PC cell-derived growth factor overexpression promotes proliferation and survival of laryngeal carcinoma

Wei-Jia Kong, Su-Lin Zhang, Xiong Chen, Song Zhang, Yan-Jun Wang, Dan Zhang and Yu Sun

PC cell-derived growth factor is a novel growth factor for tumor formation and progression. No comprehensive literature concerning PC cell-derived growth factor expression status and its biological function in squamous cell carcinoma, especially in the larynx, is, however, available. The target of this study is to evaluate the clinical significance of PC cell-derived growth factor and the potential of small interfering RNA-induced genetic silencing of PC cell-derived growth factor as a supplementary therapeutic way for laryngeal squamous cell carcinoma. A total of 146 primary laryngeal cancer, 108 adult laryngeal papilloma and 41 laryngeal leukoplakia samples, as well as 10 normal larynx tissues were investigated. The PC cell-derived growth factor mRNA level was examined by real-time polymerase chain reaction and protein localization by immunohistochemistry. The biological function of PC cell-derived growth factor was assessed by transfection of small interfering RNA PC cell-derived growth factor construction. The PC cell-derived growth factor protein levels and mRNA levels of the laryngeal squamous cell carcinomas were significantly higher than those of normal laryngeal tissues ($P<0.001$). Simultaneously, the difference in the levels of mRNA and protein between those of laryngeal precancerous lesions (papilloma/leukoplakia) and those of normal tissues was significant ($P<0.05$, $P<0.05$), whereas those of laryngeal precancerous lesions (papilloma/leukoplakia) were significantly lower than those of laryngeal squamous cell carcinomas ($P<0.05$, $P<0.05$). Strong PC cell-derived growth factor expression was associated with lymph node metastases in laryngeal squamous cell carcinoma ($P<0.05$). Functional studies on Hep-2 cell lines demonstrated that the attenuation of PC cell-derived growth factor expression levels led to diminished cell proliferation rates ($P<0.001$), anchorage-independent growth *in vitro* ($P<0.001$), tumor forming *in vivo* ($P<0.01$) and resistance to apoptosis ($P<0.001$). PC cell-derived growth factor is a pivotal autocrine growth factor in the tumorigenesis of laryngeal squamous cell carcinoma. Our findings also indicate that PC cell-derived growth factor is a logical and potential target for early diagnosis, specific therapy and prognosis of laryngeal squamous cell carcinoma.
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Introduction

Laryngeal squamous cell carcinoma (LSCC), the second most common cancer of the head and neck region worldwide [1], is a multistep and multifocal process. It involves field carcinogenesis (the phenomenon that long-term carcinogenic exposure results in 'condemned mucosa' containing many mutated cells, from which multifocal independently arising polyclonal tumors develop) and accounts for 3–5% of all cancers [2]. It is estimated that there were 121 000 new cases worldwide in 1985 [3]. Conventional treatment often results in a poor prognosis of advanced cancer (stage III and IV) [2,4]. Therefore, a comprehensive understanding of the causation and underlying pathogenesis is vital for the prevention, diagnosis, treatment and prognosis of the disease. Many preliminary studies have suggested that human papilloma virus infection, tobacco and alcohol ingestion, asbestos exposure, steroid hormone abnormality, radiation, and gastro-esophageal reflux are all intimately related to LSCC [5–10]. The multistage nature of squamous epithelial cancer development indicates that carcinogenesis involves a complex set of genetic and epigenetic changes [11,12]. It has been reported that it is the growth factors, both in autocrine and paracrine fashion, and their receptor-mediating signal cascades that enable cells to override growth controls and undergo carcinogenesis [13,14].

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PC cell-derived growth factor (PCDGF), epithelin/granulin precursor, acrogranin or progranulin is an 88-kDa glycoprotein, originally isolated from the culture medium of a tumorigenic murine teratoma PC cell line and highly expressed in aggressive cancer cell lines [15–19]. It orchestrates several physiological processes, such as cell proliferation, developmental events, wound repair and inflammation. On the other hand, it may play an important role in multistep carcinogenesis. That is, it stimulates cell proliferation, mediates cancer survival, promotes tumor invasion and angiogenesis, and renders cells antineoplastic drug resistant [20–35]. Moreover, it is distinguished from any one of the well-established growth factor families, and can bypass their classic signal pathways to sustain cell proliferation in diverse cells such as SW-13 (adrenal carcinoma line), R^- (insulin-like growth factor I receptor-deleted R^- fibroblasts) and PC, which refract to most other growth factors, but proliferate in response to PCDGF [16,18,31].

Many laboratories uncovered specifically that PCDGF was abundantly expressed in many types of tumor specimens including human breast cancer cells [22,24], glioblastoma [21], renal clear cell carcinoma [25], ovarian cancer [32], multiple myeloma [33], hepatocellular carcinoma [34] and prostatic adenocarcinoma [35]. These investigations were, however, mostly limited to adenocarcinoma, hematopoietic and neurocyte malignancy. In contrast, Daniel *et al.* [36] reported that by in-situ hybridization, normal squamous epithelium in the skin and esophagus of a rat stained strongly for PCDGF mRNA. Tumors derived from different histological types, embryonic layer and anatomic, may have different biological characteristics in terms of tissue homeostasis, proliferation and tumorigenesis pattern, and, consequently, clinical behavior. Similarly, in cell lines PCDGF significantly promoted growth and reduced anoikic cell death in SW-13 [28]. It is noteworthy that the epidermoid carcinoma A431 and the embryonic fibroblast NIH-3T3 are unresponsive to the mitogenicity of PCDGF. R^- fibroblasts are also not protected from anoikis (a form of apoptosis that occurs when cells that are normally attached to a surface release their contact with the substratum) by PCDGF, although it stimulates their proliferation [16,31,37]. This can be explained by the hypothesis that distinguished biological outcomes depend on differential contribution, duration, strength and downstream messengers of the same signal pathways or that they access different pathways owing to tissue specificity or cell background. Thus, the molecular circuitry of PCDGF may be wired differently among different tumor types. Until now, no comprehensive literature concerning PCDGF expression status and its biological function in squamous cell carcinoma, especially in the larynx, have been available.

Presently, all studies that examined the role of PCDGF in terms of tumorigenic properties and the possible therapeutic way were performed by blocking PCDGF action by neutralizing the anti-PCDGF antibody or by inhibiting PCDGF expression through antisense DNA transfection [20,22,34]. These studies have vastly increased our knowledge of the biological function and molecular mechanisms of PCDGF. Nevertheless, the antibody only blocks PCDGF that has already been secreted and does not completely or persistently inactivate endogenous PCDGF expression. In addition, antisense DNA is a useful tool to study gene functions, working at the post-transcriptional stage to reduce the level of a target protein. Even though they share the same properties, RNA interference (RNAi) has a lot of advantages over antisense DNA. More importantly, small interfering RNA (siRNA) requires lower concentrations to achieve levels of knockdown that are comparable to antisense reagents, showing more effective and long-lasting inhibition at lower dose compared with antisense oligonucleotides. Moreover, unmodified siRNA is more stable than unmodified antisense oligonucleotides [38]. To date, no study on PCDGF via RNAi has been carried out.

In this study, we detected the mRNA level and the protein localization of PCDGF, using quantitative reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. This was carried out in a continuum ranging from normal larynxes through precancerous lesions (adult papilloma and leukoplakia) to LSCCs. Furthermore, by RNAi technology we successfully down-regulated PCDGF in the Hep-2 cell line with a high level of PCDGF expression. This cell line was originally established by Moor *et al.* [39] from tissues of a LSCC in 1955, as a squamous carcinoma cell model. On the basis of the aforementioned, the effect of PCDGF down-regulation was investigated in terms of proliferation, antiapoptosis, anchorage-independent growth and tumorigenicity in nude mice of the Hep-2 cell line. This study has proved that PCDGF is involved in the formation and progression of human LSCC. It also offers a potential molecular target in the clinical treatment of laryngeal cancer.

Materials and methods

Patients and specimens

One hundred and forty-six primary laryngeal cancer samples, 108 adult papilloma samples and 41 leukoplakia samples were derived from patients between May 2000 and March 2005 in the affiliated Union Hospital of Tongji Medical College. Among the LSCC patients, 128 were men and 18 were women. The ages ranged from 51 to 73 years (mean age, 59 years). Ten samples of normal laryngeal tissues were obtained from patients with larynx trauma. Distribution of the pTNM (T: tumor extent, N: node, M: metastasis) stages on the basis of the TNM

status classification (International Union Against Cancer 2002) and other clinicopathological features are listed in Table 1. All the specimens were subjected to histological diagnosis by a pathologist. Each tissue specimen, 0.5–1 cm^3, was divided into two equal parts. One was formalin-fixed and paraffin-embedded for histological and immunohistochemical studies. The other was stored at –70°C for further real-time PCR assay. No radiotherapy or chemotherapy was given to any patient before the operation. This study conforms to approved institutional guidelines. Informed consent was obtained from each patient.

Immunohistochemistry

Sections (5 μm) sections were cut from a representative paraffin block in each case. Slides were deparaffinized in xylene twice for 10 min and rehydrated through graded ethanol solution to distilled water. Antigen retrieval was performed by boiling sections in a citrate buffer (pH 6.0; 0.01 mol/l; Dako, Carpinteria, California, USA) for 20 min. Endogenous peroxidase activity was blocked by immersing in H_2O_2 for 30 min. Nonspecific binding reactions were prevented using 10% bovine serum albumin for 30 min. Tissue sections were then incubated with an affinity-purified polyclonal antibody against human PCDGF (1 : 100 dilution; 2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, California, USA) overnight at 4°C and kept wet. The biotinylated anti-goat secondary antibody was added and immunostained using a standard peroxidase-conjugated streptavidin biotin method, and finally counterstained with hematoxylin. PCDGF expression was cytoplasmic and was semiquantitatively categorized as follows: $< 5\%$ of cells staining, negative; $> 5\%$ of cells staining, positive; positive staining was graded from weak/focal (1 +) to moderate/focal or diffuse (2 +) to strong/diffuse (3 +). This method was described in detail by Ginette Serrero and Loffe [40]. Appropriate positive and negative controls were included in each run. Positive controls for the antibody consisted of a breast carcinoma case shown to be positive and specific in a pilot study. Negative controls were stained with phosphate-buffered saline.

Table 1 Clinicopathological features of LSCC in association with PCDGF expression

Clinicopathological parameters	*n*	Score 0–1	Score 2–3	χ^2	*P*
N1	65	9	56	7.93[a]	<0.05
N0	81	29	52		
G1	72	22	50	3.84	>0.05
G2	33	10	23		
G3	41	6	35		
T1	22	10	12	6.24	>0.05
T2	57	13	44		
T3	48	9	39		
T4	19	6	13		

LSCC, laryngeal squamous cell carcinoma; PCDGF, PC cell-derived growth factor.
[a]$P<0.05$.

Cell culture

A human laryngeal epithelial cell line Hep-2 was obtained from the China Center for Type Culture Collection (Shanghai, China). Hep-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, nonessential amino acids, sodium pyruvate, 50 unit/ml penicillin G, and 50 μg/ml streptomycin.

Real-time polymerase chain reaction

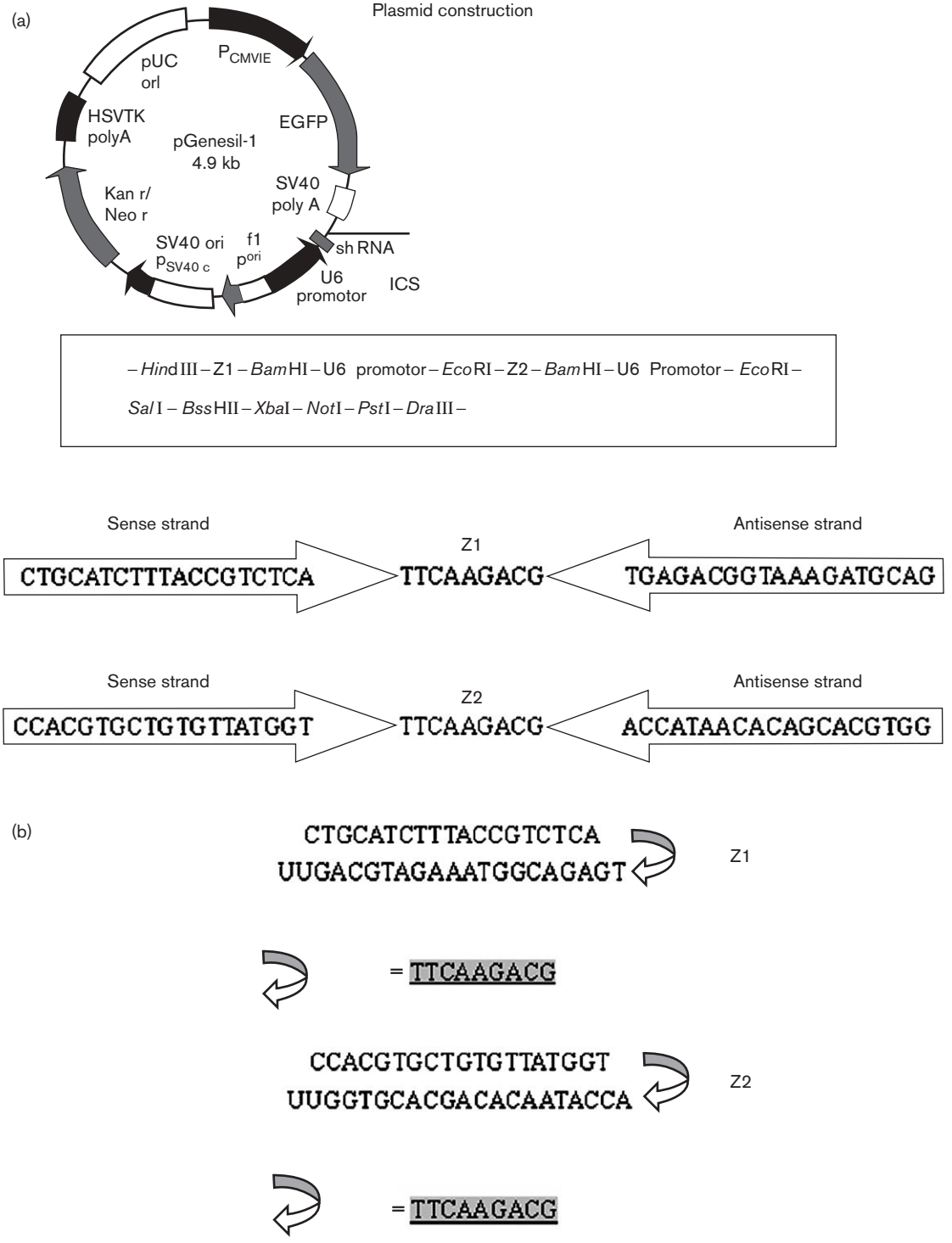
The mRNA expression of the tissues was measured by real-time PCR as described elsewhere in detail [41]. Quantification was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, California, USA). Primers and probes for PCDGF were as follows: forward (5′-GTCCCTCCTCCGATACCT-3′), reverse (5′-GCTTCCTCGCTGACACTG-3′) and probe (5′-FAM-GCTGCTGTCCAATCCCAGAGTAMRA-3′). Primers and probes for β-actin were as follows: forward (5′-GTTGCGTTACACCCTTTCTTG-3′), reverse (5′-TGTCACCTTCACCGTTCCAGT-3′) and probe (5′-FAM-TGCGCAGAAAACAAGATGAGATTGGTAMRA-3′ Invitrogen, Carlsbad, California, USA). The experiment was performed at least in triplicates. The relative expression amount of PCDGF, which was calculated by comparison between samples taking housekeeping gene β-actin's C_t as normalization control and calibrator for plate-to-plate variation, was delineated as the relative fold change in two bases: $\Delta C_t = C_t(\text{PCDGF}) - C_t(\beta\text{-actin})$ $\Delta\Delta C_t = \Delta C_t(\text{PCDGF}) - \Delta C_t(\text{control})$ mRNA relative amount $= 2^{-\Delta\Delta C_t}$. The data were normalized by subtracting the difference of the threshold cycles (C_t) between the gene of interest's C_t and the housekeeping gene β-actin's.

Plasmid construction

Design and preparation of constructs

The siRNA design targeting PCDGF is as follows: the target sequence 1 (CTGCATCTTTACCGTCTCA) corresponded to nucleotides 234–252 and the target sequence 2 (CCACGTGCTGTGTTATGGT) corresponded to 428–446 of the human PCDGF mRNA (National Center for Biotechnology Information access number: AY124489). Synthetic sense and antisense oligonucleotides (Invitrogen) constitute the template for generating mRNA composed of two identical 19-nt sequence motifs in an inverted orientation, separated by a 9-bp spacer to form a double-strand hairpin of siRNA. Two micrograms of both oligonucleotides were annealed for 3 min at 94°C, for 30 min at 37°C and 10 min at 65°C, and then ligated into 2 μg of pEGFP-C1-U6 plasmid (containing kanamycin resistance gene; the mouse U6 RNA polymerase III promoter; enhanced green fluorescence protein clone), and linearized with *Bam*HI and *Hin*dIII (Fig. 1). The construct was cloned in competent *Escherichia coli*,

Fig. 1



(a) Schematic presentation of U6 RNA polymerase III promoter-based small interfering RNA (siRNA) expression vector. Sequence encoding two siRNAs with 19 nt of homology to PC cell-derived growth factor (PCDGF) are inserted immediately downstream of U6 promoter. (b) Predicted secondary structures of the siRNA PCDGF transcripts from the expression vector.

according to the manufacturer's instructions (Invitrogen). The sequence of the insert was confirmed by automated sequencing and by analyzing the fragments generated from digestion with *Bam*HI. The target sequence of unrelated siRNA controls (HK) is GACTTCATAAGGCGCATGC.

Transfection

Hep-2 cells were grown to a confluency of 30–50%, and then were transfected with pGenesil-1 carrying siRNA using Lipofectamine 2000 (Invitrogen) on six-well plates according to the manufacturer's instructions. Hep-2 cells transfected with pGenesil-1 carrying HK plasmid DNA were used as control. Transfected cells were selected for neomycin resistance in DMEM containing G418 (800 μg/ml; control siRNA: cells transfected with vector-HK; siRNA: cells transfected with siRNA PCDGF construct). Stable clones were isolated after 4 weeks and screened using Western blot to measure the PCDGF protein expression level.

Immunoprecipitation and Western blot

The PCDGF protein expression was examined in the conditioned medium of all cells tested, because PCDGF is secreted immediately after being synthesized [18]. The conditions for immunoprecipitation and concentration of antibody to be used in the assay had been previously determined [20]. Proteins were electrophoresed in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Nonspecific reaction was blocked in 5% fat-free dry milk in TBST (10 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 0.05% Tween-20) for 1 h at room temperature. The membranes were incubated with the first and second antibody. Protein detection was performed using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, UK). The relative amount of PCDGF expression was normalized to the same cell number (5×10^6 cells).

Cell proliferation assay

As a modified 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay, a nonradioactive Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) [42] was used to assess cell proliferation viability. After stable transfection, 2×10^3 cells per well were seeded in 96-well culture plates and maintained in DMEM in the presence or absence of 10% fetal bovine serum at 37°C in a 5% CO_2 humidified incubator for 7 days with the medium replaced every 3 days. According to the instruction manual, the number of living cells at the indicated time points in triplicate wells were calculated as the absorbance at 450 nm of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) through an automatic microplate reader.

Colony formation in soft agar

Anchorage-independent growth was examined by soft agar colony-forming assay. The bottom agar layer of 1.5 ml in a six-well plate was prepared by mixing an equal volume of 1.2% low-melting agar (USB) and 2 × DMEM supplemented with 20% fetal bovine serum. The upper agar layer was a mixture containing 0.7% low-melting agar (USB), 2 × DMEM supplemented with 20% fetal bovine serum and 5000 cells were harvested after stable transfection. G418 (400 μg/ml) was incorporated into these two kinds of agar for selection. Colony formation was monitored daily under the microscope, and 10 or more cells in a cluster were defined as a colony.

Xenograft analyses

Male BALB/c-*nu*/*nu* (4 weeks old) athymic nude mice, randomly divided into three groups, were used to evaluate the in-vivo tumorigenicity potential of the transfectants. 5×10^6 cells, transfected siRNA, control vector, and untransfected Hep-2, suspended in 200 μl of serum-free DMEM, were inoculated subcutaneously at the dorsal region of the trunk of the experimental and control groups. The appearance and weight of the tumors were monitored twice weekly. The mice were killed 60 days after injection to measure the tumor weight and size. Each of the experimental groups contained five mice. The Animal Care and Use Committee of the TongJi Medical College approved this experimentation on animals.

Recovery of Hep-2 transfectants from mice tumor

Tumors were excised and chopped into fine pieces (0.5 mm^3) with a surgical scalpel. These pieces were then incubated with 30 ml of digestion buffer containing 60 mg of Collagenase IV (Sigma Aldrich, St Louis, Missouri, USA) at 37°C. Two hours later, the suspensions were spun at 800 r.p.m. for 10 min; then the supernatant was removed. The collected cells were then cultivated in 10% DMEM containing 800 μg/ml G418 for selecting siRNA PCDGF or control vector transfectants, which contained the neomycin-resistant gene. Contaminating murine cells, which do not carry the neomycin-resistant gene, were removed by treating the cultures with G418.

Reverse transcription-polymerase chain reaction

Total RNA was extracted by TRIzol reagent (Sigma Aldrich) according to the manufacturer's protocol. RNA was then dissolved in Rnase-free water and quantified by an ultraviolet spectrometer. Two micrograms of total RNA was reversibly transcribed with oligo-dT, deoxynucleoside triphosphates and M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA) in a 20-μl reaction mixture, following the instruction manual. PCR primer sets were designed by means of computer software (Primer Premier 5.0; Premier Biosoft International, Silicon Valley, California, USA) according to published sequences of PCDGF and β-actin mRNA (GenBank): PCDGF primers (sense 5′-CCACGGACCTCCTCACTAA-3′; antisense 5′-CGCTTCCTCGCTGACACT-3′) and β-actin primers (sense 5′-ACGAGAGAATGGAAAGTC-3′; antisense 5′-ATGCTGCTTACATGTCTCGAT-3′). The final multiplex PCR reaction volume was 25 μl, including 2.0 μl of

cDNA, 2.0 mmol/l of Mg^{2+}, 0.4 mmol/l of each dNTP, 0.4 μmol/l of each primer and 2.5 U of Taq DNA polymerase. Thirty cycles of touchdown PCR [43] were performed consisting of an initial melt at 94°C for 5 min, a 0.5° decline in annealing temperature per cycle of 30 s at 94°C, 30 s at 61°C and 30 s at 72°C. Fifteen cycles of 94, 46 and 72°C for 30 s, with a final extension at 72°C for 7 min, completed each reaction. A single reaction void of a template was performed with each experiment as a negative control. Preliminary experiments were performed to ensure that the amplifications were not saturating under these conditions. PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet radiation. PCDGF mRNA levels were normalized with β-actin for MRNA amount variation.

Anoikis Annexin V/propidium iodide assay

Anoikis is a form of apoptosis that occurs when cells are denied attachment to a substratum. When testing for anoikis, the cells are in suspension and may be lacking cell–matrix interaction. Cells (10×10^4/ml) were detached from a culture dish with 0.02% ethylenediaminetetraacetic acid and resuspended in serum-free DMEM and seeded in polypropylene tubes (Falcon, Beckton Dickinson Labware, Bedford, Massachusetts, USA) at 37°C, 5% CO_2. After 48 h, cell suspensions were analyzed by flow cytometry, as described [16]. The apoptosis of cells transfected in experimental groups was measured by an Annexin V–FITC Apoptosis Detection Kit (Sigma Aldrich). According to the manufacturer's protocol, 1×10^6 cells were resuspended in 100 μl of Annexin V binding buffer without NaN_3 (BaculoGold; San Diego, California, USA) and incubated with 5 μl of Annexin V–FITC and 10 μl of propidium iodide (20 μg/ml) for 15 min at room temperature. Next, a 400-μl aliquot of Annexin V binding buffer was added to each tube and the flow cytometry analysis was performed within 60 min.

Statistical analysis

Statistical analysis was carried out with statistical software (SPSS version 10.0 for Windows; SPSS, Chicago, Illinois, USA). The comparison of categorical variables was assessed by the χ^2 test or nonparametric statistics. Data are expressed as mean ± SEM from at least three independent experiments. We compared expression levels of both PCDGF mRNA and protein as continuous variables in different tissues using the two-sample Mann–Whitney test. We used the χ^2 test to examine the association between PCDGF protein expression and various clinicopathological features in LSCCs. The difference in mice tumor weight and the cell number of proliferation assays between groups was assessed by the *t*-test. $P < 0.05$ was taken as the level of significance. All *P* values were corrected for multiple comparisons.

Results

PC cell-derived growth factor protein expression in tissues

To examine PCDGF protein levels, immunohistochemical staining was performed on all the collected samples, as well as on the 10 normal larynges. In cell cytoplasm, the PCDGF protein level in the LSCCs was found to be significantly higher than those of the papilloma, leukoplakia and normal tissues ($P < 0.001$; Table 2 and Fig. 2). We also found statistically significant differences with respect to the PCDGF protein levels when papilloma/leukoplakia and normal tissues ($P < 0.05$, $P < 0.05$; respectively) were compared, whereas laryngeal precancerous lesions (papilloma/leukoplakia) were significantly lower than those of LSCCs ($P < 0.05$, $P < 0.05$; respectively).

The χ^2 test showed that there was no significant difference in PCDGF expression among the samples with different histologic grades ($\chi^2 = 3.84$; $P > 0.05$) or samples from patients with different T stages ($\chi^2 = 6.24$; $P > 0.05$). In contrast, a significant difference was found between specimens from patients with N0 and N1 stages ($\chi^2 = 7.93$; $P < 0.05$; Table 1).

PC cell-derived growth factor mRNA level in tissues

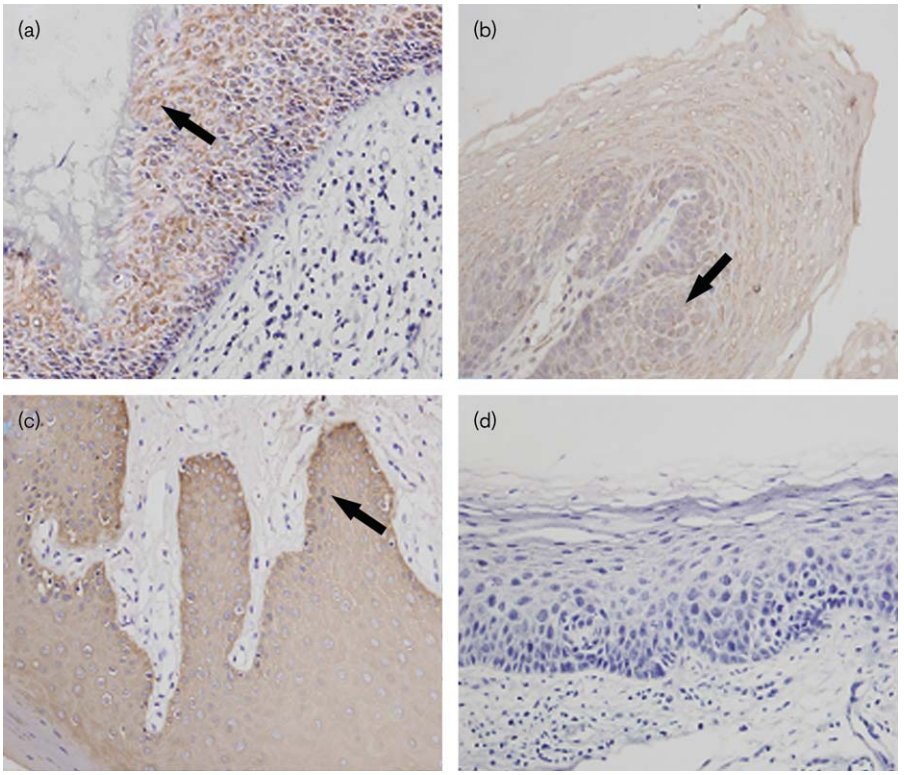
We recruited the sample set (50 LSCCs, 50 papilloma, 41 leukoplakia tissues and 10 larynx tissues) and applied real-time RT-PCR to measure the transcript levels (Fig. 3). The results indicated that the LSCCs (median 7.5, interquartile range, 5.9–8.8) documented a significantly higher PCDGF transcript level than did papilloma, leukoplakia and normal tissues (median 5.3, interquartile range 4.3–6.9; median 5.7, interquartile range 4.7–6.4; median 4.0, interquartile range 2.9–4.8; $P < 0.001$). Simultaneously, there was also significant difference between those of papilloma/leukoplakia and those of normal tissues ($P < 0.05$, $P < 0.05$, respectively), whereas those of laryngeal precancerous lesions (papilloma/leukoplakia) were significantly lower than those of LSCCs ($P < 0.05$ and $P < 0.05$, respectively). The data were normalized by subtracting the difference of the threshold cycles (C_t) between the gene of interest's C_t and the housekeeping gene β-actin's C_t.

Table 2 Expression of PCDGF in papilloma, leukoplakia, LSCC and normal tissues by immunohistochemical staining

		PCDGF staining *N* (%)			
Tissues	*N*	Negative (0)	Weak (1+)	Moderate (2+)	Strong (3+)
LSCC	146	26 (17.8)	12 (8.2)	46 (31.5)	62 (42.5)
Papilloma	108	61 (56.5)	22 (20.4)	25 (23.1)	0
Leukoplakia	41	21 (51.2)	9 (22.0)	11 (26.8)	0
Normal	10	9 (90)	1 (10)	0	0

LSCC, laryngeal squamous cell carcinoma; PCDGF, PC cell-derived growth factor.

Fig. 2



Immunohistochemical staining of PC cell-derived growth factor (PCDGF) protein in human larynx samples (squamous epithelium). (a) Squamous cells in laryngeal squamous cell carcinoma (LSCC) with strong positive staining. (b) Squamous cells in papilloma with positive staining. (c) Squamous cells in leukoplakia with positive staining. (d) Squamous cells in normal tissue with negative staining. Arrows refer to positive staining of cells. Photographs were $\times$ 400 magnification.

Inhibition of PC cell-derived growth factor protein expression by siRNA stable transfection

siRNA-transfected cells and control cells were assayed for the level of PCDGF protein expression by Western blot analysis. The PCDGF protein expression was examined in the conditioned medium of all the cells tested because PCDGF is secreted immediately after being synthesized [18]. Tested siRNA transfected clones exhibited significantly inhibited PCDGF protein expression in conditioned media when compared with controls. Studies presented here were performed with one representative clone. PCDGF expression was 73.7% lower in the siRNA clone than in the control vector cells (Fig. 4a). Similar data were obtained with other randomly picked clones (Fig. 4b).

Growth properties of siRNA cells in in-vitro studies

To evaluate the in-vitro proliferative ability of siRNA and control transfected cells, we examined the growth rate of transfected cells in serum-supplemented and in serum-free conditions. Cell numbers were calculated by the CCK-8 assay via the mitochondrial activity. After propagation for 7 days, only the siRNA transfectants had a dramatic decrease in cell numbers by 51.9 and 75.3% with or without serum ($P < 0.001$, $P < 0.001$, respectively). The control vector transfectants depicted a growth rate similar to the untransfected cells in both conditions (Fig. 5).

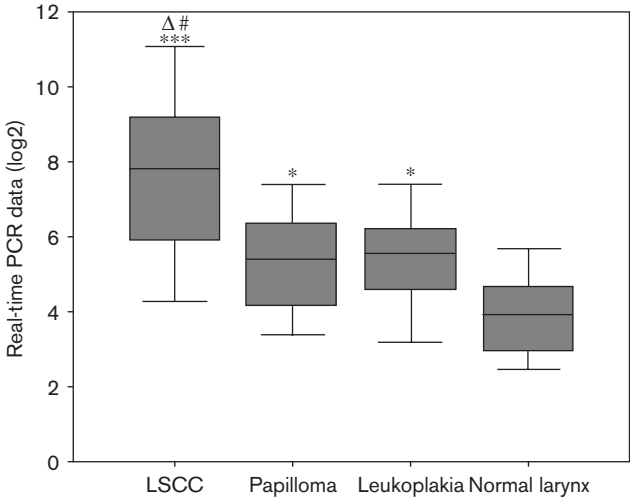
Colony formation studies

To determine whether inhibition of PCDGF expression in laryngeal carcinoma cells would affect their capacity to anchorage independence, which was the aggressiveness and metastasis potential believed to be an in-vitro parameter reflective of the transformed phenotype, we compared the colony formation capacity of transfected cells. siRNA PCDGF transfectants showed impaired clonogenicity in semisolid agar compared either with control-transfected/G418-selected cells or with untransfected cells ($P < 0.001$, Fig. 6).

In-vivo tumorigenicity studies

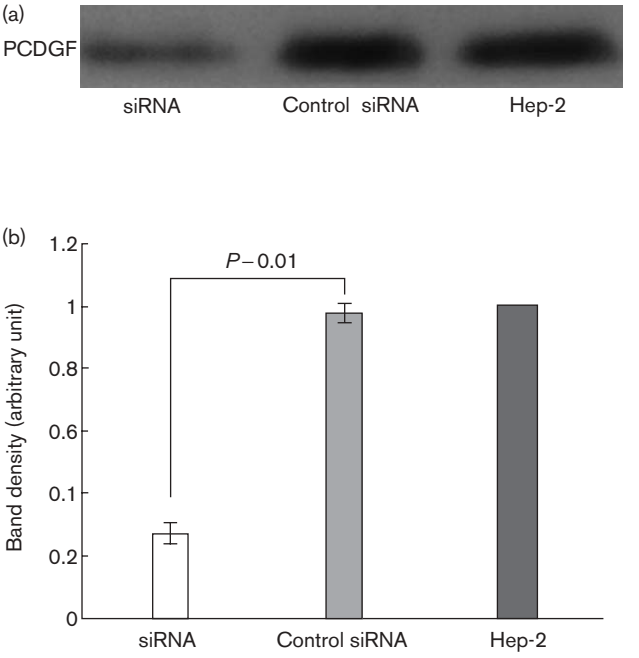
To assess whether inhibition of PCDGF expression in laryngeal carcinoma cells would influence tumorigenicity in nude mice, 5×10^6 cells, transfected siRNA, control vector, and untransfected Hep-2 were injected subcutaneously into nude mice (as described in Materials and methods). Tumor formation was monitored daily and the mice were killed after 60 days to examine the weight of

Fig. 3



PC cell-derived growth factor (PCDGF) transcript levels in human larynx tissues. Real-time polymerase chain reaction (PCR) data presented in boxplot. The top and bottom horizontal lines of the box represent the 25th and 75th percentiles, respectively. The lines within the box represent the median values. The top and bottom horizontal bars represent data within 1.5 times the interquartile range. *$P<0.05$ vs. normal larynx, ***$P<0.001$ vs. normal larynx; △$P<0.05$ vs. papilloma; #$P<0.05$ vs. leukoplakia.

Fig. 4



Inhibition of PC cell-derived growth factor (PCDGF) protein expression after small interfering RNA (siRNA) PCDGF stable transfection by Western blot. The expression of PCDGF protein was examined in the conditioned medium of all cells tested, because PCDGF is secreted immediately after being synthesized [18]. Transfected cells were cultivated in DMEM medium supplemented with 10% fetal bovine serum. When cells reached confluency, medium was replaced by fresh culture medium. The conditioned medium was collected 24 h later. Immunoprecipitation and Western blot analysis were performed to measure PCDGF expression in the medium (a). The relative expression amount of PCDGF was normalized to the same cell number (5×10^6 cells) (b).

tumor. These in-vivo studies were repeated three times. The results of one representative experiment are provided in Table 3. Tumors were palpable as early as 12 days after injection for both the control transfectants and untransfected Hep-2. In contrast, transfected siRNA cells showed both reduced tumor incidence and tumor weight. siRNA PCDGF transfectants developed smaller tumors only in two of five mice examined (40% incidence), whereas the control vector transfectants developed larger tumors in all of the five mice tested (100% incidence). The tumor weight of the siRNA group was significantly decreased by 87% compared with the control vector group (0.46 ± 0.6 and 2.2 ± 0.5 g, respectively; $P < 0.01$).

To confirm that the siRNA transfectants continue to express reduced levels of PCDGF mRNA after growth *in vivo*, cells recovered from the tumor xenografts through digestion with collagenase and grown in monolayer culture were measured by RT-PCR. The Hep-2 siRNA transfectants continued to show a 67.5% diminution in PCDGF mRNA expression compared with controls (Fig. 7).

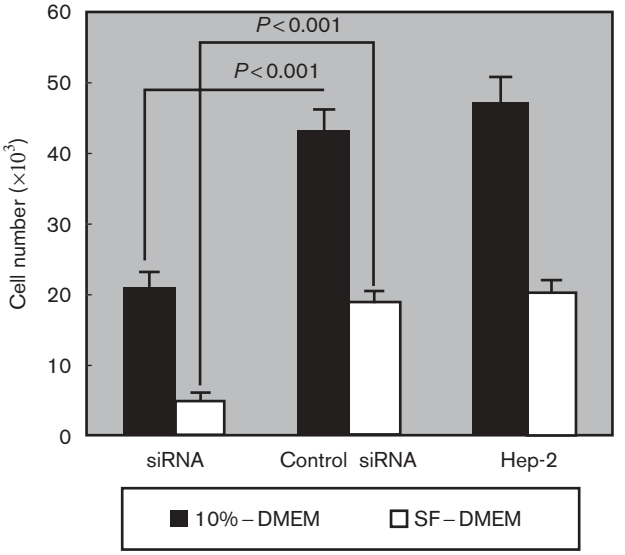
Anoikis resistance of small interfering RNA cell studies

To determine anoikis resistance of siRNA cells, cells (10×10^4/ml) were detached from a culture dish with 0.02% EDTA and resuspended in serum-free DMEM and seeded in polypropylene tubes (Falcon) at 37°C, 5% CO_2. After 48 h, cell suspensions were analyzed by flow cytometry. siRNA-transfected cells exhibited elevated apoptosis sensitivity, whereas control vector cells and untransfected cells were equally resistant to anoikis. The results of one representative experiment are shown in Fig. 8. After the transfection of siRNA PCDGF, the proportion of apoptosis cells was increased from 21.40 to 71.72% ($P < 0.001$).

Discussion

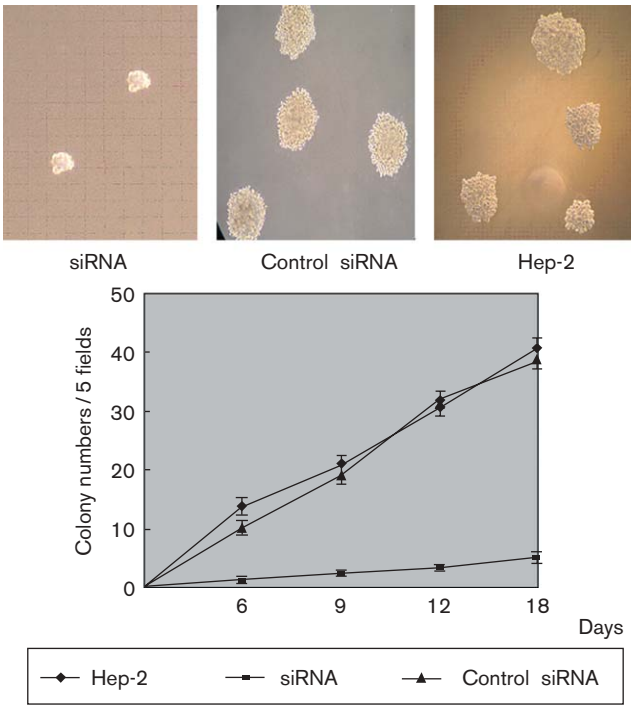
In this study, we have provided pilot evidence that PCDGF is overexpressed in LSCC. Both the mRNA and protein levels in the LSCCs were significantly higher than those in normal laryngeal tissues ($P < 0.001$), whereas expression of PCDGF was infrequent and much weaker in normal laryngeal tissues. Moreover, both the mRNA and protein levels had corresponding expression patterns. Previous studies carried out with other kinds of malignant tumors, such as breast cancer cells, ovarian cancer, multiple myeloma and renal carcinoma [24,25,32,33], have shown that the expression level of

Fig. 5



Cell proliferation rate of small interfering RNA (siRNA) PC cell-derived growth factor (PCDGF) transfectants compared with control transfectants and untransfected cells in monolayer culture. siRNA PCDGF transfectants (siRNA), control transfectants (Control siRNA), and untransfected cells (Hep-2) were assayed for their growth after 7 days in monolayer in DMEM medium with or without 10% fetal bovine serum (three independent experiments; each data point represented as mean; bars, SE).

Fig. 6



Clonogenicity of small interfering RNA (siRNA) PC cell-derived growth factor (PCDGF) transfectants vs. control transfectants and untransfected Hep-2 cells in soft agar. For Hep-2 cells, the differences in anchorage independence between siRNA PCDGF transfectants and control vector transfectants achieved statistical significance from day 6 (three independent experiments, $P<0.001$). No significant difference was found between control transfectants and untransfected Hep-2 cells.

PCDGF is low in nontumorigenic cells and increases in malignant tumor cells. These data cumulatively corroborate to demonstrate that the levels of PCDGF expression appear to increase with increasing degrees of tumorigenicity [15,16,20,22,25,30]. The present study indicates that the up-regulation of PCDGF is a distinct event in the pathogenesis of LSCC. It has been reported that the mutation form of P53, as an oncogene, induces the development of malignancy. P53 positivity in LSCC ranged from 44 to 73% by immunohistochemical approach [44–46]. The positive staining of PCDGF by immunohistochemical study ranged from 77.3 to 100% in nonsquamous tumors [21,25,32–35]. Our data documented that the positivity of PCDGF in LSCC was 82.2%. Therefore, PCDGF can be assumed to be a more specific biomarker for malignancy diagnosis.

Our results show that the levels of PCDGF mRNA and protein are frequently overexpressed in laryngeal precancerous lesions (papilloma/leukoplakia tissues), whereas those of laryngeal precancerous lesions (papilloma/leukoplakia) were significantly lower than those of LSCCs ($P < 0.05$, $P < 0.05$, respectively). Laryngeal papilloma is one of the most common benign tumors of the larynx. In adults, they are included in preneoplastic diseases for their potential malignant conversion. Moreover laryngeal leukoplakia is also considered a precancerous lesion [47]. It has been reported that benign tumors

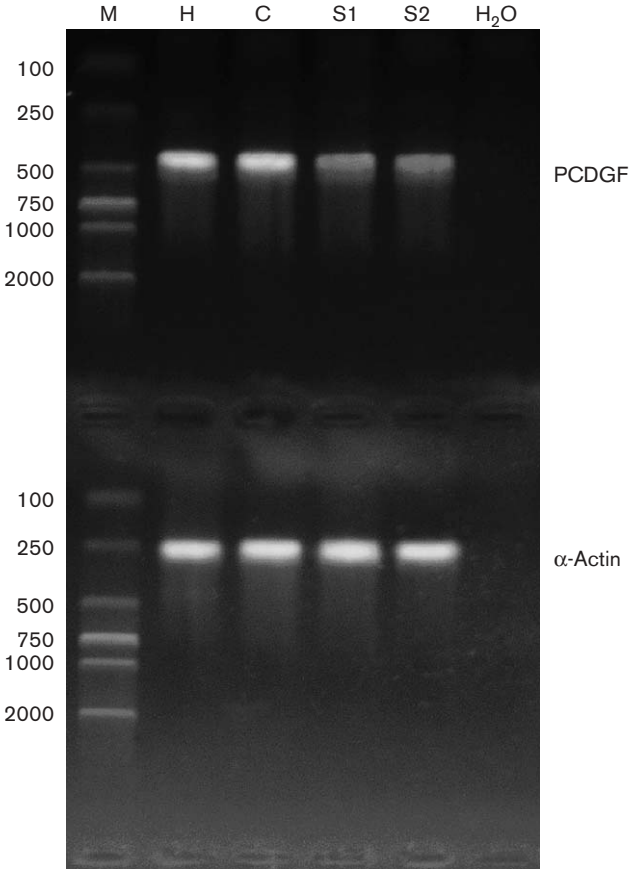
Table 3 In-vivo tumorigenicity of Hep-2 cells transfected with siRNA PCDGF, with control vector and with untransfected cells

Cell injected	Day of appearance (±SD)	Mice with tumors	Weight (g±SD)
Hep2	12±4	5/5	2.3±0.5
siRNA	30±6	2/5	0.46±0.6[a]
Control siRNA	13±3	5/5	2.2±0.5

siRNA, small interfering RNA; PCDGF, PC cell-derived growth factor.
[a]$P<0.01$.

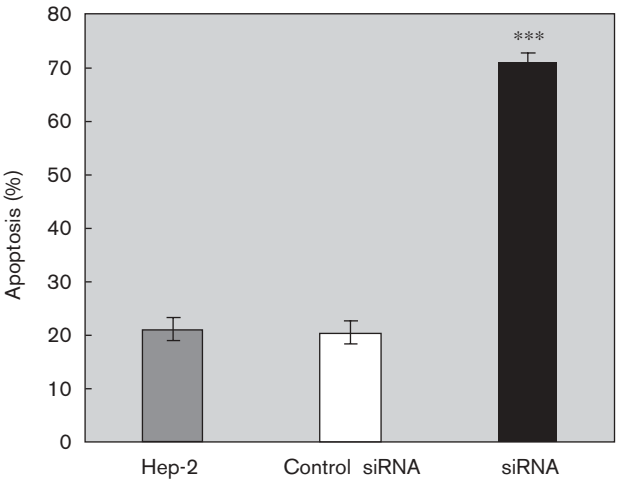
present different expression patterns of PCDGF. PCDGF gene expression was negative in breast benign tumor epithelium [40], but presented higher expression in Warthin's tumors, the second most common benign tumor of the salivary glands, than in nontumor salivary gland tissue [48]. In contrast, Pan *et al.* [35] found that prostatic intraepithelial neoplasia, a precancerous lesion in the prostate, similarly presented PCDGF overexpression to invasive prostatic adenocarcinoma. Low malignant potential, referred to as borderline ovarian tumor, was detected with a focal and punctuated presence of PCDGF protein staining [32]. Taken together, PCDGF overexpression is not exclusively restricted to neoplastic cells, but is also present in precancerous lesions. Our results suggested

Fig. 7



PC cell-derived growth factor (PCDGF) mRNA level in cells recovered from tumor xenografts. M, marker; H, Hep-2; C, control small interfering RNA (siRNA) transfectants; S1, siRNA PCDGF transfectants of mouse 1; S2, siRNA PCDGF transfectants of mouse 2; H_2O, H_2O vacuity control siRNA PCDGF transfectants PCDGF mRNA level relative to vector control transfectants after normalization by α-actin levels.

Fig. 8



Depletion expression of PC cell-derived growth factor (PCDGF) by small interfering RNA (siRNA) PCDGF transfection attenuated Hep-2 cells resistance to anoikis. Proportion of the apoptosis in total cells. ***$P<0.001$ vs. Hep2 (each data point represented as mean; bars, SE).

that PCDGF overexpression may contribute to the early phases of LSCC development and that PCDGF may be involved in the first transformation stage. This was supported by the observations that forced to overexpress PCDGF, a transformed, but poorly tumorigenic, adrenal carcinoma line, SW-13 acquired escalated tumorigenicity [28]. The development of head and neck cancer, derived from a multistep process of tumorigenesis, is closely correlated with an accumulation of genotypic and phenotypic alterations. The phenotypic changes could be the result of dysregulation of growth-determining genes such as epidermal growth factor receptor [49]. In the same way, PCDGF overexpression may confer a proliferation advantage in the cells. Thus, PCDGF may be a sensitive candidate for the early diagnosis of LSCC.

It has been reported that strong PCDGF expression was related to aggressive hepatocellular carcinoma clinicopathological features including large tumor size, presence of venous infiltration and early intrahepatic recurrence [34]. The data presented here showed a high incidence of PCDGF expression in LSCC. No statistically significant difference was, however, found in PCDGF expression among the samples with different histological grades and the ones with different T stages. A significant difference was, however, found between specimens from the patients with N0 and N1 stages. Limited pericellular digestion of the extracellular matrix and angiogenesis are crucial cascades for a solid tumor to acquire an invasive phenotype. In SW-13 cells, PCDGF augmented the expression of matrix metalloproteinase (MMP)-17, MMP-13. The latter was markedly associated with highly malignant head and neck squamous carcinomas [28,50]. Overexpressed PCDGF also induced secretion and activity of MMP-9 in MCF-7 cells (breast tumor cell line; human, Caucasian female breast adenocarcinoma). As well, PCDGF stimulates vascular endothelial growth factor expression in MCF-7 cells. Moreover, MMP-9 and vascular endothelial growth factor promote the expression of each other reciprocally [30]. Therefore, PCDGF may act on the tumor directly, in autocrine fashion, or on the nontransformed stroma to execute supplementary functions such as neovascularization, in paracrine fashion. Taken together, PCDGF could play a pivotal role in lymph node metastases in LSCC. In light of our study, PCDGF maybe a potential candidate as a biomarker of LSCC for prognosis and disease monitoring.

In the present study, the expression of PCDGF in Hep-2 cells was inhibited by using RNAi technology. The growth of Hep-2 cells with siRNA transfection, in serum-free

medium, fell to one-fourth of the untransfected cells. In comparison, the growth of Hep-2 cells with siRNA transfection, in serum medium, fell to one-half of the untransfected cells. This indicated that serum could partially restore cell growth, but was unable to completely compensate for the down-regulation of PCDGF mRNA levels. These results implied a strong dependence of these cells on autocrine PCDGF. It also showed that some other factors in the serum would be involved in the PCDGF function. It has been demonstrated, in this study, that a lowered PCDGF mRNA level by siRNA transfection on Hep-2 cells decreased anchorage-independent growth *in vitro* and tumor formation *in vivo*. These effects were consistent with the results that sensitivity to anoikis of siRNA transfectants was elevated. Therefore, our functional tests revealed that PCDGF positively modulated the cell proliferation, colony-forming ability in an anchorage-independent environment, tumorigenicity in nude mice and resistance to anoikis. For a tumor to progress, invasive cells must escape anoikis without attachment to their normal substrate and actively proliferate in the newly encountered matrix environment. PCDGF may be involved in these discrete reciprocal scenarios of laryngeal tumorigenesis. Previous studies on other malignant tumors illustrated that PCDGF underwent biological functions, in cell growth and apoptosis, through exciting p44/42 mitogen-activated protein kinase in the extracellular regulated kinase pathway, phosophatidyl inositol-3-kinase/AKT protein kinase B pathway and focal adhesion kinase in the adhesion/motility pathway [16,23,32]. Activation of the above three signal pathways has been depicted in human laryngeal neoplasia [51–56]. Therefore, control of LSCC growth and apoptosis by PCDGF could be mediated through these pathways. For many years, the multimodal treatment for laryngeal cancers has incorporated surgery, radiotherapy and chemotherapy. Nevertheless, the overall 5-year survival rate for patients with LSCC is among the lowest for major tumors and has not changed during the past 30 years [57]. Investigations of biological markers and novel biological and genetic techniques are in progress. As a consequence, these measures will result in further improvements in the rate of survival and of laryngeal preservation, and offer patients earlier diagnosis, easier therapy and better prognosis. One intriguing advantage of RNAi is its extreme efficiency; this is because only a few trigger double-strand RNA molecules delivered into the cells suffice to continuously silence a transcribed cognate target mRNA for long periods of time. Furthermore, at the optimal duration and dosage, siRNA can circumvent the nonspecific and toxic effects of siRNA. Finally, siRNA can be quite stable in serum and highly active in cultured mammalian cells without the need for further chemical modification [38,58–61]. Therefore, RNAi has emerged as a powerful tool for investigating gene function and as a platform for therapeutic development in mammalian cells [62]. The main challenge for developing siRNA *in vivo* is delivering duplex RNA intact to a target tissue. As with any compound, issues of adsorption, distribution, metabolism and excretion are significant obstacles [38]. Our work suggests that the siRNA-inducing genetic silencing of PCDGF might be a potential supplementary therapeutic way for the treatment of LSCC.

In conclusion, we have demonstrated that PCDGF is a pivotal autocrine growth factor in the tumorigenesis of LSCC, because (1) PCDGF was specifically up-regulated in clinical samples and the cell line of LSCC, (2) PCDGF overexpression was also present in precancerous lesions of the larynx, and (3) attenuating endogenous expression of PCDGF retarded key stages in tumor formation and progression. These findings also indicate that PCDGF is a logical and potential target for the early diagnosis, specific therapy and prognosis of LSCC. It will be intriguing to clarify the mechanisms of PCDGF up-regulation, its true functional receptor, and its different functions in diverse cell types and in different local microenvironments.

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