

Claudin-4 Expression Decreases Invasiveness and Metastatic Potential of Pancreatic Cancer¹

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ABSTRACT

Claudin-4 has been identified as an integral constituent of tight junctions and has been found to be highly expressed in pancreatic cancer. The aim of the present study was to elucidate the effect of claudin-4 on growth and metastatic potential in pancreatic cancer cells, as well as the regulation of claudin-4 by oncogenic pathways. Claudin-4 was stably overexpressed in SUII-2 pancreatic cancer cells, and its effect on invasion and growth *in vitro* was examined by using two-chamber invasion assays, soft agar assays, and fluorescence-activated cell sorter analysis. Claudin-4 localization was characterized by light and electron microscopy, and pulmonary colonization was analyzed *in vivo* after injection of claudin-4 overexpressing cells into the tail vein of nude mice. Overexpression of claudin-4 was associated with significantly reduced invasive potential *in vitro* and inhibited colony formation in soft agar assays. *In vivo*, tail vein-injected claudin-4 overexpressing cells formed significantly less pulmonary metastases in comparison with mock-transfected cells. These effects were not caused by changes in proliferation, cell cycle progression, or matrix metalloproteinase gelatinolytic activity, but were paralleled by increased cell contact formation. Moreover, proinvasive transforming growth factor β was able to down-regulate claudin-4 in PANC-1 cells. Inhibition of Ras signaling by using dominant-negative Ras and specific inhibitors of both downstream effectors mitogen-activated protein/extracellular signal-regulated kinase kinase and phosphatidylinositol 3'-kinase also decreased claudin-4 expression. Our findings identify claudin-4 as a potent inhibitor of the invasiveness and metastatic phenotype of pancreatic cancer cells, and as a target of the transforming growth factor β and Ras/Raf/extracellular signal-regulated kinase pathways.

INTRODUCTION

Tight junctions are the most apical component of intercellular junctional complexes, thereby establishing cell polarity and functioning as major determinants of paracellular permeability (1). The family of claudins, which form integral constituents of tight junctions (2), consists of at least 20 transmembrane proteins and represents a major factor in establishing the intercellular barrier (3). Many members of the claudin family show a distinct organ-specific distribution pattern within the human body (4).

Claudin-4 consists of 209 amino acids and contains four putative transmembrane segments (5). In previous studies, we identified claudin-4 as overexpressed in pancreatic cancer in various expression profiling approaches using representational difference analysis and

DNA array technology (6, 7). To date, the physiological relevance of this finding remains unknown.

Interestingly, claudin-4 and, with a lesser affinity, claudin-3 have been described to function as receptors for CPE³ (5). CPE is known to injure intestinal epithelial cells by an increase in membrane permeability resulting in loss of the osmotic equilibrium and subsequent cell death (8). In pancreatic cancer cells, we observed a cytotoxic effect of CPE *in vitro* and *in vivo*, which was restricted to claudin-4 expressing cells. These findings might open a novel treatment approach for claudin-4 expressing solid tumors using CPE (9). Our observations were confirmed recently by Long *et al.* (10), who described a cytotoxic effect of CPE in prostate cancer cells overexpressing claudin-3 and claudin-4.0. Claudin-4 was also found recently to be overexpressed in ovarian cancer (11) underlining the therapeutic potential of the CPE-claudin-4 interaction in a variety of solid tumors.

Several claudins have been shown to be regulated by oncogenes and tumor promoting growth factors. Transfection of oncogenic Raf-1 into a salivary gland epithelial cell line resulted in down-regulation of claudin-1 and loss of tight junction function (12). In Ras-transformed MDCK cells, claudin-1 was absent from cell-cell contacts but was reassembled to the cell membrane after blocking the mitogen-activated protein kinase pathway, paralleled by tight junction formation (13). TGF- β , a potent modulator of invasion and metastasis in pancreatic cancer (14, 15), has been demonstrated to perturb the tight junction permeability and assembly of claudin-11 in Sertoli cells (16). Furthermore, claudins have been shown to modify tumor invasion by the regulation of MMPs. Several members of the claudin-family such as claudin-2 and claudin-5 are able to activate membrane-type 1-MMP-mediated pro-MMP-2 processing (17). These reports indicate that members of the claudin-family are modulated during the process of oncogenic transformation and may play an important role in influencing tumor progression and invasion.

On the basis of our previous observations, the aim of the present study was to characterize the biological role of claudin-4 up-regulation in several solid tumors including pancreatic carcinoma (9). Therefore, we investigated the effect of claudin-4 on proliferation, invasion, and metastatic behavior of pancreatic cancer cells, and examined the regulatory effects of both TGF- β and oncogenic Ras including its major downstream effectors on claudin-4 expression.

MATERIALS AND METHODS

Materials and Cell Lines. Human tissue from patients with ductal adenocarcinoma of the pancreas were provided by the Department of Surgery at the University of Ulm and by the Hungarian Academy of Sciences (Budapest, Hungary). All of the tissues were obtained after approval by the local ethics committees.

³ The abbreviations used are: CPE, *Clostridium perfringens* enterotoxin; TGF, transforming growth factor; MMP, matrix metalloproteinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; PI3K, phosphatidylinositol 3'-kinase; FACS, fluorescence-activated cell sorter; EM, electron microscopy.

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The human pancreatic cancer cell lines were obtained from the indicated suppliers: PANC-1 (European Collection of Animal Cell Cultures, Salisbury, United Kingdom), SUIT-2 clones S2-007 and S2-028 (Takeshi Iwamura, Miyazaki Medical College, Miyazaki, Japan; Ref. 18). PANC-1 cells were stably transfected with human dominant-negative H-Ras (S17N) cloned into the pEGFP-C expression vector (Clontech, Palo Alto, CA). PANC-1 cells stably overexpressing TGF- β 1 were generated as described previously (19). All of the cell lines were incubated in DMEM (Life Technologies, Inc., Karlsruhe, Germany) and 10% FCS (Life Technologies, Inc.).

The rabbit polyclonal antibody against claudin-4 was obtained from S. Tsukita and was used as described previously (20). TGF- β was purchased from R&D Systems (Minneapolis, MN). The inhibitors UO126 and LY294002 were purchased from Sigma (St. Louis, MO).

Overexpression of Claudin-4 in S2-007 Cells. A full-length claudin-4 cDNA was PCR-amplified from normal pancreas tissue using the 5'-primer CGGGTACCGTGGACGCTGAACAATGG and 3'-primer GGACTAGTG-GAGCCGT GGCACCTTA. A 673-bp PCR product containing nucleotides 183–812 of human claudin-4 (Accession No. NM_001305) was sequence-verified and cloned into *kpn*I and *spe*I restriction sites of the mammalian expression vectors pBIG2R (21) and pBIG2i (tetracyclin-inducible). Stable transfection into the S2-007 pancreatic cancer cell line was performed using the Lipofectin reagent (Life Technologies, Inc.). Claudin-4 expressing clones were selected with hygromycin (300 μ g/ml) and screened by Northern blot analysis.

Immunohistochemistry and Immunocytochemistry. For immunohistochemical analysis, snap-frozen sections of pancreatic cancer tissues were fixed in 3% paraformaldehyde, blocked for 1 h in 1% BSA/PBS, and subsequently stained with a rabbit polyclonal antibody for claudin-4 at a dilution of 1:1000 as described previously (20). As secondary antibody, a Cy3-labeled anti-rabbit goat IgG was used. Microscopic analysis was performed with a Zeiss fluorescence microscope. For immunocytochemical analysis, S2-007 cells were grown on a four-chamber slide and subsequently treated as described above for frozen tissues.

RNA Extraction and Northern Blot Analysis. RNA from cell lines was extracted using the RNeasy kit (Qiagen, Hilden, Germany). RNA from fresh-frozen pancreatic tissue was prepared as described previously (22). Thirty μ g of total RNA were size-fractionated and blotted as described previously (6). Hybridization was performed with a digoxigenin-11 dUTP-labeled probe for claudin-4 using the Dig-Labeling kit (Roche Diagnostics, Mannheim, Germany). Specific bands were detected using the CDP-Star chemiluminescence substrate (Roche Diagnostics).

Western Blot Analysis. For protein analysis, cells were lysed in radioimmunoprecipitation assay protein lysis buffer. Identical amounts of protein were size-fractionated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany) as described previously (23). For immunodetection, blots were incubated with a rabbit polyclonal antibody against mouse claudin-4 (1:1000; Ref. 20) followed by peroxidase-coupled secondary antiserum (Pierce, Rochester, NY). Antibody detection was carried out using an enhanced chemiluminescence reaction system (Pierce, Bonn, Germany).

Proliferation Assay. [3 H]Thymidine incorporation was measured to determine cellular proliferation as described previously (24). Each assay was repeated four times.

FACS Analysis. After harvesting, cells were centrifuged at 1500 rpm for 3 min and washed once with PBS. After resuspending in 500 μ l PBS, 5 ml 80% ethanol (-20° C) were added dropwise while vortexing, and cells were incubated on ice for 30 min. Cells were centrifuged at 1500 rpm for 5 min and resuspended in 320 μ l PBS. After RNase treatment cells were stained with propidium iodide according to standard methods. To minimize aggregates, the suspension was passed several times through a syringe and subsequently filtered through a 53 μ m nylon mesh before analyzing in a FACScan flow cytometer (Becton Dickinson). Remaining aggregates were gated out before analysis. Doublets were excluded using the doublet discrimination method of the Cellfit software (Becton Dickinson). The percentage of apoptotic nuclei was determined as described by the method of Nicoletti *et al.* (25). Cell cycle analysis was done using the WinMDI software, as well as the Modfit software (Becton Dickinson).

Invasion Assay. The invasive potential of the parental S2-007 and S2-028 cell lines, two claudin-4 overexpressing S2-007 clones and one vector-trans-

fecting S2-007 clone, was determined using a modified two-chamber invasion assay as described previously (14). Briefly, 12-well Transwell-plates (COSTAR, Cambridge, MA) with 8- μ m pore size were coated with 1:2 diluted Matrigel (Becton Dickinson, Heidelberg, Germany). The lower chamber contained DMEM-medium with 10% FCS, and the upper chamber was filled with 1.5×10^5 tumor cells in DMEM with 1% FCS. Incubation times were 6, 12, and 28 h. After the incubation period, cells on the upper side of the membrane were wiped off, and the membrane was fixed in 4% paraformaldehyde and 0.25% glutaraldehyde. Cells on the lower side of the membrane were stained with 0.5% methylenblue in 50% methanol and counted. All of the invasion assays were done in triplicate.

Soft Agar Assay. Soft agar assays were performed as described previously (23). Two $\times 10^4$ cells of each S2-007 and S2-028 parental cells, as well as two claudin-4 overexpressing S2-007 clones and one mock-transfected S2-007 clone were seeded in DMEM/0.33% bacto-agar onto a bottom layer of DMEM/0.5% bacto-agar. Anchorage-independent growth was measured 2 weeks later by counting the colony number.

Zymography. The gelatinolytic activity of MMPs was determined in the supernatants of S2-007 cells with tetracyclin-inducible claudin-4 expression as described previously (15). For this purpose, cells were treated in serum-free or 5% FCS-containing DMEM in the presence or absence of 2 μ g/ml doxycyclin. After 24 h, supernatants were concentrated and the protein content was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). Equal amounts of protein (20 μ g/lane) were mixed with SDS sample buffer without reducing agents and incubated for 20 min at 37°C. Subsequently, samples were separated on a 7.5% PAGE containing 1 mg/ml gelatin for the detection of MMP activity (Invitrogen, Karlsruhe, Germany). After electrophoresis, gels were soaked for 1 h in 2.5% Triton X-100 to remove SDS and incubated for 16 h at 37°C in 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 10 mM CaCl $_2$, and 0.02% NaN $_3$. Gels were stained for 1 h in 45% methanol/10% acetic acid containing 0.5% Coomassie brilliant blue G250 and destained. Proteolytic activity was detected as clear bands on a blue background of the Coomassie blue-stained gel. Zymographic analyses were performed in at least three independent experiments.

Mouse Experiments. NMRI-*v/v* mice were propagated and maintained in a pathogen-free environment according to the guidelines of the local animal welfare committee. One $\times 10^6$ cells of claudin-4 overexpressing S2-007 cells or mock-transfected cells dissolved in PBS (2 clones each; 6 mice/group) were injected into the tail vein of 5-week-old female NMRI *v/v* mice each. Five weeks after injection, the animals were sacrificed. Lungs, liver, and spleen were paraffin-embedded and examined histologically. Colonizations of the lungs were counted in 10 serial sections of each lung.

EM. EM analysis was performed as described previously (26). In brief, SUIT-2 cells were fixed in an iced solution of buffered 5% paraformaldehyde, embedded in Epon, postfixed, and contrasted with osmium, uranyl, and lead. Semithin sections were studied by light microscopy and thin sections evaluated on a Philips EM10 electron microscope.

RESULTS

Expression of Claudin-4 in SUIT-2 Pancreatic Cancer Cells and Pancreatic Carcinomas. Claudin-4 expression was studied in the SUIT-2 pancreatic cancer cell lines, which represent subclones from the same primary pancreatic tumor, but display different spontaneous metastatic potential in nude mice (18). Interestingly, subclone S2-028, which is known to have low metastatic and invasive potential (18), exhibited strong claudin-4 expression, whereas claudin-4 expression was very weak in the highly invasive subclone S2-007 (Fig. 1A). On the basis of this observation we hypothesized that expression of claudin-4 might be inversely correlated and modulate invasion. To elucidate the effect of claudin-4 on invasion, we stably transfected claudin-4 in the highly metastatic subclone S2-007 with weak endogenous claudin-4, and selected clones with a strong claudin-4 expression on the RNA and protein level (Fig. 1A) for additional analysis.

Immunocytochemistry of cell monolayer cultures grown on chamber slides showed a strong claudin-4 signal located at cell-cell contact sites in claudin-4 overexpressing cells consistent with tight junctions.

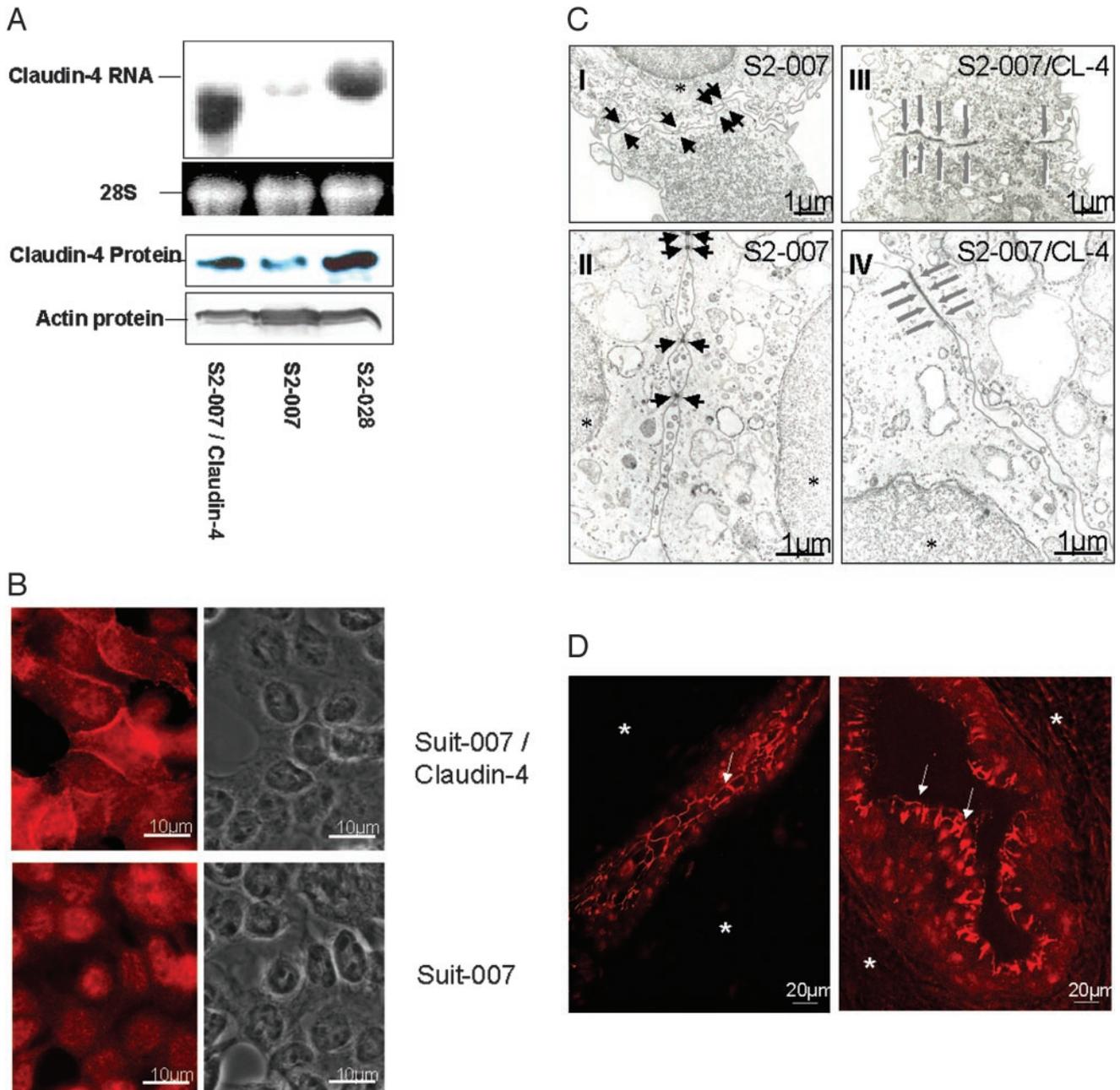


Fig. 1. A, Northern blot (top panels) and Western blot (bottom panels) analysis of claudin-4 expression in the invasive S2-007 and noninvasive S2-028 pancreatic cancer cell lines, as well as in a claudin-4 overexpressing S2-007 clone, representative for three selected stably transfected clones used for additional analysis. B, immunocytochemistry of claudin-4 overexpressing S2-007 cells and mock-transfected S2-007 cells, using a polyclonal rabbit anti-claudin-4 antibody, together with the corresponding phase-contrast microscopic images on the right hand panels. Original magnification, 63-fold. C, EM of mock-transfected controls (I and II) and claudin-4 overexpressing (III and IV) S2-007 cells. Black arrows in I and II indicate sparsely present adhesions at the cell contact sites between mock-transfected S2-007 cells, which are mainly composed of adherens junctions and desmosomes, whereas the gray arrows in III and IV indicate abundantly present tight junctions. * denote tumor cell nuclei, and bars indicate 1 μm. D, immunohistochemistry of two ductal pancreatic adenocarcinomas, using a polyclonal rabbit anti-claudin-4 antibody. Note the predominant claudin-4 staining of apical tumor cell layers (arrows), whereas the stroma shows no specific staining (*). Original magnification, 40-fold.

In contrast, parental or mock-transfected cells showed no claudin-4 labeling at these intercellular adhesion sites (Fig. 1B). When S2-007 cells were studied by EM, parent or mock-transfected cells were loosely connected by interspersed and sparsely present cell contacts that, at higher magnification, could be identified as adherens junctions and desmosomes (Fig. 1C, panels I and II), whereas tight junctions were rarely found. Claudin-4 expressing S2-007 cells, on the other hand, were tightly connected by a network of tight junctions that often occupied most of the cellular adhesion sites (Fig. 1C, panels III and IV).

To confirm these findings that were obtained by overexpressing

claudin-4, we examined a series of six pancreatic adenocarcinomas immunohistochemically. As depicted in two representative tissues shown in Fig. 1D, claudin-4 immunostaining in each tissue was restricted to tumor cells, whereas no claudin-4 staining was found in the surrounding extracellular matrix compartment. Moreover, in tumors exhibiting ductal-like structures, claudin-4 was predominantly localized in the apical cell layers adjacent to the ductal lumen (Fig. 1D). Interestingly, claudin-4 expression tended to be stronger in well-differentiated tumors as compared with poorly differentiated tumors, a finding that has to be confirmed in a study using a larger series of tumor samples.

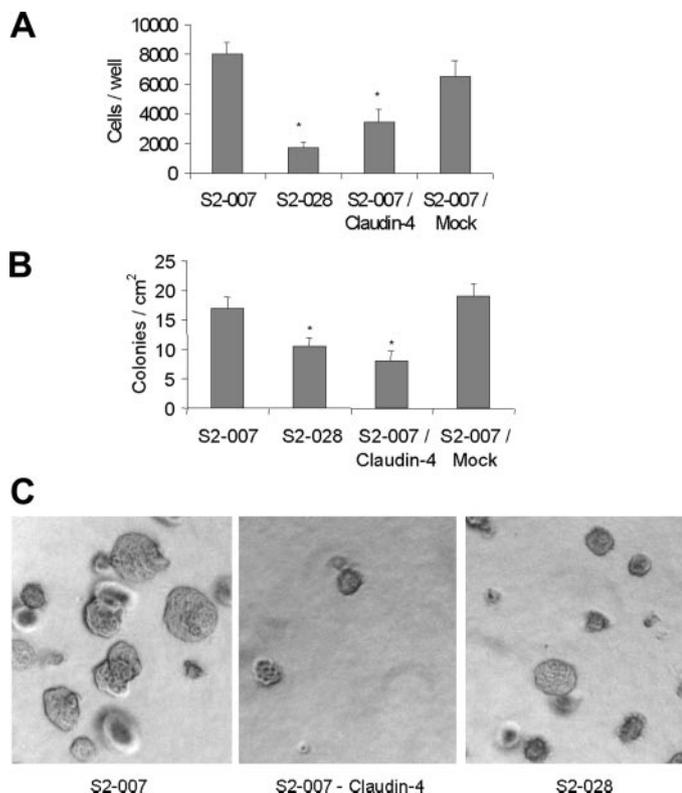


Fig. 2. **A**: Two-chamber invasion assay of parental, mock-transfected, and claudin-4 overexpressing S2-007 cells, as well as parental S2-028 cells. All assays were done in triplicate with at least two different claudin-4 overexpressing clones. **B**: quantitative evaluation of soft agar assays performed with parental S2-007 cells, mock-transfected S2-007 cells, claudin-4 overexpressing S2-007 cells, and parental S2-028 cells. All assays were done in triplicate. **C**: representative colonies formed by mock-transfected S2-007 cells, claudin-4 overexpressing S2-007 cells, and parental S2-028 cells, documented 10 days after seeding. Original magnification, 4-fold. Statistical data are expressed as mean and are representative of three independent experiments; bars, \pm SE. * indicates $P < 0.05$ as compared with parental S2-007 cells using unpaired double-sided t test with Bonferroni correction for multiple sampling, where indicated.

Effect of Claudin-4 Expression on Cell Invasion and Anchorage-independent Growth *in Vitro*. To investigate the effect of claudin-4 on the invasive potential of S2-007 cells, we performed two-chamber assays. In all three of the examined claudin-4 overexpressing S2-007 clones, invasion was significantly reduced as compared with both parental S2-007 cells and mock-transfected S2-007 cells. The invasive potential of the claudin-4 overexpressing S2-007 cells, as assessed by the two-chamber assays, was closer to the invasive

potential of the low-invasive S2-028 cells with high endogenous claudin-4 levels than to the parental S2-007 cells (Fig. 2A).

To examine whether claudin-4 overexpression also alters the ability for anchorage-independent growth as a measure for tumorigenic potential, soft agar assays were performed. Interestingly, claudin-4 transfected cells showed a significantly reduced colony formation, both in number and in size, as compared with parental or mock-transfected cells (Fig. 2B). Again, claudin-4 overexpressing S2-007 cells more closely resembled the low-invasive S2-028 cells with high endogenous claudin-4 levels than the parental S2-007 cells (Fig. 2C).

Effect of Claudin-4 Expression on Lung Colonization *in Vivo*. To examine whether the anti-invasive effect of claudin-4 *in vitro* has a physiological relevance *in vivo*, we injected two S2-007 clones stably overexpressing claudin-4 into the tail vein of ν/ν mice and compared the occurrence of pulmonary colonization in claudin-4 overexpressing versus mock-transfected S2-007 clones after a period of 5 weeks. Interestingly, claudin-4 overexpression significantly reduced the number of pulmonary colonizations by $\sim 50\%$, as compared with mock-transfected cells (Fig. 3A). Furthermore, 5 of 16 mice injected with claudin-4 overexpressing cells did not show any evidence of lung metastases, as determined by 10 serial sections of each lung. In contrast, none of the control mice was free of lung metastasis upon sacrifice (Fig. 3B). Thus, the anti-invasive effect of claudin-4 could be confirmed *in vivo*.

Effect of Claudin-4 Expression on Proliferation, Cell Cycle Progression, and MMP Activity. To examine the possibility that overexpressed claudin-4 alters proliferation or cell cycle progression, we performed proliferation assays and FACS analysis with stably transfected claudin-4 overexpressing clones and mock-transfected clones. [³H]Thymidine incorporation assays revealed no significant alterations of the proliferation rate of claudin-4 overexpressing clones (data not shown). In addition, FACS analysis showed neither altered cell cycle progression nor a significant fraction of apoptotic cells, neither in subconfluent or confluent cell populations (Fig. 4A).

Because a direct activating effect of several claudins including claudin-2 and -5 on pro-MMP-2 has been postulated recently (17), we wanted to rule out a modulatory effect of claudin-4 overexpression on activity of MMPs. Therefore, we performed gelatin zymographies as a screening test for the activity of gelatinolytic MMPs. However, claudin-4 overexpressing clones showed no altered pattern of MMP activity, particularly no alteration in MMP-2 at M_r 63,000 (Fig. 4B). Therefore, a direct or indirect modulation of MMP activity by claudin-4 appears unlikely.

Modulation of Claudin-4 by TGF- β and Oncogenic Ras. Tight junction structure and integrity are modulated by a variety of cyto-

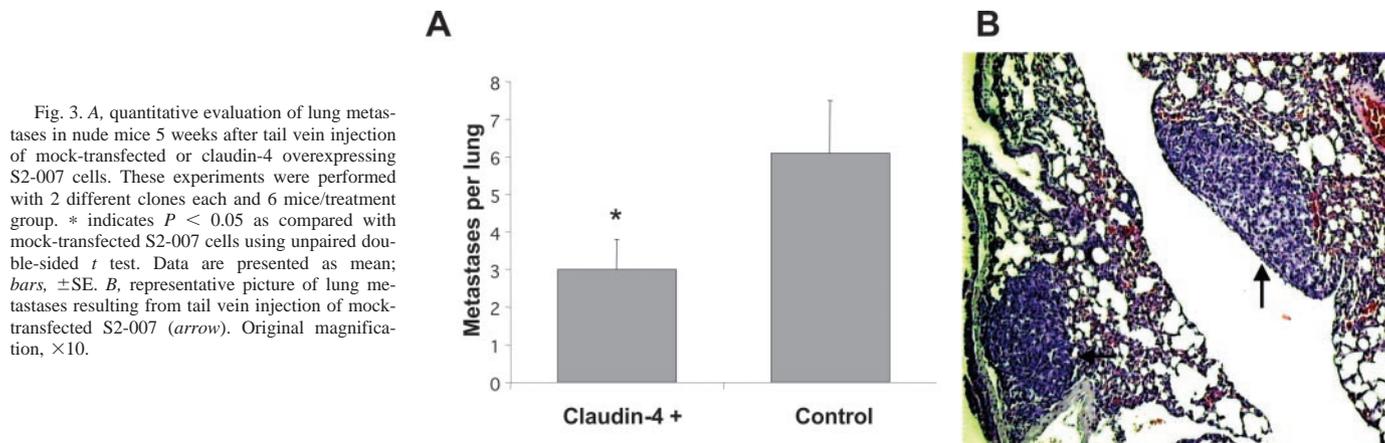


Fig. 3. **A**, quantitative evaluation of lung metastases in nude mice 5 weeks after tail vein injection of mock-transfected or claudin-4 overexpressing S2-007 cells. These experiments were performed with 2 different clones each and 6 mice/treatment group. * indicates $P < 0.05$ as compared with mock-transfected S2-007 cells using unpaired double-sided t test. Data are presented as mean; bars, \pm SE. **B**, representative picture of lung metastases resulting from tail vein injection of mock-transfected S2-007 (arrow). Original magnification, $\times 10$.

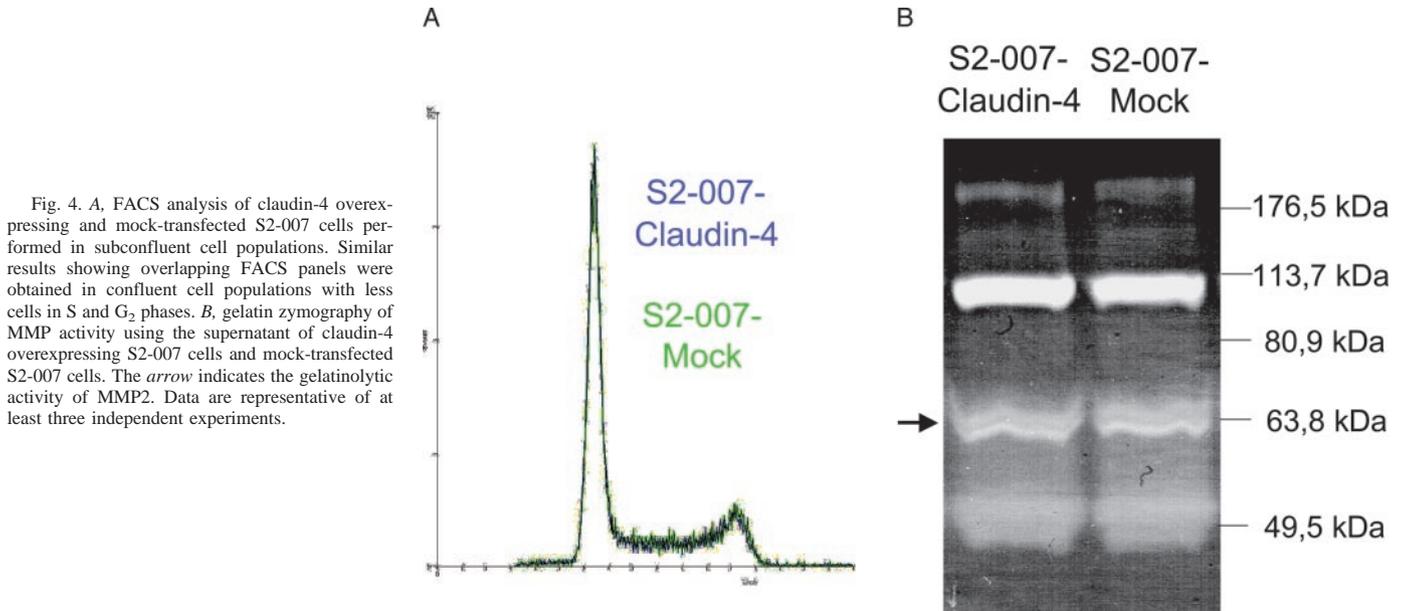


Fig. 4. A, FACS analysis of claudin-4 overexpressing and mock-transfected S2-007 cells performed in subconfluent cell populations. Similar results showing overlapping FACS panels were obtained in confluent cell populations with less cells in S and G₂ phases. B, gelatin zymography of MMP activity using the supernatant of claudin-4 overexpressing S2-007 cells and mock-transfected S2-007 cells. The arrow indicates the gelatinolytic activity of MMP2. Data are representative of at least three independent experiments.

kines and growth factors. Both the TGF- β and the Ras cascade have been shown to be of paramount importance for the development of the metastatic phenotype of tumor cells and tumor progression. In pancreatic carcinoma, TGF- β has been demonstrated as an important modulator of tumor progression (15). Despite of its direct growth-inhibitory effects *in vitro*, TGF- β is also known to strongly promote invasion of pancreatic cancer cells. To study the TGF- β responsiveness of claudin-4 we used exogenously added TGF- β as well as

PANC-1 cells stably overexpressing TGF- β . Interestingly, in both systems claudin-4 expression was down-regulated by TGF- β (Fig. 5A). After addition of exogenous TGF- β , claudin-4 RNA decreased after 4 h, which was followed by a decrease in claudin-4 protein levels after 12 h (Fig. 5C).

More than 90% of pancreatic carcinomas have activating K-ras mutations (27). Therefore, we examined whether inhibition of downstream effectors of the Ras signaling pathway had an effect on claudin-4 expression. Furthermore, we used PANC-1 cells stably overexpressing dominant-negative H-Ras (S17N). In both systems, inhibition of Ras signaling was associated with decreased claudin-4 expression. Interestingly, both MEK and PI3K inhibition using the inhibitors UO126 and LY294002 led to a decrease in claudin-4 transcription within 4 h suggesting that several signaling effectors downstream of Ras are positively regulating claudin-4 (Fig. 5B). These effects were also observed on claudin-4 protein levels 12 h after addition of both inhibitors (Fig. 5C), after addition of UO126 the effect was present up to 24 h.

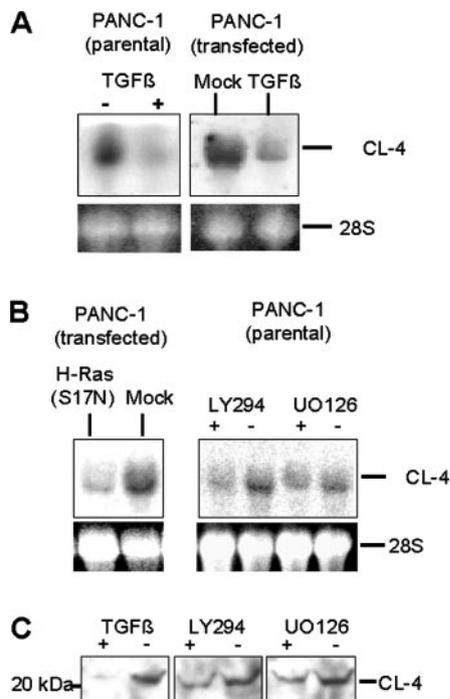


Fig. 5. A, Northern blot analysis of the effect of a 4 h treatment of parental PANC-1 cells with TGF- β (10 ng/ml; \pm) and of stable overexpression of transfected TGF- β on claudin-4 RNA expression in PANC-1 cells. B, Northern blot analysis of the effect of dominant-negative H-Ras (S17N) expression or a 4 h treatment with UO126 (20 μ M) or LY294002 (20 μ M) on claudin-4 RNA expression in PANC-1 cells. Results are representative for three independent experiments. C, Western blot analysis of the effect of TGF- β (10 ng/ml), or UO126 (20 μ M) or LY294002 (20 μ M) treatment on claudin-4 protein levels in PANC-1 cells 12 h after the addition of the growth factor or inhibitors in serum-free conditions. Results are representative of three independent experiments.

DISCUSSION

We have reported previously that claudin-4, one of the integral constituents of tight junctions, is overexpressed in the majority of pancreatic carcinomas and functions as a receptor for cytotoxic CPE, which is able to exert a cytotoxic effect specifically on claudin-4 positive tumor cells. Therefore, claudin-4 overexpressing tumors might represent a potential target for CPE-based therapeutic approaches (9). Thus far, increased claudin-4 levels have also been shown in prostate cancer (10), ovarian carcinoma (11), and in several other tumor cell lines (28), mainly by using high-throughput expression profiling approaches. On the basis of these observations, claudin-4 overexpression appears to be typical for adenocarcinomas with ductal, cystic, or glandular structures such as pancreatic, ovarian, and prostate cancer. However, the functional relevance of these findings is largely unknown.

In this study, we focused on the biological role of up-regulated claudin-4 in pancreatic cancer cells. We first demonstrated that overexpressed claudin-4 is mainly concentrated at cell-cell contacts. An increase in these cell-cell adhesion sites is ultrastructurally paralleled by an increased number of tight junctions in claudin-4 overexpressing

cells. These findings are in accordance to observations by Van Itallie *et al.* (29) in claudin-4 overexpressing MDCK cells, who described an increase in the total content of TJ strands with a more elaborate and complex pattern of strands.

Interestingly, our functional *in vitro* data indicate that overexpression of claudin-4 is associated with decreased invasiveness, as demonstrated by two-chamber invasion assays. In addition, claudin-4 overexpression was also associated with a markedly diminished ability of S2-007 cells to anchorage-independent growth in soft agar. We could confirm these observations *in vivo* and showed a significantly decreased lung colonization rate after tail vein injection of claudin-4 overexpressing cells in nude mice. By examining proliferation and cell cycle progression, we could rule out that the described effects are secondary to altered proliferation, cell cycle progression, or significantly increased apoptosis.

A role of tight junctions as a prerequisite for tumor cell invasion has been described in endothelial cells. The reduction of tight junctions in these cells was found to decrease paracellular resistance and to facilitate invasion of epithelial tumor cells through endothelial cell layers (30, 31). In epithelial tumor cells, the formation of junctional complexes including tight and adherent junctions has been shown to reduce the invasive potential (32). However, to our knowledge, the direct effect of claudins on invasion of tumor cells has not been examined systematically. Our data show for the first time that overexpressed claudin-4 confers a less invasive phenotype *in vitro* and *in vivo*. We were able to show that this is not likely to be caused by alterations in the activity of gelatinolytic MMPs, as it has been described previously for other members of the claudin family (17). In our ultrastructural studies we found an increase in tight junctions between neighboring claudin-4 overexpressing tumor cells. This leads to the conclusion that an increase in the density of cell-cell adhesions formed by tight junctions may represent a crucial impediment against the dissociation of pancreatic cancer cells from their original tumor. This, in turn, would prevent the invasion into neighboring tissues or the formation of distant metastases. A similar mechanism has been proposed for the E-cadherin-mediated development of epithelial polarity and the suppression of invasiveness of cancer cells, which is associated with increased cell contact formation (32).

Considering that claudin-4 is highly expressed in many pancreatic carcinomas (9) and several other solid tumors (10, 11), our *in vitro* and *in vivo* findings demonstrating claudin-4 as an anti-invasive factor appear somewhat surprising. On the basis of the immunohistochemical findings in our series of pancreatic carcinoma tissues, in which claudin-4 expression was higher in well-differentiated carcinomas than in undifferentiated tumors, one can hypothesize that claudin-4 expression is mainly a phenomenon of differentiated, less invasive tumors with ductal-like structures. In undifferentiated, highly invasive tumors claudin-4 expression is less pronounced. However, to unequivocally prove a correlation among tumor grading, invasiveness, and claudin-4 expression, a study with a larger series of tumor specimens is warranted.

In support of our data, we identified TGF- β as a negative modulator of claudin-4. Both exogenous TGF- β and endogenously overexpressed TGF- β decreased claudin-4 levels markedly. Despite its antiproliferative effects in the early phase of tumorigenesis, TGF- β is known as a potent mediator of tumor progression by inducing cell spreading, migration, angiogenesis, and tumor cell invasion (33, 34). TGF- β -induced down-regulation of membrane proteins involved in cell-cell contact formation, such as claudin-4, therefore might be an important mechanism through which TGF- β promotes tumor invasion.

In addition to the important role of TGF- β in pancreatic cancer, up to 90% of pancreatic carcinomas have an activating K-Ras mutation

(27). Surprisingly, we found that specific inhibition of both major downstream effectors of Ras, MEK and PI3K, is associated with a decrease in claudin-4 expression in PANC-1 cells. In addition, claudin-4 was also transcriptionally down-regulated in dominant-negative H-Ras (S17N) overexpressing cells. Therefore, transcriptional up-regulation of claudin-4 by Ras-activation could account for the observed high expression of claudin-4 in the majority of pancreatic carcinomas. In the literature, the effect of Ras-activation and its downstream effectors on claudin expression and tight junctions has been discussed controversially thus far. Kinugasa *et al.* (35) reported a decrease in interleukin 17-induced claudin-2 expression in intestinal epithelial cells after the addition of the MEK inhibitor PD98059. In mammary epithelial cells, both MEK inhibition by PD98059 and PI3K inhibition by LY294002 were able to diminish glucocorticoid-induced transepithelial resistance, which is mainly maintained by the tight junctions (36). In contrast, Ras-transformed MDCK cells treated with the MEK inhibitor PD98059 showed recruitment of claudin-1 to the cell membrane and increased assembly of tight junctions (13). In addition, recent results in pig thyrocytes suggest that TGF- β exerts its effect on epithelial-mesenchymal transition, which includes down-regulation of claudin-1 and decreased transepithelial resistance through a MEK-dependent mechanism (37). Thus, it seems likely that the constituents of the tight junctions are regulated in a complex manner, which depends on the cellular context and might also vary among different members of the claudin family. Additional studies are necessary to elucidate the regulatory network modulating claudin expression and tight junction formation and integrity.

In summary, we showed that claudin-4 is overexpressed in pancreatic cancer and associated with decreased invasiveness *in vitro* and *in vivo*. This effect was morphologically associated with increased formation of tight junctions between tumor cells. Claudin-4 is negatively regulated by TGF- β and down-regulated by inhibition of the Ras signaling pathway. Our data clearly identify claudin-4 expression as an event that impairs the invasiveness and the metastatic potential of pancreatic cancer cells. Additional studies are warranted to investigate the correlation among claudin-4 expression, tumor invasion, and clinical outcome in pancreatic cancer patients. This might also characterize the groups of patients, which might benefit from a possible CPE-based anticancer therapy targeting claudin-4.

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