Markedly enhanced intratumoral spread and antitumor effect of oncolytic adenovirus expressing decorin

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Abstract

With the aim of improving viral distribution and tumor penetration, we have engineered decorin expressing replication-incompetent (dl-LacZ-DCNG) and -competent (Ad-ΔE1B-DCNG) adenoviruses. In both tumor spheroids and established solid tumors *in vivo*, administration of dl-LacZ-DCNG resulted in greater transduction efficiency and viral spread throughout the tumor mass. Ad-ΔE1B-DCNG also enhanced viral distribution and tumor spread, leading to an increased anti-tumor effect and survival advantage. Upon histological analysis, Ad-ΔE1B-DCNG also elicited greater percentage of apoptotic cells and extensive necrosis compared to those from untreated or control virus-treated tumors. Furthermore, Ad-ΔE1B-DCNG substantially decreased extracellular matrix components within the tumor tissue, while normal tissue adjacent to the tumor was not affected. Finally, intratumoral administration of Ad-ΔE1B-DCNG did not enhance but inhibited the formation of pulmonary metastases of B16BL6 melanoma cells in mice. Taken together, these data demonstrate the utility of decorin as a dispersion agent and suggest its utility and potential in improving the efficacy of replicating adenovirus-mediated cancer gene therapy.

Introduction

Adenoviral vectors' inefficient ability to disseminate throughout tumor tissues remains a major obstacle in the development of a successful cancer gene therapy [1-6]. Number of groups is seeking to address this problem by using oncolytic adenoviruses that selectively replicate in tumor cells. Bischoff and coworkers reported that by deleting an adenovirus early gene, E1B 55kDa, Ad selectively proliferated in cancer cells [7]. Subsequently in our previous report, we have shown that E1B 19kDa- and E1B 55kDa-double deleted replication-competent adenovirus, Ad-ΔElB19/55 (referred to as Ad-ΔE1B in this paper), induced viral cytolysis as well as E1A-mediated apoptosis, leading to an improved efficacy over E1B 55kDa-deleted replication-competent adenovirus, Ad-ΔElB55 [8].

Overall, selective replication and viral spread within cancer cells has seen some success, but the overwhelming promise that was once projected has not been fully realized mainly due to the uneven penetration and viral distribution of these engineered Ads within tumor tissues. To date, ONYX-015 (originally named dl1520), an E1B 55kD gene-deleted oncolytic adenovirus, has been tested in over 15 clinical trials in a range of tumor types [9,10], but only has reported limited success (7 to 14% local response rate) in patients with head and neck cancer, and worse no single agent efficacy was noted in other tumor types [11]. In preclinical xenograft tumor model studies, wild-type adenovirus has been shown to delay the growth of tumors, but rarely is able to eradicate established tumors. Recently, Sauthoff et al. demonstrated that high level of titratable Ads persisted within xenograft tumors for at least 8 weeks after intratumoral injection of the wild-type adenovirus [12], but the pattern of viral distribution within the tumor tissue was uneven

and patchy. The long-term viral persistence without complete responses suggests that viral spread maybe limited and that this limitation could be an important mechanism for the reported lack of efficacy.

Recent evidence has shown that connective tissue and extracellular matrix (ECM) may play a prominent role in inhibiting viral spread following their administration. First, Kuriyama et al. demonstrated that treatment with collagenase/dispase or trypsin prior to intratumoral injection of adenovirus, enhanced virus-mediated gene transduction [13]. Similarly, Maillard et al. elevated the efficiency of adenovirus-mediated gene transfer by about two-fold by treating rabbit iliac arteries with elastinase, an enzyme which dissociates elastin [14]. More recently, studies from our laboratory have demonstrated that ECM degradation by relaxin expressed from adenovirus increased viral spread throughout the tumor mass, resulting in an enhancement of transduction efficiency in solid tumors *in vitro* and *in vivo* [15]. Relaxin also demonstrated superior viral distribution of replicating oncolytic adenovirus, leading to an increased anti-tumor effect and survival advantage.

To extend our understanding of the role of the ECM barrier and connective tissue in inhibiting viral spread and penetration within tumor masses, we evaluated the utility of decorin. Decorin, a small leucine-rich proteoglycan, is a ubiquitous component of the ECM and is preferentially found in association with collagen fibrils [16]. Decorin binds to collagen fibrils and delays the lateral assembly of individual triple helical collagen molecules, resulting in the decreased diameter of the fibrils. Decorin may also affect the production of other ECM components by blocking the activity of transforming growth factor-β (TGF-β) [17]. Additionally, decorin can modulate the interactions of ECM components such as fibronectin and thrombospondin with cells. Furthermore, decorin is

capable of affecting the ECM remodeling via induction of the matrix metalloproteinase collagenase (MMP-1) [16,18]. These observations suggest that decorin regulates the production and assembly of the ECM at several levels and hence play a prominent role in remodeling connective tissues.

Given the importance of decorin's role in the ECM remodeling, we hypothesized that an increased expression of decorin would facilitate the spread of Ads within tumor tissues. We show here that expression of decorin dramatically increased the transgene expression and functionally, the dispersion of Ads within tumor tissues. As such the oncolytic potency of a decorin-expressing replicating adenovirus was significantly greater, both *in vitro* and *in vivo*. Taken together, these data demonstrate the utility of decorin as a dispersion agent, and suggest that decorin exhibits a high potential in improving the efficacy of replicating adenovirus-mediated cancer gene therapy.

Results

Construction of decorin-expressing adenoviruses and expression pattern of decorin

To evaluate role of decorin in altering tumor penetration efficiency, replication-incompetent dl-LacZ-DCNG, dl-LacZ-DCNQ, and dl-LacZ-DCNK adenoviruses expressing LacZ as a reporter were constructed. Tumor-specific replication-competent adenoviruses (Ad-ΔE1B-DCNG, Ad-ΔE1B-DCNQ, and Ad-ΔE1B-DCNK) were also constructed to enhance viral spreading into tissues. Point mutants of decorin gene, DCNK and DCNQ, contain substituted nucleotides at the region which plays a pivotal role in binding to type I collagen fibril. The first mutant, DCNQ, harbors a point mutation at the E180 amino acid site (E180Q), which is located in the sixth leucine-rich repeat of the wild type decorin core protein, and has a moderate binding affinity to type I collagen fibril. The second mutant, DCNK, also harbors an amino acid exchange at the amino acid E180 site but is substituted with lysine (K), resulting in lack of binding affinity to type I collagen fibril [19,20]. To assess the activity of the adenoviruses, U343 cells were infected with replication-incompetent dl-LacZ-DCNG, dl-LacZ-DCNQ, and dl-LacZ-DCNK adenoviruses at 20 MOI or tumor-specific oncolytic adenoviruses Ad-ΔE1B, Ad-ΔE1B-DCNG, Ad-ΔE1B-DCNQ, and Ad-ΔE1B-DCNK at 5 MOI, and medium was then recovered for Western blotting. Cells infected with dl-LacZ or Ad-ΔE1B as negative controls for replication-incompetent adenovirus and tumor-specific oncolytic adenovirus were revealed not to express decorin, whereas all those infected with dl-LacZ-DCNG, dl-LacZ-DCNQ, dl-LacZ-DCNK, Ad-ΔE1B-DCNG, Ad-ΔE1B-DCNQ, and Ad-ΔE1B-DCNK showed decorin expression (Fig. 1).

Evaluation on spreading and penetration potency of decorin-expressing adenoviruses to tumor tissue

In order to evaluate the penetration potency of decorin-expressing adenoviruses, U343 and U87MG tumor xenografts grown in nude mice were intratumorally injected with dl-LacZ or dl-LacZ-DCNG at 5 x 10^8 PFU for three injections. At 3 days post final adenoviral injection, tumor tissue was removed and stained for expression of β-galactosidase (Fig. 2A). While dl-LacZ exhibited low level of LacZ expression and the staining region was localized to the viral injection site, dl-LacZ-DCNG showed much higher LacZ expression and the stained region was found to be widely distributed other regions of the tumor. In comparison, both dl-LacZ-DCNK and dl-LacZ-DCNQ showed much lower LacZ expression was present in limited areas of the tumor. These results clearly demonstrate that tissue penetration potency of dl-LacZ-DCNG is greatly enhanced than those of dl-LacZ, dl-LacZ-DCNQ, and dl-LacZ-DCNK, and this enhanced tissue penetration effect derived from decorin-expressing adenovirus is dependent on the binding affinity of decorin to collagen fibril.

To further confirm the enhanced transduction efficiency and tissue penetration potency of wild-type decorin-expressing adenovirus, GFP-expressing adenoviruses (5 x 10^8 PFU of dl-GFP or dl-GFP-DCNG) were injected 3 times every other day into U87 tumor xenografts established in nude mice. Three days following the final injection, tumors were then harvested and photographed using a confocal microscope. As shown in Fig. 2B, we observed narrow path of fluorescence in the tumors infected with dl-GFP, which is assumed as a needle track formed when we injected viruses into tumors. In marked contrast, we observed fluorescence in more extended area of the tumor mass treated with dl-GFP-DCNG, and was not limited to sites of injections.

Furthermore, to examine whether the enhanced transduction efficiency and tissue penetration potency of decorin-expressing adenovirus can also be translated to primary human tumor tissues, tumor spheroids were prepared from human patients with brain or breast cancers. The spheroids were transduced with LacZ- or GFP-expressing adenoviruses (1 x 10^7 PFU of dl-LacZ, dl-LacZ-DCNG, dl-GFP, or dl-GFP-DCNG). Three days after viral infection, the tumor spheroids were then observed under a light or fluorescence microscope. As shown in Fig. 3, LacZ or GFP expression was limited on the surface of primary tumor spheroids treated with either dl-LacZ or dl-GFP. In contrast, LacZ or GFP was strongly and widely expressed in most of tissues of the glioma and breast primary tumor spheroids treated with either dl-LacZ-DCNG or dl-GFP-DCNG. This profile of transduction efficiency and viral spread confirmed the observations noted from the *in vivo* experiments using human xenograft models.

Assessment decorin-expressing oncolytic adenovirus' ability to kill tumor cells

To link that the increase in spreading of decorin-expressing adenovirus contributes to enhanced tumor cell killing, an *in vitro* cytopathic effect assay was carried out. Two human glioma cell lines (U343 & U87MG) and human normal cell lines (CBHEL, IMR90, and WI38) were infected with dl-LacZ, Ad-ΔE1B, Ad-ΔE1B-DCNG, Ad-ΔE1B-DCNQ, or Ad-ΔE1B-DCNK adenovirus at MOIs of 0.1- 20 for glioma cell lines and 0.1-100 for normal cell lines, and the tumor cell killing effect was analyzed. As shown in Fig. 4, as expected, dl-LacZ had no effect in killing glioma cell lines. In contrast, Ad-ΔE1B-DCNG exhibited about 10-20 fold higher tumoricidal effect than Ad-ΔE1B control oncolytic adenovirus. This result was consistent over several independent experiments, indicating that decorin expression does not decrease

the replication competency of adenoviruses and contributes to the dramatic increase in tumoricidal effect of adenoviruses as well. Interestingly, the tumoricidal effects of Ad-ΔE1B-DCNQ and Ad-ΔE1B-DCNK were decreased upon lowering the binding affinity to collagen compared to that of Ad-ΔE1B-DCNG. In marked contrast, Ad-ΔE1B-DCNG in normal cells showed no significant changes in cell killing effect compared to Ad-ΔE1B, highlighting the cancer specificity of this Ad.

Evaluation on anti-tumor efficacy of decorin-expressing oncolytic adenovirus in vivo

Wild-type decorin-expressing oncolytic adenovirus, Ad-ΔE1B-DCNG, was next examined for its ability to suppress the growth of human tumor xenografts, U343 and U87MG glioma models established in nude mice. Tumors were generated by subcutaneous injection of cells into the mice abdominal region. When tumors reached an average size of 70-100 mm^3, 5 x 10^8 PFU of viral load were injected intratumorally 3 times, every other day. As shown in Fig. 5A, the growth of oncolytic adenovirus-treated tumors was substantially delayed in all human xenograft models examined. For U343 xenograft model, the control mice treated with PBS resulted in the considerable tumor growth to 2118.17 ± 144.96 mm^3 at day 53 post-treatment, whereas Ad-ΔE1B and Ad-ΔE1B-DCNG led to the significant suppression of tumor growth to 916.00 ± 191.78 mm^3 and 329.80 ± 126.74 mm^3, showing 57% ($P < 0.001$ versus PBS) and 84% ($P < 0.001$ versus PBS; $P < 0.05$ versus Ad-ΔE1B) tumor growth inhibition, respectively. Similarly, Ad-ΔE1B-DCNG-treated U87MG glioma tumors also showed remarkable anti-tumor effect than those treated with Ad-ΔE1B, demonstrating that the decorin-expressing oncolytic adenovirus has stronger anti-tumor potency than Ad-ΔE1B. At 34 days after treatment for U87MG xenograft models, more specifically, Ad-ΔE1B and

Ad-ΔE1B-DCNG adenoviruses led to the tumor volume of 591.30 ± 180.44 and 38.33 ± 36.03, respectively, showing a decrease of 94% ($P < 0.05$ versus Ad-ΔE1B) in tumor volume in Ad-ΔE1B-DCNG-treated mice relative to Ad-ΔE1B-treated mice. Moreover, Ad-ΔE1B-DCNG adenovirus completely eradicated tumors in 5 out of 7 mice.

Figure 5B shows survival curve for PBS-, Ad-ΔE1B-, and Ad-ΔE1B-DCNG-treated tumor bearing mice. For U343 tumor bearing mice, 53 days after the beginning of the treatment, 100% of the animals treated with Ad-ΔE1B-DCNG were still viable, whereas 75% of Ad-ΔE1B-treated mice were viable in the same time period. Similarly, Ad-ΔE1B-DCNG-induced survival benefits were obtained in U87MG xenograft models. Throughout the course of the study, no systemic toxicity, such as diarrhea, loss of weight, or cachexia was observed. These results demonstrate that Ad-ΔE1B-DCNG can confer significant survival advantage and tumor reduction *in vivo*.

Change of tumor characteristics induced by decorin-expressing oncolytic adenovirus

The enhanced anti-tumor effect of decorin-expressing oncolytic adenovirus was further investigated by histological examination. Tumors were harvested from each treatment group at 3 days after the three sequential treatments. Hematoxylin and eosin (H & E) staining revealed that the majority of remaining tumor mass treated with Ad-ΔE1B-DCNG was necrotic, whereas necrotic lesions were only detectable in the limited region of tumors treated with Ad-ΔE1B (Fig. 6A). Viral distribution within the tumor mass was then confirmed by immunohistochemistry using an antibody specific to adenoviral hexon protein. Marked increase in number of adenoviral particles was detected in wider areas of Ad-ΔE1B-DCNG-treated U343 and U87MG tumors in

comparison to Ad-ΔE1B-treated tumors. To analyze the degree to which Ad-ΔE1B-DCNG induced apoptosis *in vivo*, TUNEL assay was carried out. As seen in Fig. 6A, apoptotic level was significantly higher in Ad-ΔE1B-DCNG-treated tumor tissue than in PBS- or Ad-ΔE1B-treated tumor tissue, thus demonstrating that Ad-ΔE1B-DCNG adenovirus replicates actively in the viral injection site and spreads widely, contributing to the induction of apoptosis and necrosis.

Change of ECM components in tumor mass treated with decorin-expressing adenovirus

Tumor section slides from the tumor-bearing nude mice treated with Ad-ΔE1B or Ad-ΔE1B-DCNG were first stained using biebrich's scarlet acid fuchsin (Masson's trichrome stain) to analyze the distribution of collagen fibers (stained blue color), a major component of extracelluar matrix. As shown in Fig. 6B, U343 tumors treated with Ad-ΔE1B consisted of high content of collagen fibers (blue staining). In marked contrast, tumors treated with Ad-ΔE1B-DCNG appeared to be devoid of collagen fibers, indicating that decorin expression dramatically reduced the content of collagen fibers within the tumor mass. We further stained the tumor tissues with antibodies specific to collagen type I, collagen type III, and elastin. All these ECM components were prominent in the PBS- and Ad-ΔE1B-treated control tumor tissues, whereas almost no immunopositivity was observed in Ad-ΔE1B-DCNG-treated tumors, mirroring the findings with the Massons's trichrome staining. These results closely parallel and support the increased viral spread in the tumor mass and enhanced anti-tumor effect of decorin-expressing oncolytic adenovirus.

Inhibition of tumor metastasis by decorin-expressing oncolytic adenovirus

Since degradation of extracellular matrix has the potential to increase tumor metastasis, the impact of decorin expression on metastatic potential was examined using a spontaneous metastasis model. B16BL6 melanoma cells (1.5×10^5/mouse) were implanted subcutaneously to form a local primary tumor on the right hind footpad of C57BL/6 mice, and metastases to distant organs were measured after the removal of the primary tumor. As shown in Fig. 7, B16BL6 metastatic tumor nodules in the lung were considerably reduced by treatment with Ad-ΔE1B or Ad-ΔE1B-DCNG. The average number of metastasized nodules in the lung from mice treated with Ad-ΔE1B and Ad-ΔE1B-DCNG was 8.3 ± 2.4 and 2.3 ± 0.9 compared to PBS-treated control group (41 ± 8.2), translating to 80% ($P < 0.01$) and 94% ($P < 0.01$) inhibition, respectively. Moreover, treatment with Ad-ΔE1B-DCNG further decreased pulmonary metastasis relative to Ad-ΔE1B, showing 72% inhibition ($P < 0.05$). These data indicate that the intratumoral injection of oncolytic adenovirus expressing decorin in primary tumor site greatly reduced and not enhanced the formation of metastatic lesions at distal sites compared to its cognate control oncolytic adenovirus in this model system.

Discussion

An important issue that has received little attention, but is fundamentally critical in improving viral-based cancer gene therapy, is the limited distribution of viruses within solid tumor tissues. Recent evidence has shown that connective tissue and ECM may play a prominent role in inhibiting viral spread. Harrison et al. showed that even though viable replicating viruses persisted within the bulk of the tumor, the connective tissue within established tumors hindered its spread and thus blocked complete tumor eradication [6]. Similarly, it has been shown that viral spreading is impaired by the presence of tumor-supporting structures [12]. Moreover, in a phase I clinical trial for recurrent glioma, it has been demonstrated that the zone of transduced cells did not extend beyond 5 mm from the site of injection [21]. To infect cells that are distally located from the site of viral inoculation, replicating adenovirus must transverse the extracellular space between cells and connective tissue. Therefore, strategies aimed at increasing the extent of anatomic distribution of replicating virus within a tumor mass would be desirable and timely.

To address this issue, we explored the use of decorin to enhance viral spreading and tumor tissue penetration. The enhanced gene transfer efficiency and viral spreading of dl-LacZ-DCNG was evident in solid tumor mass *in vivo*, where intratumoral injection with dl-LacZ-DCNG resulted in much more extensive LacZ staining throughout the entire tumor tissue than that with dl-LacZ. Further, mutant decorin-expressing adenoviruses, dl-LacZ-DCNQ and dl-LacZ-DCNK, lost their ability to enhance gene transfer efficiency and tissue penetration in tumor tissues. In accordance with these results, GFP-expressing dl-GFP-DCNG also increased viral spreading and tumor tissue

penetration in U87MG xenograft models as well as human primary tumor tissues, confirming the decorin-mediated increase in transduction efficiency. Taken together, these lines of evidence demonstrate that decorin expression significantly enhanced the transgene expression carried by adenoviral vectors and facilitated the viral dispersion throughout the tumor tissue both *in vitro* and *in vivo*.

The role of decorin in markedly enhancing the transduction efficiency of decorin-expressing adenoviruses (dl-LacZ-DCNG & dl-GFP-DCNG) is likely due to its role in degrading the ECM of tumors/spheroids. We observed that the gene transfer efficiency of dl-LacZ-DCNG in comparison to dl-LacZ was more intensified when these engineered adenoviruses were injected multiple times (Data not shown). Since tumor spheroids were cultured for 3 days in the culture medium, residual adenovirus can infect cells continuously into the core of the spheroid. Similarly *in vivo*, established xenografts were treated repeatedly, allowing ample time for decorin to reduce the ECM content of the tumor tissue by the initial exposure and subsequent penetration and spread of Ads by the repeated treatment. In both cases, previous exposure to dl-LacZ-DCNG seems to make the tumor environment much more favorable to subsequent viral infections.

Utilization of decorin also successfully enhanced viral spread of replicating adenovirus, leading to an increased anti-tumor effect. In CPE assay, Ad-ΔE1B-DCNG showed increased cell killing potency compared to Ad-ΔE1B. Of note, the cell killing effect of Ad-ΔE1B-DCNQ and Ad-ΔE1B-DCNK was decreased as the binding affinity to collagen was decreased, demonstrating that the increase of cell killing effect is driven by decorin's action on extracellular matrix. The enhanced cell killing potency of decorin-expressing adenovirus also translated into *in vivo*, as greater anti-tumor and

survival advantage was observed for Ad-ΔE1B-DCNG treated tumor bearing mice. In particular, the anti-tumor activity of Ad-ΔE1B-DCNG in the U87MG xenograft models was striking, as 5 out of 7 mice underwent complete regressions. Viral persistence and distribution within tumor tissues were confirmed by immunohistohemistry, where a marked increase in number of adenoviral particles in Ad-ΔE1B-DCNG-treated tumor was detected as compared to control. Ad-ΔE1B-DCNG also substantially decreased ECM components within tumor tissues, while normal tissue adjacent to the tumor cells was not affected. These findings provide further support for the ECM's role in inhibiting the viral spread what we have previously demonstrated with relaxin [15].

Decorin has a high affinity binding site for TGF-β, so that binding of decorin to TGF-β neutralize its biological activity [17]. TGF-β is a pro-fibrotic cytokine that induces the accumulation of ECM via the inhibition of proteolytic enzymes and the enhancement of their tissue inhibitors. We found that TGF-β protein levels were reduced by decorin-expressing adenoviruses (dl-LacZ-DCNG & Ad-ΔE1B-DCNG) not only in U343 glioma cells but also in tumor tissues, supporting the notion that decorin is a natural inhibitor of TGF-β (Supplement Data. 1 & 2). In addition, the proteoglycan decorin is known to bind collagen fibrils near the d bands and delays the lateral assembly of collagen fibrils [16]. In accordance with these results, we observed that the level of collagen fibrils was significantly decreased in the tumor tissues treated with decorin-expressing adenovirus.

Finally, intratumoral administration of Ad-ΔE1B-DCNG did not enhance but inhibited the formation of pulmonary metastases of B16BL6 melanoma cells in mice. Treatment with Ad-ΔE1B-DCNG resulted in a 94% inhibition ($P < 0.01$) of pulmonary metastases versus 80% ($P < 0.01$) for Ad-ΔE1B relative to PBS treatment, suggesting

that decorin-expressing oncolytic adenovirus can elicit potent anti-tumor efficacy not only against solid tumor but also against metastatic tumor lesions. Moreover, the therapeutic advantage of treatment with decorin-expressing oncolytic adenovirus demonstrated in this study using a murine spontaneous metastatic model would be greatly enhanced in a human system because human tumor cells are much more permissive to adenoviral infection and replication.

The pressing challenge for contemporary gene therapy is to deliver enough therapeutic genes to enough cancer cells *in vivo*. The application of decorin to enhance the ability of adenovirus to facilitate cell-to-cell dispersion resulted in significantly enhanced anti-tumor effect of replicating adenovirus in a variety of tumor xenograft models. This study is the first to report utilization of decorin to achieve therapeutic benefit with increased distribution of viral vectors in tumor tissues. In addition, we also demonstrate the usefulness of replication-incompetent adenovirus-mediated transfer of decorin as well as that of oncolytic adenovirus-mediated transfer of decorin. In summary, our findings presented here indicate that the expression of decorin may be a useful strategy in enhancing the efficacy of virus-mediated cancer gene therapy.

Materials and Methods

Cell lines and cell culture

U343 and U87MG human glioma cell lines and HEK293 (human embryonic kidney cell line expressing the adenoviral E1 region) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (50 μg/ml). The murine B16BL6 melanoma cells [15] were grown in MEM supplemented with 5% FBS, vitamin solution (1 mM), sodium pyruvate (100 mM), nonessential amino acid (10 mM), penicillin (500 IU/ml), and streptomycin (50 μg/ml). All cell lines were maintained 37^{o}C in a humidified atmosphere at 5% CO_2.

Experimental animals

In vivo anti-tumor experiments were conducted using 6- to 8-week-old male nude mice (BALB/c-nu) and C57BL/6 mice purchased from Charles River Korea (Seoul, Korea). All mice were maintained in a laminar air-flow cabinet under specific pathogen-free conditions. All facilities are approved by AAALAC (association of assessment and accreditation of laboratory animal care), and all animal experiments were conducted under the institutional guidelines established for the Animal Core Facility at Yonsei University College of Medicine.

Generation of decorin-expressing adenoviruses

To generate adenoviruses expressing decorin at the E3 region, wild-type and mutant decorin genes were first excised from pcDNA3.1-DCNG (containing the wild-

type decorin cDNA), pcDNA3.1-DCNQ (containing mutant-type decorin cDNA having weaker binding affinity to collagen), and pcDNA3.1-DCNK (containing mutant-type decorin cDNA lacking binding affinity to collagen) [19,20] using *EcoR*I-*Xba*I and resulting 1 kb DNA fragments were subcloned into the pCA14 vector (Microbix, Ontario, Canada), generating pCA14-DCNG, pCA14-DCNQ, and pCA14-DCNK vector, respectively. The mutant decorin DCNQ harbors a point mutation of E180 amino acid to E180Q in the sixth leucine-rich repeat of the wild type decorin core protein. The mutant decorin DCNK harbors an amino acid exchange at amino acid E180, which is an important site for the interaction of decorin with type I collagen fibrils. Each of the pCA14-DCNG, pCA14-DCNQ, and pCA14-DCNK vectors were digested with *Bam*HI, and the CMV-decorin-polA expression cassettes were then subsequently cloned into the adenoviral E3 shuttle vector pSP72-E3 [22] resulting in pSP72-E3/CMV-DCNG, pSP72-E3/CMV-DCNQ, pSP72-E3/CMV-DCNK adenoviral E3 shuttle vectors. The adenoviral E3 shuttle vectors thus prepared were linearized with *Xmn*I and cotransformed into *E. coli* BJ5183 together with the adenoviral total vector, pdl-LacZ linearized with *Spe*I for homologous recombination, generating the replication-incompetent adenoviral vectors dl-LacZ-DCNG, dl-LacZ-DCNQ, and dl-LacZ-DCNK which express the LacZ and decorin simultaneously. We also generated tumor-specific oncolytic adenoviruses expressing the decorin gene. Specifically, each of dl-LacZ-DCNG, dl-LacZ-DCNQ, and dl-LacZ-DCNK was linearized with *Xmn*I and cotransformed into *E. coli* BJ5183 together with the E1B19 kDa/E1B55 kDa-deleted pAdΔE1B19/55 adenovirus vector linearized with *Spe*I [23] for homologous recombination, generating the Ad-ΔE1B-DCNG, Ad-ΔE1B-DCNQ, and Ad-ΔE1B-DCNK adenovirus vectors, respectively. To verify the occurrence of homologous recombinants, the recombinant adenoviral vectors were

digested with *Hind*III, and the digestion pattern was analyzed. The proper homologous recombinant adenoviral vectors were digested with *Pac*I and transfected into 293 cells to generate adenoviruses. All adenoviruses were propagated in 293 cells and their titration was performed according to limited dilution assay, followed by concentration using CsCl gradient and purification. Viral particle (v.p.) numbers were also calculated from measurements of optical density at 260 nm (OD_{260}), where 1 absorbency unit is equivalent to 10^{12} viral particles per milliliter.

Examination of decorin expression

To examine the decorin expression pattern induced by generated recombinant adenoviruses, replication-incompetent adenoviruses and oncolytic adenoviruses harboring the decorin gene (dl-LacZ-DCNG, dl-LacZ-DCNQ, dl-LacZ-DCNK, Ad-ΔE1B-DCNG, Ad-ΔE1B-DCNQ, and Ad-ΔE1B-DCNK) and dl-LacZ and Ad-ΔE1B adenovirus as negative controls were infected to U343 at 20 MOI for replication-incompetent adenoviruses and at 5 MOI for oncolytic adenoviruses. At 48 hr after infection, the medium used was recovered and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins on the gel were electro-transferred to PVDF membrane, incubated with the primary anti-decorin antibody (AF143; R & D Systems, Minneapolis, MN) and anti-β-actin antibody (Sigma, St. Louis, MO), and then incubated with the HRP (horse radish peroxidase)-conjugated secondary antibody (6160-05; Southern Bio Technology Associates, Inc. Birmingham, AL), after which the expression patterns of decorin were revealed using the ECL detection kit (sc-2004; Santa Cruz Biotechnology, Santa Cruz, CA).

Evaluation on spreading and penetration of adenoviruses in vivo

To determine the extent of viral spreading and tumor penetration *in vivo*, U343 and U87MG human glioma xenografts were established subcutaneously by injecting cells into the abdomen of 6- to 8-week-old male nude mice. Once the tumors reached to 100-200 mm^3 in volume, mice were randomized and dl-LacZ, dl-LacZ-DCNG, dl-LacZ-DCNQ, or dl-LacZ-DCNK adenovirus at 5 x 10^8 PFU in 50 μl was intratumorally injected into the tumors three times, every other day. On day 3 after the last injection, animals were sacrificed and tumors were taken, after which they were fixed in 4% paraformaldehyde (PFA) at 4□ for 4-8 hr and dehydrated in 30% sucrose solution for 12 hr. The dehydrated tumor tissues were embedded in O.C.T. compound and snap frozen. Observance of blue staining with a light microscopy under low power was considered positive for β-galactosidase expression. Each experiment was carried out three to four times, and data from a representative experiment are shown.

Evaluation on transduction efficiency and tissue penetration potency of decorin-expressing adenoviruses using tumor tissues from breast cancer and glioma patient

Tumor tissues from breast cancer and glioma patients were freshly collected, cut into 1-2 mm sections and then plated onto HydroCell 12 Multi-well plates (CellSeed, Japan) after which they were cultured for 4 hr in IMDM (Isocove's Modified Dulbecco's Medium, Gibco BRL) supplemented with 5% FBS, 10 μM/L insulin and 1 μM/L hydrocortisone. Each of adenoviruses (dl-GFP or dl-GFP-DCNG for breast cancer spheroids and dl-LacZ or dl-LacZ-DCNG for glioma spheroids) at 1 x 10^7 PFU (breast cancer spheroids) or 1 x 10^8 PFU (glioma spheroids) were added into the plates containing primary tumor tissues, and incubated at 37□ in 5% CO_2 incubator for 11

(breast cancer spheroids) and 3 (glioma spheroids) days. For the observation on penetration of adenovirus into tumor spheroid, the X-gal stained tumor spheroids were embedded in O.C.T. compound (Sakura Finetec) and snap frozen, followed by performing X-gal staining described above. The glioma spheroids were observed under a fluorescence microscope.

Anti-tumor effects and survival rates of decorin-expressing oncolytic adenovirus in vivo

Tumors were implanted on the abdomens of 5 to 6 week-old male nude mice by subcutaneous injection of 1 x 10^7 U343 and U87MG glioma cells in 100 μl of Hank's balanced salt solution (HBSS; Gibco BRL). When tumors reached a range of 70~100 mm^3, animals were randomly assigned to one of three groups to receive PBS, Ad-ΔE1B, or Ad-ΔE1B-DCNG (seven or eight mice per group). The first day of treatment was designated as day 0. Adenovirus or PBS was administered intratumorally (5 x 10^8 PFU in 50 μl of PBS) on days 0, 2, and 4. Tumor growth delay was assessed by talking measurements every 2 or 3 days. The volume of tumors was calculated with the major axis and minor axis measured using a caliper: tumor volume = (minor axis mm)^2 x (major axis mm) x 0.523. The percentage of surviving mice was determined by monitoring the death of mice (for U87MG xenografts) or tumor growth related events (tumor size > 1500 mm^3 for U343 xenografts)

Change of metastatic potential over decorin expression using spontaneous metastasis model

To assess changes of metastatic potential over decorin expression, B16BL6 cells (1.5 x 10^5/mouse) were administered subcutaneously into the right hind foot pad of 6

week-old male C57BL/6 mice (Charles River Korea, Seoul, Korea) to form primary tumors. When the primary tumor reached a volume of about 200 mm^3, animals were randomly assigned to one of three groups to receive PBS, Ad-ΔE1B, or Ad-ΔE1B-DCNG (six to eight mice per group). The first day of treatment was designated as day 0, and then 5 x 10^8 PFU per tumor in 50 μℓ of PBS, mixed with Lipofectamine and Plus solution (Gibco BRL) at a 2 : 6 ratio or PBS was injected directly into the tumor on days 0, 2, and 4. On day 7, primary tumors were surgically removed by amputating the right hind leg below the knee under mild anesthesia. On day 28 after primary tumor lesions in the lungs of the mice was assessed.

Observation of the change of tumor characteristics induced by the administration of decorin-expressing adenovirus

Tumor tissue was fixed in 10% formalin, embedded in paraffin and cut into 3-μm sections (Wax-it; Vancouver, Canada). Representative sections were stained with hematoxylin-eosin or with Masson's trichrome and then examined by light microscopy. We used reagents supplied by DAKO ARK (Dako, Carpinteria, CA) to detect adenoviral antigens, as described previously [24]. The same paraffin slides were also used to identify apoptotic cells in tumors according to the instructions in the ApopTag kit for detecting cleaved deoxyribonucleic acids in situ with the TUNEL method. For the detection of collagen type I, collagen type III, and elastin in tumor tissues, paraffin tumor sections were first treated with monoclonal anti-collagen type I antibody (sc-25974; Santa cruz biotechnology), monoclonal anti-collagen type III antibody (C7805; Sigma, St. Louis, MO), or monoclonal anti-elatin antibody (E4013; Sigma), and then with goat anti-mouse IgG-Biotin (sc-2039; Santa Cruz Biotechnology) as a secondary

antibody. Finally, diaminobenzidine/hydrogen peroxidase (Dako) was used as the chromogen substrate. All slides were counterstained with Meyer's hematoxylin.

Statistical Analysis

The data were expressed as mean ± standard error of the mean (SEM). Statistical comparison was made using Stat View software (Abacus Concepts, Inc., Berkeley, CA) and the Mann-Whitney test (nonparametric rank sum test). The criterion for statistical significance was taken as $P < 0.05$.

Acknowledgments

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Figure Legends

Fig. 1. Representative Western blot analysis of human decorin from medium collected at day 2 after infection. The results showed a significant expression of the virally-transduced decorin gene.

Fig. 2. Enhanced transduction efficiency and viral spreading of decorin-expressing adenoviruses. (A) U343 and U87MG xenografts were established in nude mice, and then intratumorally injected with dl-LacZ , dl-LacZ-DCNG, dl-LacZ-DCNQ, or dl-LacZ-DCNK adenovirus (5 x 10^8 PFU) three times every other day. At 3 days after the last injection of adenovirus, tumors were harvested and processed for LacZ staining. Original magnification: x 40 and x 400. (B) The U87MG xenografts established in nude mice was injected three times with dl-GFP or dl-GFP-DCNG (each at 5 x 10^8 PFU). Three days after the last injection of adenoviruses, tumors were harvested and photographed with a confocal microscope. In the tumors treated with dl-GFP, we observed a narrow path of fluorescence that appeared to follow the needle track when we injected the viruses. In contract, in the tumors treated with dl-GFP-DCNG, fluorescence was distributed through the tumor mass. Original magnification: x 100.

Fig. 3. Tumor spheroids prepared from a human patient with glioma (A) and breast cancer (B). Spheroids were transduced with either dl-LacZ or dl-LacZ-DCNG for glioma spheroids (1 x 10^8 PFU) and either dl-GFP or dl-GFP-DCNG for breast cancer spheroids (1 x 10^7 PFU). The glioma spheroids were photographed with a light microscope for LacZ expression at 3 days post-infection and breast cancer spheroids were photographed with a fluorescence microscope for GFP expression at 11 days post-

infection. Increased gene transfer, as shown by green fluorescence or LacZ staining, was achieved with decorin-expressing adenoviruses than with the control adenoviruses. Original magnification: x 40.

Fig. 4. Enhanced cytopathic effect of decorin expressing oncolytic adenovirus. Cells were infected with dl-LacZ, Ad-ΔE1B, Ad-ΔE1B-DCNG, Ad-ΔE1B-DCNQ, or Ad-ΔE1B-DCNK at the indicated MOI. At 4-8 days post-infection, surviving cells were stained using crystal violet. Replication-incompetent adenovirus dl-LacZ served as a negative control. Each cell line was tested at least three times, and data shown are from representative experiments.

Fig. 5. Enhanced suppression of the growth of established tumors (A) and survival (B) following intratumoral injection with Ad-ΔE1B-DCNG compared to Ad-ΔE1B oncolytic adenovirus. Human glioma tumors were grown subcutaneously in the abdomen of nude mice and were subsequently injected directly with PBS (◆), Ad-ΔE1B (◇), or Ad-ΔE1B-DCNG (○). Tumor volume was monitored regularly following treatment. The arrow indicates when treatment was given (5 x 10^8 PFU). Values represent the mean ± SEM (n=8 animals per group for U343; n=7 animals per group for U87MG). There was a statistically significant enhanced anti-tumor effects and longer survival in Ad-ΔE1B-DCNG-treated mice compared to Ad-ΔE1B treatment ($P< 0.05$).

Fig. 6. Representative photomicrographs of U343 and U87MG tumor tissues demonstrating histological changes in response to administration of PBS, Ad-ΔE1B, or Ad-ΔE1B-DCNG. (A) H & E-stained sections of PBS-, Ad-ΔE1B-, or Ad-ΔE1B-

DCNG-treated tumor tissue (H & E). Immunohistochemical staining of adenoviral hexon protein to localize adenovirus in tumor tissue (Ad hexon). Adenovirus-infected cells stain brown. Induction of apoptosis in tumor tissue was examined by TUNEL assay (TUNEL). Original magnification: x400. (B) Masson's trichrome staining of ECM (blue staining) present in the tumor tissue sections (M & T). Tumors treated with Ad-ΔE1B-DCNG were significantly smaller than those treated with PBS or Ad-ΔE1B. Note the extensive bundles of collagen fibrils that stained blue with Masson's trichrome in the tumors treated with PBS or Ad-ΔE1B (arrow). Masson's trichrome-stained section of tumor tissue from Ad-ΔE1B-DCNG-treated animal was devoid of parenchymal trichrome staining, but murine normal tissue adjacent to tumor tissue was stained for collagen (arrow). Original magnification: x1, x40 and x400; A higher magnification (x400) of the boxed area in x40 magnification. Immunostaining for collagene type I, collagen type III, and elastin. The content of ECM components are also significantly reduced in Ad-ΔE1B-DCNG-treated tumor tissues.

Fig. 7. Therapeutic efficacy of Ad-ΔE1B-DCNG on spontaneous pulmonary metastasis. When the B16BL6 primary tumor reached a volume of about 200 mm^3, PBS, Ad-ΔE1B, or Ad-ΔE1B-DCNG was administered intratumorally three times every other day. On day 7 after viral injection, the primary tumors were then surgically removed. On day 28 after primary tumor removal, the number of metastasized nodules in the lungs of the each mouse was counted and plotted. Each point represents the tumor nodules for every individual mouse (6 mice for PBS group, 7 mice for Ad-ΔE1B, and 8 mice for Ad-ΔE1B-DCNG) and the mean number of metastasized nodules for each group is shown with a line. $*P < 0.01$ vs. PBS-treated control and $*P < 0.05$ vs. Ad-ΔE1B-treated group.

Supplement Data Legends

Supplement Data 1. Suppression of TGF-β expression in decorin-expressing adenovirus-treated glioma cells. U343 cells were transduced with either dl-LacZ or dl-LacZ-DCNG at different MOIs, and TGF-β expression was measured in the culture medium by conventional ELISA kits. The results showed a significant suppression of the TGF-β expression upon transduction with decorin-expressing adenovirus, whose levels decreased in a dose-dependent manner.

Supplement Data 2. Immunohistochemical analysis for TGF-β. Formalin-fixed tumor tissues were embedded in paraffin and sectioned. Paraffin tumor sections were stained with anti-TGF-β monoclonal antibody, MAB1032 (Chemicon). Representative histological features of U343 tumors at x400 magnification were documented. The level of TGF-β expression was significantly decreased in the tumor tissues treated with decorin-expressing oncolytic adenovirus, Ad-ΔE1B-DCNG.

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Fig. 1

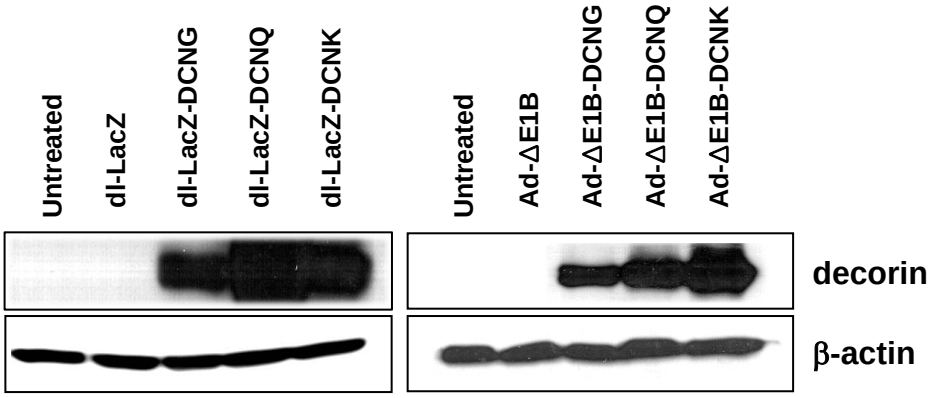
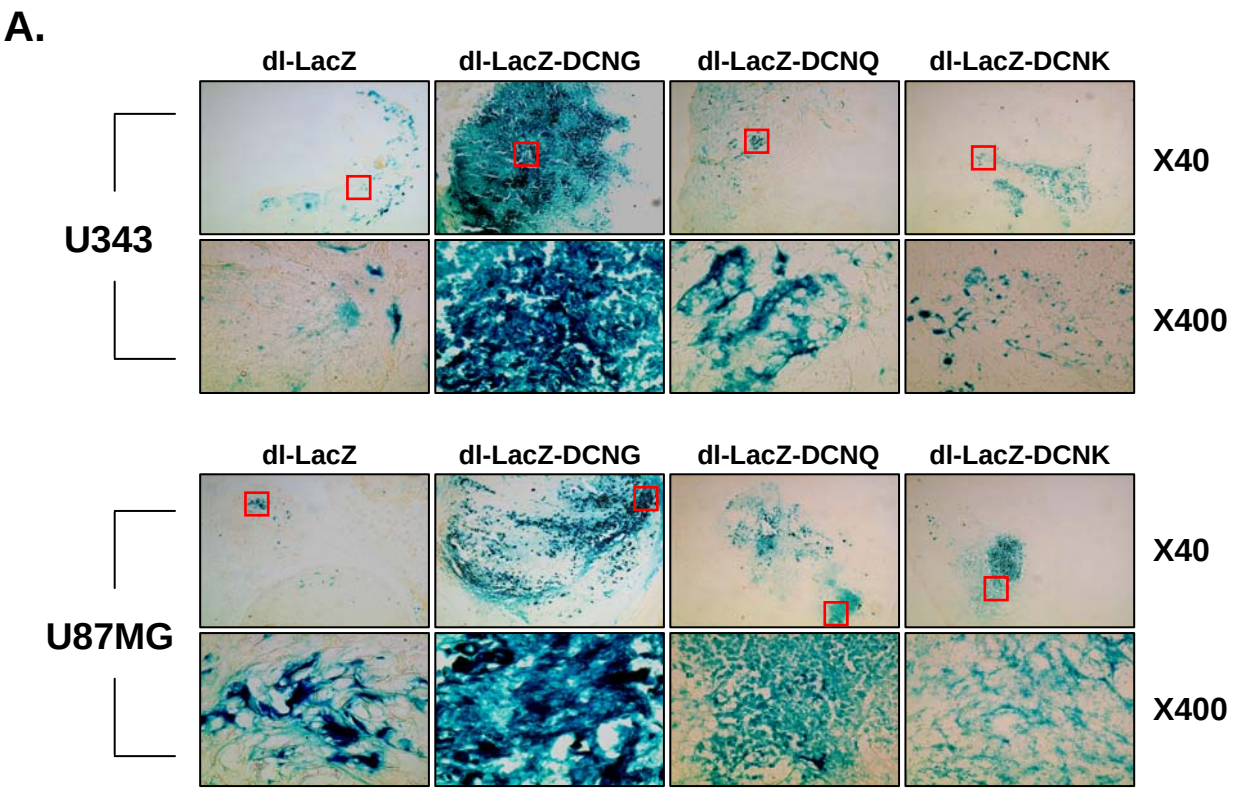


Fig. 2



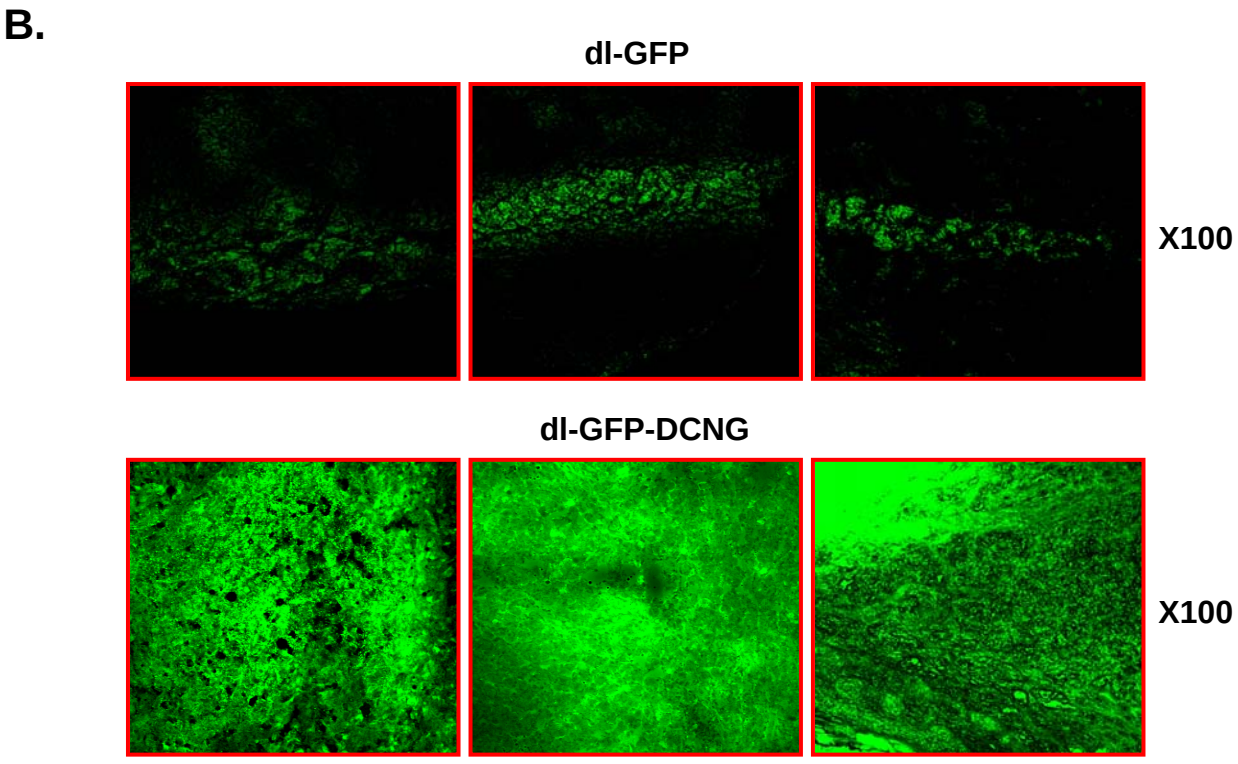


Fig. 3

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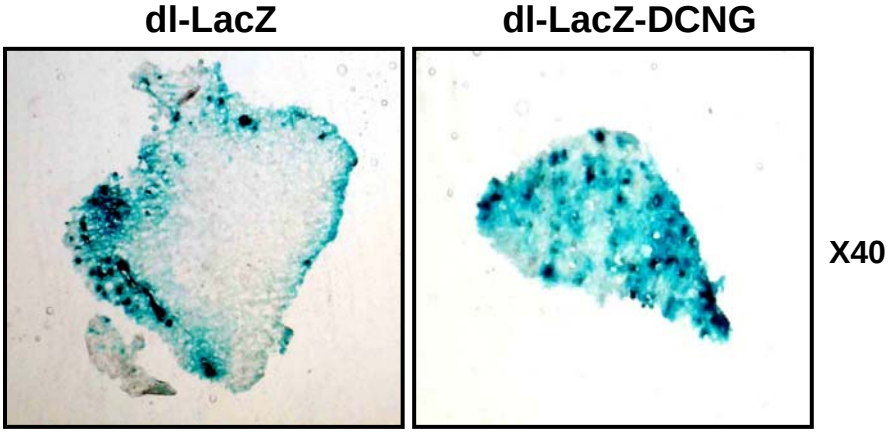

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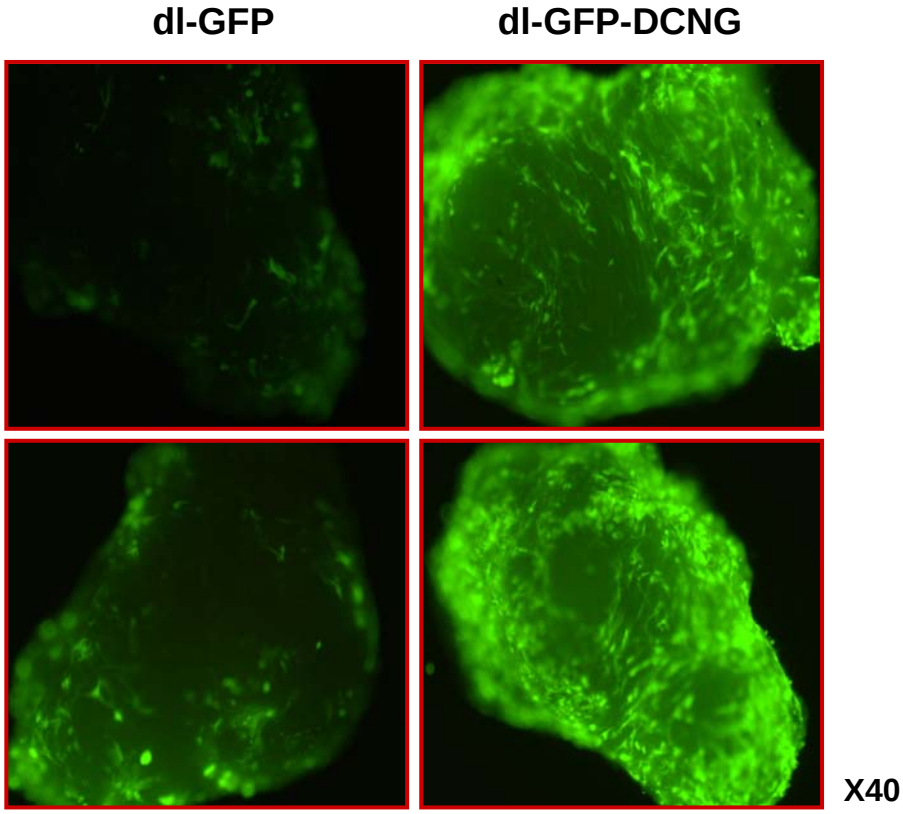

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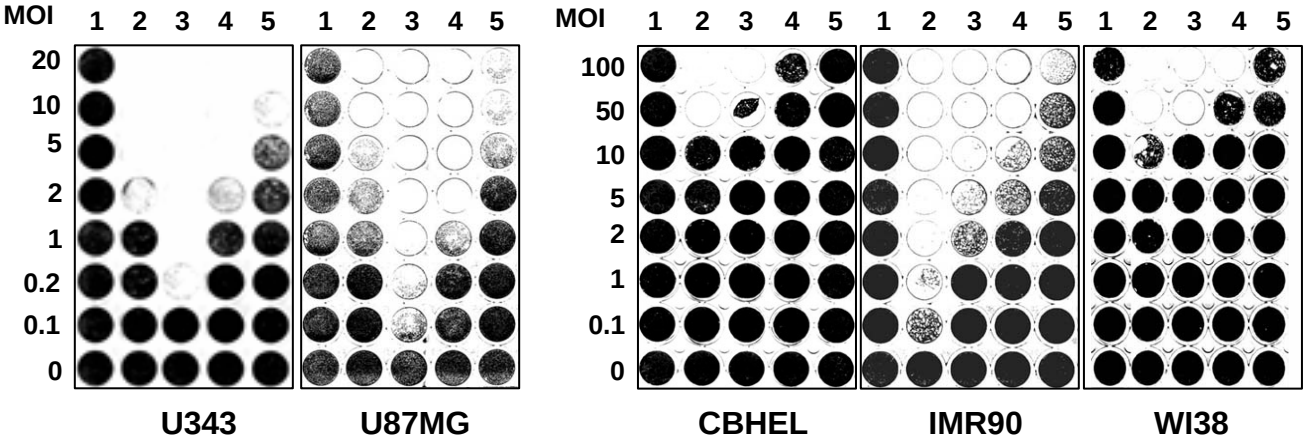

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Fig. 5

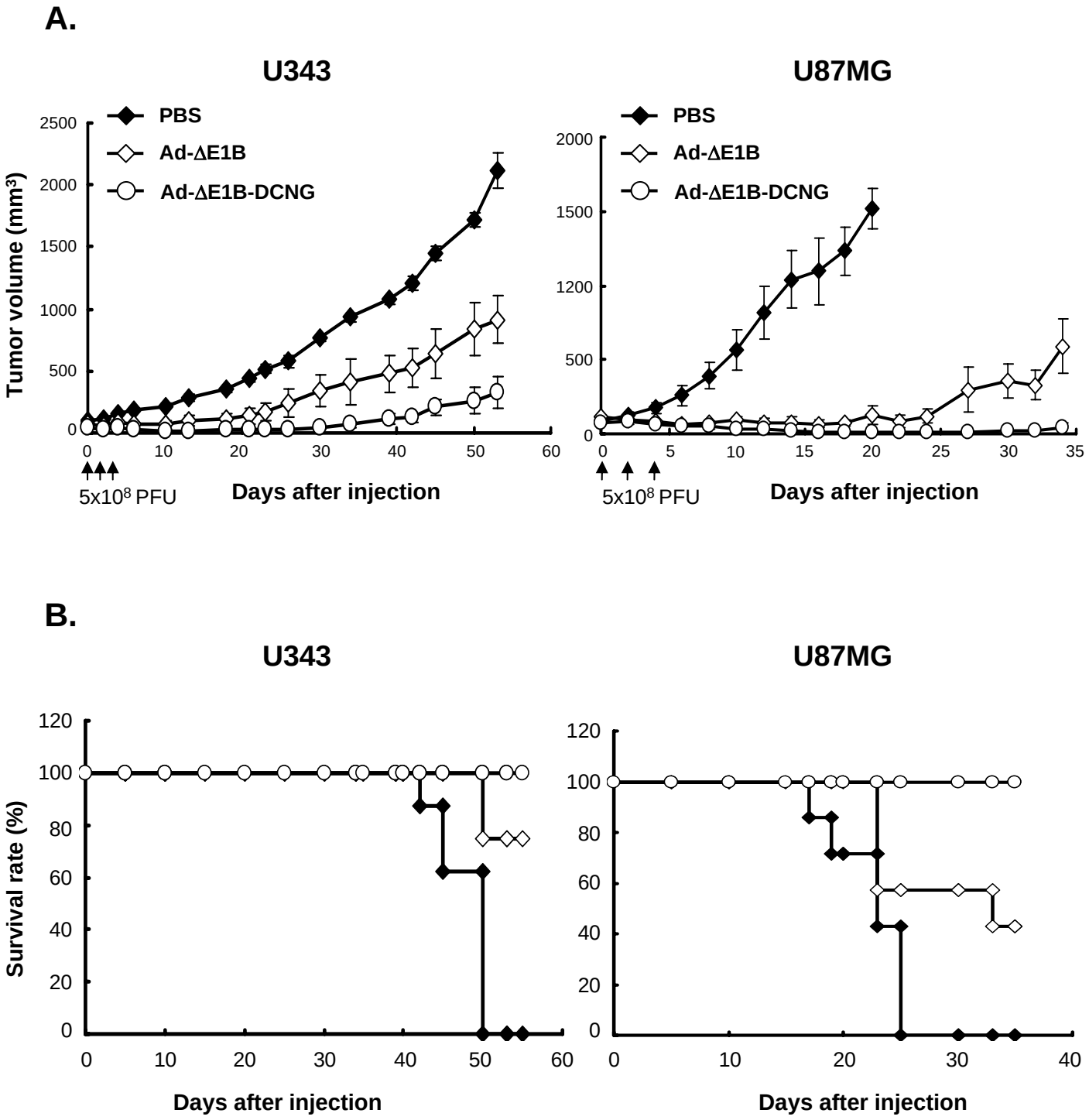


Fig. 6

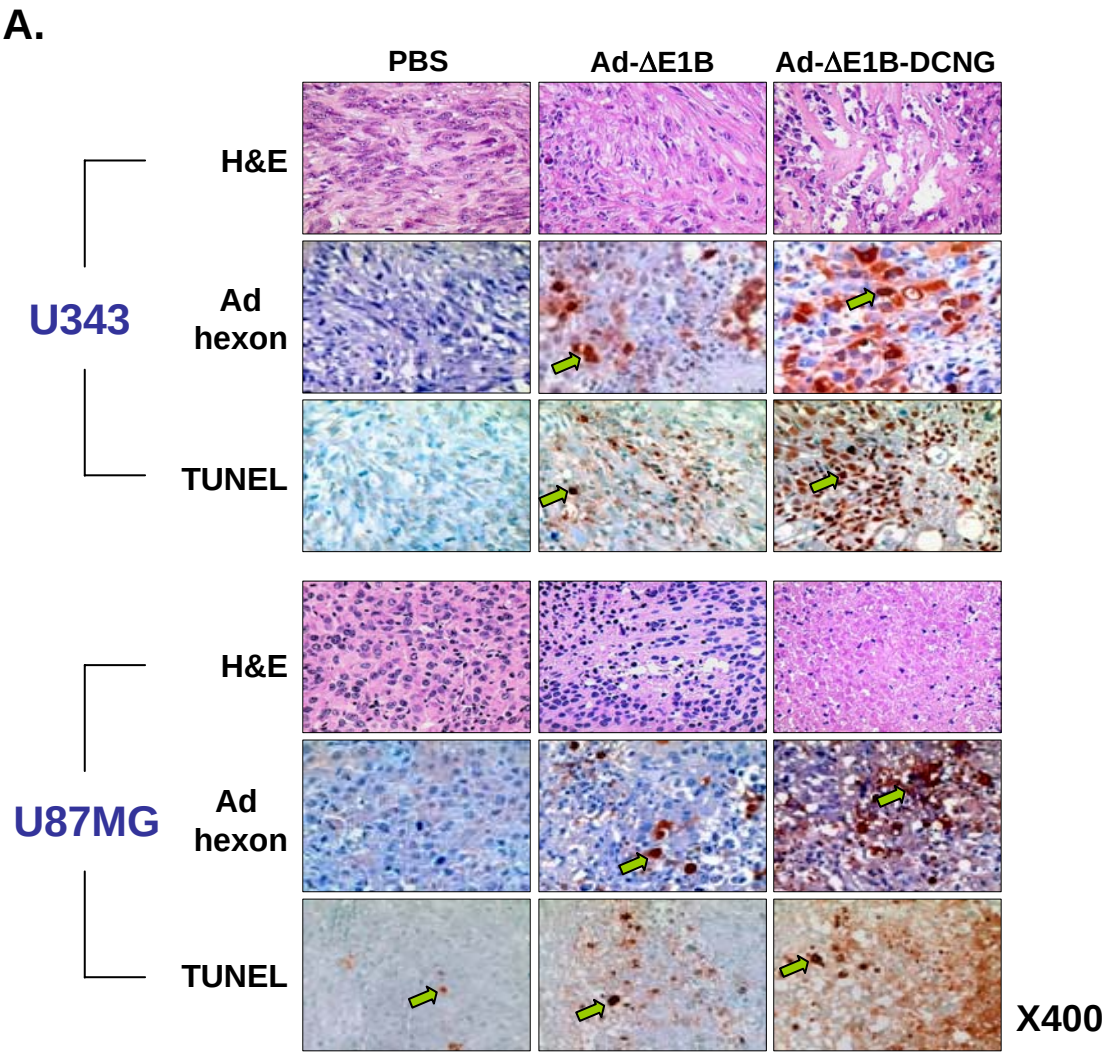


Fig. 6

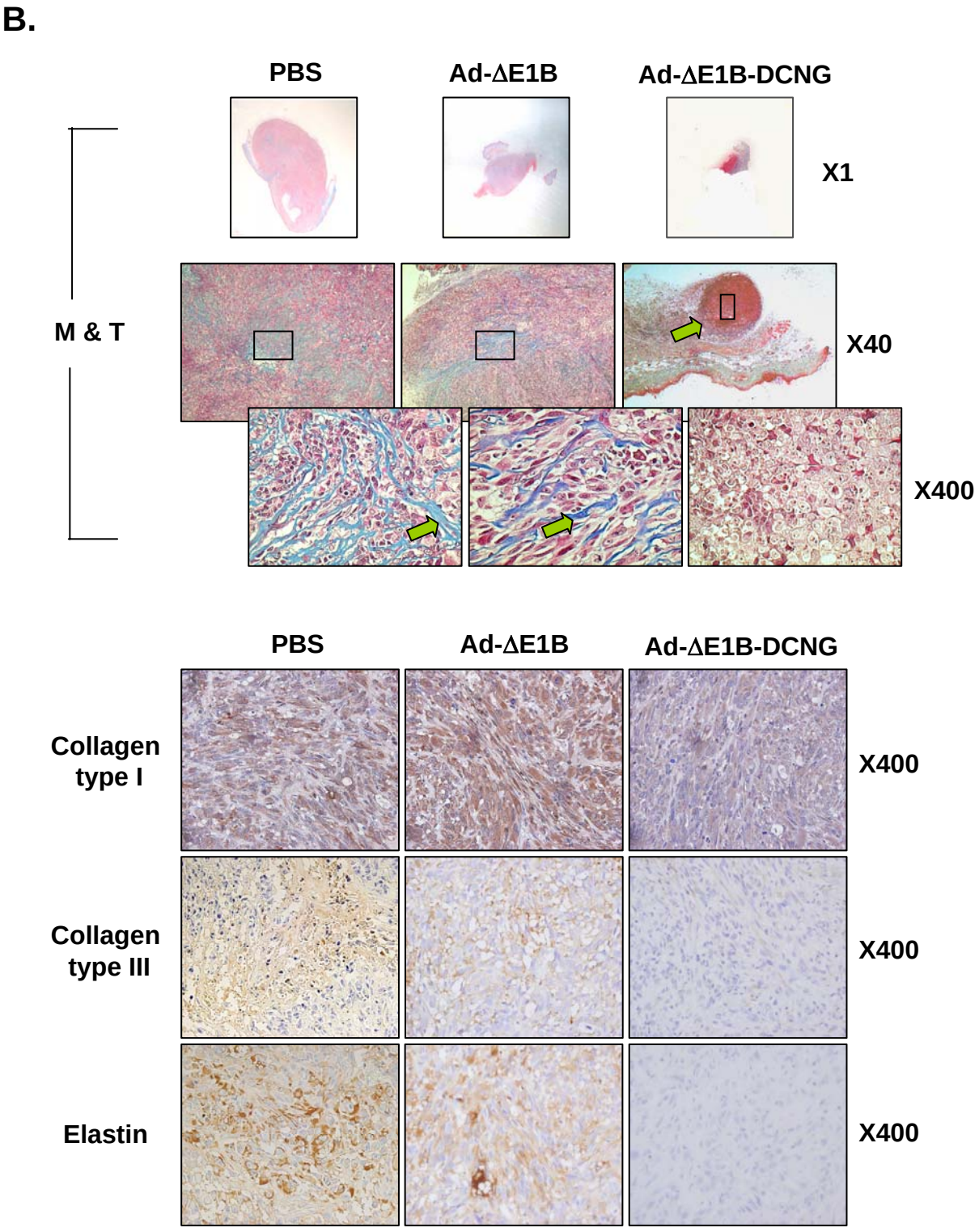
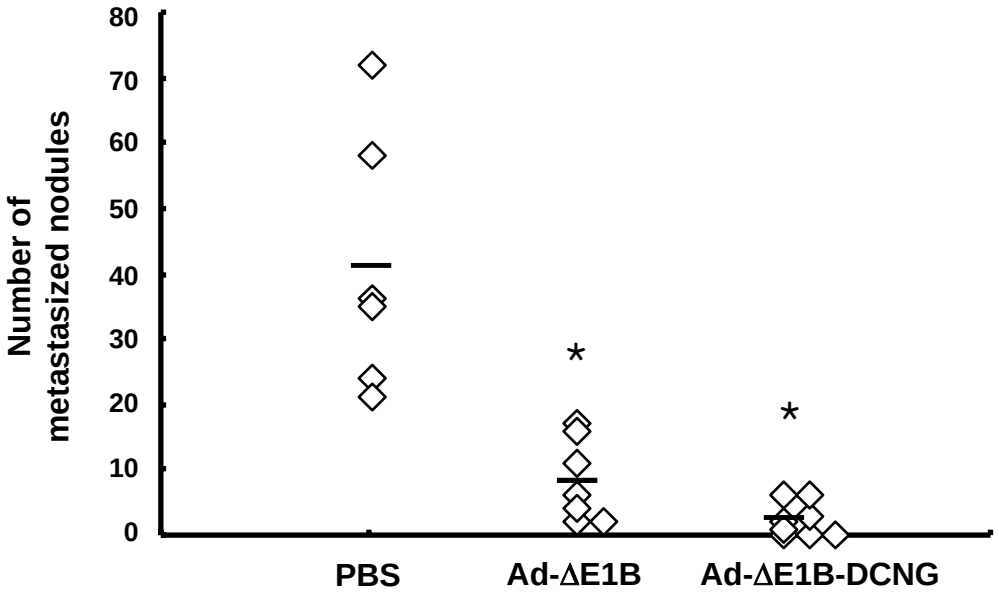
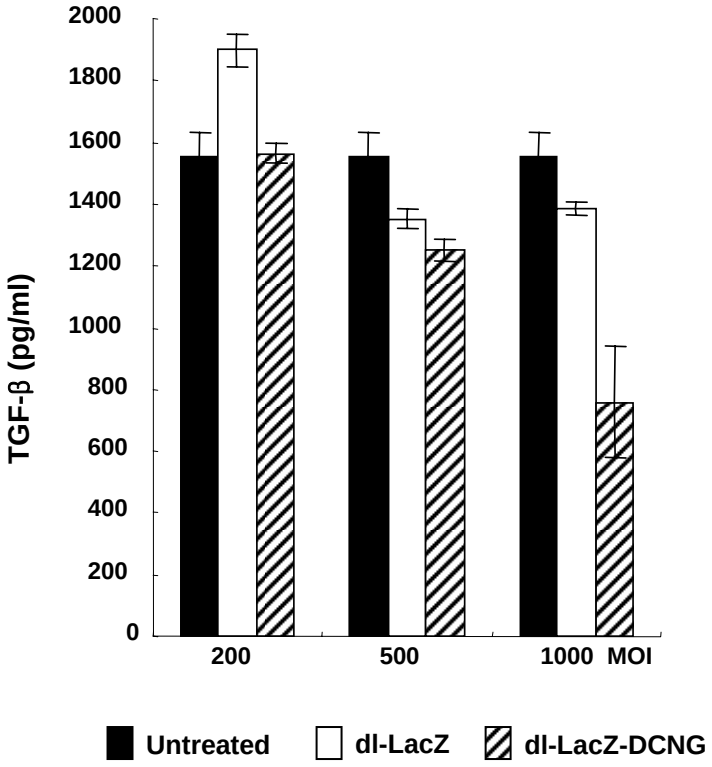


Fig. 7



Supplement Data. 1



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