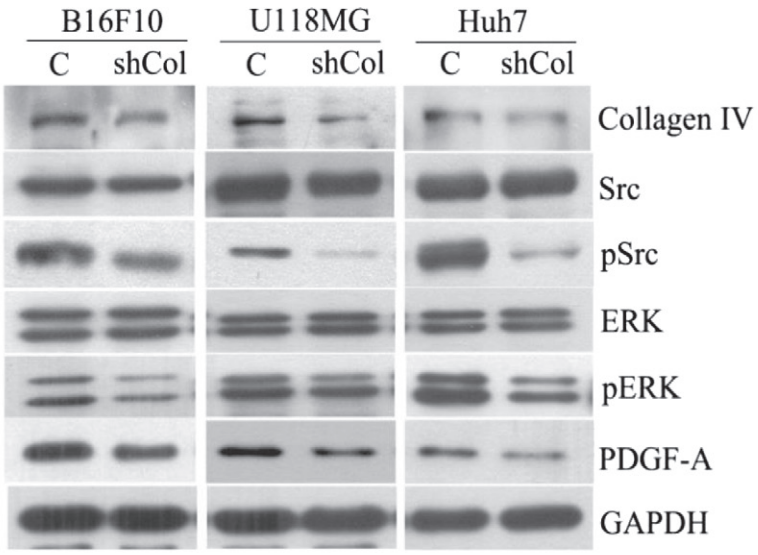
The authors declare that they have no competing interests.

Author's contributions

SYC and JSL participated in the study design, carried out the molecular and biochemical experiments, and drafted the manuscript. HCL and YJC performed immunofluorescence staining and contributed to the study design. YSS provided some tumor samples. BCY conceived the study, coordinated the work, interpreted the data, and finalized the manuscript. All authors read and approved the final manuscript.

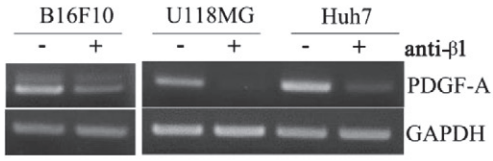
Fig. 6. Contribution of integrin signaling to collagen-associated PDGF-A expression. The B16F10-, U118MG-, and Huh7-derived shCol cells were established as described in Fig. 1. (A) Protein levels of type IV collagen, pSrc, Src, pERK, ERK, and PDGF-A were determined by Western blot analysis. GAPDH served as the loading control. (B) Parental cells of B16F10, U118MG, and Huh7 were cultured in fresh medium for 24 h and then treated with 10 μg/ml β1-integrin-blocking antibody for additional 24 h. Transcripts of PDGF-A were analyzed using RT-PCR. Protein levels of pSrc, Src, pERK, ERK, and PDGF-A were determined by Western blot analysis. (C) To validate the integrin effect, B16F10 cells were transfected with plasmids encoded for β1 integrin wild-type (WT), constitutively active (G429N), and autoclustering (V737N) variants. PDGF-A transcripts were analyzed using RT-PCR. Integrin (CD29), pSrc, Src, pERK, ERK, and PDGF-A were detected by Western blot analysis.

A) Knocking down type IV-α1 collagen

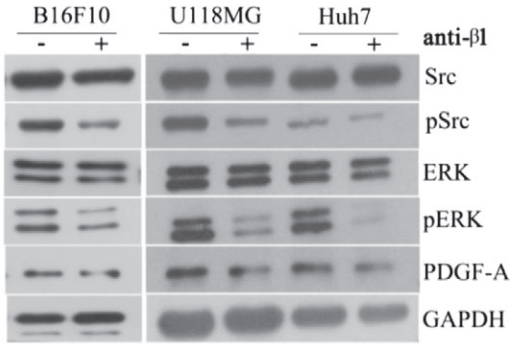


B) Treatment of β1-integrin blocking antibody

RT-PCR

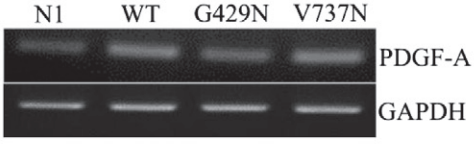


Western blot analysis

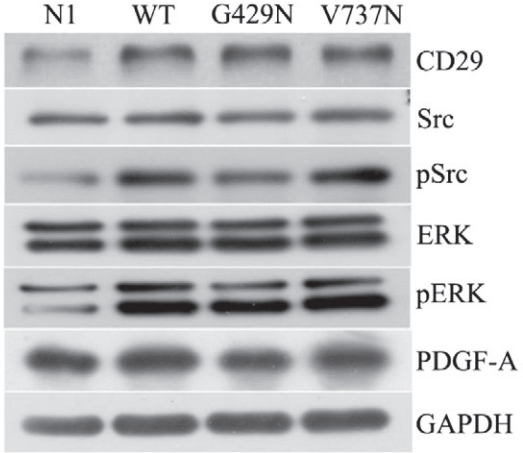


C) Overexpression of β1-integrin

RT-PCR



Western blot analysis



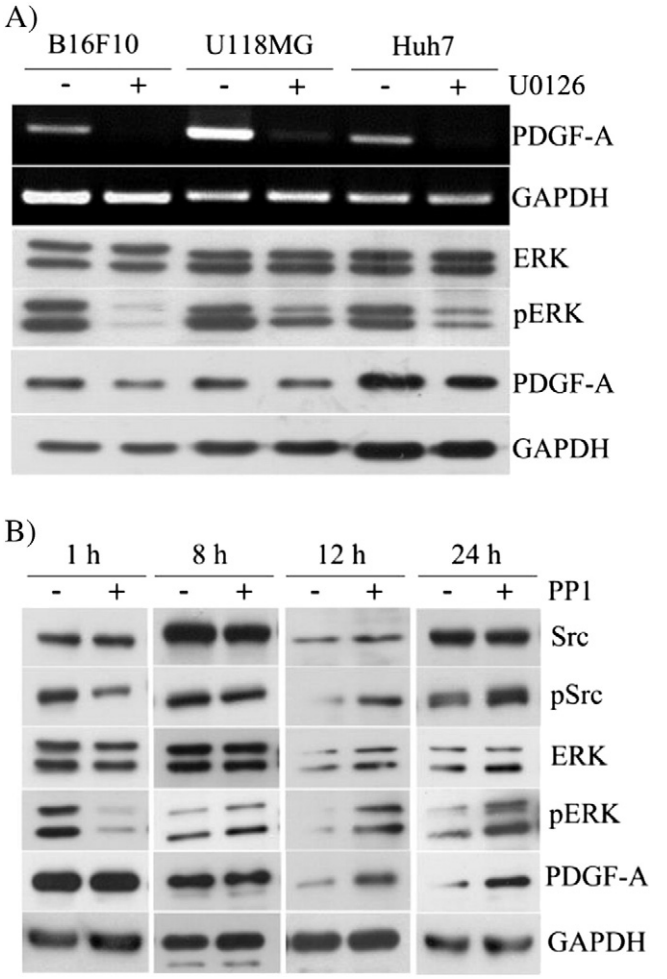


Fig. 7. Suppression of PDGF-A expression by ERK and Src inhibitors. Parental cells were treated with 25 μM U0126 for 24 h (A) or 10 μM PP1 for the indicated time periods (B). Protein levels of ERK, pERK, Src, pSrc, and PDGF-A were determined by Western blot analysis. Upper panel of (A) shows the transcripts of PDGF-A of cells treated with U0126 detected by RT-PCR.

Transparency Document

Transparency Document associated with this article can be found, in the online version.

List of abbreviations

α-SMA	alpha smooth muscle actin
DMEM	Dulbecco's Modified Eagle's Medium
DDR1	discoidin domain receptor family member 1
ECM	extracellular matrix
ERK	extracellular signal regulated kinase
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GTP	guanosine-5′-triphosphate
HRP	horseradish peroxidase
MMLV	Moloney murine leukemia virus
MTT	3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PDGF-A	platelet-derived growth factor A
PBS	phosphate buffered saline
RT-PCR	reverse-transcription polymerase chain reaction
shRNA	small hairpin ribonucleic acid or short hairpin ribonucleic acid
TGF-β	transforming growth factor beta
TAE	tris-acetate-EDTA
UKCCCR	United Kingdom Coordinating Committee on Cancer Research
VSMC	vascular smooth muscle cell

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.02.004.

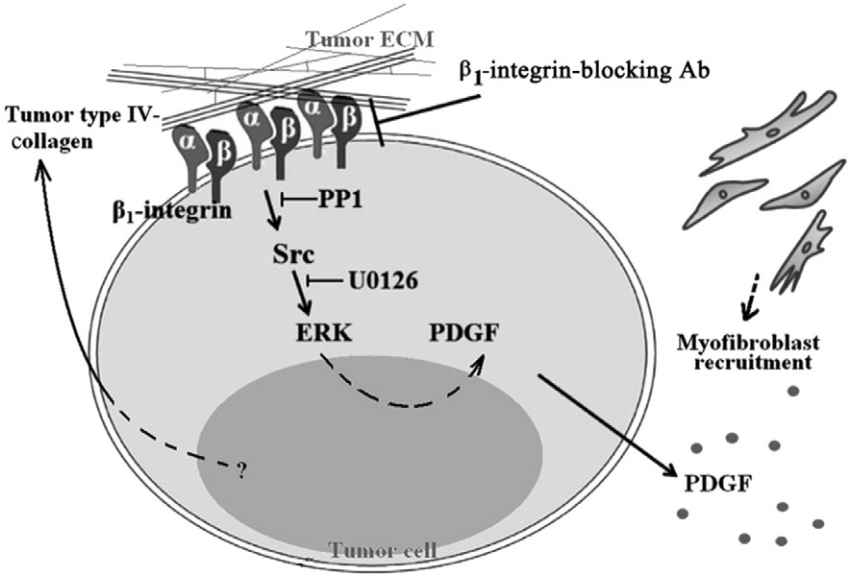

Fig. 8. Proposed model for tumor fibroblast infiltration initiated by deposition of collagen through enhanced PDGF production. In this scenario, tumor type IV collagen engages with β1-containing integrin receptors on the tumor cell surface or extracellular space, resulting in abnormal clustering. This aberrant signal triggers PDGF expression through the activation of Src and ERK. In response to the elevated PDGF, fibroblasts are recruited into the tumor region and promote tumor growth.

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