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*IN VIVO* SELECTIVE AND DISTANT KILLING OF CANCER CELLS  
USING ADENOVIRUS-MEDIATED DECORIN GENE TRANSFER

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**ABSTRACT**

Decorin is a well known, ubiquitous proteoglycan that is a normal component of the extracellular matrix. Upon transgenic expression of decorin, tumor cells with diverse histogenetic background overexpress p21<sup>WAF1</sup>, a potent inhibitor of cyclin-dependent kinase activity, become arrested in G1, and fail to generate tumors in immunocompromised animals. Since decorin is a secreted protein, it has been recently suggested that decorin could act as an autocrine and paracrine regulator of tumor growth. Here we demonstrate that adenovirus-mediated transfer and expression of human decorin cDNA induced *in vivo* apoptosis of xenograft tumor cells in nude mice. This oncolytic activity was observed when the adenovirus vector encoding the decorin cDNA was injected intratumorally or intravenously. Importantly, intratumoral injection of the decorin adenovirus vector led to growth inhibition of the injected tumor associated with similar growth inhibition of a distant contralateral tumor, demonstrating a distant decorin antitumoral effect. Immunohistochemistry against human decorin and decorin quantitation in tumors confirmed that decorin migrated to the tumor distant site. Furthermore, decorin effect was specific to tumor cells, since neither apoptosis nor growth inhibition were observed in non-tumoral human cells such as hepatocytes, endothelial cells, and fibroblasts, despite p21 overexpression.

## INTRODUCTION

Decorin is a member of the small leucine-rich proteoglycan gene family that has recently become a focus in several areas of cancer research. It is secreted in all types of connective tissue, where it plays an important role in intercellular contact modulation, migration and cellular proliferation by modulating the interactions of cell-surface receptors with extracellular matrix proteins (1). The growth-suppressive properties of decorin are supported by a number of observations, including: decorin levels are markedly elevated during quiescence; decorin is rarely expressed by actively proliferating or transformed cells; decorin expression is abrogated by viral transformation; and decorin gene transcription is suppressed in a variety of tumor cell lines and tumour tissues by methylation of its control regions (2). Upon transgenic expression of decorin, tumor cells with diverse histogenetic background overexpress p21<sup>WAF1</sup>, a potent inhibitor of cyclin-dependent kinase activity, become arrested in G1, and fail to generate tumors in immunocompromised animals (3).

Recently, Reed et al. demonstrated that transient transgene expression of replication-deficient adenovirus-containing decorin caused a significant growth inhibition of colon and squamous carcinoma tumor xenografts (4). Since decorin is a secreted protein, it has also been recently suggested that decorin could act as an autocrine and paracrine regulator of tumor growth (5). In this study, we hypothesized that adenovirus-mediated decorin gene transfer could lead not only to local but also to remote tumor growth inhibition. Furthermore, we demonstrated that decorin overexpression induced tumor cell apoptosis and that decorin-induced apoptosis was specific for tumor cells and no such effect was observed in normal cells.

## MATERIALS AND METHODS

### **Animals, cell culture and recombinant adenoviral vectors**

Four-week old female nu/nu mice (Janvier) were maintained under standard conditions. The human lung cancer cell lines A549 (ATCC, CCL 185) and H1299 (ATCC CRL-5803), the human hepatocyte cancer cell lines Hep3B (ATCC, HB 8064), HUH7, and HepG2 (ATCC HB-8065), the human colorectal adenocarcinoma cell line Caco-2 (ATCC CRL-2102), and the human cervical adenocarcinoma cell line HeLa (ATCC CCL-2) were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% FBS, or as described (6-8). Human fibroblasts HS27 (ATCC, CRL 1634), human umbilical vein endothelial cells, and human hepatocytes were cultured as described(9-12). Two first generation, E1a- replication-deficient adenoviral vectors were used: Ad.DCN which contains human decorin cDNA (13) and has already been used in *in vivo* studies conducted by J. Gauldie's group (4, 13-15), and Ad.null which is also E1a- and replication-deficient, and contains no exogenous gene and was used as a control (16). The Ad vectors were amplified in 293 cells, purified by two ultracentrifugation steps on cesium chloride gradients, and stored as previously described (16).

### **Tumor induction and *in vivo* gene therapy**

A549 tumor xenografts were established in the subcutaneous (sc) space. Five days after sc injection of  $10^7$  A549 cells, animals were palpated every two days to monitor sc tumor growth. When the tumor reached a volume of 50-150 mm<sup>3</sup>, animals received an intratumoral or i.v. (tail vein) injection of Ad.DCN or control vector Ad.null ( $5 \times 10^9$  plaque-forming units/injection) at days 0, 3, and 6. In experiments with bilateral tumors, A549 cells were inoculated into the left and right hind flanks of each animal. When tumor

nodules on both flanks reached 50-150 mm<sup>3</sup>, an intratumoral injection of Ad.DCN or of control vector Ad.Null, at  $5 \times 10^9$  pfu/animal at days 0, 3 and 6, was performed into the left tumor nodule, and the right contralateral tumor remained uninjected. The largest and smallest diameters of each tumor were measured with an external caliper. Tumor volume was calculated as:  $axb^2/2$ , where a=larger diameter and b=smaller diameter (17).

### **Northern blot analysis**

Total cellular RNA was extracted using RNazol (Bioprobe systems). Total RNA (10-20 µg) was fractionated on a 1% agarose gel and transferred onto a nylon membrane. The membrane was hybridized with randomly labeled <sup>32</sup>P cDNA probes (full-length human decorin cDNA or human p21 cDNA). Human GAPDH cDNA probe, and in some experiments human γ-actin and human β-actin probes were used as controls for equal loading of the gel.

### **Western-blot, quantification of decorin in plasma, in tumors and in cell supernatants**

Plasma was collected 3 days after a single Ad vector or PBS injection. Decorin was partially purified by adsorption to and elution from DEAE-Trisacryl and visualized by Western blots after SDS-PAGE or dot blots as described previously (18). In detail, decorin was extracted with 50 mM sodium acetate, pH 6.0, 4 M guanidinium chloride, 0.1% Triton X-100 and protease inhibitors at 4°C for 18h (18). After centrifugation, tumor lysates (diluted to give 0.2 M guanidinium chloride as final concentration and made 7 M with respect to urea by adding solid substance), plasma samples, cell supernatants and the appropriate standard solutions were mixed with DEAE Trisacryl M (100 mg wet weight, Life Technologies, Karlsruhe, Germany), equilibrated with 20 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 0.1% Triton X-100 and 7 M urea and protease

inhibitors (buffer 1) and processed as described (18). The samples were washed sequentially with 3 ml of buffer 1, 3 ml of urea-free buffer 1, and 3 ml of urea-free buffer 1 containing 0.3 M NaCl. Elution was achieved with 1.5 ml urea-free buffer 1 containing 1 M NaCl. Upon adding 5 vol of methanol and 1 vol of chloroform followed by freezing on dry ice, proteoglycans were collected after thawing at the interphase between chloroform and aqueous methanol. The upper phase was removed and proteoglycans were precipitated to the bottom of the tube by adding 5 vol of methanol. The proteoglycans were digested with chondroitin ABC lyase (Seikagaku Kogyo, Tokyo, Japan) to remove chondroitin sulfate and dermatan sulfate chains. According to the analysis of [<sup>35</sup>S]sulfate-labelled decorin from fibroblast secretions as an internal standard, the recovery after the ion exchange chromatography step averaged  $85 \pm 10\%$ . Untreated and chondroitin ABC lyase-treated samples from tumors were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting (18). Proteoglycans from plasma samples and cell supernatants were transferred to nitrocellulose membranes using the Bio-Dot Microfiltration Apparatus (BioRad, Hercules, CA, USA). The membranes were blocked with 3% casein, 1% goat serum and 0.002% Tween 20 in 10 mM Tris/HCl, pH 7.4/0.15 M NaCl (TBS). Western and dot blot membranes were incubated with an affinity-purified rabbit anti-decorin antibody, which was raised against the core protein of human decorin and quantified as described previously (18). The concentration of decorin extracted from homogenates of whole tumors was normalized to the DNA content (19).

### **Histology and immunohistochemistry**

Paraffin sections were stained by immunoperoxidase or APAAP (alkaline phosphatase anti-alkaline phosphatase) techniques (18). Primary antibodies included: affinity purified rabbit  $\alpha$ -PG-II (1:1000) which was raised against the core protein of human decorin (18),

LF-113 (1:500), a rabbit anti-murine decorin antiserum kindly provided by Dr. L.W. Fisher (NIDCR, NIH, Bethesda, MD, USA) and rabbit anti-p21<sup>WAF1/CIP1</sup> (1:50, Santa Cruz, Heidelberg, Germany). Staining for decorin was performed in serial sections under the same conditions in parallel for human and mouse decorin using both techniques (immunoperoxidase and APAAP). In APAAP staining mouse anti-rabbit IgG (1:50, DAKO, Hamburg, Germany) was used as a secondary antibody and a soluble complex of rabbit APAAP (Sigma, Munich, Germany) was added to complete the sandwich technique. The enzyme label was visualized with naphthol AS-MX-phosphate (Sigma) and fast red dye (Sigma) in the presence of levamisole (Sigma) in order to block endogenous alkaline phosphatase. The slides were counterstained with Mayer's hemalaun (Sigma). For immunoperoxidase staining endogenous peroxidase was blocked and tissue sections were incubated with a goat anti-rabbit-horseradish peroxidase-conjugated secondary antibody (1:1000, Bio-Rad, Munich, Germany) and developed with the diaminobenzidine. Substrate Kit (Vector Lab, Burlingame, CA, USA). Counterstaining was performed with methyl green. The specificity of immunostaining was tested by omitting the primary antibody, by using non-immune serum/"unspecific" IgG, and by preabsorption of antisera with antigens. Immunocytochemistry using primary antibodies against human decorin and mouse decorin were performed in parallel.

For detection of apoptosis, tissue sections were labeled by *in situ* nick labeling of nucleosomal DNA (TUNEL) as described (12). Immunocytochemistry and TUNEL analyses were performed on tissue samples 3 days after a single injection of the Ad vector or PBS.

### **Tumor Cell Inhibition Assays**

Cell proliferation was measured using the Alamar-Blue assay (Bio Source International). Cells were seeded into flat-bottomed 96-well plates and incubated with the Ad vector at different multiplicities of infection (MOI). Alamar-Blue assay (Bio Source International) (10  $\mu$ l/well) was added and incubated at 37°C for 3 hours. Plates were then read at 590 nm using a plate reader (Cytofluor<sup>TM</sup> 2350 / fluorescence measurement system). All data points were measured in quadruplicates and each experiment was performed at least three times.

#### **Analysis of cell cycle by flow cytometry**

Cells infected with Ad.DCN or Ad.null were collected by trypsinization, washed twice with PBS, fixed with 70% ethanol for 30 min, incubated with 100  $\mu$ g/ml RNase for 30 min at 37°C, stained with propidium iodide (40 $\mu$ g/ml), and analyzed on a FACScalibur flow cytometer (Becton Dickinson). Data were analyzed using the ModFit LT program (Becton Dickinson).

#### **Statistical analysis**

All values were expressed as mean  $\pm$  SEM. Mean tumor volumes and decorin amounts in treated versus control animals were compared using simple or multifactorial analysis of variance. Differences were considered significant for  $p < 0.05$ .

## RESULTS

### ***In vivo* distant anti-tumoral effect of adenovirus-mediated decorin gene transfer.**

To evaluate *in vivo* decorin anti-tumoral effect we used a xenograft model, in which A549 cells (a human lung epithelial cell line derived from a non small cell lung carcinoma) were inoculated subcutaneously (sc) to the right hind flank of nude mice. After establishment of a sizable tumor 7-15 days later (mean tumor volume  $98 \pm 36 \text{ mm}^3$ ; range 50-134  $\text{mm}^3$ ), mice were injected intratumorally at day 0, 3, 6 with a first generation adenovirus (Ad) vector encoding the human decorin cDNA (Ad.DCN), or a similar Ad vector containing no exogenous gene and used as a control (Ad.null), or with phosphate buffer saline (PBS). No statistical difference was observed between Ad.null and PBS groups at any time. In contrast, a significant reduction in tumor growth was observed in the Ad.DCN group as compared to the Ad.null control group ( $p < 0.003$ ; Fig. 1, graph I). We then evaluated tail vein injections of Ad vectors, since such administrations into the venous circulation target most of the viral particles to the liver without significant intratumoral infection (20). Consequently, any inhibition of tumor growth on the flank due to protein produced in the remote site (i.e., liver) would presumably occur by a systemic mechanism (21). Growth inhibition of subcutaneous xenografts was observed, similar to growth inhibition secondary to intratumoral Ad injections, demonstrating a decorin antitumoral effect remote from decorin expression site ( $p < 0.001$ ; Fig. 1, graph II). To confirm decorin remote antitumoral effects, and to avoid potential toxic effects due to i.v. Ad injections (22), a xenograft model composed of one subcutaneous tumor on each hind flank was established. Only one tumor was injected with the Ad vector and both were measured over time. Intratumoral injections of Ad.DCN induced growth inhibition of the injected tumor together with parallel growth inhibition of the contralateral uninjected tumor ( $p < 0.001$ ; Fig. 1, graph III). Finally, 5 repeated intra-tumoral injections of Ad.DCN vector led, after each injection, to

significant reduction in tumor volume, similar to the initial one (Fig. 1, graph IV), demonstrating that no short-term resistance against decorin apoptotic effect was induced.

Northern analysis of liver and tumors from Ad.DCN mice confirmed the local expression of decorin at the injected site and showed absence of decorin expression at distant sites, e.g. in non-injected tumors when the Ad vector had been injected i.v. or into the contralateral tumor (Fig. 2, human decorin cDNA probe). This suggests that distant decorin anti-tumoral effect was not due to Ad vector spreading.

Plasma measurements showed slight increase in decorin levels in Ad.DCN injected animals either i.t. or i.v. over background in Ad.null or PBS injected animals, but with no significant statistical difference (Table 1). Decorin delivery to the contralateral tumor site or to the distant tumor after Ad.DCN i.v. injection was demonstrated by western blot analysis on tumor lysates with an antibody against human decorin (Fig. 3). Human decorin was not detected in any control tumors injected with Ad.null (Fig. 3, lanes 1,2,6), but significant productions of decorin, a ~46-kDa broad band with a migration similar to purified decorin protein (Fig.3, lane 7) were detected in tumors injected with Ad.DCN i.t. (Fig. 3, lane 3) or i.v. (Fig. 3, lane 5), and a weaker signal but still obvious was present in contralateral tumors from i.t. Ad.DCN injected animals (Fig. 3, lane 4). Importantly, quantitative measurements of decorin levels in whole tumors showed great decorin protein increase over background in i.t. injected tumors ( $P < 0.0001$  vs Ad.null), i.v. injected tumors ( $P < 0.0008$  vs Ad.null), and slight increase, although not significant, in contralateral tumors (Table 1). Decorin delivery to the contralateral tumor site or to the distant tumor after Ad.DCN i.v. injection was further confirmed using immunochemistry (Fig. 4), with specific staining of the extracellular matrix (Fig. 4. b,c,d).

Moreover, decorin delivery to the distant tumors was confirmed by analysis of p21 expression, a well-known downstream mediator of decorin effects (23). Using

Northern analysis with a human p21 cDNA probe, we observed p21 overexpression either in decorin expression sites or in tumors of animals injected with Ad.DCN i.v. or in contralateral uninjected tumors of animals injected intratumorally with Ad.DCN (Fig. 2, human p21 cDNA probe). p21 overexpression was further confirmed using immunochemical analysis of human p21, in tumors injected i.t. with Ad.DCN (Fig. 4. g) and in contralateral uninjected tumors (Fig. 4. h).

#### **Adenovirus-mediated decorin gene transfer induced tumor cell apoptosis *in vivo*.**

Because the reduction in tumor volume after Ad.DCN intratumoral injection was rapid but transient, followed by tumor growth starting again at day 5 after a single Ad.DCN injection (not shown), we suspected a rapid extinction of decorin overexpression. Northern analysis of decorin overexpression over time in Ad.DCN injected tumors confirmed this hypothesis, with a 50% decrease in decorin mRNA at day 6 compared to day 2, and a 47% correlated decrease in p21 mRNA (not shown). Subsequently, we suspected that decorin overexpression was inducing tumor cell apoptosis, and that apoptosis of tumor cells overexpressing decorin occurred by an autocrine mechanism, leading to rapid disappearance of decorin expression. Because of low gene transfer efficiency, persistence of non-transduced cells after apoptosis of decorin overexpressing cells may induce rapid recovery of tumor growth. TUNEL analyses of Ad.DCN injected tumors were performed together with immunochemistry using an antibody against human decorin. Less than 1% of the tumor cells were transduced following a single intratumoral injection of Ad.DCN (Fig. 5. a,b), a very low level of gene transfer efficiency confirmed by experiments using an Ad vector expressing the  $\beta$ -galactosidase reporter gene (not shown). TUNEL analyses on subsequent slides revealed tumor cell apoptosis (Fig. 5. c,d), in large regions surrounding the decorin-positive cells, demonstrating an *in vivo* paracrine anti-tumoral effect of decorin,

and no apoptosis in Ad.null injected tumors ((Fig. 5. g,h). Despite very low gene transfer efficiency, a  $-11\pm 13\%$  decrease in tumor volume was observed at day 3 following a single Ad.DCN injection (versus  $118\pm 65\%$  increase in Ad.null mice), showing the importance of decorin-induced apoptosis. Finally, apoptosis was also observed in tumors from animals injected i.v. with Ad.DCN, and in contralateral uninjected tumors in animals injected intratumorally with Ad.DCN, but not in tumors injected i.v. with Ad.null (not shown). These results confirm a proapoptotic effect of decorin, distant from its expression site.

### **Decorin pro-apoptotic effect was specific to tumor cells.**

Surprisingly, when decorin was expressed in livers from mice injected i.v. with Ad.DCN, apoptosis was not observed in liver cells (Fig. 5. i,k). We first hypothesized that human decorin may have effects specific to human cells. This was suspected because previous work from our laboratory showed that human decorin did not induce any *in vitro* cell growth inhibition of several mouse or rat tumor cell lines (J.G. Tralhao and P. Lemarchand, unpublished data). However, an increase in p21 expression was observed by Northern analysis in livers from mice overexpressing human decorin (Fig. 2), demonstrating that human decorin could upregulate mouse p21 expression, and suggesting that decorin has a specific proapoptotic effect on cancer cells. To further verify this hypothesis, we tested both *in vitro* and *in vivo* a human hepatocyte cell line, Hep3B, in parallel with *in vitro* studies of human hepatocytes in primary culture. A dose-dependent growth inhibition was observed *in vitro* upon decorin overexpression in Hep3B cells (Fig. 6A), and intratumoral Ad.DCN injections in Hep3B xenografts induced an *in vivo* growth inhibition similar to that observed in A549 xenografts (Fig. 1, graph IV). In marked contrast, when normal human hepatocytes were incubated *in vitro* with Ad.DCN, there was no reduction in cell survival (Fig. 6B), indicating that decorin

may have a selective anti-tumoral activity. Because normal hepatocytes are quiescent and are not comparable to proliferating tumor cells, and to evaluate whether we could take advantage of decorin selective oncolytic property, we analyzed the *in vitro* decorin effect on A549 human tumor cells in parallel with primary (nonimmortalized) human proliferating cells (human endothelial cells and human fibroblasts). Cells were incubated with Ad.DCN at different MOI and cell proliferation was measured overtime. Apoptosis was assessed by analysis of cell cycle using flow cytometry. While A549 (Fig. 6A) underwent proliferation inhibition and apoptosis (presence of a 40% sub-G1 peak at day 6 in A549, Fig. 6B) upon decorin expression (Fig. 6C), non-immortalized human cells were shown to be highly resistant to decorin, with no inhibition of cell growth (Fig. 6A), and no apoptosis (Fig. 6B), despite both decorin and p21 overexpression (Fig. 6C). Finally, we evaluated proliferation and apoptosis in several human tumor cell lines with a genetic background and cellular origin different from that of A549 and Hep3B cells. While A549 cells are human lung epithelial cells with p16<sup>-/-</sup>, p53<sup>+/+</sup>, Rb<sup>+/+</sup> genetic background (24), and Hep3B are human hepatocarcinoma p53<sup>-/-</sup>, Rb<sup>-/-</sup> cells (6), we also used human hepatocarcinoma cells with p53 mutations on both alleles (HUH7 (6)), human hepatocellular carcinoma liver cells with normal p53 expression (HepG2 (6)), human colorectal adenocarcinoma cells (Caco-2), human lung adenocarcinoma p16<sup>-/-</sup>, p53<sup>-/-</sup>, Rb<sup>+/+</sup> cells (H1299 (24)), and human cervical adenocarcinoma cells with low p53 and Rb levels (HeLa (8)). Each tested tumor cell line, when transfected with Ad.DCN, demonstrated proliferation inhibition (Fig. 6A) and/or a sub-G1 peak using FACS analysis (Fig. 6B), suggesting that decorin pro-apoptotic effect was not dependent of a specific genetic background. Decorin-induced apoptotic effect was further confirmed by quantitation of the activity of caspase 8, a prototypic initiator of the death-domain receptor pathway. Caspase-8 activity was significantly increased in A549 and HeLa cells

overexpressing DCN, but not in HS27 fibroblasts, confirming a pro-apoptotic effect specific to tumor cells (Fig. 6D).

## DISCUSSION

The inhibitory effects of decorin on tumor cell growth have been previously demonstrated both *in vitro* and *in vivo* (4). Here we confirmed that adenovirus-mediated decorin gene transfer induces significant tumor volume reduction and tumor growth inhibition in another human xenograft model as compared to previous studies. Importantly, we demonstrated that decorin acts through a paracrine and endocrine pathway, inducing tumor growth inhibition distant from the injection site. Finally, we showed that decorin induces both *in vitro* and *in vivo* tumor cell apoptosis, and this effect was specific to tumor cells but independent from cellular origin or genetic background.

Although human decorin was detectable in serum from mice injected i.v. and i.t. with Ad.DCN, no significant increase in decorin serum levels was observed compared to background after i.t. injection. This result was anticipated, because of the short half-life of circulating decorin (25). Others, using decorin gene transfer to skeletal muscle in a rat kidney fibrosis model, did not detect any decorin in the plasma but could demonstrate decorin protein in the kidney using immunochemistry, together with regression of the kidney fibrosis (25). Nevertheless, our quantitative results in the i.v. model demonstrated that decorin can be detected in a significant amount at a distance from its production location, e.g. in distant tumors. Taken together with the western blot and immunochemistry data, these results confirmed that decorin could migrate to the distant tumor site.

This rapid decrease in decorin overexpression could not be explained by immunological reaction against the Ad infected cells, since exogenous gene expression using Ad-mediated gene transfer in nude mice usually lasts for several months (26). Similarly, an immunological mechanism responsible for the volume reduction of the uninjected tumor is unlikely, since experiments were performed in nude mice that are

deprived of T lymphocytes. Furthermore, this reduction in tumor volume was immediate and parallel to that in the Ad.DCN injected tumors, in contrast with immunological responses which are usually delayed by a few weeks. Decorin-induced apoptosis suggests that cells overexpressing human decorin undergo apoptosis through an autocrine mechanism, leading to rapid decrease in decorin overexpression and in vivo only transient tumor volume reduction.

Among the human cancer lines we tested for decorin-induced apoptosis, the HeLa cell line had already been used in a study demonstrating cell growth inhibition and p21 overexpression upon decorin gene transfection and decorin addition to cell supernatant (3). Nevertheless, no decorin pro-apoptotic effect had been observed in cellular clones overexpressing decorin after plasmid transfection (23, 27). One hypothesis is that adenovirus-mediated gene transfer leads to very high production of decorin, and that decorin pro-apoptotic effect requires high decorin concentrations. To evaluate this hypothesis, we quantitated decorin amounts in cell supernatants from A549 cells transfected with Ad.DCN at 50 pfu/cell, a low dose which is usually used for Ad experiments and which does not induce any cytopathic effect with Ad.null, and which was used for both our proliferation and apoptosis evaluations (Figures 6A and 6B). Decorin amounts in cell supernatants ranged from 180 to 200  $\mu\text{g/ml}$ . These concentrations were higher than those used in experiments with purified decorin (27). Furthermore, when adding purified DCN to cell supernatant instead of using Ad.DCN gene transfer, we were able to induce apoptosis only when decorin concentrations in cell supernatants reached 300  $\mu\text{g/ml}$  (Figure 6B). At lower concentrations, although cell growth was much slower, no sub-G1 peak could be observed (not shown). Our hypothesis is that transfecting cell lines by conventional plasmid transfection methods leads to rapid disappearance of cell clones producing very high decorin quantity because of apoptosis, and to selection of “low” decorin-producing clones, unable to induce

apoptosis. This hypothesis is further strengthened by the fact that A549 cells transfected with Ad.DCN at 50 pfu/cell synthesized human decorin at levels around 100  $\mu\text{g}/\text{day}/1 \times 10^6$  cells, while the clones used in previous studies synthesized 60-fold less decorin (1.5  $\mu\text{g}/\text{day}/1 \times 10^6$  cells(23)).

As previously observed, decorin overexpression led to p21 overexpression. Several of the principal actors of cell cycle control can affect apoptosis regulation, and no clear-cut pro- or anti-apoptotic effects can be described for any of the cyclins, Cdks and their inhibitors, including p21(29). Overexpression of decorin in human endothelial cells has already been shown as protecting endothelial cells from apoptosis (30). In our study, since p21 is a well-known downstream mediator of decorin effect (23), p21 expression was primarily used as a “reporter” of decorin biological effect, confirming a decorin biological effect distant from its expression site, and confirming that decorin was biologically active in non-tumoral cells, since p21 expression was increased while no apoptosis was observed in such cells. Besides p21 overexpression, we observed an increase in caspase-8 activity upon decorin overexpression in tumor cells. Caspase –8 contributes to cell death both by direct activation of effector caspases and by the initiation of other major cascades of the apoptotic process (31). Increases in caspase-8 activity upon decorin overexpression confirm decorin-induced apoptosis specific to tumor cells, since no such increase was observed in fibroblasts. Furthermore, caspase-8 is activated through the death-domain receptor pathway, including TRAIL and TRAIL receptors DR4 and DR5 (32, 33). Recent studies demonstrated that tumor cells express high levels of DR4 and DR5 and may be more sensitive to apoptosis than normal cells through activation of the TRAIL pathway (34, 35). Studies are currently underway to evaluate if such mechanism may be responsible for the tumor cell specificity of decorin-induced apoptosis.

Numerous studies using Ad-mediated gene transfer have been published with direct i.t. Ad injections in the human xenograft model, mainly with transfer and overexpression of tumor suppressor genes (p53 (36-38), FHIT (39), pHyde (40)), or genes encoding proteins associated with cdk (p21 (8, 36), p27 (41), p16 (37)). Because the human cell lines, the number and the doses of Ad injections were different in each study, results are variable and difficult to compare, but most of them showed significant slower growth rates, similar to that obtained in our present study. Decorin has the advantage that it is a secretory protein being delivered to the extracellular space and can therefore reach distant sites (25). Experiments using i.t. metalloprotease gene transfer or i.v. angiostatin gene transfer showed slower growth rates and prevention of lung metastases formation (42-44). To our knowledge, only studies with cytokine gene transfer used a graft model with two distant tumors comparable to ours. In this model Ad-mediated transfer of IL-2 and IL-12 genes induced stabilization or slow progression of both i.t. and contralateral tumors (45), but with very high toxicity, including high mortality (46). In our present study using i.t. decorin gene transfer no mortality was observed, and one might expect low toxicity since decorin pro-apoptotic effect was specific to tumor cells. Finally, all forms of cancer therapy carry the danger of selection for resistance. Repeated intra-tumoral injections of Ad.DCN vector led, after each injection, to significant reduction in tumor volume, demonstrating that no short-term resistance against decorin apoptotic effect was induced in our model. Regardless of efficiency in cell killing, the success of restoring apoptotic response in tumor cells depends also on the extent to which such therapies confine death to the cancer cells, and allow survival of normal tissue (47). Many conventional chemotherapies induce significant toxicity, whereas our results demonstrate that overexpression of decorin, a natural ubiquitous protein, led to no apoptosis in healthy tissues, and *in situ* injections of Ad.DCN into an established tumor can result in significant, though temporary, local and

distant growth suppression. These results suggest the potential of decorin gene therapy as a novel approach to the treatment of primary and metastatic cancer.

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**TABLE AND FIGURE LEGENDS****Table 1** - Decorin quantification

**Figure 1.** Evidence for a major local and distant antitumoral effect *in vivo*, using Ad-mediated decorin gene transfer. Nude mice were injected sc with human lung A549 or human liver Hep3B xenograft cells. When tumors reached volume  $>50 \text{ mm}^3$ , an Ad vector encoding human decorin cDNA (Ad.DCN), a control vector (Ad.null), or phosphate buffer saline (PBS) was injected *in vivo* (arrows) intratumorally (graphs I, III, IV) or intravenously (graph II). In graph III, 2 distinct xenografts were concomitantly established (one on each flank), and only one tumor was injected with the Ad vector (intratumoral, IT) whereas the contralateral (CL) tumor remained uninjected.

**Figure 2.** Northern analysis of human decorin and p21 expression. Total RNA was extracted from tumors 3 days after intratumoral Ad injection, or from livers and tumors 3 days after i.v. Ad injection. Northern analysis was then performed, using human decorin, human p21, human GAPDH, human  $\gamma$ -actin and human  $\beta$ -actin cDNA probes. p21/x is the ratio between p21 and x mRNAs quantified by Phosphorimager.

**Figure 3.** Western immunoblotting of tumor lysates using an antibody against human decorin protein core. Lysates from tumors injected with Ad.null i.t. (lane 1) or contralateral (lane 2) or i.v. (lane 6), and lysates from tumors injected with Ad.DCN i.t. (lane 3) or contralateral (lane 4) or i.v. (lane 5) were migrated together with purified decorin protein (lane 7).

Figure 4. Evaluation *in vivo* of human decorin and human p21 expressions by immunochemical analysis. Staining using a primary antibody against human decorin, a monoclonal secondary antibody with an APAAP rabbit complex and Mayer's hemalaun counterstaining, showed human decorin in Ad.DCN tumors injected i.t. (panel a), in Ad.DCN tumors injected i.v. (panel b), and in contralateral tumors from i.t. Ad.DCN injected tumors (panel c), but not in Ad.null controls, injected either i.t. (panel g) or i.v. (panel f). In panel d, the same tumor slide as in panel c was stained with the same primary antibody against human decorin but with a polyclonal peroxidase-labelled secondary antibody and methyl green counterstaining. Immunochemistry using a primary antibody against mouse decorin showed light staining only in the connective tissue surrounding the tumor (not shown). Similarly, staining using a primary antibody against human p21, a polyclonal peroxidase-labelled secondary antibody and methyl green counterstaining, showed human p21 overexpression in Ad.DCN tumors injected i.t. (panel g) and in contralateral tumors from i.t. Ad.DCN injected tumors (panel h), as compared to Ad.null controls, injected either i.t. (panel i) or i.v. (panel j). Magnification x1000 (a), x400 (b - j).

Figure 5. Evaluation *in vivo* of human decorin by immunochemical analysis and of apoptosis by TUNEL on subsequent slides, in tumors 3 days after intratumoral Ad injection (a-h), and in liver 3 days after i.v. Ad injection (i-l). a-d: Ad.DCN injected tumor, e-h: Ad.null injected tumor, i and k: liver after Ad.DCN i.v. injection, j and l: liver after Ad.null i.v. injection. Magnification x100 (a,c,e,g), x400 (b,d,f,h), x600 (i, j), x200 (k,l). a and c represent histological sections of Ad.DCN injected tumors in which most of the tumor mass has been replaced by necrotic tissue, e and g are sections of Ad.null tumors with very few areas of necrosis. The arrows in a and b show cells expressing human DCN, the rectangles in a,k,l show the same areas that are shown in the squares in b,i,j, respectively.

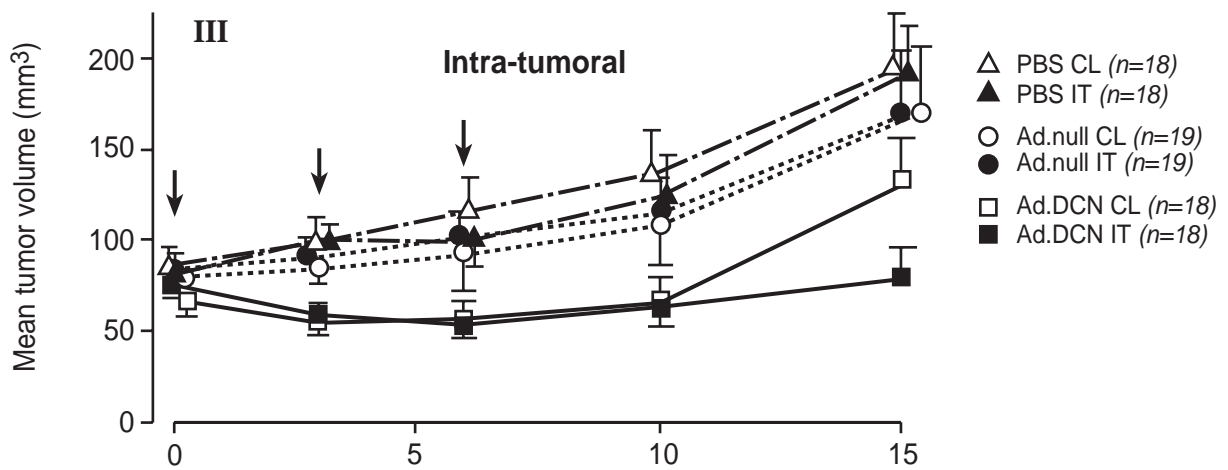
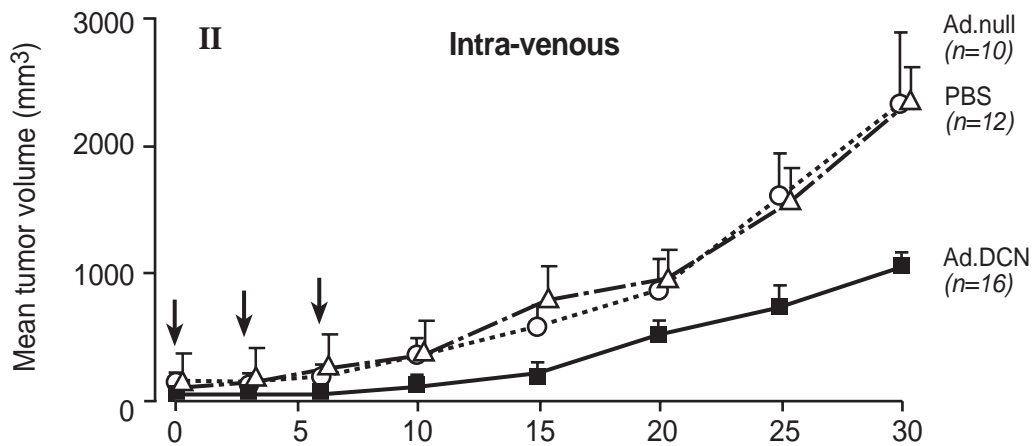
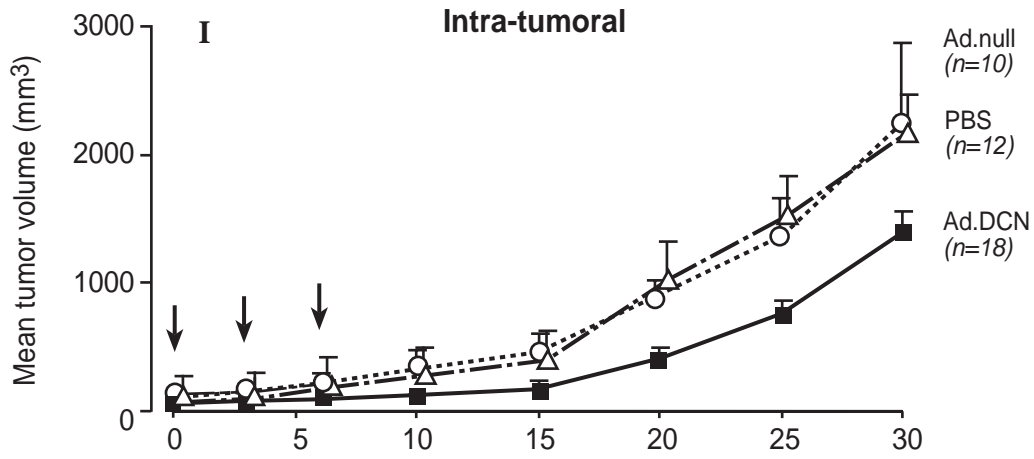
Figure 6. *In vitro* selective killing of tumoral cells, using Ad-mediated decorin gene transfer. Human lung A549, H1299 tumor cells, human liver Hep3B, HUH7, HepG2 tumor cells, human colorectal adenocarcinoma Caco-2 cells, human cervical adenocarcinoma HeLa tumor cells, human hepatocytes in primary culture, human umbilical vein endothelial cells (HUVEC), and human fibroblasts were incubated *in vitro* with an Ad vector encoding human decorin cDNA (Ad.DCN) or a control vector (Ad.null) at different MOI. A). Cell proliferation was measured overtime using Alamar Blue Assay. B). Facs analysis of cell cycle, 6 days after *in vitro* incubation with Ad vectors. The arrow indicates a peak of cells in sub-G1 phase. The top right panel shows A549 cells incubated with 300  $\mu\text{g/ml}$  purified decorin. C). Northern analysis using human decorin, human p21, and human GAPDH cDNA probes, 2 days after *in vitro* incubation with Ad vectors. p21/GAPDH is the ratio between p21 and GAPDH mRNAs quantified by Phosphorimager. D). Quantitation of caspase-8 activity in whole cells lysates, 4 days after *in vitro* Ad.DCN or Ad.null transduction.

**Table 1 - Decorin quantification**

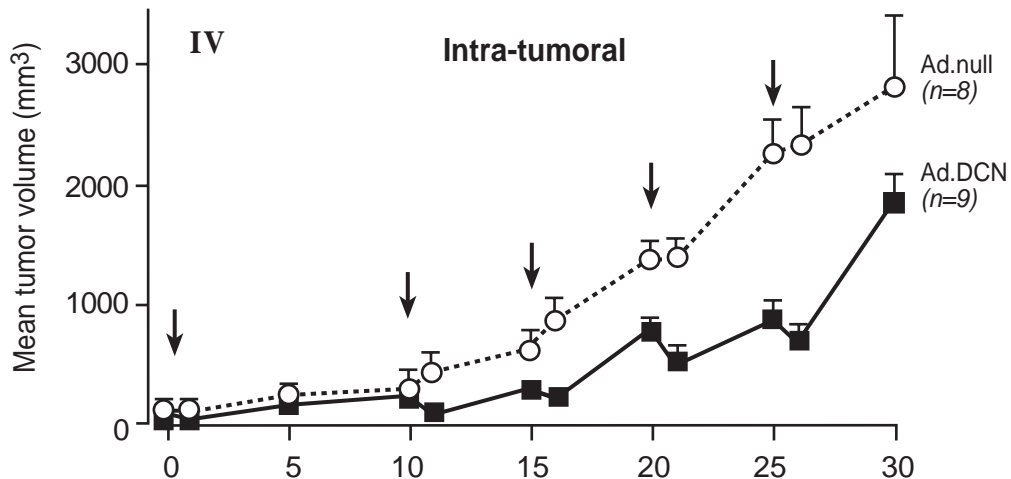
	PBS		Ad.null			Ad.DCN		
	IT	CL	IT	CL	IV	IT	CL	IV
<b>Plasma</b> ng/ml* (n)	17 ± 4 (7)		22 ± 5 (5)		20 ± 7 (4)	28 ± 8 (11)		37 ± 8 (12)
<b>Tumor</b> ng/mg DNA* (n)	41 ± 4 (4)	43 ± 2 (4)	44 ± 10 (6)	43 ± 4 (5)	50 ± 4 (6)	263 ± 26 (8)	56 ± 7 (8)	115 ± 5 (8)

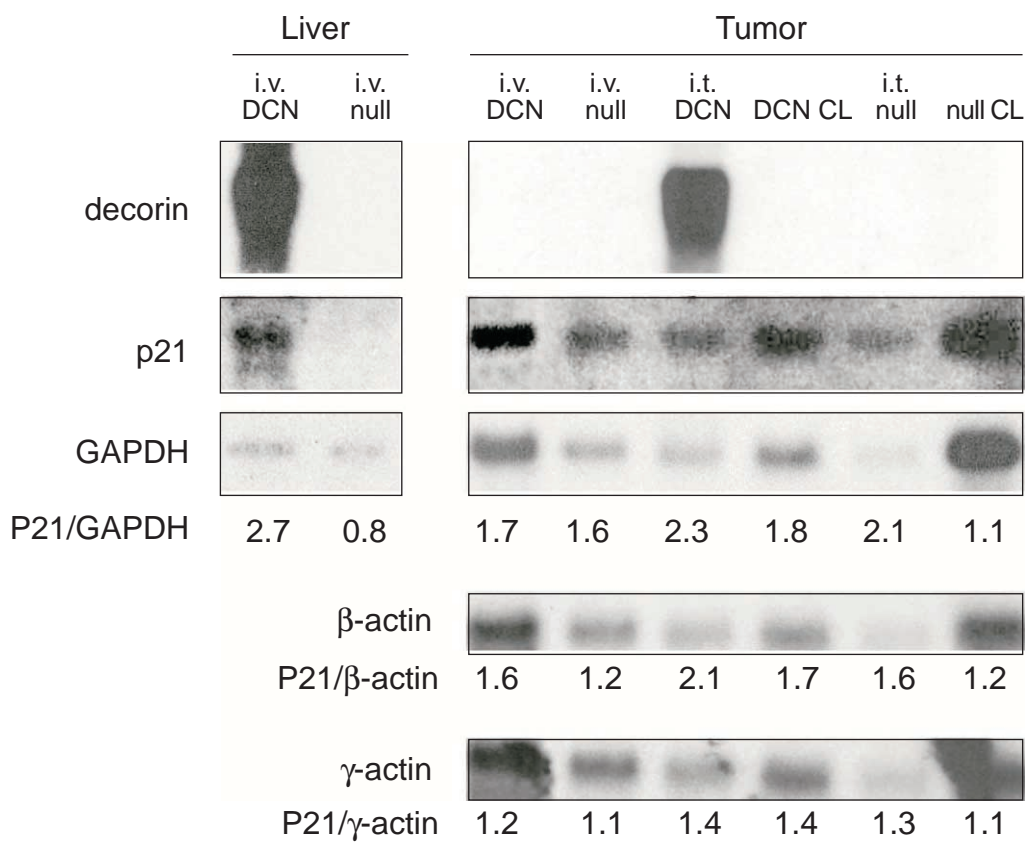
\*m±SEM

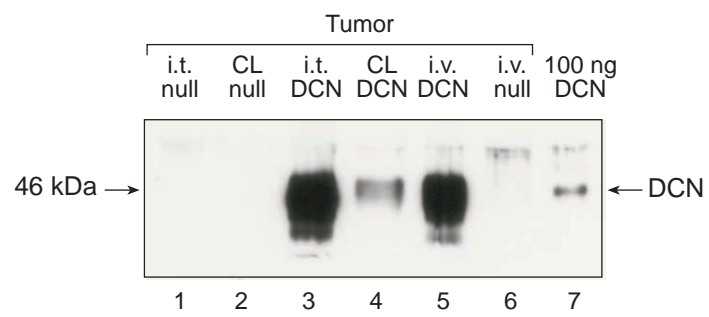
## A549 tumor

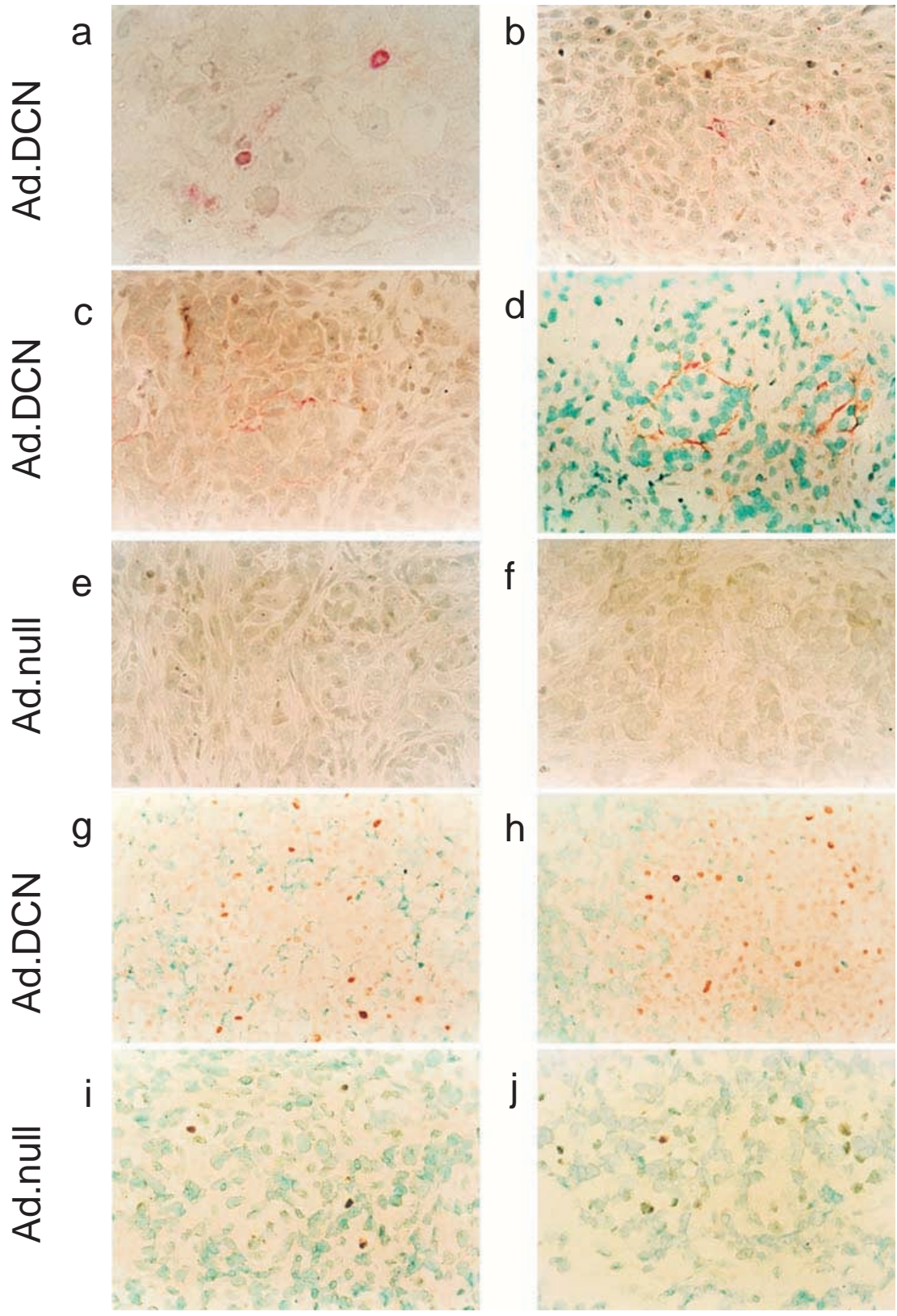


## HEP3B tumor

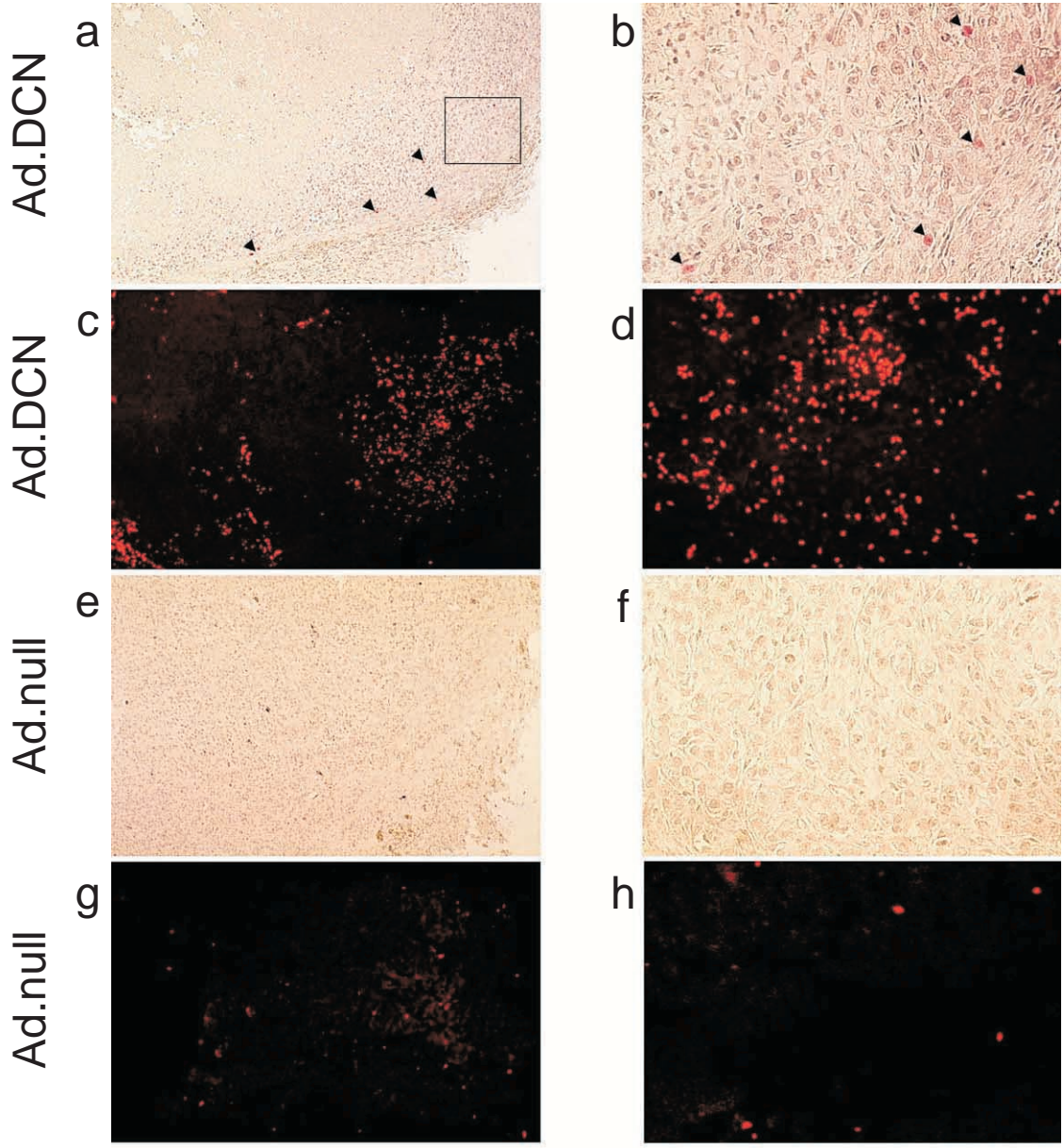




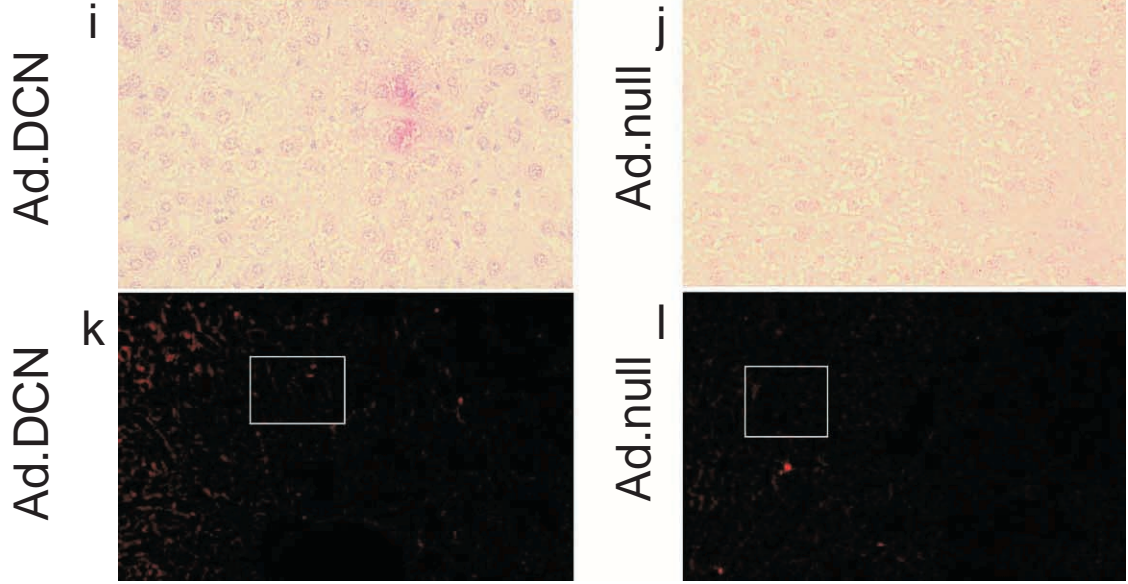




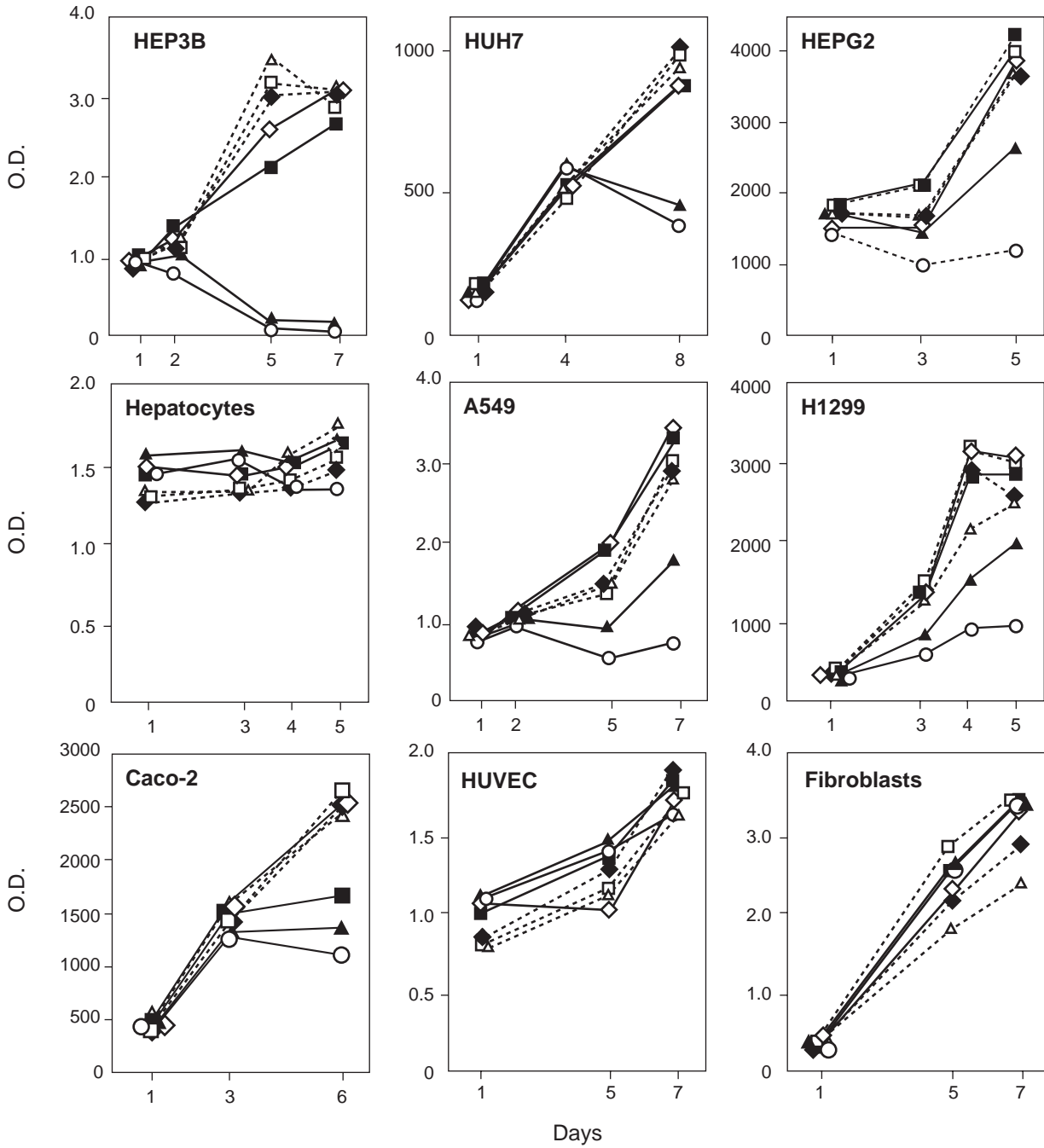
# Tumor



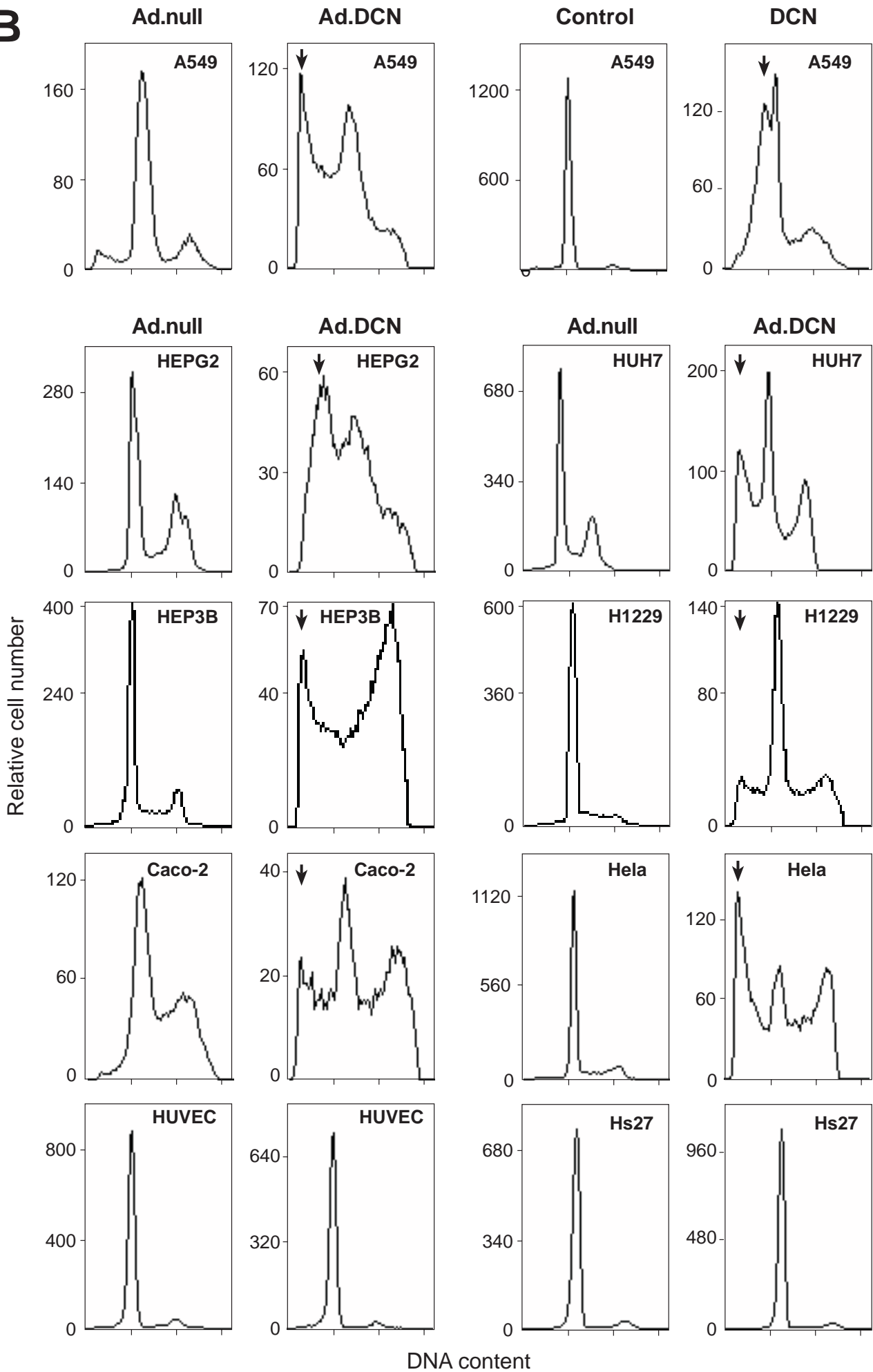
# Liver



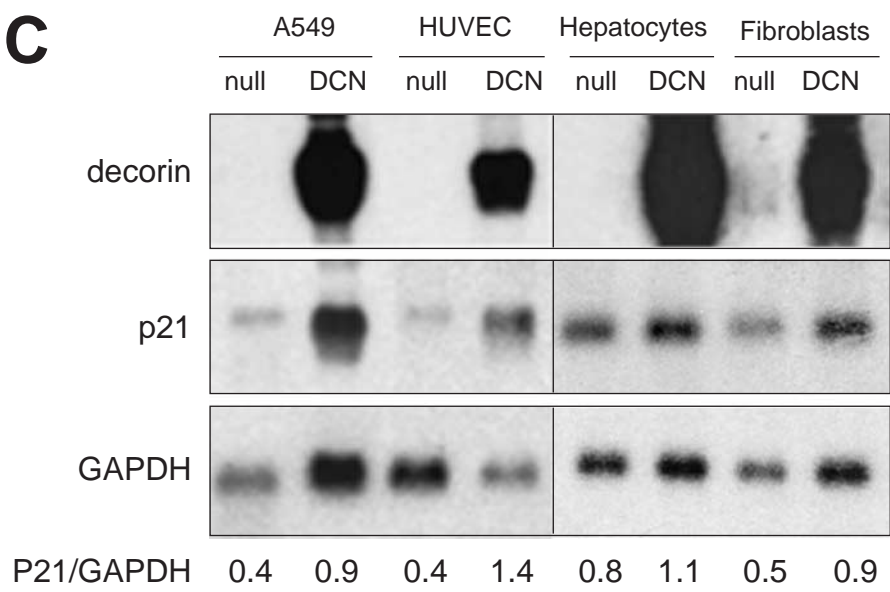
# A



- ◇— Control
- Ad.DCN5
- ▲— Ad.DCN50
- Ad.DCN100
- - □ - - Ad.null 5
- - ◆ - - Ad.null 50
- - ▲ - - Ad.null 100

**B**

**C**



**D**

