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13. ABSTRACT (Maximum 200 Words)

A significant proportion of human breast cancers overexpress ErbB2, a member of the receptor tyrosine kinase gene family that also includes the epidermal growth factor receptor (EGFR). Overexpression of ErbB2 correlates with increased metastatic potential and poorer prognosis. Agents that antagonize the activity of ErbB family members have obvious clinical implications. We have previously demonstrated that decorin is a novel ligand for the EGFR, whose interaction with EGFR/ErbB family members ultimately leads to significant growth suppression. In the preliminary data that support the basis of this proposal, we discovered that decorin causes a functional inactivation of the oncogenic ErbB2 protein in mammary carcinoma cells overexpressing ErbB2. In this final report, we demonstrate that transient transgene expression of decorin delivered by an adenoviral vector causes a significant growth inhibition of multiple tumor xenografts. These cytostatic effects correlated with a reduced proliferative index and an attenuation of EGFR phosphorylation *in vivo*.Further, direct treatment of metastatic tumors with decorin protein can abrogate spreading, as can expression of decorin in an inducible cell system. The effects clearly demonstrate decorin's effectiveness in not only suppressing primary tumor growth, but also metastatic spread via it interaction with ErbB family members.

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Decorin, proteoglycan, adenovirus, EGFR, ErbB2, breast carcinoma, gene therapy, colon carcinoma, swuamous carcinoma, adeno-associated virus, inducible promoter

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INTRODUCTION

Aggressive human breast cancers are strongly linked to increased activity of receptor tyrosine kinase, specifically to members of the ErbB family (1). Overexpression of EGFR and ErbB2 correlates with increased metastatic potential and poorer prognosis. Therapeutic interventions focused on attenuating either the expression or the intrinsic activity of these receptors could represent useful anti-cancer treatments (2). Decorin, a small leucine-rich proteoglycan (SLRP), is involved in a number of cellular processes including matrix assembly, fibrillogenesis and the control of cell proliferation (3-5). Decorin is a biological ligand of the epidermal growth factor receptor (EGFR) (6;7), and its interaction with EGFR leads to a transient activation of the receptor tyrosine kinase, followed by a protracted down-regulation of activity. We have previously shown that decorin exerts an anti-oncogenic activity in various tumors, including squamous and adenocarcinomas, by suppressing both EGFR (8-10) and ErbB2 (11) activity. In either case, decorin causes a profound down-regulation of these receptors both *in vitro* and *in vivo*. The cytostatic effects of decorin occur in a wide variety of tumor cell lines with a diverse histogenetic background (8;12). Decorin can also indirectly retard tumor growth by blocking transforming growth factor-β (13), by inhibiting tumor cell production of angiogenic factors such a VEGF (14), or by interacting with anti-angiogenic compounds such as thrombospondin-1 (15).

Agents that antagonize the activity of ErbB family members have obvious clinical implications. Our working hypothesis, validated by multiple independent studies (12;16), is that decorin is a natural anti-cancer agent and enhancement of decorin gene expression should lead to a retardation of tumor growth. We hypothesize that expression of decorin by breast carcinoma cells in an *in vivo* tumor model will result in inhibition of tumor growth. We further hypothesize that exogenous administration of human decorin by any of several delivery systems will lead to growth slowdown and/or growth inhibition of already established xenograft tumors of human breast carcinomas.

This final report will summarize results from all work related to this grant.

BODY

We have achieved significant progress and success in our understanding of decorin's effects in metastatic breast cancer, as well as a functional delivery system. In addition, the construction of a cell line expressing decorin under the control of an inducible promoter has allowed us to perform a series of biologically relavent experiments on decorin expression *in vitro* and *in vivo*, and will continue to be a useful tool as we explore the temporal effects of decorin induction in tumor models. In summary, we have 1) successfully used and adenoviral vector containing the decorin transgene to retard tumor growth in multiple *in vivo* cancer

models, 2) found an appropriate breast cancer tumor model that mimics metastatic spread *in vivo*, 3) generated an inducible cell line from the metastatic tumor cells that conditionally expresses decorin, and 4) used decorin in several delivery methods to successfully slow both primary tumor and metastatic growth and spread *in vivo*. The use of an AAV-[derived expression system, as well as the use of liposome-mediated gene transfer, however, remained stumbling blocks.

Adenoviral decorin vector

We have been successful in using decorin packaged in a replication-deficient adenoviral construct to treat multiple types of tumor models *in vivo*. This work was published in Oncogene and was the subject of a poster presentation. We demonstrate that transient transgene expression of replication-deficient adenovirus-containing decorin causes a significant growth inhibition of colon and squamous carcinoma tumor xenografts. Cytostatic effects were achieved with relatively low viral titers and correlated with a reduced proliferative index and an attenuation of the EGFR phosphorylation *in vivo*, as determined by immunohistochemistry. A reprint of this work is appended to the report.

Breast cancer tumor model

The MTLn3 cell line, a rat breast adenocarcinoma cell line that spontaneously metastasizes *in vivo* (a kind gift from J.E. Segall, Albert Einstein Medical Center) has been found to express releatively high level of ErbB-2. Its in vivo metastatic properties make it ideal for a breast cancer tumor model. We use this cell line in further work to study decorin's effects on ErbB-2 levels, activity, and subsequent *in vitro* and *in vivo* effects on cell growth, proliferation, and spread.

Inducible decorin promoter

We have created MTLn3 cells expressing decorin under the control of a Tet responsive element with the RevTet On System (Clontech). MTLn3 cells express decorin only in the presence of doxycycline, a harmless antibiotic. Growth inhibition tests *in vitro* are pending, as is Western blotting to confirm the amounts and phosphorylation states of EGFR and ErbB-2. *In vivo* tests will begin shortly if results are favorable.

Treatment of metastatic breast cancer cell line with decorin

We show that decorin causes marked tumor suppression both *in vitro* and *in vivo* in a metastatic breast cancer cell line. Treatment with decorin protein core reduced primary tumor growth by 70% and eliminated observed metastases. Further, an adenoviral vector containing the decorin transgene was used to treat the same tumor model and also caused primary tumor retardation of 70%, in addition to greatly reducing observed metastases. Lastly, we demonstrate that ErbB2 phosphorylation and total receptor protein levels are reduced in this model system upon *de novo* expression of decorin under the control of the Tet On promoter. Primary tumor growth *in vivo* was reduced by up to 67% upon decorin induction, and subsequent pulmonary

metastases were likewise reduced in induced animals. These results were featured in a poster presentation and will be published in an upcoming issue of *Oncogene*. The appendix also contains a copy of the accepted paper, describing these results in detail.

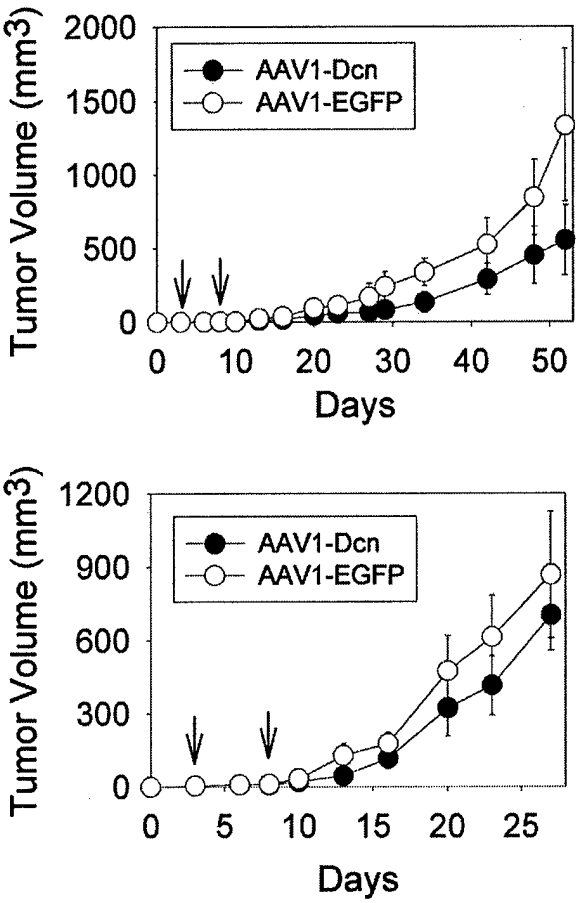

Figure 1. Treatment of WiDr colon carcinoma in vivo tumor model (above) and A431 squamous carcinoma in vivo tumor model (below) with AAV1-Decorin constructs. $1x10^6$ cells of the respective types were injected into the flanks of nu/nu mice (n=5 for each group). AAV-Decorin or AAV-EGFP (control) was injected twice ($1x10^{12}$pfu/injection), indicated by arrows.

<u>AAV decorin vector</u>

Generation of an AAV vector contaqining decorin has proven to be consistently problematic. AAV (adeno-associated virus) is a novel viral delivery method for gene therapy (18,19). Despite the use of several different AAV serotypes (AAV1, AAV2, AAV5) and several different decorin constructs (using CMV and CAG promoters, WPRE elements to prolong the life of the message, and other methods), we have been unable to produce detectable amounts of decorin from any *in vivo* transduction model, and have likewise been unable in most cases to even detect decorin expression after viral transduction *in vitro*. The plasmid constructs, however, have shown normal expression in vitro after transfection into mammalian cells by standard electroporation. The AAV system, as we have been able to utilize it, unfortunately has not been a functional tool for decorin transduction in this study. Effects *in vivo*, where AAV is supposedly most effective, have been unremarkable. Most *in vivo* trials produced no change in tumor growth. Some growth inhibition was observed in one set of experiments, but the effects were not significant (Figure 1). We were never able to detect decorin expression after *in vivo* AAV transduction. While, to date, the AAV vector system has proven to be problematic for decorin expression, we intend to remain informaed about advances in AAV vectorology and to attempt AAV-based decorin expression when improved vectors are available.

<u>Liposome-mediated gene transfer</u>

Use of DC-Chol lipsoomes for *in vivo* delivery of decorin plasmid constructs failed to show any expression either *in vitro* or *in vivo*. These experiments were discontinued due to lack of progress and the unlikelihood of liposome-based therapies ever being used in preclinical or clinical settings. We were interested, in this grant, in using methods of gene transfer that have some relavence to those settings.

KEY RESEARCH ACCOMPLISHMENTS

- Successfully used an adenoviral construct containing the decorin transgene to treat multiple tumor cell lines both *in vitro* and *in vivo*. Downregulation of ErbB receptors was observed, and correlated with marked growth suppression. Successfully treated tumor lines included squamous and colon carcinomas. This work resulted in both a poster presentation at the 2002 Gordon Conference on Proteoglycan in Andover, New Hampshire, and a 2002 publication in the journal *Oncogene*.

- Use of MTLn3 breast carcinoma tumor model to generate tumors *in vivo* that spontaneously metastasize to the lungs.

- The MTLn3 tumor model was successfully treated with adenoviral decorin. Reduction in tumor size, ErB-2 levels and phosphorylation status was observed, as well as greatly reduced metastatic spread.

- Creation of a metastatic cell line (MTLn3) which conditionally expresses decorin, controlled by a tetracycline/doxycycline-inducible promoter (Tet On System). Once induced, cells exhibited greatly reduced growth rates, suppressed tumorigenicity, and lower ability to metastasize. This activity correlated with greatly reduced ErbB-2 levels and phosphorylation status.

- Successful treatment of MTLn3 tumor model in vivo with direct administration of decorin core protein. Metastatic spread was completely abolished in these experiments.

- Research with the MTLn3 tumor model was recently accepted for publication in *Oncogene*, and was the subject of a poster at the 2004 American Society of Matrix Biology Annual meeting in San Diego.

REPORTABLE OUTCOMES

This work resulted in several important discoveries, and advanced the role of decorin as a possible natural therapeutic agent for breast cancer. Additionally, several delivery systems, including viral and direct administration of protein, were used and evaluated for relative efficacy.

- Publication: Reed, C.C., Gauldie, J., Iozzo, R.V.: Suppression of tumorigenicity by adenovirus-mediated gene transfer of decorin. Oncogene 21: 3688-3695, 2002.

- Poster Presentation: Reed, C.C., Gauldie, J., Iozzo, R.V.: Suppression of tumorigenicity by adenovirus-mediated gene transfer of decorin. Gordon Research Conference, Andover, NH, 2002.

- Poster Presentation: Reed, C.C., Waterhouse, A., Kirby, S., Kay, P., Owens, R.T., McQuillan, D.J., and Iozzo, R.V.: Decorin prevents metastatic spreading of breast cancer, American Society of Matrix Biology 2nd Annual Meeting, San Diego, CA, 2004.

- Publication: Reed, C.C., Waterhouse, A., Kirby, S., Kay, P., Owens, R.T., McQuillan, D.J., and Iozzo, R.V.: Decorin prevents metastatic spreading of breast cancer, Oncogene, in press, 2004.

CONCLUSIONS

The ultimate goal of this research has been to prove that human decorin is a viable primary or adjunctive candidate for treatment of certain breast cancers, and that it is possible to deliver decorin or the decorin transgene by one or more well-established transfer methods to achieve a positive clinical response. The use of a relevant tumor model which can metastasize proved extremely valuable in determining decorin's effects and biology in slowing or halting tumor growth and spread. In addition, the successful trial of a viral delivery system for the decorin transgene proved that it is possible to deliver an active transgene to tumor tissue and that expression of therapeutic levels of decorin can be achieved in this manner. Use of direct decorin core protein to treat the MTLn3 metastatic tumor model showed that, even in small quantities, decorin was able to not only significantly slow the growth of primary tumors, but also to abrogate metastases. This is an important observation, as it is possible to produce large amounts of recombinant proteins today for clinical use. Direct protein treatment is a delivery method that must be considered in research into decorin's antitumor benefits.

We will continue to investigate decorin's pronounced antitumor and antimetastatic effects. We are exploring improved decorin delivery methods, including other viral vectors, among them retroviral vector systems that can stably transduce cells at high efficiency. The inducible decorin Tet On system will continue to be a valuable tool, allowing us to study the temporal effects of decorin, specifically the effects of decorin after metastatic spread has occurred, a more clinically relevant model. Other metastatic tumor models will be investigated, also. In particular, a mouse cell line, 4T1, is known to be highly metastatic and could be used for future *in vivo* experiments. It is syngeneic with BALB/c mice, allowing for a possible tumor model in an immunocompetent animal. A proprietary human salivay gland tumor cell line, Hep3 (available from Scripps), is another possible model system for the future. Many head and neck tumors are known to express high levels of ErbB family members, making this tumor another possible target for decorin therapeutic research. Several possible new directions for research have been opened, but our overriding goal to effectively evaluate decorin's efficacy as an antitumor therapy remains the same.

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APPENDIX

Attached is a reprint of Reed et al *Oncogene* publication from 2002. A copy of this, however, should already be on file. Also, a copy of Reed et al *Oncogene* paper from 2004, which is currently in press, is attached.

Oncogene (2002) 21, 3688–3695

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Suppression of tumorigenicity by adenovirus-mediated gene transfer of decorin

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There is mounting evidence that decorin inhibits the growth of various tumor cell lines when either over-expressed *in vitro* or provided as a recombinant protein. The mechanism of action is primarily via a protracted inactivation of the epidermal growth factor receptor (EGFR) tyrosine kinase. In this study we explored the possibility of retarding the growth of tumor xenografts by decorin gene delivery into the growing neoplastic tissues. We demonstrate that transient transgene expression of replication-deficient adenovirus-containing decorin causes a significant growth inhibition of colon and squamous carcinoma tumor xenografts. These cytostatic effects were achieved with relatively low viral titers and correlated with a reduced proliferative index and an attenuation of the EGFR phosphorylation *in vivo*. Thus, decorin gene therapy helps in retarding the growth of human tumors in immunocompromised animals and could represent a new independent or adjunctive therapeutic modality against cancer.
Oncogene (2002) **21,** 3688–3695. DOI: 10.1038/sj/onc/1205470

Keywords: decorin proteoglycan; adenovirus gene therapy; colon carcinoma; squamous carcinoma

Introduction

Decorin is a prototype member of an expanding family of small leucine-rich proteoglycans, ubiquitous molecules characterized by 10–12 tandem arrays of leucine-rich repeats (Iozzo, 1997, 1998). The decorin protein core folds into an arch-shaped structure capable of interacting with various extracellular matrix proteins, growth factors and their receptors (Weber *et al.*, 1996). Targeted deletion of the decorin gene (Danielson *et al.*, 1997) and bitransgenic animals with deficiency of both decorin and p53 (Iozzo *et al.*, 1999a) have provided genetic evidence for a role of decorin in matrix assembly and tumorigenesis, respectively. The results of these studies have confirmed and corroborated earlier observations implicating decorin protein core in modulating collagen fibrillogenesis (Vogel *et al.*, 1984) and cell growth via a block of TGF-β^1 activity (Yamaguchi *et al.*, 1990).

Our working hypothesis is that decorin represents a natural antagonist of tumor growth. This hypothesis is based on several key observations that have shown a marked elevation of decorin expression within the tumor stroma of colon cancer (Adany *et al.*, 1990), an increased decorin gene transcription in serum-starved and quiescent cells (Coppock *et al.*, 1993; Mauviel *et al.*, 1995), and a marked suppression of decorin gene expression in most viral- or spontaneously-transformed cells (Coppock *et al.*, 1993; Adany *et al.*, 1990). Moreover, decorin expression is differentially down-regulated in hepatocellular carcinomas (Miyasaka *et al.*, 2001) and in ~70% of ovarian carcinomas and ovarian cancer cells (Shridhar *et al.*, 2001) as compared to their normal counterparts.

The mechanism of action of decorin has only recently begun to be elucidated by the discovery that decorin is a novel biological ligand of the EGFR (Moscatello *et al.*, 1998; Patel *et al.*, 1998; Iozzo *et al.*, 1999b). This interaction with the EGFR differs from that induced by EGF, since decorin leads to a protracted down-regulation of EGFR tyrosine kinase (Csordás *et al.*, 2000), and other members of the ErbB family of receptor tyrosine kinase (Santra *et al.*, 2000), and causes an attenuation of the EGFR-mediated mobilization of intracellular calcium (Csordás *et al.*, 2000). The decorin/EGFR interaction induces the expression of the endogenous cyclin-dependent kinase inhibitor $p21^{WAF1}$ (De Luca *et al.*, 1996) and a subsequent arrest of the cells in the G1 phase of the cell cycle (Santra *et al.*, 1995). These cytostatic effects of decorin occur in a wide variety of tumor cell lines with a diverse histogenetic background (Santra *et al.*, 1997; Nash *et al.*, 1999) and can also affect murine tumor cells (Santra *et al.*, 1997) and normal cells such as endothelial cells (Schönherr *et al.*, 2001) and macrophages (Xaus *et al.*, 2001).

Because decorin is a highly soluble compound, and because the activity of decorin occurs in an autocrine and paracrine fashion, we sought to

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determine whether adenovirus-mediated gene delivery into solid tumors could affect the behavior of the tumor xenografts. We demonstrate that transient transgene expression of replication-deficient adenovirus-containing decorin causes a significant growth inhibition of colon carcinoma and squamous carcinoma tumor xenografts. The cytostatic effects of decorin correlated with a markedly reduced proliferative index and an attenuation of the EGFR phosphorylation *in vivo*. Thus, decorin gene therapy helps in retarding the growth of human tumors in immunocompromised animals and could represent a new independent or adjunctive therapeutic modality against cancer.

Results

Efficient transduction of various tumor cell lines with Ad-Dcn

To determine whether Ad-Dcn was capable of efficiently transducing WiDr and A431 tumor cells, we added increasing amounts of Ad-Dcn vector or empty vector, and determined decorin expression by Northern blotting. The results showed a significant expression of the virally-transduced decorin gene transcript, a single ~1.9 kb band (Figure 1a), whose levels increased in a dose-dependent manner. In agreement with previous results (Santra *et al.*, 1995; Moscatello *et al.*, 1998), no decorin message was detected in either control tumor cell (Figure 1a). Real time PCR showed that the expression of the decorin transcript was time-dependent in A431 cells (Figure 1b), and could also be demonstrated in Cos-7 and HeLa cells (Figure 1c). Moreover, following a 48 h-incubation, the message levels induced by Ad-Dcn were higher than those induced by a bacterial expression vector (pcDNA) harboring the potent CMV promoter (not shown). Western immunoblotting showed significant production of decorin proteoglycan (Figure 1d), a ~100-kDa broad band, with a migration similar to that described before for stable expressing clones of WiDr and A431 (Santra *et al.*, 1995, 2000), and typical of recombinant decorin proteoglycan (Hocking *et al.*, 1998; Ramamurthy *et al.*, 1996; Mann *et al.*, 1990). To further confirm the biological activity of decorin, we reacted Ad-Dcn-transduced cells with a monoclonal antibody (mAb E12120) that specifically recognizes the phosphorylated (activated) form of the EGFR. The results showed a marked suppression of the EGFR phosphorylation in the A431 cells when compared to either control (no virus) or the empty viral vector (Figure 1e). Thus, our Ad-Dcn vector is capable of inducing high levels of decorin expression that can be detected at least 10 days after transduction in several cell lines. Moreover, decorin is biologically active since it can down-regulate the EGFR kinase as in stably transfected tumor cell lines (Csordás *et al.*, 2000; Santra *et al.*, 2000).

Suppression of tumor growth by direct intra-neoplastic injection of Ad-Dcn

The high efficiency of transduction and the relatively high levels of transgene expression mediated by the adenoviral vector in cultured cells prompted us to investigate the anti-oncogenic properties of decorin *in vivo* by direct intra-neoplastic injection of the Ad-Dcn. To this end, we performed two sets of experiments. In the first set of experiments, animals were injected s.c. with a relatively large inoculum of tumor cells (2×10^6 cells/animal), and the mice were observed for 5–7 days until a palpable tumor of ~30 mm^3 emerged. At this time, each tumor xenograft was directly injected with 50 μl of PBS containing 7×10^7 pfu of either Ad-Dcn or empty viral vector. A second treatment was done 4–5 days later. Interestingly, in both WiDr (Figure 2a) and A431 (Figure 2b) tumor xenografts there was a significant reduction of tumor growth ($P<0.02$). However, the effect was rather transient since the slope of the curve was nearly identical in both treated and untreated animals at later times. In addition, because of the large inoculum size, the growth rate of both xenografts was very rapid and, thus, several tumors in both groups had necrotic centers, thereby preventing an accurate assessment of the decorin's effects.

In a second set of experiments we tried to duplicate a more clinically relevant situation by injecting smaller inocula, and by treating the preestablished tumor xenografts with higher doses and multiple injections of recombinant Ad-Dcn. The results showed a marked inhibition of tumor growth in both the colon carcinoma (Figure 2c) and squamous carcinoma (Figure 2d) xenograft models. In both cases, a progressive inhibition of tumor burden was observed. At the end of the experiments, the decrease in tumor volume for the Ad-Dcn-treated colon carcinoma xenografts reached 67% ($P<0.001$) (Figure 2c), and 52% inhibition ($P<0.002$) for the squamous carcinoma xenografts (Figure 2d). Thus, topical delivery of Ad-Dcn has substantial cytostatic effects in two animal models of aggressive human tumors.

Adenoviral-mediated expression of decorin causes morphological changes in tumor xenografts

We studied the morphology of the tumor xenografts generated with smaller inocula and multiple Ad-Dcn injections. While the wild-type WiDr showed solid tumors (Figure 3a) with invasion of the deep fascia and skeletal muscle (not shown), the Ad-Dcn-treated neoplasms showed well demarcated margins (Figure 3b) and often showed areas of glandular differentiation (Figure 3c). Similarly, the A431 squamous carcinoma xenografts showed invading margins (Figure 3d), whereas the Ad-Dcn-treated counterparts showed sharp, non-invading borders (Figure 3e), and areas of squamous differentiation characterized by the formation of numerous keratin pearls (Figure 3f). Thus, the decorin-induced growth inhibition is also associated

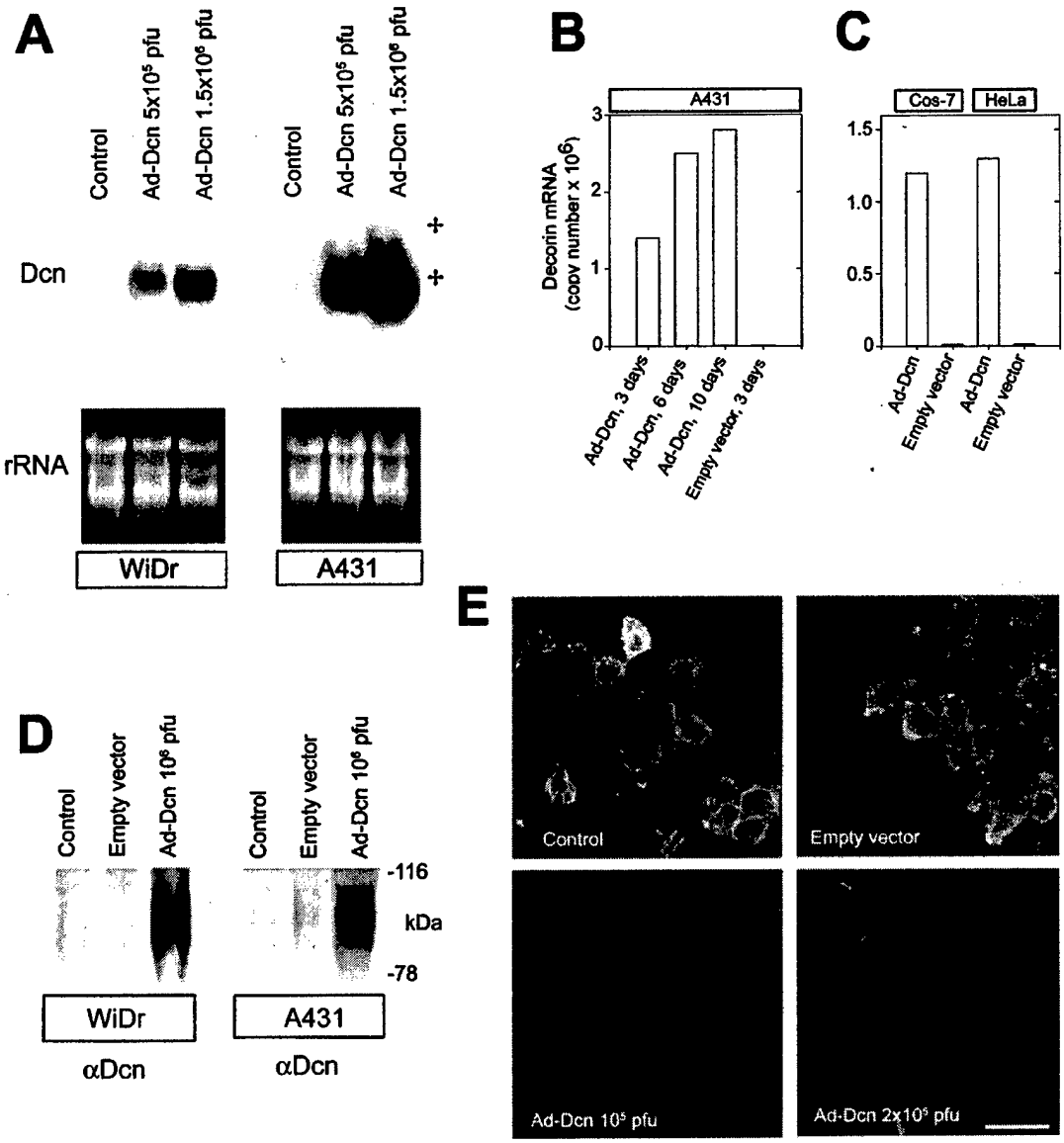

Figure 1 Efficient transduction of various tumor cell lines with Ad-Dcn. (**a**) Northern blotting of total RNA extracted from the designated cells 2 days after transduction with either 2.5×10^6 pfu of empty viral vector (Control) or with increasing amounts of Ad-Dcn as indicated in the top margin. The autoradiographs were exposed for 2 h. The asterisks in the top right panel correspond to the migration position of the two ribosomal RNAs. (**b**) Real time PCR values of decorin gene expression 2 days following transduction with either Ad-Dcn or the empty viral vector (10^6 pfu each) for the indicated times. Values represent the mean of duplicate determinations and are based on a standard DNA curve generated with known quantities of human decorin full-length cDNA, as per manufacturer's protocol (Applied Biosystems 7700 ABI Prism Sequence Detector). (**c**) Transduction of monkey Cos-7 or human squamous carcinoma HeLa cells with either Ad-Dcn or empty viral vector as indicated. (**d**) Western immunoblotting of media conditioned for 48 h by WiDr or A431 carcinoma cells following transduction with Ad-Dcn viral vector as indicated. The precipitated media aliquots were reacted with an anti-decorin antibody (αDcn) directed against the N-terminal region (Fisher *et al.*, 1995). The migration of pre-stained molecular weight markers is shown in the right margin. (**e**) Immunofluorescence staining of A431 squamous carcinoma cells following transduction for 72 h with Ad-Dcn at the designated concentrations, control (no virus) or empty vector, using a monoclonal antibody directed against the phosphorylated form of the EGFR (BD Transduction Laboratories). Cells were fixed for 15 min in 3% paraformaldehyde, permeabilized for 10 min with digitonin, and incubated with the primary antibody (1 : 500). Slides were washed and incubated with secondary goat anti-mouse IgG-FITC (1 : 1000) antibody. Immunofluorescence was performed on an Olympus IX70 inverted epifluorescence microscope coupled to a high quantum efficiency cooled CCD camera. The four panels were exposed for the same amount of time. Notice the significant reduction in EGFR signal in both decorin-transduced cells. Scale bar = 50 μm

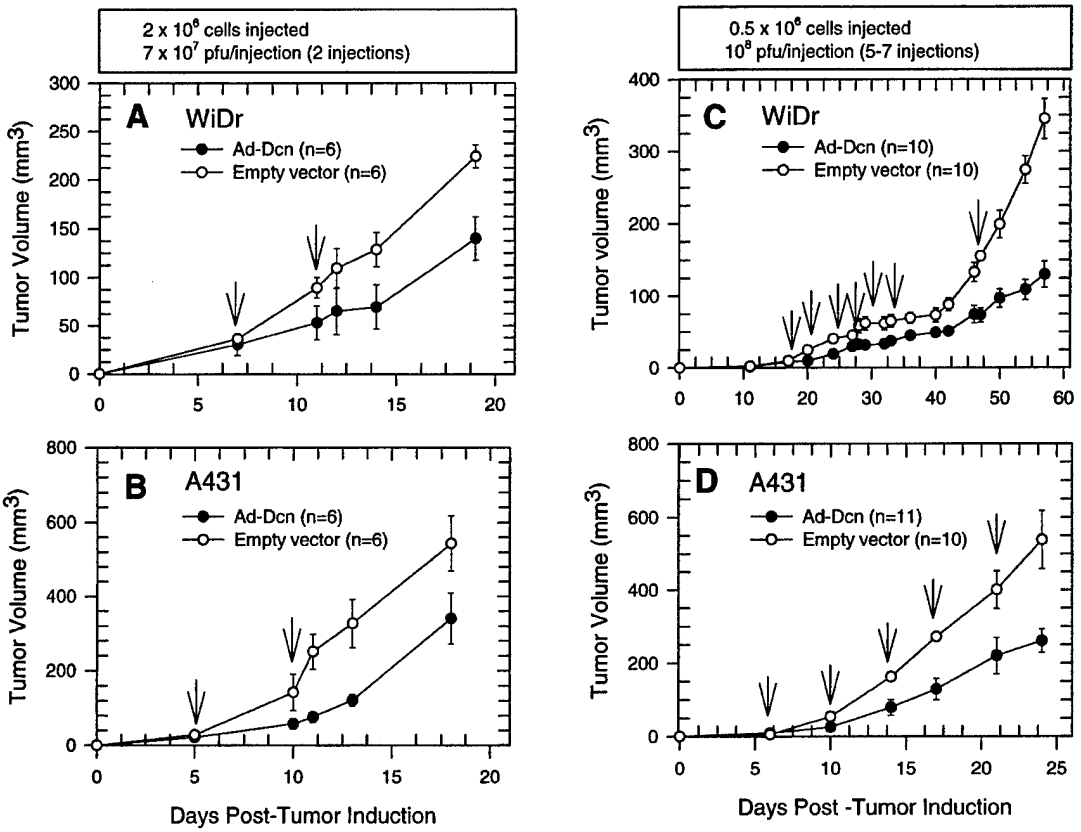

Figure 2 Intra-neoplastic injections of Ad-Dcn reduce the growth of preestablished tumor xenografts. (**a** and **b**) About 2×10^6 WiDr or A431 cells were injected s.c. into the dorsal region of *nu/nu* mice ($n=6$ for each group). At the indicated time points (vertical arrows) the tumors were directly injected with 50 μl of PBS containing 7×10^7 pfu of either recombinant Ad-Dcn or empty viral vector, as indicated. The values represent the mean$\pm$s.e.m. (**c** and **d**). In these experiments a smaller inoculum of $\sim0.5\times10^6$ cells was injected and several, higher-titer injections were performed at the designated times. The values represent the mean$\pm$s.e.m. of 10–11 animals per group as indicated

with changes in the architecture and differentiation of the tumor xenografts.

Decorin gene expression correlates with a decreased proliferative index and an attenuation of EGFR activation

Next, we sought to determine whether decorin gene transfer would correlate with a reduced proliferative index and a reduced activation of the EGFR. To test these possibilities we stained tumor samples with antibodies directed against Ki-67, an established proliferation marker, and a monoclonal antibody (mAb E12120) that specifically recognizes the phosphorylated (activated) form of the EGFR (see above). There was a marked reduction of the Ki-67 staining in the treated tumor xenografts (Figure 4b,d) as compared to the vector controls (Figure 4a,c). To obtain the proliferative index, we estimated the percentage of tumor cell nuclei positive for Ki-67 marker in 10 high-power ($\times400$) fields per animal ($n=3$ animals per group). The proliferative index for the WiDr and A431 controls was $36\pm6\%$ and $28\pm5\%$, respectively (mean$\pm$s.e.m.). In contrast, in the Ad-Dcn-treated samples, the Ki-67 proliferative index was $6\pm2\%$ and $5\pm3\%$, respectively ($P<0.001$ in both cases).

The staining for the activated EGFR was also diminished in the treated samples (Figure 4f,h) when compared to vector controls (Figure 4e,g). In the WiDr xenografts there was a linear staining outlining the cellular contours (Figure 4e). In agreement with their high content of EGFR, A431 cells reacted more intensely with the anti-EGFR antibody. Figure 4g shows a pocket of intensely-stained A431 tumor cells just below the unreactive mouse epidermis. These data are also in agreement with the *in vitro* data shown in Figure 1e.

When the tumor xenografts were reacted with a polyclonal antibody specific for the N-terminal peptide of the human decorin protein core, we detected decorin epitopes in pericellular pools mostly located near the periphery of the tumor xenografts (Figure 4j,l), while the vector controls were unreactive (Figure 4i,k).

Collectively, these results provide strong evidence for the *in vivo* anti-proliferative effects of decorin and suggest that, at least in part, decorin inhibits the proliferation of tumor cells by interfering with EGFR activity.

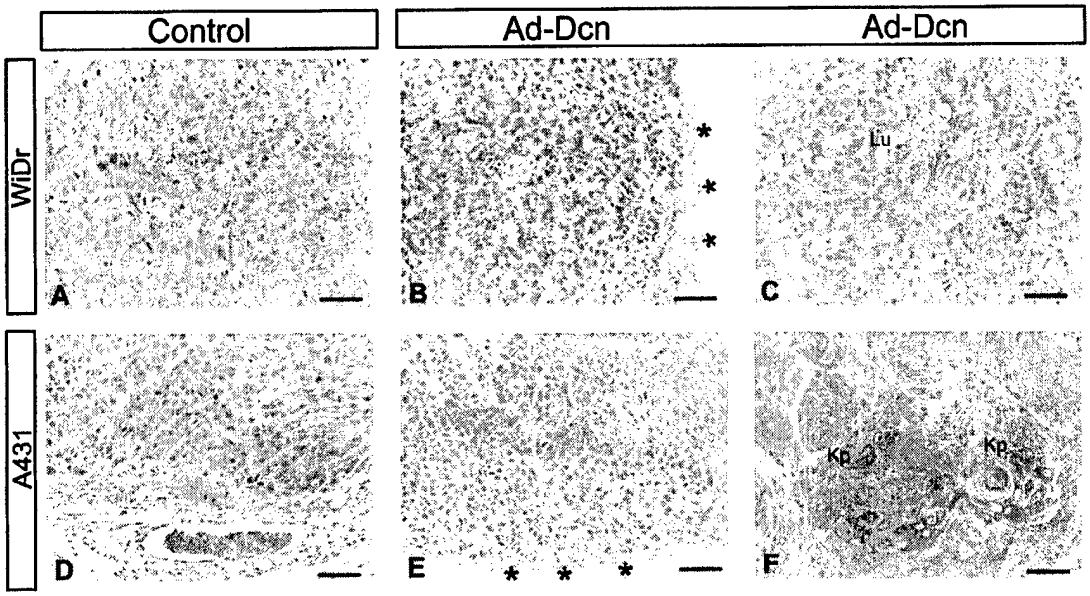

Figure 3 Adenoviral-mediated expression of human decorin causes morphological changes in human tumor xenografts. Gallery of light micrographs highlighting the morphology of the tumor xenografts generated by injection of WiDr colon carcinoma cells (**a**–**c**) or A431 squamous carcinoma cells (**d**–**f**) into the dorsal subcutaneous tissue of *nu/nu* mice. These sections are from the animals in Figure 2c,d and show representative fields of tumor xenografts of mice treated with multiple injections of either empty adenoviral vector (Control) or Ad-Dcn as indicated. Asterisks in **b** and **e** point to the sharper margins of the Ad-Dcn-treated tumor xenografts; *Lu*, lumen; Kp, keratin pearl. Scale bars = 125μm

Discussion

The inhibitory effects of decorin on TGF-β activity have been exploited in experimental glomerulonephritis (Isaka *et al*., 1996), atherosclerosis (Fischer *et al*., 2001), lung morphogenesis (Zhao *et al*., 1999), and pulmonary fibrosis (Kolb *et al*., 2001). This is the first report of a successful *in vivo* treatment of two human xenograft models using adenovirus-mediated decorin gene transfer. The results showed a marked inhibition of tumor growth in both the colon and squamous carcinoma xenograft models. In both cases, a progressive inhibition of tumor burden was observed. In Ad-Dcn-treated colon carcinoma xenografts this reduction was greater than squamous carcinoma xenografts (67% versus 52% of control values) but in both cases the difference was statistically significant with P values <0.001 and <0.002, respectively). We should point out that the total amount of Ad-Dcn injected is still below what has been used in most animal experiments and recent clinical trials. In fact, the highest total dose/animal (see Figure 2c) reached 7×10^8 pfu/22 g (the average weight of a *nu/nu* mouse), or equivalent to a dose of $\sim3.2\times10^{10}$ pfu/kg in a human. Thus, topical delivery of Ad-Dcn has a significant cytostatic effect in two animal models of aggressive human tumors.

Interestingly, adenoviral-mediated expression of decorin caused morphological changes in the human tumor xenografts. These changes included sharper border, reduced invasiveness, reduced angiogenesis and evidence of cyto-differentiation. Whether these changes are the direct results of decorin treatment or a consequence of reduced tumor growth is not clear, but it warrants future investigation. The morphologic appearance of the transduced tumors is reminiscent of that obtained with xenografts produced by co-injection of wild-type and decorin-expressing A431 cells (Csordás *et al*., 2000). In the latter case, tumors grew only when the ratio of wild-type to decorin-expressing cells was quite high ($\geqslant1:2$), and in the tumors generated at lower (1 : 4 or 1 : 8) ratios there was evidence of cyto-differentiation and sharper, non-invasive tumor boundaries. Thus, the Ad-Dcn vector can produce effects similar to those induced by a constant source of decorin derived from the stably expressing tumor cell clones (Santra *et al*., 2000). The present study extends these observations and supports the feasibility of decorin gene transfer in a clinically relevant setting, either as primary or combined therapy. The latter possibility is supported by the synergistic action of decorin and carboplatin in the *in vitro* inhibition of human ovarian cancer cells (Nash *et al*., 1999).

Human carcinomas frequently express high levels of EGFR and over-expression of EGFR and ErbB2 has been correlated with a more aggressive phenotype (Carpenter, 2000). Accordingly, therapeutic modalities directed at preventing the function of these receptors have been pursued as useful anti-cancer treatments (Mendelsohn and Baselga, 2000). A key finding of our study is that decorin gene expression in the treated tumor xenografts correlated well with a decreased proliferative index and an attenuation of EGFR activation. The latter observation is a novel and

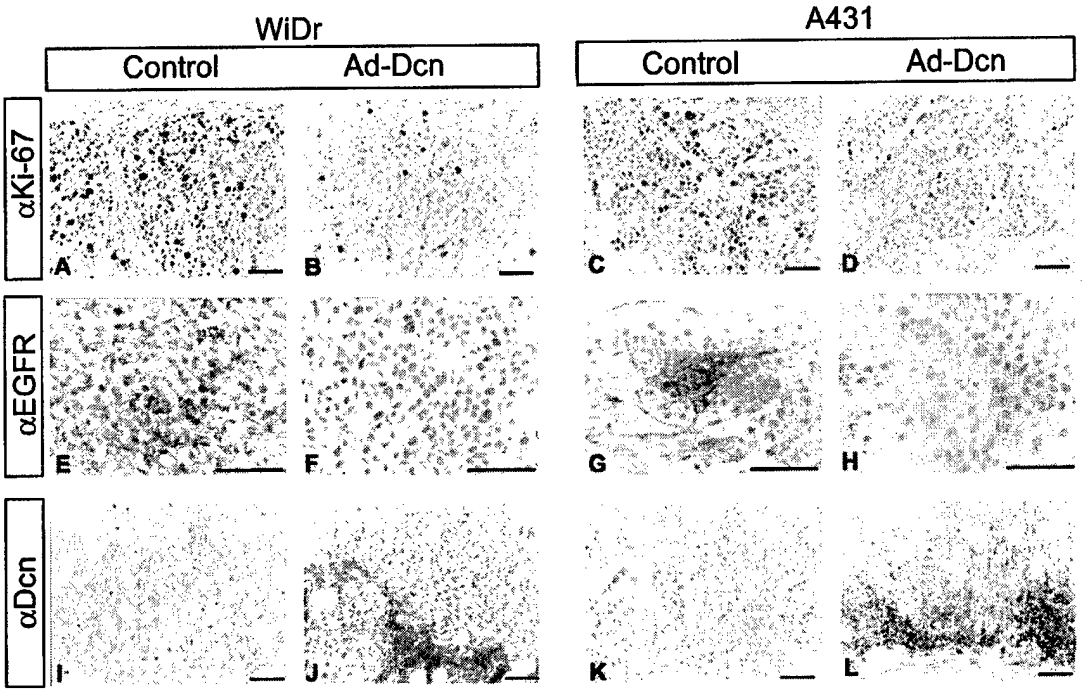

Figure 4 Expression of Ki-67, activated EGFR and decorin in WiDr colon carcinoma and A431 squamous carcinoma xenografts treated with either empty adenoviral vector (Control) or Ad-Dcn. (**a–d**) Representative photomicrographs of sections stained with a mouse monoclonal antibody (B126.1) against the nuclear proliferation marker Ki-67 (Biomeda Corp.), used at 1 : 100 dilution. Notice the markedly reduced number of cells with positive nuclear staining in the Ad-Dcn treated samples. (**e–h**) Representative photomicrographs of sections stained with a mouse monoclonal antibody (E12120) raised against the activated (phosphorylated form) of the human EGFR (Transduction Laboratories), used at 1 : 500 dilution. Notice the linear staining outlining the contours of WiDr cells (**e**) and the markedly diminished staining in the Ad-Dcn-treated xenograft (**f**). In A431 xenografts there is a more intense staining because of the large number of EGFRs ($1-2\times10^6$ receptors/cell) expressed by these neoplasic cells. Notice the presence of a strongly reactive group of tumor cells just below the unreactive mouse epidermis (**g**) and the diminished reactivity in the Ad-Dcn treated tumor xenograft. (**h**). (**i–l**), representative photomicrographs of sections stained with a rabbit affinity-purified antiserum raised against the N-terminal peptide of human decorin (Fisher *et al*., 1995), used at 1 : 500 dilution. Notice the mostly perpendicular deposits of decorin-containing epitopes only in the Ad-Dcn treated xenografts (**j** and **l**). Scale bars = 140 μm

important finding since this is the first report that decorin gene delivery alters EGFR activity *in vivo*. There is strong genetic evidence to support this important pathway of EGFR inactivation (Moghal and Sternberg, 1999; Carraway and Sweeney, 2001). In *Drosophila melanogaster*, for instance, there is a protein, named Kekkon1 (Musacchio and Perrimon, 1996), that acts in a feedback loop to negatively regulate the *Drosophila* EGFR during oogenesis (Ghiglione *et al*., 1999). Kekkon1 contains six leucine-rich repeats homologous to decorin and accumulates in the dorsal-anterior follicle cells in an EGFR-dependent manner (Ghiglione *et al*., 1999; Musacchio and Perrimon, 1996). Notably, loss and gain of Kekkon1 augments or suppresses EGFR activity, respectively, and the two proteins interact physically when co-expressed in cells (Ghiglione *et al*., 1999). These findings suggest a model where Kekkon1 may interfere with ligand activation or may facilitate the negative regulation of EGFR following activation (Carraway and Sweeney, 2001). It is likely that mammalian cells may also utilize similar leucine-rich structures to counteract the EGFR. In contrast to Kekkon1, which is a trans-membrane protein and, thus, able to interact only with EGFR molecules on the same or adjacent cells, the soluble decorin could diffuse distantly and potentially interact with numerous target cells expressing varying levels of EGFR.

While adenovirus vectors induce immuno-reactivity in humans, thereby limiting their multiple use, other gene delivery systems, such as adenovirus associated viral vectors, are being rapidly developed. Thus, we believe that the safe and repeatable gene transfer of decorin-containing viral vectors could represent a novel therapeutic tool against cancer. There are a number of advantages of using decorin as a primary or adjuvant therapy. Decorin, being a secreted proteoglycan, could interact with a number of cell-surface receptors at distant sites of transduction (Iozzo, 1999). Conceivably, a single decorin-transduced cell could affect several neighboring cells, thereby rendering this therapeutic modality effective even in systems with low or unpredictable transduction efficiency. An increase in decorin content in the pericellular environment of solid tumors, such as colon and squamous carcinomas, could trigger a functional interaction with the EGFR and other members of the ErbB family of receptor tyrosine kinase, thereby initiating a signaling cascade

that would lead to growth retardation or arrest. Decorin is a natural product and, therefore, there should not be any immune response to it. Moreover, over-expression of decorin has profound cytostatic effects preferentially on malignant cells independent of their histogenetic origin (Santra *et al.*, 1997). At least *in vitro*, smooth muscle cells appear to react differently to decorin, primarily by responding to the decorin-mediated block of TGF-β activity (Fischer *et al.*, 2001), while in macrophages recombinant decorin can enhance cell survival (Xaus *et al.*, 2001). Thus, although the pleiotropic effects of decorin need to be elucidated in future studies, our results provide support to the hypothesis that decorin could be used as a therapeutic modality to combat cancer, either as a primary or an adjuvant compound. The lack of toxicity, its solubility and its mode of action, i.e. an action on multiple cells and at a distance, make decorin gene therapy an attractive mode of intervention for numerous forms of human malignancy.

Materials and methods

Cell lines, antibodies and adenovirus production

Human WiDr colon carcinoma, A431 squamous carcinoma cells, 293 embryonic kidney cells, HeLa squamous cell carcinoma and monkey Cos-7 cells were obtained from the American Type Culture Collection. The following antibodies were used: a monoclonal antibody (E12120) raised against the activated (phosphorylated form) of the human EGFR (BD Transduction Laboratories); a rabbit affinity-purified antiserum raised against the N-terminal peptide of human decorin (Fisher *et al.*, 1995); a mouse monoclonal antibody (B126.1) against the nuclear proliferation marker Ki-67 (Biomeda Corp., Foster City, CA, USA). Full-length human decorin cDNA was cloned into a shuttle vector containing the human CMV promoter and the E1 region of the human adenovirus type 5 genome. The recombinant adenovirus-decorin (Ad-Dcn) was generated by homologous recombination after co-transfection with the shuttle vector and a virus-rescuing vector as described before (Zhao *et al.*, 1999). Samples of both decorin and control vectors were step-amplified in increasing sizes of low passage 293 cells, using successive rounds of freezing and thawing, and centrifugation. High titers of Ad-Dcn or empty vector were purified by CsCl density gradient ultracentrifugation and chromatography on pre-packed G-25 columns. Purified viruses were plaque titered in 293 cells and expressed as plaque-forming units (pfu). Alternatively, virus was purified with the Viraprep Kit (Virapur, Carlsbad, CA, USA), and viral titer was determined by $TCID_{50}$ assay.

Viral transduction, Northern blotting, real time PCR and immunofluorescence

WiDr and A431 cells were cultured in a 6-well tray. While sub-confluent, Ad-Dcn (5×10^5, and 1×10^6 pfu) was added to the media. Cells were grown for 2–10 days without media change before harvesting for RNA. In addition, Cos-7 and HeLa squamous carcinoma cells were transduced. As a further control, human decorin cDNA in pcDNA3 plasmid (Invitrogen, Carlsbad, CA, USA) was transfected in cells using Transit LT-1 (PanVera, Madison, WI, USA), Cells were drained of media and RNA was extracted with Tri Reagent (Sigma, St. Louis, MO, USA). RNA was run on a 1% agarose gel with 10% formaldehyde in 1× MOPS and stained with ethidium bromide for photography. RNA was transferred onto a nylon membrane (Osmonics, Westborough, MA, USA) and cross-linked with UV Strata linker (Stratagene, Cedar Creek, TX, USA). A human decorin cDNA probe was produced by PCR of full-length human decorin cDNA. Forward primer AAGAACCTTCACG-CATTG and reverse primer TCCAACTACAGAGATATT were used to generate a 611 bp fragment which was labeled with ^{32}P-dCTP using the Random Primer DNA Label System (Life Technologies, Rockville, MD, USA). Blots were hybridized with a radio-labeled probe in 1 M NaH_2PO_4 at pH 7.2, 1 mM EDTA, 7% SDS, and 1% BSA overnight, thoroughly washed, and exposed to film for 2–4 h at room temperature. Reverse transcription reaction was performed on RNA with SuperScript II Reverse Transcriptase (Life Technologies, Rockville, MD, USA). Real time PCR was carried out on samples as per manufacturer's protocols (Applied Biosystems 7700 ABI Prism Sequence Detector) using forward primer AGTGACTTCTGCCCACCCTGG and reverse primer CCGGGTTGCTGAAAAGACTC. Quantization of message was based on standard DNA curve generated with known quantities of human decorin cDNA. For immunofluorescence, cells were cultured in chamber slides (Nunc, Napervile, IL, USA), and, while sub-confluent, they were transduced with adenoviral decorin (1×10^5 pfu and 2×10^5 pfu) or control vector (2×10^5 pfu) for 72 h. Following a 15 min fixation in 3% paraformaldehyde, the cells were permeabilized for 10 min with digitonin (30 μg/ml), blocked with 1% BSA for 1 h, and incubated for 30 min with the primary antibody (1 : 500) against the phosphorylated (activated) form of the human EGFR (BD Transduction Laboratories, Franklin Lakes, NJ, USA). Slides were washed with 1% BSA and incubated with secondary goat anti-mouse IgG-FITC antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at a 1 : 1000 dilution for 30 min. Immunofluorescence microscopy was performed on an Olympus IX70 inverted epifluorescence microscope coupled to a high quantum efficiency cooled CCD camera. Digital images were imported into Adobe® Photoshop 6.0.

Animal experimemts, morphology and immunohistochemistry

Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23) and the Institutional Animal Care and Use Committee of Thomas Jefferson University. Human colon and squamous cell carcinoma xenografts were established in athymic nude *nu/nu* mice, 6–8 weeks of age (Charles River Laboratories) through subcutaneous inoculation of $0.5-2\times10^6$ cells into the dorsal flank of each mouse. Mice were carefully examined every 2 or 3 days and any tumor growth was measured with a micro-caliper according to the following formula: $V=a\ (b^2/2)$, where a and b represent the larger and smaller diameters, respectively. When tumors had reached 2–3 mm in greater diameter, each mouse received direct intra-neoplastic injections ($\sim$50 μl containing $0.7-1\times10^8$ pfu) of replication-incompetent adenovirus, either empty virus or virus harboring the full-length decorin gene. Student's two-sided t-test was used to compare the values of the treated and control samples. A value of $P<0.05$ was considered as significant.

Animals were sacrificed at the end of the experiments, between 19 and 58 days depending on the treatment regimen and inoculum size, and each tumor was carefully dissected. The tumors were fixed in 10% buffered formaldehyde, embedded in paraffin and processed for routine histology. For immunohistochemistry, sections placed on poly-L-lysine-coated glass slides were de-paraffinized, and epitope retrieval was performed in 10 mM EDTA buffer for 10 min in a microwave at 600 W, or by incubating the slides with pepsin for 10 min. After epitope retrieval, endogenous peroxidase was blocked in 0.03% H_2O_2, washed in PBS and incubated with the primary antibodies diluted between 1:100 and 1:500. The peroxidase-labeled polymer conjugated to either goat anti-rabbit or anti-mouse method (DAKO EnVision+ System, DAKO Corp., Carpinteria, CA, USA) was used to detect antigen-antibody reaction. Specific reactions were visualized with 3,3'-diaminobenzidine as a chromogen and images were captured with a Pixera digital camera and processed using Adobe® Photoshop 6.0. To determine the proliferative index of tumor xenografts, we estimated the percentage of tumor cell nuclei positive for Ki-67 marker in 10 high-power (×400) fields per animal ($n=3$ animals per group). Additional details are provided in the legends to figures.

Abbreviations

Ad-Dcn, adenovirus-decorin; EGFR, epidermal growth factor receptor; TGF-β, transforming growth factor-β; pfu, plaque-forming units.

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SHORT REPORTS

Decorin prevents metastatic spreading of breast cancer

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Running Title: Suppression of breast cancer metastases by decorin

Keywords: Decorin proteoglycan; adenovirus gene therapy; breast carcinoma; doxycycline inducible promoter

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Abstract

Tumor metastases in breast cancer are a vital concern in treatment, with EGFR and ErbB2 strongly implicated in mediating cellular invasion and metastatic properties. In this study we investigated the role of decorin, a member of the small leucine-rich proteoglycan family, in suppressing both primary breast carcinomas and pulmonary metastases *in vivo*. We show that decorin causes marked tumor suppression both *in vitro* and *in vivo* in a metastatic breast cancer cell line. Treatment with decorin protein core reduced primary tumor growth by 70% and eliminated observed metastases. Further, an adenoviral vector containing the decorin transgene was used to treat the same tumor model and also caused primary tumor retardation of 70%, in addition to greatly reducing observed metastases. Lastly, we demonstrate that ErbB2 phosphorylation and total receptor protein levels are reduced in this model system upon *de novo* expression of decorin under the control of a doxycycline-inducible promoter. Primary tumor growth *in vivo* was reduced by up to 67% upon decorin induction, and subsequent pulmonary metastases were likewise reduced in induced animals. The observed effects are likely occurring through decorin's long-term down-regulation of the ErbB2 tyrosine kinase cascade. Taken together, these new results demonstrate a novel role for decorin in reduction or prevention of tumor metastases in this breast cancer model and could eventually lead to improved therapeutics for metastatic breast cancer.

The aggressive behavior of human breast cancer correlates with enhanced expression and activity of receptor tyrosine kinase, primarily of the epidermal growth factor or ErbB family (1). Thus, therapeutic interventions focused on attenuating either the expression or the intrinsic activity of these receptors could represent useful anti-cancer treatments (2). Decorin, the prototype member of an expanding family of small leucine-rich proteoglycans, is involved in a number of cellular processes including matrix assembly, fibrillogenesis and the control of cell proliferation (3-5). Notably, decorin protein core is a biological ligand of the epidermal growth factor receptor (EGFR) (6;7), but unlike typical ligands, such as EGF and TGF-α, decorin/EGFR interaction leads to a transient activation of the receptor tyrosine kinase, followed by a protracted down-regulation of its activity. Indeed, we have previously shown that decorin exerts an anti-oncogenic activity in various tumors, including squamous and adenocarcinomas, by suppressing both EGFR (8-10) and ErbB2 (11) activity. In either case, decorin causes a profound down-regulation of these receptors both *in vitro* and *in vivo*, thereby providing a plausible explanation for its cytostatic effects. The cytostatic effects of decorin occur in a wide variety of tumor cell lines with a diverse histogenetic background (8;12) and can also affect murine tumor cells (13) and normal cells such as endothelial cells (14) and macrophages (15). Decorin can also indirectly retard tumor growth by blocking transforming growth factor-β (16), by inhibiting tumor cell production of angiogenic factors such a VEGF (17), or by interacting with anti-angiogenic compounds such as thrombospondin-1 (18). Our working hypothesis, validated by multiple studies from our group (9;10;19) as well as by several independent studies (12;20), is that decorin is a natural anti-cancer agent and enhancement of decorin gene expression should lead to a retardation of tumor growth. We have recently shown that transient transgene expression of a replication-deficient adenovirus (Ad)-containing decorin causes a profound growth inhibition of colon and squamous cell carcinoma xenografts (21). A key finding of our study was that decorin gene expression in the treated tumor xenografts correlated well with a decreased proliferative index and an attenuation of EGFR activation. Moreover, using the same construct it was subsequently found that directly intra-neoplastic injection of Ad-decorin

could affect not only the treated tumor xenograft, but also a distant tumor on the contra-lateral side (20).

In this study we investigated whether decorin had any effects on the metastastic spreading of breast carcinoma cells *in vivo*. Our primary goal is to determine that decorin's mechanism of action, i.e., downregulation of EGFR activity (9;10;12;19-21), has the far-reaching effect of reducing invasion of tumors and metastatic spread *in vivo*. This is a vital step to proving the relevance of decorin as a potent biological inhibitor of cancer. To this end, we utilized an established animal model in which spontaneously metastasizing rat MTLn3 breast carcinoma cells are injected in the mammary gland of virgin SCID animals to generate an orthotopic tumor xenograft. The MTLn3 express low EGFR number (~ 5×10^4/cell) and relatively high number (~ 5×10^5) of ErbB2, similar to the human counterpart. These cells have been utilized in a number of studies (22-24) and represent a valid model of orthotopic breast carcinoma xenograft. The MTLn3 grow very rapidly and within 2-3 weeks metastasize nearly exclusively to the lungs (25). One week following tumor implantation, we injected intra-tumorally highly purified decorin protein core (60 μg/animal) (Figure 1a), and repeated the same treatment for another two times in the following two weeks. The total dosage was 7.2 mg/Kg of body weight. The results showed a marked suppression of primary tumor growth ($P<0.001$) four weeks after implantation (Figure 1b). In spite of the fact that the decorin-treated tumors had a morphology similar to the untreated (not shown), and that they reached a sizable tumor of ~500 mg (Figure 1b), we found neither gross nor microscopic lung metastases (Figure 1b). All the lungs were completely analyzed by serial sectioning and we found only a few cell aggregates, possibly incipient metastases, in the decorin group. In contrast, the animals treated with vehicle alone clearly showed a significant number of metastases, which we sub-divided into three categories based on their average size (Figure 1c).

Next, we determined whether Ad-Dcn was capable of efficiently transducing the rat MTLn3 tumor cells. The results showed a significant expression of the virally-transduced decorin gene transcript, a single ~1.9 kb band detected after only 2h of exposure (Figure 2a), whose levels

increased in a dose-dependent manner. In agreement with previous results which have clearly demonstrated that decorin is a product of mesenchymal cells rather than epithelial cells (8;26-28), no decorin message was detected in the MTLn3 breast carcinoma cells, even after a long exposure (Figure 2a). Note that our human probe would recognize murine decorin after a long exposure. The *de novo* expression of decorin correlated with a reduction of *in vitro* tumor cell growth. Specifically, MTLn3 cells ($6x10^4$) were plated in triplicate and transduced with Ad-Empty or Ad-Dcn (MOI = 2), and cell number was determined 48 h later. The results showed a significant growth inhibition ($P<0.01$) (Figure 2b). The high efficiency of transduction and the relatively high levels of transgene expression mediated by the adenoviral vector in cultured cells prompted us to investigate the anti-oncogenic properties of decorin *in vivo* by direct intra-neoplastic injection of the Ad-Dcn. To this end, animals were injected directly into the orthotopic breast xenografts with Ad-Empty or Ad-Dcn ($5x10^8$ pfu/injection) at various intervals. The results showed a marked inhibition of tumor growth (Figure 2c). At the end of the experiments, the decrease in tumor volume for the Ad-Dcn-treated breast carcinoma xenografts reached ~70% ($P<0.001$) (Figure 2c). In a previous publication (21), we demonstrated by immunohistochenistry lowered levels of activated EGFR, along with a reduced proliferative index, in tumors following treatment with adenoviral decorin. These results were both confirmed and extended in an independent publication using the identical decorin viral vector (20). We have additionally observed decreased levels of activated EGFR following *in vivo* tumorigenesis experiments with cells constitutively expressing decorin (10). Thus, both topical delivery of recombinant human decorin protein core and transduction of tumor xenografts with Ad-Dcn have substantial cytostatic effects in an animal model of aggressive breast cancer.

There was also, in addition to primary tumor inhibition, a significant inhibition of metastatic spreading to the lungs (Figure 2d), albeit not as striking as with the recombinant decorin protein core. Immunohistochemistry to observe decorin in pulmonary metastases of adenoviral-treated animals was negative, due most likely to the transient and local nature of viral treatment (data not shown). We presume that the difference in observed metastases is due to differences in

decorin levels, since the Ad-Dcn would produce decorin levels in the low μg range and would only be transiently expressed, while the dosages with the recombinant decorin core were likely much higher and were presented to the nascent tumors in a bolus.

Conditional regulation of gene expression in the course of *in vivo* tumor progression provides important insights into the role of gene products during different phases of tumor development. To determine whether the endogenous production of decorin would also produce a retardation of growth and a reduced metastatic burden, we generated stably transfected clones of the MTLn3 cells expressing decorin controlled by a *Dox*-inducible promoter (29;30). We utilized the Rev Tet-On™ (BD Bioscience) system due to its high level of inducible expression, quantitative regulation of gene expression, and minimal pleiotropic and non-specific effects. We generated several stable clones that were highly inducible by exposure to *Dox* for 48 h following transfection with the pRevTRE-luciferase construct (Figure 3a). Representative immunoblotting of media from clone 13 showed a significant induction of decorin proteoglycan by doxycycline treatment with minimal promoter leakage (Figure 3b). Notably, the overall amount of ErbB2 receptor that could be immunoprecipitated with a specific monoclonal antibody was significantly reduced (Figure 3c). Quantification of several immunoprecipitation experiments revealed a mean 60% reduction in ErbB-2 levels (Figure 3d). However, the degree of phosphorylation of ErbB-2 was reduced by over 80% (Figure 3d). These results are similar to those obtained with stable expression of decorin in a human mammary carcinoma cells which also over-express ErbB2 (11). The changes in ErbB2 activity correlated with a significant *in vitro* growth inhibition (Figure 3e). Induced cells also demonstrated a greatly reduced ability to proliferate in a hydrated collagen matrix (Figure 3f), with over 75% fewer colonies formed after five days in culture (Figure 3g). Reduced ability to grow in a three-dimensional lattice demonstrates *in vitro* that cells expressing decorin exhibit reduced aggressiveness and invasive potential and correlates well with the reduced ErbB-2 activity and significant growth inhibition previously observed.

In vivo, the tumor growth inhibition was performed in two independent experiments, and demonstrated nearly identical results of nearly 70% inhibition of primary tumors (Figures 4a and c). In addition, there were 57% fewer microscopic metastases observed in the lungs in one experiment and no observable metastases in the second (Figures 4b and d). Immunohistochemical staining decorin in primary tumors of induced and uninduced animals revealed decorin in induced tumors, concentrated in pericellular pockets as well as in the in the tumor periphery (Figures 4e through 4j). The induced tumors additionally showed a more defined capsule than uninduced tumors (not shown). The pattern of decorin expression and cell morphology in tumors is amazingly similar to previously observed decorin in tumors following adenoviral decorin treatment (21). It is possible that cells in the tumor periphery, with greater access to blood supply and, by extension, doxicycline, secrete the induced gene more efficiently. Alternatively, decorin could be concentrating in these regions by a not yet understood mechanism. The overall amounts of decorin detected in induced tumors are low, underscoring the potency of decorin in growth inhibition and metastatic spread. Higher levels of expression should, we believe, correlate in even more dramatic *in vivo* inhibition.

Note that, in the decorin induction experiments, the overall amount of tumor metastases was lower than the previous *in vivo* trials, possibly due to the slight leakage of the Tet promoter. It is known that low levels of decorin in invasive breast carcinomas have been associated with larger tumor size, shortened time to progression, and poor outcome (31). These results show that decorin causes a significant growth suppression of rat breast carcinoma cells and could inhibit metastases to the lungs as a result of the decreased tumor size. Alternatively, decorin itself could be directly involved in a mechanism regulating the behavior of metastatic cells by lowering their tendency to spread. Over-expression of EGFR and ErbB2 has been shown to stimulate cell migration, which is an important step in metastasis (32). Breaking the signaling cascade of the EGFR pathway should, therefore, correlate with reduced metastatic spread and better overall prognosis *in vivo*. Decorin's proven down-regulation of EGFR/ErbB2, demonstrated both in the present study and in others {1612, 1824}, point to a role for decorin in

slowing the spread of metastases through either reduction in primary tumor growth or possibly also through direct or indirect signaling mechanisms at the sites of metastasis. These results could have important clinical implications as metastases to the lungs are lethal in patients with breast cancer. Extending decorin's effects to the reduction of a primary breast cancer tumor model which rapidly and aggressively metastasizes is an important step in elucidating the reach of decorin's proven downregulation of EGFR/ErbB2 activity {1126,1625,1626,1591,1824,1924}. Collectively, these results provide strong evidence for the *in vivo* anti-proliferative effects of decorin and suggest that, at least in part, decorin inhibits the proliferation of tumor cells by interfering with ErbB2 activity.

Our results further underscore the possibility of decorin as a viable therapeutic, either alone or in combination, for several types of cancers. Previous work with decorin delivered by an adenoviral vector showed decorin's efficacy against several *in vivo* tumor models {1824,1924}, and the results here not only reinforce these results, but extend them into the combating of a serious clinical problem- that of secondary tumor metastases. We have used several tools, including viral vector-mediated gene delivery, to induce decorin's effects. Gene transfer methods to deliver decorin in a therapeutic setting are, we believe, a distinct prospect in the future, especially in light of decorin's proven synergistic effects with other therapy modalities in inhibiting cancer cells {1591}. Decorin gene delivery offers distinct advantages, including the ability to affect many nearby cell-surface receptors from a single transduction event, since decorin is secreted into the surrounding environment and acts extracellularly (4). This is of great value in systems where effective transduction is variable or unpredictable. Decorin is also a natural product and therefore should not by itself be immunogenic. Through its interaction with ErbB tyrosine kinase family members and the subsequent functional down-regulation of such, decorin could lead to remarkable growth delay or arrest in a variety of solid tumors. In addition to this, we have presented strong evidence that not only does decorin affect primary tumors, but can potentially slow or halt distant metastases. While a great deal of work needs to be done

to adequately explore this, our results indicate that limiting distant metastatic growth with nontoxic, biological compounds is an achievable goal.

Abbreviations

Ad-Dcn, adenovirus-decorin; EGFR, epidermal growth factor receptor; pfu, plaque-forming units; HRP, horseradish peroxidase,

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Figure Legends

Figure 1 Decorin protein core causes sustained growth inhibition and abolishes observed metastases in MTLn3 tumor model *in vivo*. (**a**) SDS-PAGE of decorin protein core. Recombinant human decorin was expressed as a polyhistidine fusion protein in 293-EBNA cells using a Celligen Plus bioreactor (New Brunswick Scientific, NJ) as described previously (33). Proteoglycan and protein core was purified over a nickel-chelating column followed by elution with a gradient of 0-250 mM imidazole in 20 mM Tris-HCl, 500 mM NaCl, 0.2% CHAPS, pH8.0. Protein core was subsequently separated from proteoglycan by anion-exchange chromatography on Q-Sepharose. Differential glycosylation of decorin protein core resulted in the expected doublet migrating with an apparent molecular mass of 50-55kDa (34). Two μg of purified decorin protein core was visualized following electrophoresis on a 10% polyacrylamide and staining with Coomassie brilliant blue. (**b**) Decorin protein core inhibits the growth of an orthotopic breast carcinoma. MTLn3 cells (~10^6) were injected into the breast of female SCID

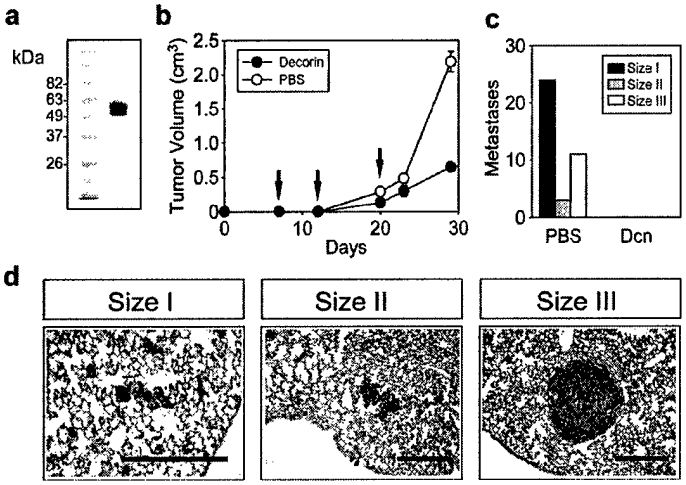
mice (n=4). Decorin protein core (60 μg protein/injection) or PBS (control) was injected directly into developing tumors. Three treatments were administered as indicated by vertical arrows. Graph indicates mean tumor volume as determined by the formula $v=axb^2/2$, where *a* is the longest axis and *b* is perpendicular to *a*. Values represent the mean ± s.e.m. (**c**) Decorin protein core eliminates pulmonary metastases *in vivo*. Upon concluding the *in vivo* protein treatment, lungs of all animals were dissected, fixed, sectioned, and examined histologically for tumor metastases. Size I indicates metastases of under 100 μm, size II from 101-200μm, and size III >200 μm. Graph indicates total metastases found in all animals. (**d**) Photomicrographs of three classes of pulmonary metastases *in vivo*. Lungs were paraffin embedded, sectioned at multiple levels, and stained with hematoxylin and eosin. Observed metastases stained differentially (blue nodules) compared to surrounding lung tissue. Scale bars = 300 μm.

Figure 2 Adenoviral decorin (Ad-Dcn) significantly slows cell growth *in vitro* as well as inhibiting primary tumor growth and metastases of MTLn3 breast carcinoma cells *in vivo*. (**a**) Northern blot indicating Ad-Dcn transduction of MTLn3 cells. MTLn3 cells (~10^6) were transduced with Ad-Empty or Ad-Dcn at the designated concentrations. RNA was extracted 48 h later, separated on a formaldehyde gel, blotted onto a nylon membrane, and probed with a radiolabeled decorin probe (Random Primer Labeling Kit, Stratagene). Film was exposed for 2 h. Asterisks indicate the migration of (28S and 18S) rRNA. (**b**) Growth inhibition of MTLn3 cells following transduction with Ad-Dcn. MTLn3 cells ($6x10^4$) were plated in triplicate and transduced with Ad-Empty or Ad-Dcn (MOI = 2). Cells were counted 48 h later and graphed showing mean ±s.d. Cell counts were analyzed by *t* test, $p<0.01$. (**c**) Ad-Dcn treatment inhibits breast carcinoma model *in vivo*. MTLn3 cells (~10^6) were injected into the breast of female SCID mice (n=5). Six days after tumor induction, direct treatment with Ad-Empty or Ad-Dcn ($5x10^8$ pfu/injection) was given. Four treatments were administered as indicated by vertical arrows. Graph indicates mean tumor volume calculated as previously stated ± s.e.m. (**d**) Ad-Dcn treatment reduces pulmonary metastases *in vivo*. Upon concluding the direct tumor treatment with Ad-Dcn lungs of all animals were fixed, sectioned, hematoxylin and eosin stained, and examined histologically for tumor metastases. Size I indicates metastases of under 100 μm, size II from 101-200 μm, and size III >200 μm. Graph indicates total metastases found in all animals.

Figure 3 De novo expression of decorin driven by a doxycycline-responsive promoter attenuates ErbB2 levels and activity. (**a**) Generation of single-stable MTLn3 clones that respond to doxycycline (Dox) induction of luciferase. Notice that all of the clones generate luciferase activity, as measured by relative light units (RLU). There is relatively low background, i.e. promoter leakage. In the y axis, - and + refer to the absence or presence of Dox (2 $\mu g\ ml^{-1}$). Clones showing induction were subsequently utilized in generating double-stable inducible decorin clones. (**b**) Western immunoblotting of conditioned media of several MTLn3 double-stable clones after exposure to Dox, 5 $\mu g\ ml^{-1}$ for 72 h. In all cases, clones show inducible activation of decorin. Note that stable decorin clones are differentially glycanated. (**c**) Immunoprecipitation (IP) and Western blotting (Wb) of cell lysates of clone 13a.7 after exposure to Dox (5 $\mu g\ ml^{-1}$) for 72 h. αErbB2 antibody (NeoMarkers) was used for immunoprecipitation, and blots were probed with either αErbB2 or α-P-Tyr (BD Biosciences) antibodies as indicated. Similar results were obtained with other clones (not shown). MDA-MB-453 cells were used as a positive control for the IP and Western blots. Ig bands indicate relative precipitation efficiency and gel loading. (**d**) IP/Western blots of clones 13a.5, 13a.7, and 13a.10 were scanned and densitometry was performed using the public domain NIH Image program (developed at NIH and available at http://rsb.info.nih.gov/nih-image/). Bands from uninduced cells were set to an arbitrary value of one for quantification and the means of scanning densitometry results from the three blots were plotted. Error bars indicate standard deviation. (**e**) Cell growth assay of clone 13a.5 in the presence or absence of Dox (5 $\mu g\ ml^{-1}$) as indicated. About10^4 cells were plated in triplicate on day 0 in the presence or absence of Dox and grown for 6 days in complete medium. Cells were counted and results plotted. Growth inhibition of 67% was observed in induced cells. Similar results were obtained with other clones (not shown). (**f**) Hydrated collagen lattice growth assay. Collagen gels (2.48 mg/ml) (BD Biosciences) were prepared with αMEM and mixed with $5x10^4$ cells of clone 13a.5. Wells were prepared in triplicate. Cells were induced with Dox (5μg/ml) for five days, after which photographs were taken colonies counted. (g) Quantification of collagen gel lattice assay. Wells were counted in triplicate. Graph displays mean, and error bars indicate SD.

Figure 4 Induction of decorin reduces growth and invasive potential *in vitro* and *in vivo*. (**a** and

c) Tumor growth *in vivo* is inhibited by induction of decorin. Two independent experiments were performed with clone 13a.5 cells (~10^6) engineered to conditionally express decorin via a Dox-sensitive promoter. Cells were injected subcutaneously into the breast of female SCID mice (n=5 for all experiments). Induced cells were exposed to Dox (5 μg ml^{-1}) and animals were likewise given Dox (500 μg ml^{-1}) and sucrose (50 mg ml^{-1}) in drinking water, or sucrose alone, 24 hours prior to injection. Dox water was replaced every two days during the course of the experiment. Graphs indicate mean tumor volume calculated as described previously, and error bars indicate S.E.M. (**b** and **d**) Decorin induction reduces pulmonary metastases *in vivo*. Upon concluding the direct tumor treatments with Ad-Dcn, lungs of all animals were fixed, sectioned, stained, and examined as already described. Graph indicates total metastases found in all animals. No size III metastases were observed in any animals. (**e-j**) Immunohistochemistry of induced tumors indicates presence of the decorin epitope. (**e**) Negative immunostain of skin with α-HRP antibody only. (**f**) Positive control α-Dcn immunostain of skin. Antibody was used at 1:2500 dilution. (**g-j**) α-Dcn immunostain of induced tumors from (c). Decorin epitope was detected primarily in the pericellular pockets and at the tumor peripheries. Uninduced tumors Scale bar = 200 μm.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kDa
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63
49
37
26
b
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1.5
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0
Decorin
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0
10
20
30
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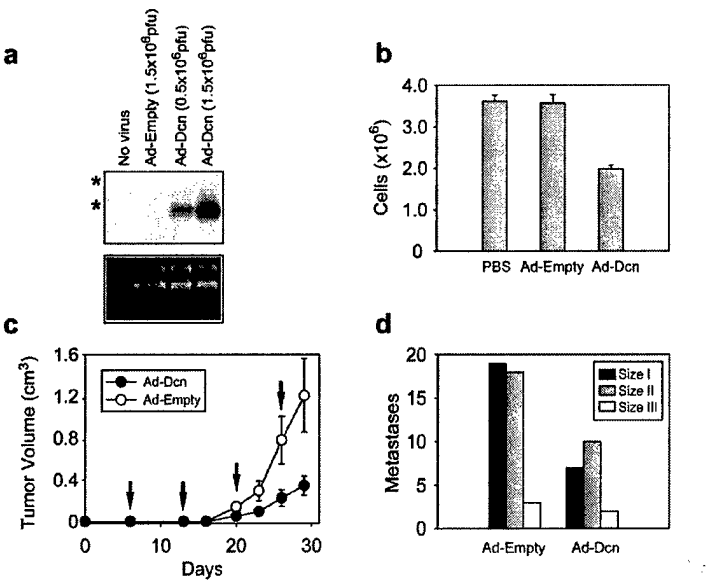
Figure 1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Cells (x10^6)
4.0
3.0
2.0
1.0
0
PBS
Ad-Empty
Ad-Dcn
c
Tumor Volume (cm^3)
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1.2
0.8
0.4
0
Ad-Dcn
Ad-Empty
0
10
20
30
Days
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Metastases
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0
Size I
Size II
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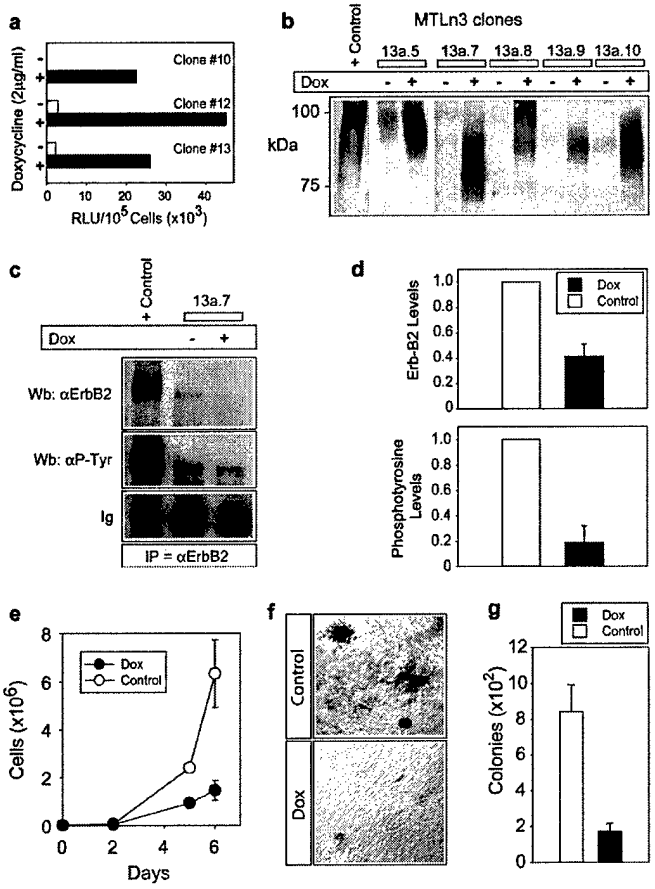
Ad-Empty
Ad-Dcn

Figure 2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Doxycycline (2µg/ml)
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Clone #13
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13a.7
13a.8
13a.9
13a.10
Dox
100
kDa
75
c
Control
13a.7
Dox
Wb: αErbB2
Wb: αP-Tyr
Ig
IP = αErbB2
d
Erb-B2 Levels
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Control
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Control
Dox
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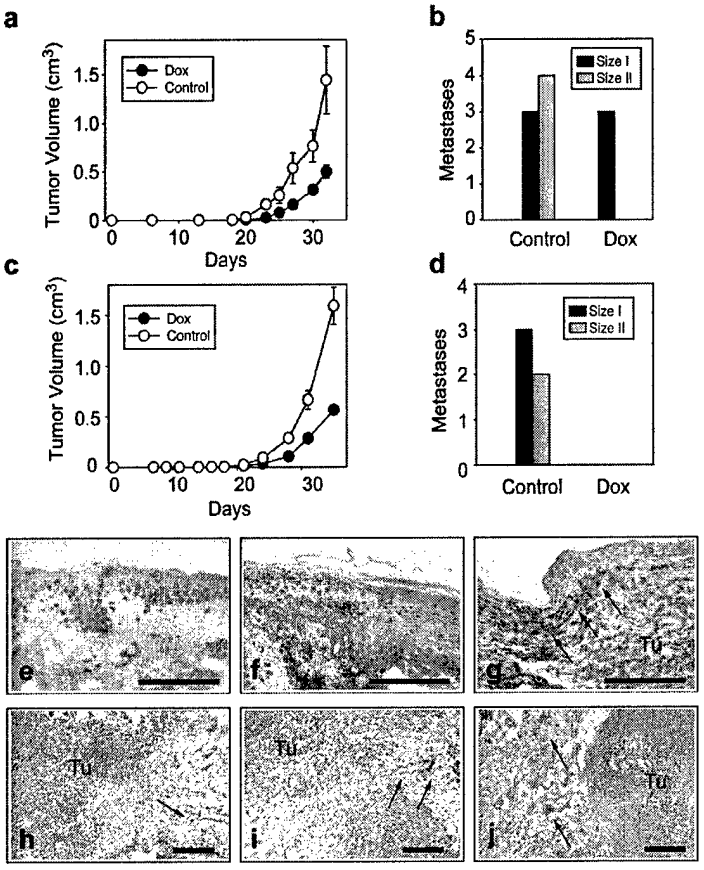
Colonies (x10^2)

Figure 3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Tumor Volume (cm3)
Dox
Control
Days
b
Metastases
Size I
Size II
Control
Dox
c
Tumor Volume (cm3)
Dox
Control
Days
d
Metastases
Size I
Size II
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e
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Figure 4