Up-Regulation, Nuclear Import, and Tumor Growth Stimulation of the Adhesion Protein p120^{ctn} in Pancreatic Cancer

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Background & Aims: **Cell adhesion proteins have been implicated as tumor suppressors because they prevent malignant cells from dissociating their cell contacts. We have studied the role of p120^{ctn}, a recently discovered member of the cadherin/catenin family, in human pancreatic cancer.** ***Methods:*** **In 32 resection specimens of pancreatic adenocarcinoma and 10 control samples the expression of p120^{ctn} was studied by Northern blot, immunocytochemistry, and immunogold electron microscopy. Patient survival data, tumor grading, and staging were correlated to the experimental results. In PaTu 8889 T pancreatic cancer cells, p120^{ctn} expression was suppressed with 21-nucleotide small interfering RNA (siRNA) duplexes and proliferation was determined by bromodeoxyuridine (BrdU) incorporation.** ***Results:*** **In pancreatic cancer p120^{ctn} messenger RNA (mRNA) was increased 3- to 4-fold. Although p120^{ctn} was localized exclusively at cell contacts in controls it was found in the cytosol and nucleus of pancreatic cancer cells. This redistribution correlated to the degree of tumor dedifferentiation but was independent of tumor stage. The mean survival of patients with predominant membrane localization of p120^{ctn} was 24 ± 7 (SEM) months vs. 9 ± 2 months for patients with predominant cytoplasmic p120^{ctn} expression ($P < 0.05$). Silencing of p120^{ctn} with siRNA duplexes reduced pancreatic cancer cell growth by 40%.** ***Conclusions:*** **Up-regulation, cytoplasmic redistribution, and nuclear import of p120^{ctn} are associated with a more malignant phenotype of pancreatic cancer. This study further represents conclusive evidence for a direct involvement of p120^{ctn} in malignant tumor cell proliferation. Both p120^{ctn}-defective tumor cell contacts and p120^{ctn}-mediated growth signals appear to contribute to the aggressive spread of pancreatic cancer.**

Pancreatic cancer is a common malignancy of men and women in Western countries and the fourth most common cause of cancer-related deaths.[1] Histologically, 90% of all pancreatic cancers are ductal adenocarcinomas that are burdened with a poor median survival of less than 6 months in patients with unresectable tumors.[2] One of the causes for the poor prognosis of pancreatic adenocarcinoma is its tendency to form micrometastases before clinical symptoms arise and before the tumor is detectable by diagnostic imaging techniques. The mechanisms that determine the highly malignant growth and dissemination pattern of pancreatic cancer are understood poorly.

Malignant tissue invasion and the formation of distant metastases require that neoplastic cells dissociate their cell-to-cell contacts with the original tumor to escape its tissue connection. This would predict that an impairment of intercellular junctions within a neoplastic tumor would increase its malignant potential. The members of the cadherin/catenin family of cell adhesion proteins are the most critical components of cell-to-cell adhesions and various mechanisms that impair their function can increase the aggressiveness of malignant tumors. These mechanisms include structural changes caused by mutations,[3–5] aberrant expression,[6] changes in the subcellular protein localization,[7] or alterations in protein phosphorylation.[8]

The proteins of the cadherin/catenin family have been proposed accordingly to represent a distinct class of tumor suppressors. In cells of epithelial origin they form homophilic, calcium-dependent interactions between neighboring cells[9] and their intracellular domains link adherens junctions to the cytoskeleton.[10] Mutations that affect the intracellular binding sites of E-cadherin, α-, or β-catenin result in a dissociation of cell contacts even when the extracellular E-cadherin domain remains intact and fully functional.[11,12] Cadherin mutants with deleted extracellular domains or transmembrane chimeras that have an intact intracellular cadherin tail continue to be targeted correctly to adherens junctions.[13]

Abbreviations used in this paper: BrdU, bromodeoxyuridine; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; UICC, union internationale contre le cancer.

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p120^{ctn} is a novel and recently discovered member in the cadherin family of cell adhesion molecules and was identified originally as a substrate for Src tyrosine kinase.[14] p120^{ctn} is highly phosphorylated on serine/threonine residues and also tyrosine phosphorylated on stimulation with various growth factors, which suggests a potential role in cell signaling. p120^{ctn} serves as an intracellular binding partner for the cadherin/catenin complex at adherens junctions[15–18] and its sequence contains an armadillo motif that shares 22% identity with β-catenin.[18] The binding of p120^{ctn} to the adhesion complex is mediated by the juxtamembrane domain of E-cadherin and no direct interaction between p120^{ctn} and either α- or β-catenin has yet been shown. The association of p120^{ctn} with the cell adhesion complex has been shown to strengthen cell-to-cell contacts[19–21] and p120^{ctn} therefore is regarded as another member of the cadherin/catenin family involved in the regulation of cell-to-cell adhesions. Because little information about a potential role of p120^{ctn} in the formation and maintenance of tumor cell adhesions is yet available we have studied its expression and subcellular localization in human pancreatic cancer and have correlated the results to the histopathology and clinical data of affected patients. Our results indicate that an up-regulation of p120^{ctn} and its redistribution into the cytosol and nucleus of pancreatic cancer cells is associated with a particularly aggressive phenotype and a very poor clinical prognosis of affected patients. We also show that p120^{ctn} is involved directly in tumor cell proliferation and malignant growth.

Materials and Methods

Patient Characteristics and Tissue Specimens

Tumor specimens from 32 patients who had undergone a Kausch–Whipple resection for ductal adenocarcinoma of the pancreas as well as pancreatic tissue from 10 organ donors were used for expression analysis. From 21 pancreatic cancer patients, tissue suitable for immunocytochemistry and sufficient clinical data were also available. The median age of the pancreatic cancer patients was 65 years (range, 52–78 years). According to the TNM classification after surgery there were 11 stage I, 2 stage II, 16 stage III, and 3 stage IVa tumors. On histopathologic tumor grading according to the union internationale contre le cancer (UICC), 7 tumors were classified as well differentiated (grade 1), 15 as moderately differentiated (grade 2), and 10 as poorly differentiated (grade 3). None of the patients had distant metastases.

Freshly resected tissue samples were divided for immediate fixation in 4% paraformaldehyde or Bouin's fixative and either embedded in paraffin or cryopreserved for electron microscopy. Untreated tissue was snap frozen in liquid nitrogen for storage at −80°C and later RNA extraction. Because pancreatic cancer often contains abundant connective tissue surrounding sparsely present tumor cells, representative H&E-stained sections from each frozen tissue block were examined microscopically to confirm the presence of sufficient tumor mass.

Specimens with less than 50% neoplastic cells were excluded from expression analysis. All studies were approved by the institutional Human Ethics Committee of the University of Bern, Switzerland.

Northern Blot Analysis

Total RNA was extracted using the single-step guanidinium isothiocyanate method, and separated by electrophoresis under denaturing conditions in a 1.2% agarose/formaldehyde gel. Gels were stained with ethidium bromide for verification of RNA integrity and loading equivalency. The RNA was electrotransferred to nylon membranes (Gene Screen; Du Pont, Boston, MA) and cross-linked by ultraviolet irradiation. Membranes were prehybridized overnight at 42°C in a buffer containing 50% formamide, 1% sodium dodecyl sulfate (SDS), 0.75 mol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 5× Denhardt's solution, 100 μg/mL salmon sperm DNA, 10% dextran sulfate, and 50 mmol/L sodium phosphate (pH 7.4). One × 10^6 cpm/mL of a ^{32}P-labeled mouse p120^{ctn} complementary DNA (cDNA) probe were added to the hybridization solution and incubated for another 12 hours, followed by 2 washes at 50°C in 2 × standard saline citrate, and 3 washes at 55°C in 0.2 × standard saline citrate, and 2% SDS. To assess equivalent RNA loading and transfer, all blots were rehybridized with a mouse ^{32}P-labeled 7S cDNA probe, which cross-hybridizes with human 7S RNA. All blots were exposed at −80°C to Fuji (Düsseldorf, Germany) radiograph film with Kodak (Stuttgart, Germany) intensifying screens for 10 days. The intensity of the radiographic bands was quantified by a computerized video imaging system and the Image-pro-plus 3.0 software (Media Cybernetics, Silver Spring, MD). The ratios of the optical densities of the RNA levels (p120^{ctn}/7S) were calculated for each sample and used for statistical analysis. Mean optical densities of the RNA levels in control tissue (p120^{ctn}/7S) were set arbitrarily as 1 and expression levels from pancreatic cancer samples were calculated as fold increase or decrease from control tissue. Only samples used in the same experiment were compared directly. Boxes in Figure 1 represent 95% confidence intervals and bars represent the SEM. Differences at the 5% level were determined by using a 2-tailed Student *t* test.

The mouse p120^{ctn} cDNA probe (GenBank accession number Z17804) used for Northern Blot analysis was a 2.8-kb fragment containing the complete sequence of the coding region of p120^{ctn} plasmid (sequence homology mouse/human p120^{ctn} 90%), which was kindly provided by A. B. Reynolds (Department of Cell Biology, Vanderbilt University, Nashville, TN). A 190-bp *Bam*HI/*Bam*HI segment of mouse 7S cDNA was subcloned into the pGEM 7ZF (+) (Promega Biotechnology, Madison, WI). Both the p120^{ctn} and the 7S

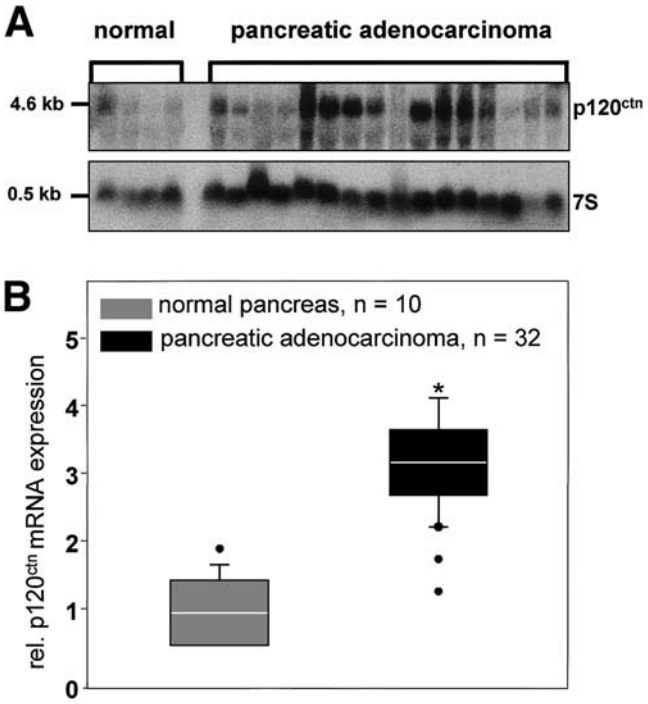

Figure 1. p120ctn RNA expression in pancreatic cancer. (*A*) Northern blots using a full-length p120ctn cDNA probe for 16 representative pancreatic adenocarcinoma specimens and 4 specimens from control pancreas. RNA was isolated from pancreatic tissue and processed as described in the Materials and Methods section and 7S RNA served as internal loading standard. (*B*) Northern blot results from all 42 investigated samples (32 pancreatic cancers and 10 controls) were used for densitometry. p120ctn expression was quantitated as the ratio of 7S RNA expression in each specimen and the graph indicates means ± 95% confidence intervals. The overall increase in pancreatic cancer was 3.3-fold (± 0.7) over control tissue.

cDNA probes were labeled with [α-^{32}P]-deoxycytidine triphosphate (Du Pont International, Regensdorf, Switzerland) by using a random primer labeling system (Boehringer-Mannheim, Mannheim, Germany).

Antibodies and Immunocytochemistry

Sections (2–4 μm) of each paraffin-embedded tissue sample were deparaffinized with xylene and rehydrated through graded alcohol into distilled water. The sections were microwaved in 10 mmol/L citrate buffer (pH 6) for 5 minutes at 900 W and for 10 minutes at 500 W. Slides where then washed in Tris-buffered saline (5 mmol/L Tris-HCl, 0.3 mol/L $NaCl_2$, pH 7.4). Endogenous peroxidase activity was quenched by incubation in 0.03% hydrogen peroxide and sodium azide, followed by washing in Tris-buffered saline. The sections were then incubated overnight at 4°C with primary monoclonal antibody against p120ctn (5 μg/mL; Transduction Laboratories, Lexington, KY), diluted in 0.05 mol/L Tris-HCl buffer containing 1% bovine serum albumin. The purified antibody has been validated previously for immunohistochemistry in different tissues.[22] Bound antibody was detected by a streptavidin-biotin horseradish-peroxidase system (Dako Diagnostics, Zurich, Switzerland) in which slides were incubated successively with biotinylated anti-rabbit immunoglobulin (Ig) G, streptavidin horseradish peroxidase, and 3-3′ diaminobenzidine. Slides were counterstained with Mayer's hematoxylin. Control slides were incubated in either the absence of primary antibody or with a nonspecific IgG antibody. All slides were evaluated by 2 independent observers who were unaware of the patient's status, followed by resolution of any differences by joint review and consultation with a third specialist. Antibody staining was classified as absent, weak, moderate, or strong and assigned a score ranging from 0 to 3 points. Expression was further assessed as predominant localization at the cell membrane or in the cytosol. For a sample to be classified as showing predominant membrane expression, at least moderate or strong membrane labeling of p120ctn and weak or absent cytosolic expression had to be present, whereas to qualify as predominant cytosolic expression the reverse relation was required. The histopathologic tumor grade (UICC) in each patient's surgical specimen was determined by an expert pathologist at the University of Bern.

Electron Microscopy

Small blocks (2 mm in diameter) of pancreatic tissue from 6 histologically characterized pancreata were fixed in 5% (wt/vol) paraformaldehyde in 0.2 mol/L piperazine-N,N′-bis [2-ethanesulfonic acid], pH 7.0, cryoprotected with polyvinylpyrollidine/sucrose, and frozen in liquid nitrogen. Ultrathin frozen sections (60 nm) were prepared using a Leica (Wetzlar, Germany) Cryoultramicrotome (block temperature −110°C, knife-temperature −100°C). The sections on formvar-coated copper grids were blocked with 5% (wt/vol) fetal calf serum (Life Technologies, Rockville, MD) in phosphate-buffered saline (PBS), pH 7.4, and then incubated with mouse monoclonal anti-p120ctn antibody (1:10–1:30; Transduction Laboratories) for 45 minutes at room temperature. After washing with PBS the sections were incubated with 10-nm gold-conjugated goat anti-mouse antibody (dilution 1:10; Dianova, Hamburg, Germany), washed again with PBS and water, and subsequently contrasted and embedded by incubation with methylcellulose/uranyl acetate on ice (9:1 mixture of 2% methylcellulose and 4% uranyl acetate). Samples were examined on a Philips (Eindhoven, Netherlands) 400 electron microscope and photographed at 30,000× magnification.

p120ctn Small Interfering RNA Transfection of PaTu 8889 T Cells

To further evaluate the biologic function of p120ctn in pancreatic cancer we used the PaTu 8988 T cell line because it corresponded to a poorly differentiated human pancreatic ductal adenocarcinoma (G3) and expressed p120ctn at comparable levels with our tumor specimens. Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 1% L-glutamine at 5% CO_2, trypsinized, seeded on chamberslides or 96-well plates, and cultured for 12 hours to subconfluency (50% to 70%). For the transfection of small interfering RNA (siRNA) to target endogenous p120ctn,

SuperFect transfection reagent (Qiagen, Hilden, Germany) was used with 60 pmol/L siRNA duplex per well (MWG AG Biotech, Ebersberg, Germany). Transfection was performed according to the manufacturer's instructions for adherent cell lines and the efficiency of transfection in protein silencing of 120^{ctn} was assayed by Western blotting and immunofluorescence labeling after 12 hours. The sequence for siRNA design was selected according to the strategy reported by Tuschl[23] with siRNA duplex encoding $p120^{ctn}$ (gene bank accession number NM 001331, position 1043–1063). For control transfection we used transfection reagent alone as well as siRNA duplex for an unrelated non–cell-adhesion protein (firefly luciferase, gene bank accession number X65324; position 153–171).

To determine protein expression PaTu 8889 T cells were lysed in iced Triton X-100 lysis buffer containing protease inhibitors (1 mL/mg tissue, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.01 mol/L sodium pyrophosphate, 0.1 mol/L sodium fluoride, 1 mmol/L dihydrogen peroxide, 1 mmol/L L-phenylmethylsulfonyl fluoride, and 0.02% soybean-trypsin inhibitor). Protein concentration was determined by a modified Bradford-assay (Bio-Rad, Hercules, CA). Samples were boiled for 5 minutes in 4× SDS sample buffer. SDS polyacrylamide gel electrophoresis was performed in a discontinuous buffer system and gels were blotted on nitrocellulose membranes (Hybond C; Amersham Pharmacia Biotech, Uppsala, Sweden). After overnight blocking in NET-gelatine 0.2%, immunoblot analysis was performed followed by enhanced chemoluminescence detection (Amersham Pharmacia Biotech) using horseradish-peroxidase–coupled sheep anti-mouse IgG (Amersham Pharmacia Biotech) or goat anti-rabbit IgG (Amersham Pharmacia Biotech).

For immunofluorescence studies cells were washed twice in PBS and fixed in 4% PBS-buffered paraformaldehyde for 30 minutes, followed by membrane permeabilization in 0.1% Triton X-100/PBS buffer for 1 minute. Nonspecific antibody binding was blocked in PBS/bovine serum albumin 1% wt/vol for 1 hour followed by overnight incubation with the primary antibody in a concentration of 5 μg/mL at 4°C.

Primary antibody binding was detected with an isospecies-specific secondary antibody conjugated to fluorescein isothiocyanate donkey anti-mouse IgG (1:100, lot 39656; Dianova). Under otherwise identical conditions controls were incubated with either species-specific nonimmune serum, purified IgG, or without primary antibody, and images were taken under the same exposure, brightness, and contrast settings. For fluorescence microscopy a high-resolution Nikon-Improvison confocal imaging system was used (Waxford, UK).

Tumor cell proliferation was determined as bromodeoxyuridine incorporation (BrdU) (10 μmol/L), which reflects total DNA synthesis. PaTu 8889 T cells were cultivated for 12 hours after siRNA transfection (at which time $p120^{ctn}$ protein expression was found to be silenced completely by Western blotting) and in Dulbecco's modified Eagle medium (10% fetal calf serum, 2% glutamin, 1% penicillin/streptomycin) before BrdU was added. Cells were then cultured for another 12 hours and subsequently assayed for BrdU-labeled DNA by an anti-BrdU peroxidase–linked monoclonal antibody, followed by enzymatic reaction with tetramethylbenzidine as substrate (detected at λ 450 nm, Dynatech 7500 ELISA reader RPN 250; Amersham Pharmacia Biotech). Whole DNA content was determined after cell lysis with propidium iodine (5 μg/mL) at 350/630 nm. Results shown represent 3 or more independent experiments and were obtained from a minimum of 28 parallel wells of which the mean was calculated.

All other chemicals were of the highest purity available and were obtained from Sigma-Aldrich (Eppelheim, Germany), Merck (Darmstadt, Germany), Amersham Pharmacia Biotech (Buckinghamshire, UK), or Bio-Rad (Hercules, CA).

Statistical Analysis

The immunocytochemically labeled specimens were classified into 2 groups: (1) those in which $p120^{ctn}$ was localized predominantly at the cell membrane and at cell contacts, and (2) those in which $p120^{ctn}$ labeling was most prominent in the cytoplasm. Patient specimens were analyzed further for a correlation between the subcellular localization of $p120^{ctn}$, the UICC tumor grade, and the TNM tumor stage. Results were expressed as means as well as medians ± SEM. For statistical analysis of differences between the 2 groups a 2-tailed Student *t* test was used.

Survival data were plotted according to the method of Kaplan and Meier and multivariant analysis was performed using the Cox regression to determine the dependence between $p120^{ctn}$ expression and tumor grade (UICC) or tumor stage (TNM). Significant differences between groups were defined as those with a P value of less than 0.05. SPSS 10.0 (SPSS, Chicago, IL) was used for statistical analysis.

Results

Northern Blot Analysis of $p120^{ctn}$

Thirty-two samples of tumor messenger RNA (mRNA) and 10 samples of mRNA from donor pancreas were used for Northern blot analysis of $p120^{ctn}$ expression. In the healthy pancreas, a 4.6-kb transcript of $p120^{ctn}$ was detectable and in 28 of the 32 pancreatic cancer samples (87.5%) a significant increase in the expression of $p120^{ctn}$ mRNA was found (Figure 1*A*). When the difference between controls and pancreatic cancer samples was quantitated by densitometry an overall 3.3-fold (± 0.7 SEM) up-regulation of $p120^{ctn}$ mRNA in pancreatic cancer compared with control pancreas was found (Figure 1*B*). In none of the investigated cancer specimens was a complete loss of $p120^{ctn}$ expression found.

Immunocytochemistry

Incubation under control conditions and without primary antibody did not result in any background la-

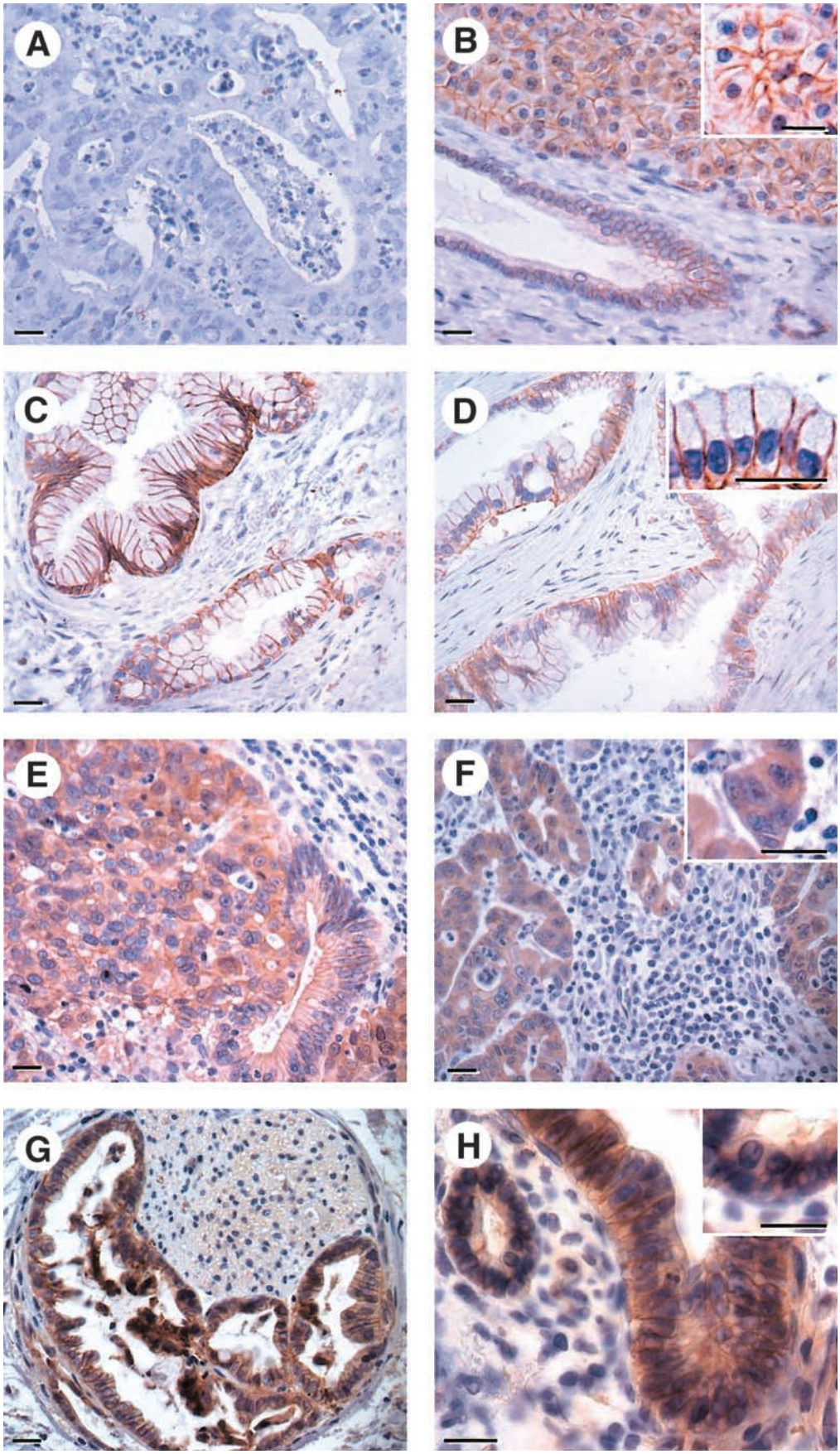

Figure 2. p120ctn immunolocalization in pancreatic cancer. (*A*) Histochemistry with control or without primary antibody resulted in no immunoreactivity. (*B*, *inset*) In sections of normal pancreas, p120ctn labeling was detected exclusively at the cell membrane of acinar and duct cells and at their respective cell contacts. (*C* and *D*, *inset*) In highly differentiated pancreatic carcinoma (UICC grade I), p120ctn labeling still was confined largely to cell adhesions (*E* and *F*, *inset*), whereas in UICC grade II cancer a cytosolic expression was predominant. (*G* and *H*, *inset*) The most prominent overall labeling and a largely cytosolic expression was found in poorly differentiated pancreatic tumors (UICC grade III). (*G*) Note the characteristic invasion pattern along the perineureum of a pancreatic nerve. Samples shown are representative for 7 or more in each group and micrographs were taken at the same exposure, brightness, and contrast setting for all samples. *Bars* indicate 200 μm.

beling (Figure 2*A*). In the normal pancreas p120^{ctn} was expressed endogenously in all epithelial cells, such as acinar and duct cells, and was detected exclusively at the cell membranes and at cell-to-cell adhesions (Figure 2*B*). In all pancreatic adenocarcinoma specimens investigated a more prominent p120^{ctn} expression was seen (Figures 2*C–H*) and its subcellular distribution was no longer strictly confined to the cell membrane or to cell contacts. In many pancreatic tumors, a much more prominent localization of p120^{ctn} in the cytosol than at the membrane was found. In none of the pancreatic tumor specimens was p120^{ctn} labeling in the cytosol absent; in 19% we found weak cytosolic staining, in 38% moderate staining, and in 43% a strong cytoplasmic expression of p120^{ctn}. The desmoplastic stroma around tumor cell islands, which is composed of extracellular matrix and mesenchymal cells, was not labeled with p120^{ctn}. When the degree of tumor differentiation was taken into account it became apparent that in highly differentiated adenocarcinomas p120^{ctn} was largely retained at the plasma membrane and cell contacts (strong membrane staining in 62.5% of G1 tumors, Figure 2*C–D*), whereas in progressively more undifferentiated tumors it was expressed prominently in the cytosol and the extent of this redistribution paralleled the degree of dedifferentiation (strong cytosolic staining in 75% of G3 tumors, Figure 2*E–H*).

Ultrastructural Localization of p120^{ctn}

Incubation without primary antibody or with nonspecific IgG resulted in virtually no background gold labeling of ultra-thin sections, and after labeling of specimens from control pancreas p120^{ctn} was detected exclusively at cell-to-cell contacts of pancreatic duct cells (Figure 3*A–B*) and acinar cells (Figure 3*C*), and neither over the cytosol, mitochondria, or nuclei (Figure 3*B*) of these cells.

In tumor cells of low-grade pancreatic cancer p120^{ctn} labeling at cell contacts was found to be reduced (Figure 3*D*, *arrow*) and became instead detectable over the cytosol and nuclei (Figure 3*D*, *asterisk*). In high-grade pancreatic cancer the morphologic appearance of cell contacts between tumor cells had changed dramatically. Rather than appearing as a closed band along the entire contact area between adjacent tumor cells, duplications and even branching of adherens junctions (Figure 3*E–F*) were common and a general distinction between tight junctions, adherens junctions, and desmosomes based on their characteristic morphologic appearance was often impossible. These atypical cell contacts sometimes were labeled prominently by p120^{ctn} but this localization was no longer exclusive. Highly abundant gold decoration was found over the cytosol and particularly over the nuclei (Figure 3*F*, *asterisk*) of the tumor cell.

Nuclear import was never detected in nonmalignant cells of the pancreas, and in the nucleus of high-grade pancreatic cancer cells it was confined to the euchromatine and spared the heterochromatine, which suggests DNA binding (Figure 3*F*, *arrowheads*).

Correlation of p120^{ctn} Distribution and Tumor Grading

A quantitative assessment of the extent of p120^{ctn} distribution to the cytosol of tumor cells vs. its localization at the cell membrane confirmed the observation that low-grade (UICC) tumors largely had retained their membrane expression of p120^{ctn} whereas in highly undifferentiated pancreatic cancers a cytosolic distribution was predominant (Figure 4). However, no significant correlation between the subcellular p120^{ctn} distribution and the tumor stage (TNM) of pancreatic cancer patients was found (χ^2 test, $r = 1.39$; data not shown).

Patient Survival and p120^{ctn} Expression

For 20 patients in whose tumor resection specimens we had characterized the p120^{ctn} expression and subcellular localization, complete follow-up data as to their postoperative clinical course were also available. The Kaplan–Meyer survival curves indicated a clear survival difference (Figure 5) of patients with a predominant membrane localization of p120^{ctn} in their cancer specimen ($n = 10$) in comparison with patients with a predominant cytosolic p120^{ctn} labeling ($n = 10$). p120^{ctn} membrane localization in the tumor was associated with a mean survival of 24 ± 7 months, whereas in patients with cytosolic p120^{ctn} expression mean survival was 9 ± 2 months (log rank test, $P < 0.05$). Median survival for predominant membrane expression was 14 ± 2 months, whereas for predominant cytoplasmatic expression it was 8 ± 3 months.

Growth Inhibition by Small Interfering RNA Silencing of p120^{ctn} in PaTu 8889 T Cells

Immunofluorescence analysis for p120^{ctn} in PaTu 8889 T cells, a poorly differentiated pancreatic adenocarcinoma cell line, revealed nearly absent membrane staining, a high cytoplasmic expression of p120^{ctn}, as well as prominent nuclear staining (Figure 6*A*). The staining pattern for p120^{ctn} in PaTu 8889 T cells thus was similar to the one found in resection specimens from patients with poorly differentiated pancreatic cancer. Transfection of PaTu 8889 T cells with siRNA duplexes targeted against endogenous p120^{ctn} led to an almost complete absence of p120^{ctn} labeling in tumor cells

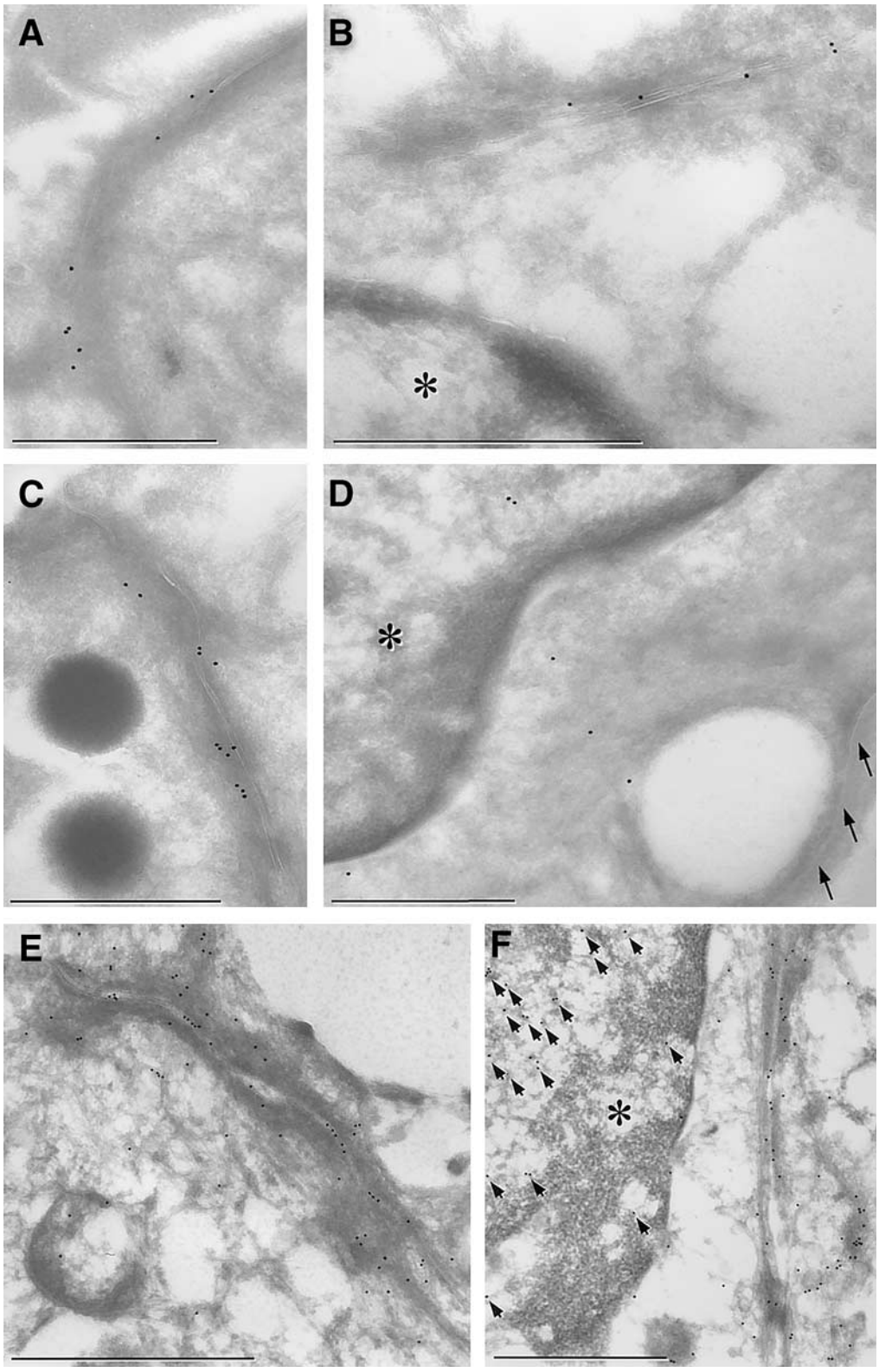

Figure 3. Ultrastructural localization of p120ctn. In control specimens of normal pancreas p120ctn was detected exclusively at cell-to-cell contacts between (*A* and *B*) duct cells and (*C*) acinar cells, but neither in the cytosol nor in the (*B*, *asterisk*) nucleus of these cells. (*D*, *arrows*) The junctions of low-grade pancreatic cancer had largely lost their p120ctn labeling and it was found instead over the cytosol and (*D*, *asterisk*) nucleus of tumor cells. (*E* and *F*) At the atypical junctions between high-grade pancreatic cancer cells (UICC III) p120ctn labeling remained present but also a prominent cytosolic and (*F*, *asterisk*) nuclear labeling were evident. Although no nuclear import was found in nonmalignant cells of the exocrine pancreas it was highly abundant in the nuclei of high-grade pancreatic cancer cells and (*F*, *arrowheads*) confined to their euchromatine while sparing the heterochromatine. Ultrathin cryosections were prepared and immunogold labeled (6 nm) as described in the Materials and Methods section and examples shown are representative for 6 specimens. *Bars* indicate 1 μm.

(Figure 6*B*). This reduction in p120ctn labeling was most prominent in the nuclear regions of PaTu 8889 T cells (Figure 6*B*). However, Figure 6*B* had to be exposed 5 times longer (890 vs. 180 ms) than Figure 6*A* to make any fluorescence visible on prints. When p120ctn protein expression in control transfected cells (Figure 6*C*, top) and p120ctn siRNA duplex-transfected cells (Figure 6*C*, bottom) was compared by Western analysis a correspond-

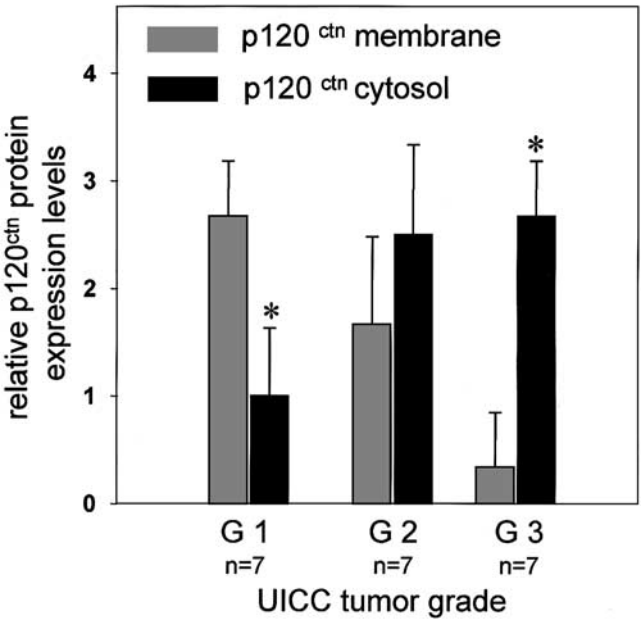

Figure 4. Cellular distribution of p120^{ctn} and tumor differentiation. p120^{ctn} antibody labeling was studied in a blinded fashion and assessed by using a 0–3 score as described in the Materials and Methods section. When correlated to the UICC grade of tumor tissue differentiation it became apparent that in low-grade tumors (UICC grade I) p120^{ctn} was localized predominantly at the cell membrane whereas in high-grade tumors (UICC grade III) it was found largely in the cytosol. Twenty-one pancreatic cancer samples were available for semiquantitative analysis. Data are expressed as means ± SEM. *Significant differences at the 5% level.

ing silencing of endogenous p120^{ctn} in PaTu 8889 T cells was found, whereas neither control siRNA nor transfection agent alone (not shown) had any effect on p120^{ctn} expression. The lower 100-kilodalton band in Figure 6*C* represents a second p120^{ctn} isoform[24] and both isoforms of p120^{ctn} were silenced by the cognate p120^{ctn} siRNA duplex. To test whether siRNA silencing of p120^{ctn} had any effect on tumor cell proliferation we studied BrdU incorporation in PaTu 8889 T cells. BrdU was added when p120^{ctn} expression was silenced completely by siRNA treatment. When BrdU incorporation was studied over 12 hours we found that tumor cell growth was reduced by 39.6% (± 2.7% SEM) in comparison with PaTu 8889 T cells that were transfected with control siRNA (Figure 6*D*). As a second line of evidence we measured total PaTu 8889 T cell DNA as an indicator of increased tumor cell numbers. In 96-well plates, 5000 cells per well were seeded and either transfected with the p120^{ctn} cognate siRNA duplex or control siRNA. Total DNA content after 24 hours was reduced to 71.3 (± 1.9% SEM) of control transfected cells by p120^{ctn} silencing. For both proliferation experiments with PaTu 8889 T cells treatment with mitomycin C (500 μg/mL) served as negative control.

Discussion

As long as a malignant tumor constitutes merely a fast-growing tissue mass it usually can be cured by surgical resection and represents only a limited threat to the affected patient. As soon as malignant cells from the tumor begin to dissociate their cell contacts with neighboring cells they acquire the potential to invade and destroy adjacent tissues and can disseminate to form metastases in distant organs. The maintenance of functional cell-to-cell adhesions is therefore one of the most effective impediments to the spread of malignant tumors and can determine its aggressiveness. Cell adhesion proteins of the cadherin/catenin family, which form calcium-dependent cell-to-cell contacts in the exocrine pancreas[25] and other epithelial organs, have been found to be involved in several processes that relate to the malignant potential of neoplastic tumors. These include tumor cell migration, local tumor invasiveness, and the transformation from adenoma to carcinoma.[26,27] A variety of mechanisms that all involve an impairment of the cadherin/catenin complex at cell-to-cell adhesions have been reported to affect the malignant potential of a variety of tumors. Somatic mutations,[5] an aberrant protein expression,[6] changes in the subcellular localization of cadherins,[7] or their phosphorylation[8] have all been found to increase the aggressiveness of neoplasms or to negatively affect the survival of affected patients.[28–32]

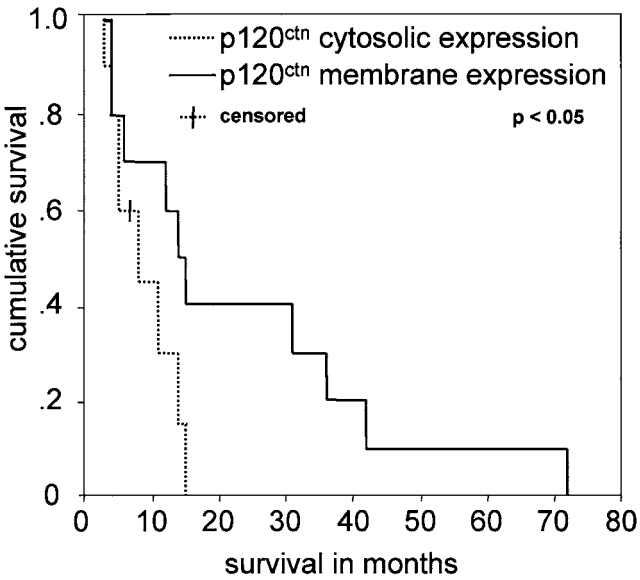

Figure 5. Kaplan–Meier representation of the cumulative survival of pancreatic cancer patients. Patients with a predominant cell membrane localization of p120^{ctn} in the tumor specimen (*solid line*) survived curative resection of pancreatic cancer for 24 ± 7 (SEM) months, whereas patients with a predominant cytosolic localization of p120^{ctn} (*broken line*) had a mean survival of 9 ± 2 months (log rank test, $P < 0.05$). One patient was lost to follow-up and accordingly is censored in the graph.

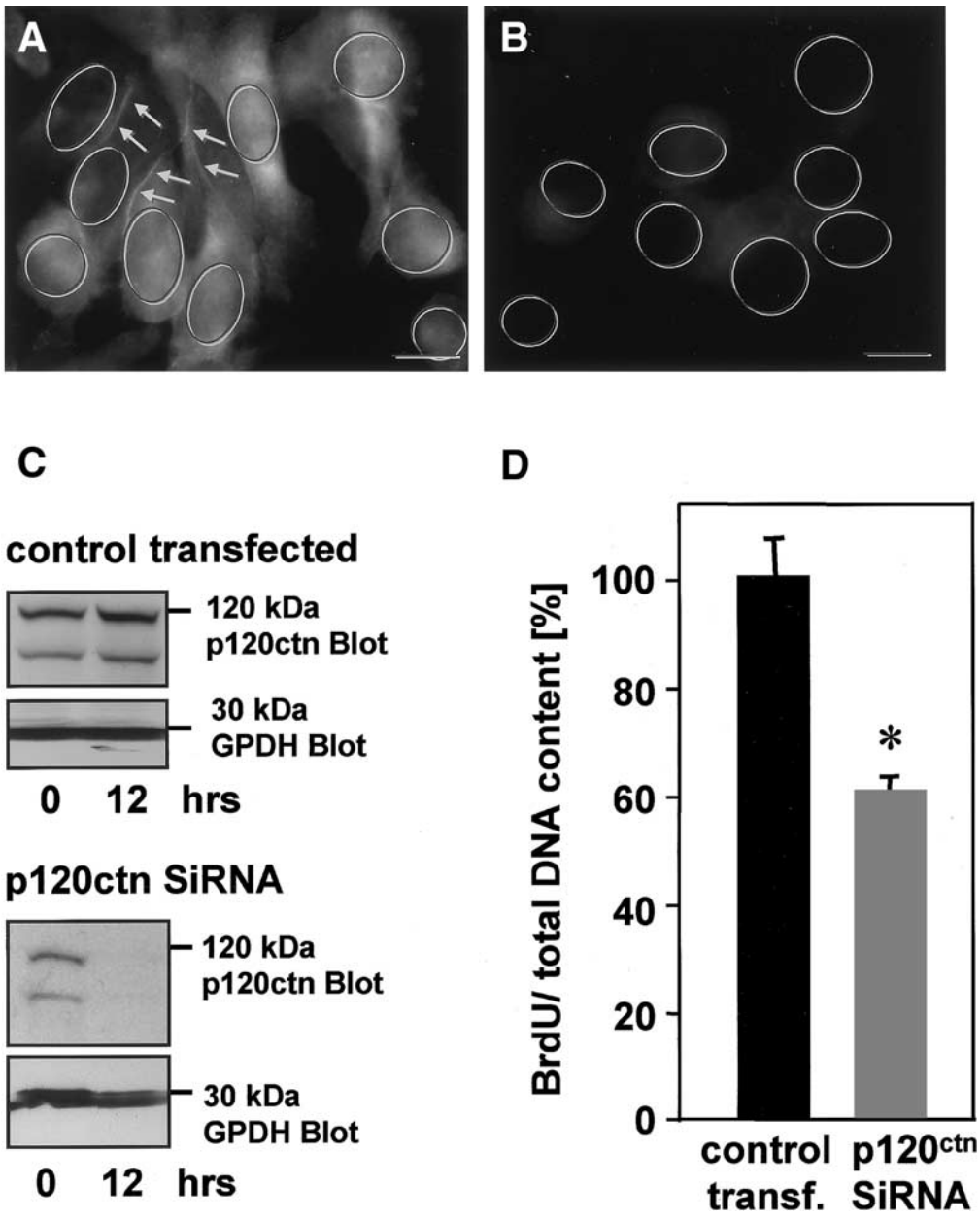

Figure 6. Silencing of p120ctn in PaTu 8889 T cells. (*A*) Immunofluorescence labeling of p120ctn in PaTu 8889 T cells after control siRNA transfection indicated expression in the nuclear region (*circles*), the cytosole, and along cell-to-cell contacts (*arrows*). (*B*) After transfection with p120ctn siRNA duplex for 12 hours, expression was reduced to barely detectable levels. Immunoblotting of PaTu 8889 T-cell homogenates indicated that (*C, top*) control transfection had no effect, whereas transfection with (*C, bottom*) p120ctn siRNA duplex for 12 hours specifically silenced p120ctn expression. (*D*) BrdU incorporation at 12 hours was reduced by 40%. *Bars* in micrographs indicate 100 μm.

p120ctn is the most recent member of the cadherin/catenin family of cell adhesion proteins, has been implicated in cell contact regulation of malignant cells,[33] and shares a 22% identity with β-catenin.[18] It was found to bind to the cadherin/catenin complex via the juxtamembrane domain of E-cadherin[15–18] and this binding contributes significantly to the function of the adhesion complex and the maintenance of cell-to-cell contacts.[19–21]

When we investigated the expression and subcellular localization of p120ctn in pancreatic cancer specimens we were surprised to find a consistent up-regulation on the mRNA and protein level in comparison with control pancreas. This up-regulation of p120ctn could have indicated that tumor cells increase the efficiency of their intercellular adhesions and this would have conferred a disadvantage for the malignant potential of the tumor—an improbable mechanism in a tumor as aggressive as pancreatic cancer. However, when we compared the localization of p120ctn in pancreatic cancer cells with that of control pancreas it became apparent that in normal acinar and duct cells p120ctn is localized exclusively at cell contacts and at the cell membrane. In malignant cells, however, p120ctn was distributed frequently over the entire cytosol and the extent of this redistribution directly paralleled the degree of dedifferentiation (UICC grade) of the tumor. Because we could not firmly distinguish between a cytosolic and a nuclear

localization of p120^{ctn} by light-level immunocytochemistry we performed electron microscopy studies using the immunogold technique. Interestingly, ultrastructural morphology indicated that high-grade dedifferentiated tumors had retained some p120^{ctn} labeling at cell-to-cell contacts. These contacts, however, were often of atypical morphology, including an aberrant branching of junctions, and had lost the characteristic appearance of the different types of cellular adhesions. Moreover, immunogold electron microscopy could not only confirm an abundant cytosolic localization but also a prominent nuclear import of p120^{ctn} in tumor cells. The question that arose from these observations was whether the subcellular redistribution and nuclear import of p120^{ctn} would directly affect the growth and aggressive behavior of pancreatic cancer or represent merely an epiphenomenon of tumor cell.

Recent developments have shown that double-stranded RNA effects a silencing of protein expression of genes that are highly homologous to either one of the RNA strands in the duplex. This phenomenon has been termed *RNA interference*. Gene silencing via RNA interference results from degradation of mRNA sequences and does not involve the actual translation of a given gene.

Elbashir et al.[34,35] have reported that silencing can be achieved most effectively by using well-defined 21-base duplex RNAs, termed *small interfering RNA* or *siRNA*. When siRNA duplexes are used for liposomal transfection into mammalian cells the 21-base RNA acts in concert with endogenous cellular components to silence the gene that has sequence homology to the siRNA strands. To study the biologic effect of p120^{ctn} silencing by siRNA duplexes we have chosen the poorly differentiated pancreatic adenocarcinoma cell line PaTu 8889 T. We not only found a dramatic reduction in p120^{ctn} protein expression and immunohistochemical labeling, that most prominently affected the tumor cell nuclei, but also a significant reduction in BrdU incorporation as an indicator of PaTu 8889 T-cell proliferation. In view of the malignant potential of pancreatic adenocarcinoma these data have several implications.

The dissociation of p120^{ctn} from intact cell-to-cell contacts or its localization to atypical, and presumably not fully functional, cell adhesions would suggest that pancreatic cancer cells indeed acquire their aggressive phenotype by early dissociation of tumor cell-to-cell contacts as seen in other types of cancer.[36] This would explain the capacity of pancreatic adenocarcinoma to form micrometastases at a clinically unapparent and still undetectable stage.[37] It would further suggest a role of p120^{ctn} in the impairment of tumor cell contacts or in the potential inability of the cadherin/catenin complex to maintain functional adhesions between pancreatic cancer cells.

The nuclear import of p120^{ctn}, which was detected exclusively in pancreatic cancer cells, and observed in an endogenous system of human tumor growth, has different implications. This would suggest that p120^{ctn} is involved in the signaling cascade that regulates growth and proliferation of pancreatic cancer. Because p120^{ctn} contains a conserved so-called arm-repeat 6-sequence, it can potentially localize to the cell membranes, such as a classic cell adhesion protein, as well as to the nucleus.[22,38] A similar dual localization has been reported previously for a number of other proteins such as ARVCF, p0071, δ-catenin, and plakophilin 1 and 2.[36,39–44] Not only has a nuclear import in cultured cells been shown for β-catenin where it was found to be associated with cell contact dissociation,[45] but also for p120^{ctn}.[46] In the case of β-catenin this process is owing to its ability to form mutually exclusive complexes with either E-cadherin, the tumor-suppressor gene *APC*, or the transcription factor lef1/TCF[47,48] via its arm domain. Depending on the intracellular localization, β-catenin can interact with each of these proteins and the according function is determined by the subcellular site of the interaction. At the cell membrane β-catenin thus acts as a cell adhesion protein and in the cytosol and nucleus it is involved in intracellular signaling.

The fact that p120^{ctn} undergoes nuclear import exclusively in malignant pancreatic cells and that it was found previously to interact in vitro with Kaiso,[22] a zinc finger transcription factor of the POZ-ZF family that is involved in DNA-methylation–dependent transcription,[49] made a role of p120^{ctn} in the regulation of pancreatic cancer growth and proliferation very likely. Other members of the POZ-ZF family of transcription factors such as the human oncoproteins BCL-6 and PLZF have already been linked to the development of neoplasms such as non-Hodgkin's lymphoma and acute promyelocytic leukemia.[50,51] In our p120^{ctn} silencing experiments using siRNA we showed that p120^{ctn} is involved, indeed, directly in tumor cell proliferation. This suggests that the subcellular redistribution and nuclear import of p120^{ctn} may represent the critical cell biologic step for p120^{ctn} to exert its growth-stimulating function in pancreatic cancer cells.

Taken together our data indicate that an up-regulation of p120^{ctn}, its redistribution to the cytosol, and its nuclear import in human pancreatic cancer cells is associated with a significantly more aggressive phenotype of pancreatic cancer and a greatly reduced survival of af-

fected patients. Moreover, p120ctn directly contributes to malignant growth by stimulating the proliferation of pancreatic cancer cells. This mechanism may be involved in carcinogenesis and malignant tumor growth in general. In regard to pancreatic cancer our data suggest that p120ctn-defective tumor cell contacts as well as p120ctn-mediated growth signals both can contribute to the aggressive spread and tissue invasion of this prognostically dreadful cancer and implicate p120ctn as a promising potential therapeutic target.

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