Oncogene (1999) 18, 3152–3158

http://www.stockton-press.co.uk/onc

DPC4/SMAD4 mediated tumor suppression of colon carcinoma cells is associated with reduced urokinase expression

Irmgard Schwarte-Waldhoff*[,1], Susanne Klein[1], Sabine Blass-Kampmann[2], Annette Hintelmann[1], Christina Eilert[1], Stephan Dreschers[1], Holger Kalthoff[3], Stephan A Hahn[1] and Wolff Schmiegel[1]

[1]*Medizinische Universitätsklinik, Knappschaftskrankenhaus, Ruhr-Universität Bochum, D44892 Bochum, Germany;* [2]*Institut für Zellbiologie (Tumorforschung), Universitätsklinik Essen, D45122 Essen, Germany;* [3]*Klinik für Allgemeine und Thoraxchirurgie, CAU Kiel, D24105 Kiel, Germany*

We recently identified *DPC4/Smad4* as a candidate tumor suppressor gene mutated or lost in one half of pancreatic carcinomas and in a subset of colon and biliary tract carcinomas. DPC4 plays a key role in signal transduction of the TGF-β superfamily of molecules and inactivation of TGF-β mediated growth inhibition is supposed to be the driving force for DPC4 inactivation in human tumors. However, DPC4 mediated tumor suppression by reconstitution of defective cells has not yet been reported. Here we show suppression of tumorigenicity in nude mice by stable reexpression of DPC4 in SW480 colon carcinoma cells. *In vitro* growth of *DPC4*-transfected cells was not affected and resistance towards TGF-β mediated growth inhibition was retained. Instead, cells exhibited morphological alterations and adhesion and spreading were accelerated. These phenotypic changes were associated with reduced expression levels of the endogenous urokinase-type plasminogen activator (*uPA*) and plasminogen-activator-inhibitor-1 (*PAI-1*) genes, the products of which are implicated in the control of cell adhesion and invasion. In patients, high expression levels of uPA and PAI-1 correlate with poor prognosis. Thus, reduced expression of uPA and PAI-1 is consistent with suppression of tumorigenicity in DPC4 reconstituted cells. These results demonstrate DPC4's tumor suppressive function and suggest a potential role for DPC4 as a modulator of cell adhesion and invasion.

Keywords: DPC4/Smad4; tumor suppressor gene; TGF-β; urokinase; colon carcinoma, SW480

Introduction

The *DPC4* gene (for Deleted in pancreatic carcinoma, locus 4) has been identified as a candidate tumor suppressor gene implicated in pancreatic tumorigenesis (Hahn *et al*., 1996b). *DPC4* is located on chromosome 18q21.1, a region characterized by a high frequency of loss of heterozygosity (LOH) in pancreas and colon carcinomas (Hahn *et al*., 1996a; Vogelstein *et al*., 1988). *DPC4* was found to be functionally inactivated in about 50% of pancreatic carcinomas (Hahn *et al*., 1996b), in 15% of colon carcinomas (Moskaluk and Kern, 1996; Thiagalingam *et al*., 1996) and in a subset of biliary tract carcinomas (Hahn *et al*., 1998). Though several other tumor types frequently exhibit 18q loss, biallelic inactivation of *DPC4* seems to be rare in tumors from outside of the gastrointestinal tract (Schutte *et al*., 1996). Germline mutations of *DPC4* were recently reported in a subset of families afflicted with familial juvenile polyposis (Howe *et al*., 1998). The product of *DPC4* belongs to the evolutionary conserved family of SMAD proteins which are involved in TGF-β signal transduction pathways (Derynck and Feng, 1997; Heldin *et al*., 1997; Massagué *et al*., 1997). Vertebrate SMADs may currently be distinguished into three classes: (i) The receptor-activated SMADs (SMAD 1, 2, 3, 5 and presumably SMAD 8 and 9) are pathway-restricted with respect to the ligand belonging to the TGF-β superfamily. (ii) The inhibitory SMADs 6 and 7 serve antagonistic functions. (iii) The shared SMAD DPC4/SMAD4 serves as a common functional partner for the receptor-activated SMADs. Upon ligand binding to the respective receptors, cytoplasmic class 1 SMADs are phosphorylated, bind to DPC4/SMAD4 and translocate into the nucleus. Heteromeric nuclear SMAD4/SMADx complexes associate with DNA-binding proteins and activate gene transcription (Derynck and Feng, 1997; Heldin *et al*., 1997; Massagué *et al*., 1997).

Developing resistance towards TGF-β is a frequent phenomenon in tumorigenesis (Polyak, 1996) and abrogation of TGF-β-induced growth inhibition was suggested to be the driving force for functional inactivation of DPC4 in human tumors. However, this assumption needs further investigation for two reasons.

First, TGF-β regulates a broad range of biological processes. In addition to its function as a negative growth factor TGF-β stimulates extracellular matrix formation, regulates differentiation and maintenance of tissue homeostasis and modulates the immune response (Massagué, 1990; Roberts and Sporn, 1990). Abrogation of either of these functions may contribute to tumorigenesis. It is not known, whether *DPC4* is dispensable for any of these pleiotropic TGF-β effects.

Second, DPC4 has been shown to be involved in signaling of other cytokines of the TGF-β superfamily, such as the activins/inhibins and bone morphogenetic proteins (Derynck and Feng, 1997; Heldin *et al*., 1997; Massagué *et al*., 1997). The ligand(s) that trigger(s) DPC4 mediated tumor suppression has not yet been identified.

*Correspondence: I Schwarte-Waldhoff, Immunologisch-Molekularbiologisches Labor, Knappschaftskrankenhaus, In der Schornau 23-25, D 44892 Bochum, Germany
Received 11 September 1998; revised 11 November 1998; accepted 18 December 1998

To further address these questions and in order to analyse the role of *DPC4* as a tumor suppressor gene, we reconstituted the DPC4 function in defective SW480 human colon carcinoma cells. Tumor suppression in this cell line after transfer of a normal chromosome 18 had been shown earlier (Goyette *et al*., 1992). Here we report that expression of physiological levels of the DPC4 protein is sufficient for tumor suppression in nude mice.

Expression analysis of TGF-β target genes in DPC4 reconstituted cells surprisingly revealed reduced mRNA levels of the endogenous plasminogen-activator-inhibitor-1 (*PAI-1*). In parallel, expression levels of the urokinase-type plasminogen-activator (*uPA*) were down-regulated as well. The serine protease urokinase converts plasminogen into the active protease plasmin, a broad specificity protease, that can degrade most extracellular matrix proteins. Moreover, both proteases are involved in proteolytic activation of growth factors including TGF-β (Andreasen *et al*., 1997). Importantly, high levels of uPA and PAI-1 predict poor patient prognosis in many tumor types (Duffy, 1996). Thus, reduced uPA and PAI-1 expression in the DPC4 reconstituted clones is consistent with the cells' phenotype as tumor suppressed revertants.

Results

Reconstitution of DPC4 *in SW480 colon carcinoma cells*

Northern blot analysis of *DPC4* in a number of colon carcinoma cell lines revealed two *DPC4* transcripts in most of the cell lines analysed. No *DPC4* specific signals were detected in SW480 RNA (Figure 1a). SW480 cells are known to harbor a number of genetic changes and have been used in several studies aimed at reversing the tumorigenic phenotype by reconstitution of a tumor suppressor gene (p53 (Baker *et al*., 1990) and APC (Groden *et al*., 1995), respectively). Interestingly, loss of tumorigenicity had been demonstrated after transfer of a normal human chromosome 18 into SW480 cells and had been interpreted to be due to reexpression of the DCC gene (Goyette *et al*., 1992). Recents results obtained with DCC deficient mice, however, did not support a role of DCC as a prevalent tumor suppressor in colon carcinogenesis (Fazeli *et al*., 1997).

Here we used stable transfection analysis of *DPC4* to assess its role as a tumor suppressor gene. Similar numbers of G418 resistant clones were obtained after transfection of a *DPC4* expression construct and empty vector DNA into SW480 cells, indicating the absence of growth inhibitory or toxic effects of DPC4. Recombinant *DPC4* transcripts were found in all of 20 clones analysed by Northern blot hybridization and three clones were chosen for further analysis (Figure 1b). The DPC4 protein product was found to be expressed at a level comparable to the endogenous protein levels in DPC4 positive cell lines (Figure 1c). To control for potential transfection or selection induced effects, about 300 G418 resistant clones derived from SW480 transfections with vector control DNA were propagated as a pool. These cells were included in all experiments in addition to the parental cell line and are designated 'vector controls' in the following paragraphs.

Restoration of TGF-β signaling as analysed by transient reporter expression assays

To assess functional activity of the transferred *DPC4* gene we analysed reconstitution of TGF-β signaling by transient transfections with the p3TPlux luciferase reporter plasmid, the most commonly used reporter to measure TGF-β responsiveness. In this vector the luciferase reporter gene is cloned under control of an artificial promoter, which is composed of a concatemerised region from the collagenase promoter with AP-1 sites, a region from the PAI-1 promoter and an adenovirus E4 promoter fragment (Wrana *et al*., 1992). Transient transfections of this reporter plasmid in parental SW480 cells, vector control cells and DPC4 reconstituted clones revealed slightly increased constitutive luciferase activity in the DPC4 positive cells. This effect may be explained by serum derived or endogenously produced TGF-β (data not shown). Addition of exogenous TGF-β moderately increased luciferase activity in the DPC4 positive cells, only, but still the effect was not significant. To analyse, whether TGF-β receptor levels may be limiting DPC4 mediated luciferase induction, cotransfection analysis of p3TPlux

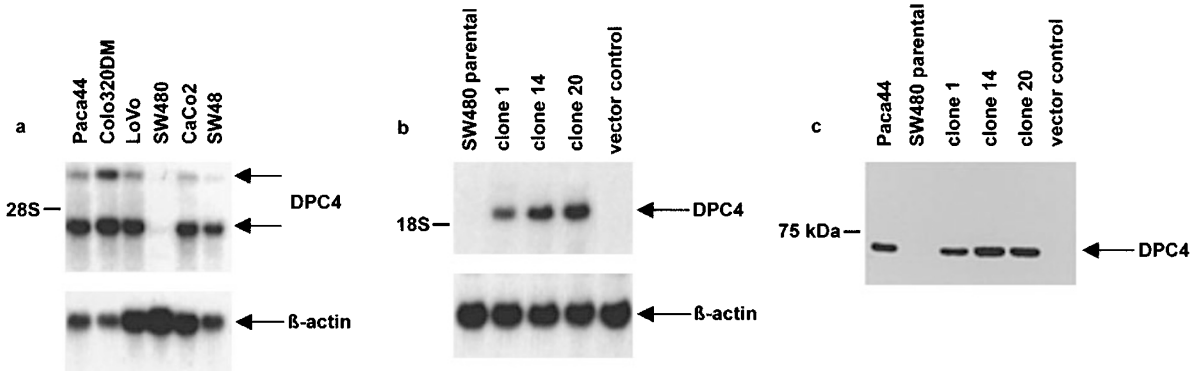

Figure 1 DPC4 expression in pancreas and colon tumor cell lines and DPC4 reexpression in SW480 colon carcinoma cells. (**a**) Northern blot analysis of DPC4 in cell lines. Both DPC4 transcripts are indicated. Relative levels of RNA loading are shown by rehybridization with a β-actin probe. (**b**) Northern blot analysis of recombinant DPC4 transcripts in stably transfected SW480 clones. (**c**) Western blotting of DPC4. Proteins (30 μg for Paca44 and 100 μg for all SW480 derivatives) were separated on 10% gels. DPC4 was detected with a monoclonal antibody

with a constitutively active mutant form of the type I receptor was performed. These experiments revealed a strong increase of luciferase activity in DPC4 reconstituted clones (Figure 2). Again, the response could be further elevated by addition of exogenous TGF-β, a finding, that may be interpreted as an additive effect of the constitutively active mutant receptor plus TGF-β activated endogenous receptors. These data support the functional reconstitution of DPC4 in the stable SW480 transfectant lines.

In vitro *growth properties of DPC4 reconstituted cells*

Analysis of *in vitro* growth of the DPC4 reconstituted and control cells revealed no difference in doubling times neither in full medium nor under reduced serum concentrations (0.5% FCS) (Table 1), though SW480 cells express high levels of endogenous TGF-β (Coffey *et al*., 1987 and unpublished data; Coffey *et al*., 1986). To assess the question whether lack of growth inhibition might be due to insufficient activation of endogenous TGF-β, we monitored the proliferation of *DPC4* transfected and control cells in the presence of exogenous TGF-β. Neither control cells nor DPC4 reconstituted cells were significantly growth inhibited (Table 1). Correct performance of the assay was confirmed by strong inhibition of TGF-β sensitive cell lines (data not shown).

Analysis of anchorage dependence for growth in semisolid medium showed that control cells formed colonies of up to 1 mm in diameter upon prolonged incubation albeit at low frequency. DPC4 reconstituted SW480 clones, however, ceased to grow after reaching a size of about 0.2 mm. Most of these small soft agar colonies exhibited irregular borders while clones from control cells displayed well-defined even margins (data not shown).

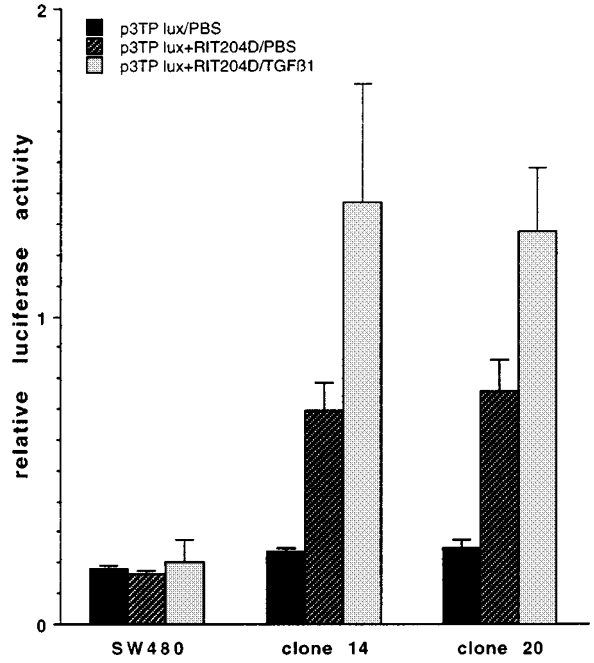

Figure 2 Ligand-dependent and -independent transcriptional activation of the p3TPlux reporter in DPC4 negative and in DPC4 reconstituted SW480 cells. Cells were transiently transfected with the p3TPlux reporter plasmid (black bars) or cotransfected with p3TPlux and pCMV-TβRI^{T204D} coding for a constitutively active TGF-β type I receptor and incubated without (hatched bars) or with TGF-β (1 ng/ml) (shaded bars) for 24 h. Luciferase activity in cell lysates was plotted as the average and standard deviation for triplicate determinations

Alterations of cell morphology, adhesion and spreading

DPC4 transfected cells were flatter and more spread in subconfluent cultures (Figure 3a) and resembled epithelial sheets at higher cell densities while parental cells readily piled up (Figure 3b). In addition vesicles of varying sizes showed up in about 5–20% of the cells (Figure 3a), a feature, which is stably maintained by the *DPC4* transfectants for about 70 passages or 200 population doublings, now. Though the nature of these vesicles is not yet known, their presence may point to a shift in the differentiation pattern.

To address a potential adhesion dependent effect upon growth we plated revertants and control cells on precoated cell culture dishes. DPC4 reconstituted clones adhered and spread much faster than the control cells on collagen type I and IV (Figure 3c), fibronectin and laminin coated plates. No significant effect upon population doubling times was exerted by these matrix molecules on either cells.

Suppression of tumorigenicity in nude mice

We tested *in vivo* growth properties of DPC4 reconstituted clones by analysis of tumor formation in nude mice. Injection of 1×10^7 cells into the flanks of 6-week-old nude mice yielded rapidly growing tumors in the case of SW480 parental cells as well as control transfectants (Table 1). Animals were sacrificed after a mean growth period of 58 and 38 days for SW480 parental cells and control transfectant cells, respectively, when the tumor diameter had reached 10 mm.

Table 1 Characterization of DPC4 transfected cells

	Population doubling time (10% FCS) (h)	*Population doubling time (0.5% FCS) (h)*	*Soft agar cloning efficiency[a] Colonies/10^5 cells*	*Tumorigenicity Number of tumors/ number of injections*	*Latency[b] (days)*	*TGF-β response[c] cell count, (% of control)*
SW480 parental	36	37	36	8/8	58	92, 103
Vector control	34	37	20	4/4	38	92, 95
Clone 1	36	36	–	2/8	>90	108, 92
Clone 14	37	40	–	0/8		87, 101
Clone 20	36	39	–	0/8		91, 87

[a]Cells (1×10^5) were plated in culture medium plus 0.3% agarose. Colonies of more than 0.5 mm in diameter were scored after 4 weeks. [b]Latency is here defined as the time needed for tumors to reach 10 mm in diameter. [c]Logarithmically growing cells (1×10^5 in 60 mm dishes) were seeded and grown in medium with 0.5% FCS with or without TGF-β (10 ng/ml). Cells were counted after 4 days. Numbers are means of duplicate determinations, each from two independent experiments

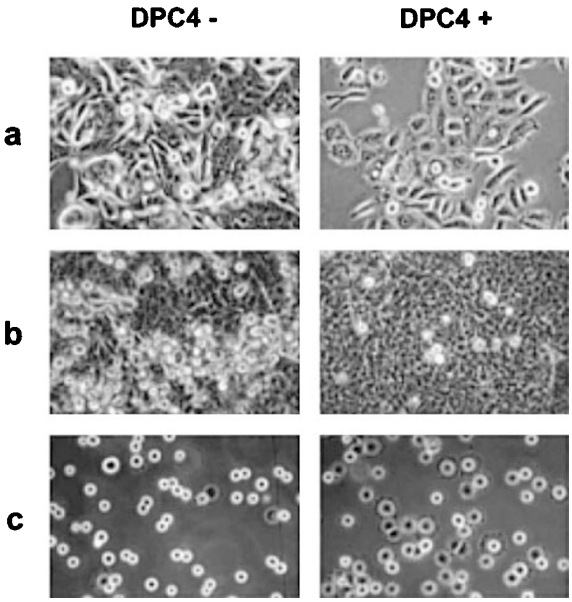

Figure 3 Morphology and spreading of DPC4 negative and DPC4 reconstituted SW480 cells. (**a**) Logarithmically growing cultures. Note the flatter, spread apprearance and the presence of vacuoles in DPC4 positive cells. (**b**) After reaching confluency, DPC4 positive cells form a dense and even cell layer, whereas DPC4 negative cells pile up. (**c**) Cells from logarithmically growing cultures were trypsinised and replated on collagen coated dishes (collagen type IV shown as an example, here). Photographs were taken 30 min after plating (phase contrast, $\times 100$)

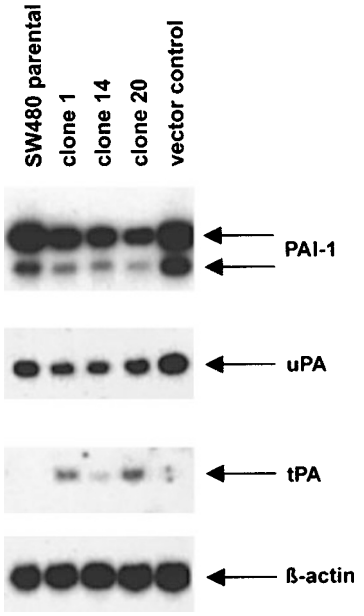

Figure 4 Effect of DPC4 on the expression of components of the plasminogen-activator system. Northern blot analysis with probes for plasminogen-activator-inhibitor-1 (PAI-1), urokinase-type plasminogen-activator (uPA) and tissue-type plasminogen-activator (tPA). Filters were exposed for 2 h (PAI-1), 24 h (uPA) and 5 days (tPA). Even RNA loading was confirmed by hybridization with a β-actin probe

No tumor was formed following injection of DPC4 reconstituted clones 14 and 20 over an observation time of 3 months. Clone 1 yielded two small tumors at a total of eight injection sites, which were first detected after more than 2# months and reached a maximum diameter at the end of the experiment of 5 and 7 mm respectively.

Reduced expression of uPA and PAI-1 and induced expression of tPA in DPC4 reconstituted cells

In order to find molecular pathways indicative of the observed biological effect of DPC4 induced tumor suppression, we started to look for the endogenous expression of TGF-β target genes. Surprisingly, Northern blot analysis of DPC4 reconstituted clones and controls showed, that constitutive expression of the endogenous PAI-1 gene, a classical TGF-β induced target gene, was reduced in the DPC4 positive clones (Figure 4). Thus, the activity of the p3TPlux promoter, which includes a PAI-1 promoter fragment does not correlate with steady state mRNA levels of the endogenous PAI-1 gene (see Discussion).

Further investigation of the plasmin/plasminogen system revealed, that mRNA levels of the urokinase-type plasminogen activator were reduced as well (Figure 4). Quantitation of uPA and PAI-1 mRNA by densitometry consistently revealed reductions by a factor of two to three in independent experiments. Determination of uPA levels in cell culture supernates by ELISA independently confirmed reduction of uPA expression levels to about half the values of DPC4 negative control cells (data not shown). Consistent with this finding, negative regulation of urokinase expression by TGF-β had been shown earlier (see Discussion).

Ultimately, expression of the tissue-type plasminogen activator (tPA) was detected in DPC4 positive clones, only. Interestingly, whereas high uPA and PAI-1 expression indicate poor prognosis in human tumors, high tumor levels of tPA correlate with a favorable outcome (Duffy, 1996).

These data provide evidence for a DPC4 impact on transcriptional regulation of the urokinase system of proteinases, which may be involved in DPC4 induced changes of phenotype and tumorigenic potential.

Discussion

Reintroduction of a candidate tumor suppressor gene in tumor derived cells which had been selected for functional inactivation of this gene during the tumorigenic process is the most direct approach for assessing its tumor suppressor function. Here we report suppression of tumorigenicity in nude mice by functional reconstitution of DPC4 in human colon tumor cells.

Consistent with the reported results from chromosome transfer studies, expression of DPC4 did not affect *in vitro* growth rates of SW480 cells. As these cells endogenously express high levels of TGF-β (Coffey *et al*., 1986, 1987) unrestrained cell growth already indicated that DPC4 reconstitution was not sufficient to restore a TGF-β induced growth inhibitory response. This was confirmed by lack of growth inhibition through exogenously added TGF-β. It is important to bear in mind that SW480 cells have accumulated a number of genetic alterations like mutational inactivation of the 'gatekeeper' APC

(Nishisho *et al*., 1991) as well as mutations of Ki-*ras* (Capon *et al*., 1983) and p53 (Baker *et al*., 1990), both of which are implicated in the control of TGF-β pathways (Blaydes *et al*., 1995; Filmus *et al*., 1992; Gerwin *et al*., 1992; Kurokawa *et al*., 1989; Reiss *et al*., 1993; Winesett *et al*., 1996).

We do not yet know, whether DPC4 induced tumor suppression is caused by restoration of other TGF-β functions or is triggered by other TGF-β superfamily ligands. DPC4 is presumably involved in signaling of every TGF-β superfamily member and is supposed to integrate the cellular response to competing or antagonistic signals (Candia *et al*., 1997). Thus, unravelling pathways crucial for DPC4's tumor suppressor function is a complex challenge. Routes for the investigation of this issue are indicated by DPC4 induced phenotypic and molecular alterations in tumor suppressed cells.

DPC4 reconstitution was associated with distinct morphological changes *in vitro* and adhesion and spreading of the cells were accelerated. These effects suggest a potential role of DPC4 in differentiation, adhesion and migration of colon cells, a hypothesis consistent with the proposed role for TGF-β in intestinal epithelial functions (Kurokawa *et al*., 1989; Winesett *et al*., 1996).

Northern blot analyses of TGF-β target genes revealed reduced expression levels of PAI-1 and uPA and induced expression of tPA in DPC4 reconstituted cells. uPA expression had been reported earlier to be subject to TGF-β dependent negative control (Laiho *et al*., 1986). This transcriptional suppression had been shown to work via a TGF-β inhibitory element (TIE) in the 5′ region of the gene (Kerr *et al*., 1990). TIE-like sequences were found in a number of genes known to be inhibited by TGF-β (Kerr *et al*., 1990). In contrast, PAI-1 is known as a classical TGF-β induced target gene (Massagué, 1990; Roberts and Sporn, 1990) and sequences derived from its promoter region are widely used to investigate TGF-β mediated transcriptional induction (Wrana *et al*., 1992). Our transient transfection analysis also indicated increased p3TPlux activities in DPC4 reconstituted cells. Thus, reduction of endogenous PAI-1 levels in DPC4 reconstituted cells was initially surprising. However, in the transient transfection assays an unequivocal increase in reporter activity was obtained after cotransfection with the TGF-β receptor encoding plasmid, only. Furthermore, it has been shown, that Smad dependent TGF-β induced p3TPlux promoter activity can be mediated by the AP1 sites in the composite promoter (Yingling *et al*., 1997).

Our findings do not imply a direct effect of DPC4 on the promoters of the genes analysed. Addressing the molecular mechanisms which ultimately result in changes of steady-state RNA levels in DPC4 reconstituted cells will require more sophisticated analyses. This study is confined to indicate DPC4 dependent expression changes, which are consistent with suppression of tumorigenicity.

The uPA system is strongly involved in processes of tumor cell directed tissue remodelling including invasion, desmoplasia and angiogenesis. Several growth factors including hepatocyte growth factor/scatter factor, basic fibroblast growth factor and, importantly, TGF-β are proteolytically activated by urokinase and/or plasmin (Andreasen *et al*., 1997 and references therein) factors which exert a variety of autocrine and paracrine functions.

Thus, control of the uPA system may contribute to the recently proposed 'landscaping' function for DPC4 (Kinzler and Vogelstein, 1998). The 'landscaper model' was based on the identification of germline *DPC4* mutations in familial juvenile polyposis (Howe *et al*., 1998). Afflicted persons develop multiple hamartomatous polyps of the colon at a young age and have an increased risk of colorectal cancer.

Findings in *APC*/*DPC4* compound knock-out mice (Takaku *et al*., 1998) are also in line with this model. APC deficient mice have been established as a model for human familial adenomatous polyposis (FAP) and like human patients they develop numerous intestinal polyps (Oshima *et al*., 1995). Codeletion of *DPC4* induced an increase in polyp size and a much more marked submucosal invasion of the more advanced tumors (Takaku *et al*., 1998).

In conclusion, the biological significance of DPC4 induced tumor suppression in SW480 colon tumor cells is emphasised by its association with reduced uPA and PAI expression levels *in vitro*. This finding sheds new light upon physiologically crucial pathways through which DPC4 inactivation may contribute to tumorigenesis. Moreover, DPC4 reconstituted cells provide ideal material for further investigations on the regulation of the uPA system, the importance of which far exceeds the restricted set of tumor types with DPC4 loss.

Materials and methods

Vector construction

The full-length coding sequence of *DPC4* was derived by ligation of two fragments from the originally isolated cDNA clones. The *DPC4* sequence was PCR amplified using *Vent* polymerase and *DPC4* specific primers containing a Kozak consensus sequence and a *Nhe*I restriction site at the 5′-end and an *Eco*RI restriction site at the 3′-prime site. The 1.6 kb PCR-fragment was cut, purified and cloned into the pBK-CMV expression vector (Stratagene) to yield *pBK-DPC4*. This vector also codes for the neomycin phosphotransferase gene, which under control of an SV40 promoter confers geneticin/G418 resistance. The construct was confirmed by direct sequencing (Sequitherm Cycle Sequencing, Epicentre). For control transfections we used an empty vector DNA or DNA derived from aliquots of a cDNA library constructed with antisense RNA from a rat fibroblast cell line (REF52) in the pBK-CMV vector. The average insert size of this library was 1.5 kb, corresponding to the *DPC4* insert size, and, as in the *DPC4* expression construct, the vector derived β-galactosidase gene was disrupted. The mixture of cDNA clones is very complex (corresponding to 20 000 independent clones) excluding specific effects due to insert sequences in these transfection assays. Plasmid DNA's used for transfections were CsCl purified followed by Proteinase K digestion, phenol extraction and precipitation.

Cell culture and gene transfer

SW480 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Pancreatic carcinoma cell line Paca44 was obtained from M Löhr, Rostock, Germany. Cells were maintained in Dulbecco's modified Eagles Medium (DMEM) supplemented with antibiotics and 10% fetal calf

serum (FCS) (Gibco). Cells were transfected by a standard calcium phosphate co-precipitation method. Positive clones were obtained after 3 weeks of cultivation in medium containing 800 μg/ml geneticin (G418). Pools of geneticin resistant clones were passaged, or single colonies were isolated with cloning cylinders and expanded for RNA and DNA isolation and analysis of growth parameters. For adhesion analysis we used precoated 'Biocoat' six-well dishes (Falcon).

Northern blot analysis

Total RNA was isolated from parental cells and transfectants by acid phenol extraction as described by Chomczynski and Sacchi (1987). Six μg of total RNA were electrophoresed using formaldehyde-containing agarose gels (1%), and capillary blotted onto Hybond N nylon membranes (Amersham). Hybridization was performed as described (Schwarte-Waldhoff *et al*., 1994). The blots were stripped and probed for β-actin.

Western blot analysis

Expression of the DPC4 product was analysed by Western blotting of lysates. Briefly, cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4/150 mM NaCl/0.5% TritonX 100/ 1 mM EDTA) containing a proteinase inhibitor cocktail (Wrana *et al*., 1992). Proteins were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and transferred to Immobilon membranes (Millipore). DPC4 was detected using a primary anti-DPC4 monoclonal antibody, raised against bacterially expressed DPC4 (Hahn *et al*., unpublished), followed by exposure to peroxidase-conjugated secondary antibody and developed using the enhanced chemoluminescent detection system (ECL, Amersham Buchler).

Luciferase assays

Cells (6.5×10^4/well in 24 well plates) were cotransfected with p3TPlux DNA (0.1 μg/well) and pCMV-TβRI^{T204D} DNA (0.2 μg/well) using Dac 30 transfection reagent (Eurogentec) according to the manufacturer's protocol. Six hours after transfection TGF-β1 (1 ng/ml, R&D Systems) was added and luciferase activity was assayed after 24 h. All transfections were normalized to β-galactosidase activity by parallel transfections of pCDNA3-lacZ.

Tumorigenicity assay

Suspensions of 1×10^7 cells in a volume of 0.1 ml of phosphate-buffered saline were injected subcutaneously into the flanks of 6-week-old female athymic nude mice (Balb/ c01aHsd-nu/nu). Cell populations were considered to be nontumorigenic if no tumors were seen by 3 months after injection.

Acknowledgements
We thank I Neumann and M Becker for expert technical assistance, J Massagué, J Wrana and T Gress for providing plasmids and probes, P Lorenz for help with antibody production and MF Rajewsky for support. We gratefully acknowledge R Schäfer and W Doerfler for critical review of the manuscript. This work was supported by grants from the Dr Mildred Scheel Stiftung für Krebsforschung (10-1137-HaI), from the BMBF (01 KV 9529) and from the Ruhr-Universität Bochum.

References

Andreasen PA, Kjoller L, Christensen L and Duffy M. (1997). *Int. J. Cancer,* **72,** 1–22.
Baker SJ, Markowitz S, Fearon ER, Willson JKV and Vogelstein B. (1990). *Science,* **249,** 912–915.
Blaydes JP, Schlumberger M, Wynford-Thomas D and Wyllie FS. (1995). *Oncogene,* **10,** 307–317.
Candia AF, Watabe T, Hawley SHB, Onichtchouk D, Zhang Y, Derynck R, Niehrs C and Cho KWY. (1997). *Development,* **124,** 4467–4480.
Capon DJ, Seeburg PH, McGrath JP, Hayflick JS, Edman U, Sevinson AD and Goeddel DV. (1983). *Nature,* **304,** 507–513.
Chomczynski P and Sacchi N. (1987). *Anal. Biochem.,* **162,** 156–159.
Coffey RJJ, Goustin AS, Mangelsdorf Soderquist A, Shipley GD, Wolfshohl J, Carpenter G and Moses HL. (1987). *Cancer Res.,* **47,** 4590–4594.
Coffey RJJ, Shipley GD and Moses HL. (1986). *Cancer Res.,* **46,** 1164–1169.
Derynck R and Feng X-H. (1997). *Biochim. Biophys. Acta,* **1333,** F105–F150.
Duffy MJ. (1996). *Clin. Cancer Res.,* **2,** 613–618.
Fazeli A, Dickinson SL, Hermiston ML, Tighe RV, Steen RG, Small CG, Stoeckli ET, Keino-Masu K, Masu M, Rayburn H, Simons J, Bronson RT, Gordon JI, Tessier-Lavigne M and Weinberg RA. (1997). *Nature,* **386,** 796–804.
Filmus J, Zhao J and Buick RN. (1992). *Oncogene,* **7,** 521–526.
Gerwin BI, Spillare E, Forrester K, Lehman TA, Kispert J, Welsh JA, Pfeifer AM, Lechner JF, Baker SJ, Vogelstein B and Harris CC. (1992). *Proc. Natl. Acad. Sci. USA,* **89,** 2759–2763.
Goyette MC, Cho K, Fasching CL, Levy DB, Kinzler KW, Paraskeva C, Vogelstein B and Stanbridge EJ. (1992). *Mol. Cell. Biol.,* **12,** 1387–1395.
Groden J, Joslyn G, Samowitz W, Jones D, Bhattacharyya N, Spirio L, Thliveris A, Robertson M, Egan S, Meuth M and White R. (1995). *Cancer Res.,* **55,** 1531–1539.
Hahn SA, Bartsch D, Schroers A, Galehdari H, Becker M, Ramaswamy A, Schwarte-Waldhoff I, Maschek H and Schmiegel W. (1998). *Cancer Res.,* **58,** 1124–1126.
Hahn SA, Hoque ATM, Moskaluk CA, da Costa LT, Schutte M, Rozenblum E, Seymour AB, Weinstein CL, Yeo CJ, Hruban RH and Kern SE. (1996a). *Cancer Res.,* **56,** 490–494.
Hahn SA, Schutte M, Hoque ATM, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH and Kern SE. (1996b). *Science,* **271,** 350–353.
Heldin C-H, Miyazono K and ten Dijke P. (1997). *Nature,* **390,** 465–471.
Howe JR, Roth S, Ringold JC, Summers RW, Järvinen HJ, Sistonen P, Tomlinson IPM, Houlston RS, Bevan S, Mitros FA, Stone EM and Aaltonen LA. (1998). *Science,* **280,** 1086–1088.
Kerr LD, Miller DB and Matrisian LM. (1990). *Cell,* **61,** 267–278.
Kinzler KW and Vogelstein B. (1998). *Science,* **280,** 1036–1037.
Kurokawa M, Lynch K and Podolsky PK. (1989). *Biochem. Biophys. Res. Comm.,* **142,** 775–782.
Laiho M, Saksela O, Andreason PA and Keski-Oja J. (1986). *J. Cell. Biol.,* **103,** 2403–2410.
Massagué J. (1990). *Annu. Rev. Cell. Biol.,* **6,** 597–641.
Massagué J, Hata A and Liu F. (1997). *Trends Cell. Biol.,* **7,** 187–192.

Moskaluk CA and Kern SE. (1996). *Biochim. Biophys. Acta,* **1288,** M31 – M33.

Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, Koyama Y, Utsunomiya J, Baba S, Hedge P, Markmann A, Krush AJ, Petersen G, Hamilton SR, Nilbert MC, Levy DB, Bryan TM, Preisinger AC, Smith KJ, Su L, Kinzler K and Vogelstein B. (1991). *Science,* **253,** 665 – 669.

Oshima M, Oshima H, Kitagawa K, Kobayashi M, Itakura C and Taketo M. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 4482 – 4486.

Polyak K. (1996). *Biochi. Biophys. Acta,* **1242,** 185 – 199.

Reiss M, Vellucci VF and Zhou ZL. (1993). *Cancer Res.,* **53,** 899 – 904.

Roberts AB and Sporn MB. (1990). In: *Peptide growth factors and their receptors.* Sporn MB and Roberts AB (eds.). Springer-Verlag: Heidelberg, pp. 419 – 472.

Schutte M, Hruban RH, Hedrick L, Cho KR, Molnar G, Weinstein G, Bova S, Isaacs WB, Cairns P, Nawroz H, Sidransky D, Casero B, Meltzer PS, Hahn SA and Kern SE. (1996). *Cancer Res.,* **56,** 2527 – 2530.

Schwarte-Waldhoff I, Martin W, Willecke K and Schäfer R. (1994). *Oncogene,* **9,** 899 – 909.

Takaku K, Oshima M, Miyoshi H, Matsui M, Seldin MF and Taketo MM. (1998). *Cell,* **92,** 645 – 656.

Thiagalingam S, Lengauer C, Leach FS, Schutte M, Hahn SA, Overhauser J, Willson JKV, Markowitz S, Hamilton SR, Kern SE, Kinzler KW and Vogelstein B. (1996). *Nature Genetics,* **13,** 342 – 346.

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM and Bos JL. (1988). *N. Engl. J. Med.,* **319,** 525 – 532.

Winesett MP, Ramsey GW and Barnard JA. (1996). *Carcinogenesis,* **17,** 989 – 995.

Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF and Massagué J. (1992). *Cell,* **71,** 1003 – 1014.

Yingling JM, Datto MB, Wong C, Frederick JP, Liberati NT and Wang X-F. (1997). *Mol. Cell. Biol.,* **17,** 7019 – 7028.