Role of RGD-containing ligands in targeting cellular integrins: Applications for ovarian cancer virotherapy (Review)

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Abstract. The purpose of this article was to review the current strategies of targeted therapy to integrins and define the best course of future research in ovarian cancer targeting. Cell surface integrin targeting has been used as a strategy for targeted therapy of several diseases with some success. The combination of virotherapy and integrin-targeting shows promise as a method for targeting ovarian cancer. More specifically, targeting of ovarian cancer with integrin-directed adenoviruses may lead to therapy with fewer toxicities and side effects. This article offers a review of the benefits of integrin-specific targeted therapy for several diseases and proposes a unique anti-ovarian cancer strategy involving the combination of the above with virotherapy as a potential anti-ovarian cancer treatment.

Contents

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1. Introduction

Targeted therapy is an attempt to biologically treat diseases by focusing on the delivery of a drug, chemical, or other treatment modality to a specific pathway or molecule in a pathobiologic system in an effort to alter its natural course. In the past two decades, strides have been made in this area of research as the human genome has been solved and the genetic and molecular bases of more and more diseases have been uncovered. These advances in knowledge have resulted in a vast increase in the number of genes, proteins, and pathways that can be targeted for therapy.

One such discovery is the role of integrins in several pathobiological processes. Scientists have attempted to exploit this finding with some success. In this article, we review the role of integrins in the pathobiology of human diseases as well as integrin-targeting as an advanced therapeutic option. We specifically discuss ovarian cancer as a disease for which integrin-targeting therapy has been a promising option, as integrins are also expressed on ovarian cancer cells. In this regard, we provide an overview of the previous attempts to target adenoviral (Ad) vectors to ovarian cancer. We also discuss the possibilities offered by combining integrin-targeting and virotherapy, and the potential benefit for ovarian cancer patients.

2. Integrins

Integrins are a family of cell surface receptors comprised of 18-α and 8-β subunits that combine to form at least 24 different heterodimers to mediate the attachment of cells to the extracellular matrix (ECM) as well as other cells (1,2). Integrins are not only responsible for interactions with the ECM or other cells, but are also responsible for the activation of many signaling pathways, which are necessary to support cell proliferation, migration and viability (3,4). Integrin heterodimers have overlapping specificity for ECM proteins, indicating some redundancy of their roles, which makes it more difficult to delineate the pathways to which they contribute.

Integrin $\alpha_v\beta_3$ is known as a receptor for fibrinogen, fibronectin, von Willebrand's factor, vitronectin, thrombospondin (Tsp), osteopontin and bone sialoprotein 1 (Bspl) (5,6).

It is not typically found on epithelial cells, but is minimally expressed on endothelial cells (7), some B-cells, platelets, monocytes, intestinal cells, and smooth muscle cells, as well as a small percentage of activated leukocytes, macrophages, and osteoclasts (8). Integrin $\alpha_v\beta_3$ mediates platelet aggregation and endothelial cell adhesion to ECM proteins and is also known as a vitronectin receptor, VNR (9). Integrin $\alpha_v\beta_3$ is known to be involved in several physiological pathways, including angiogenesis and osteoclast-mediated bone resorption.

Unlike $\alpha_v\beta_3$, $\alpha_v\beta_5$ is less promiscuous and binds preferentially to vitronectin (10). It is expressed on hepatoma cells, fibroblasts, and carcinoma cells. The exact physiological role of $\alpha_v\beta_5$, also known as αv/βS and αv/β3B (11), is unclear. One known role of $\alpha_v\beta_5$ is to support angiogenesis initiated by vascular endothelial growth factor (VEGF) or transforming growth factor-α. To date, no selective ligand has been identified for integrin $\alpha_v\beta_5$. However, most small molecules that bind to the $\alpha_v\beta_3$ integrin also bind to integrin $\alpha_v\beta_5$ (9).

3. Integrins in pathology

Two integrins that play a major role in the pathogenesis of several cancers are $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (12). These integrins are present on the surface of several cancer cell types (13-15). Bello *et al* reported the presence of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in gliomas, in which expression of these molecules correlated with the histological grade of the tumors (16). Invasive melanoma, but not benign nevi or normal melanocytes, also expresses integrin $\alpha_v\beta_3$ (17). Furthermore, increased rates of melanoma metastases correlate with elevated $\alpha_v\beta_3$ expression (18,19). Analysis of metastatic breast tumors showed from 10- to 15-fold higher expression levels of $\alpha_v\beta_3$ vs. normal tissue counterparts (20). Additionally, expression of $\alpha_v\beta_3$ was determined to be a prognostic factor for the clinical outcome for 197 patients involved in the above clinical study (20). For ovarian cancer, studies have revealed an increased presence of $\alpha_v\beta_3$ on three different cell lines, which is consistent with the effective treatment of intraperitoneal (i.p.) tumors in mice using a fully humanized anti-$\alpha_v\beta_3$ antibody (21).

Tumor metastasis, rheumatoid arthritis and osteoporosis are on the list of pathologies in which $\alpha_v\beta_3$ plays a key role (8). Importantly, $\alpha_v\beta_3$ has been shown to have relatively limited cellular distribution in humans, but is expressed at high levels in the inflamed synovial tissues of rheumatoid arthritis patients (22,23). Integrin $\alpha_v\beta_5$ is often found in the same pathological contexts as $\alpha_v\beta_3$. Integrin $\alpha_v\beta_5$ was found to be overexpressed on the surface of several of 15 tested lung cancer cell lines and to be implicated in the natural mechanism of adenovirus internalization in infected cells, which correlated with the efficiency of gene transfer by the vector monitored by expression of a reporter transgene (24).

The fibrotic response to lung injury is mediated by several factors, including $\alpha_v\beta_5$ (25). The role of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins in neoangiogenesis is underscored by their known association with ocular diseases such as diabetic retinopathy and macular degeneration (26-28). Both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are typically overexpressed on ovarian cancer cells (29) and tumor vasculature (30). For this and other reasons discussed below, ovarian cancer lends itself to the study of targeted therapy involving integrin-targeting.

4. Ovarian cancer

Ovarian cancer is the leading cause of gynecological disease-related death (31). While diagnostic and therapeutic tools have improved significantly over the past 30 years, the 5-year survival rate of women diagnosed with ovarian cancer is still less than 50% (32). There is a 90% cure rate for women with the disease localized exclusively to the ovary at the time of diagnosis. However, only 20% of ovarian cancers are diagnosed at this stage. Due to a lack of ovarian cancer pre-screening methods (such as the ones available for breast and cervical cancers) and because ovarian cancer patients present with common gastrointestinal and genitourinary symptoms, early detection of this disease is difficult, resulting in more than 60% of women being diagnosed when already at the metastatic stage (33). Although ovarian cancer typically disseminates from its original location in the ovary, the metastases are most often localized to the intraperitoneal cavity of the patients. Owing to the unique pathogenesis of this disease, novel ovarian cancer therapies have attempted to capitalize on the physical confinement of metastases to this anatomic compartment to augment the efficacy of targeted therapies.

One approach to simultaneous treatment of the primary tumor and intraperitoneal metastases found at diagnosis is a combinatorial administration of chemotherapy intravascularly (i.v.) and i.p., as opposed to i.v. administration alone. While this treatment regimen has resulted in severe toxicities and side effects, it has also shown a small, but statistically significant, increase in patient survival (34). Armstrong *et al* reported an additional 5 months of disease-free survival and a 16-month increase in life span of patients who received standard of care taxane- and platinum-based chemotherapeutics i.p. vs. those patients who received one drug i.p. and the other i.v. Despite these modest improvements made to conventional ovarian cancer treatments, more effective therapies are needed in order to increase the percentage of patients reaching the 5-year disease-free survival mark (34). To this end, novel anti-cancer treatments are continuously being developed and tested in pre-clinical and clinical studies.

5. Targeted therapies for treatment of ovarian cancer

Tumor survival and progression to metastasis is dependent upon complex changes in the interaction of tumor cells with other cells and the ECM (35). Normal cell-cell interactions must be altered and individual tumor cells must be able to interact with the ECM to migrate and establish distant metastases. Therefore, it is plausible to expect that cell surface motifs needed for abnormal interaction with other cells or with the ECM would be present on the surface of cancer cells. Targeting these structural, genetic, or molecular differences of tumor cells that allow them to behave differently than normal cells may be an effective means to anti-cancer treatment (13). In this regard, scientists have used several strategies to target cancer cells, including: engineered antibodies (16-18), 'homing' peptides that can bind to a targeted organ or tumor cells specifically (30,39,40), and infectivity-enhanced Ads (41,42). Some of the above methods have been used to target receptors present on ovarian cancer cells as well as on other types of cancer cells.

6. Integrin targeting as an anti-cancer therapy

The arginine-glycine-aspartate (RGD) sequence motif is a ligand for many types of integrins (1). However, integrins are capable of distinguishing between different RGD-containing proteins based on the amino acid sequences flanking the RGD motif or the structural context of the RGD presentation (8). In this regard, scientists have designed RGD-containing ligands that specifically bind to certain integrins (43). Integrins, particularly $\alpha_v\beta_3$ and $\alpha_v\beta_5$, are increasingly being used for various tumor-targeting and imaging applications. Recently, targeting of nanoparticles to integrin $\alpha_5\beta_1$ allowed noninvasive NMR-based imaging of tumor-induced angiogenesis owing to preferential expression of this RGD-binding integrin by the endothelium of tumor vasculature. These findings supported the utility of $\alpha_5\beta_1$ for effective and selective targeting of tumor vasculature (44).

Targeting of $\alpha_v\beta_3$ with either antibodies or small molecules has led to the inhibition of migration, adhesion, motility, angiogenesis and proliferation of several cell types *in vitro* and *in vivo* (45-49). Several anti-$\alpha_v\beta_3$ humanized antibodies, such as Vitaxin for melanoma and prostate cancers (50) and Cilengitide for glioblastoma and other cancers (51-53), have been studied in Phase I/II human trials, and have been shown to be clinically safe. Pharmaceutical companies Smithkline Beecham (54), Hoescht (8) and Dupont (55) have also manufactured RGD-containing peptides and peptidomimetics that are all being tested for activity in pre-clinical models. Additionally, blocking of the RGD-integrin signaling pathway reduces gene transfer by adenovirus several-fold (56,57). It has also been reported that inefficiency of gene transfer to human pulmonary epithelia was due to the lack of $\alpha_v\beta_5$ integrin on those cells (58). Several potential problems with antagonists of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ include inefficient oral bioavailability and short half-life of the peptides/peptidomimetics (8). Another approach, involving targeting of adenovirus to integrins by incorporation of RGD-containing peptides in the viral capsid, may be sufficient to circumvent the inadequacies of the above approaches, and could result in improved delivery of anti-cancer therapy to tumor cells.

7. Adenovirus as a targeted therapy for ovarian cancer

Human adenoviruses (Ads) of serotype subgroup C (Ad1, 2, 5 and 6) initially bind to cells via the coxsackie and adenovirus receptor (CAR) (59). Internalization, an independent step of Ad infection, is mitigated by interaction of the RGD motif on the Ad caspid with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on the cell surface (60-63). Deficiency of CAR and/or integrins on the surface of target cells results in their relative resistance to Ad vector infection (59,64-66). The ability to circumvent this deficiency, by allowing infection of CAR-deficient cells via alternate pathways, has been the focus of extensive Ad research for the past decade.

Despite the paucity of CAR on many types of cancer cells, Ads are preferred for anti-cancer targeted therapy for several reasons. They can be relatively easily produced in high titers and are capable of infecting both dividing and non-dividing cells with unparalleled efficiency. Additionally, the genomes of the human serotype 2 and 5 Ads can accommodate insertion of large size immunomodulatory transgenes such that the Ad-infected tumor cells may evoke an immune response, which could facilitate eradication of the tumor (67). Furthermore, the ability to manipulate Ads in order to re-target them from the natural receptors to specific cell types is of particular utility for targeted therapy applications. Several requirements must be met in order to achieve clinical efficacy with any Ad-based therapy. These requirements include the ability of the virus to maintain viability within the host and its capability for efficient tumor cell infection and selective replication in tumor cells. The latter is critical for reducing the toxicity of the treatment.

8. Strategies to improve transduction of ovarian tumors by adenoviral vectors

Two problems related to targeting ovarian cancer with Ads must be overcome for effective treatment to occur: i) the deficiency of CAR, the receptor for Ads on most tumor cells including ovarian cancer cells, and ii) Ad persistence in patients with ovarian cancer. One impediment to effective Ad-based virotherapy is the resistance of ovarian tumors (42,68,69) and many other tumor types (64,70-74) to Ad infection due to CAR deficiency. To address this problem, a number of strategies to increase Ad infectivity have been endeavored (42,75-77). Among the approaches used in an effort to target ovarian cancer cells has been targeting of Ad5 to the Ad serotype 3 receptor by using a chimeric (Ad5/3) fiber protein (78,79) and to heparan sulfates (a linear polysaccharide) via an Ad-fiber incorporated hepta-lysine (pK7) (80).

Another approach, originally reported by Dmitriev *et al* (42), is the construction of a modified Ad vector containing the RGD-4C peptide (CDCRGDCFC) in the HI loop region of the Ad5 fiber, which allows the fiber knob to bind integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (63) in addition to CAR, thereby expanding its tropism. Thus, additional RGD motifs added to the viral capsid can enhance infectivity of Ad. Infectivity of Ad vectors for several cancer types (81,82), including ovarian cancer (83), has been improved by the use of Ad containing an RGD-modified fiber knob (29,42,69,70,74). The CRAd version of the RGD-modified virus, Ad5Δ24RGD, showed enhanced oncolytic effects in lung adenocarcinoma, prostate cancer, and ovarian cancer cells (84,85). The presence of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on ovarian and other cancer cells allows for direct targeting of the RGD-modified Ad vectors to those receptors. This approach, therefore, proved to be promising for therapy of ovarian and other cancers.

Genetic technologies have previously been developed to incorporate the RGD ligand as well as other small ligands into various locales of the Ad5 capsid (86,87). Two different ligands such as RGD and pK7 peptides have been simultaneously incorporated at the HI loop and the C-terminus of the Ad5 fiber knob, respectively, resulting in a robust synergy in Ad vector infectivity enhancement (88). In one of our studies, we generated a battery of RGD-modified Ad5/3 vectors to determine the optimal locale for the RGD ligand on the Ad3 fiber knob. The results demonstrated that a short RGD peptide could also be incorporated into the HI loop or the C-terminus of the Ad3 knob. Interestingly, whereas the HI loop was an optimal locale for the functional incorporation of

the RGD-ligand in the Ad5 knob, the C-terminus was found to be superior to the HI loop for targeting the functionality of the same peptide in the Ad3 knob context, suggesting structural differences between the two knob domains (89). More recently the same RGD-peptide was found to be effective in infectivity enhancement when inserted into the HI loop of the Ad41 (subgroup F) fiber knob domain in the context of a chimeric (Ad5/41) fiber (90). *In vivo* studies of the RGD-modified CRAd Ad5Δ24RGD to determine potential toxicities showed that vector toxicity was minimal in a rat tumor model (91). Because of the improved targeting and safety profile of the RGD-modified CRAd in ovarian cancer, Alvarez *et al* carried this virus forward to clinical trial in 2007.

9. Adenovirus persistence in patients with ovarian cancer

General confinement of ovarian cancer to the intraperitoneal cavity makes it an ideal disease target for Ad-based virotherapy treatment. Ad is notorious for extensive intrahepatic sequestration as well as rapid clearance from the blood stream by pre-existing anti-Ad immunity in humans, compromising its efficient dissemination throughout the body. The ability to avoid systemic distribution, thereby bypassing the liver and delivering the therapeutics directly into the intraperitoneal cavity, provides a great advantage for ovarian cancer treatment by Ad vectors. Use of warfarin has been reported to decrease Ad uptake in the liver of mice, administered i.v. with a bolus of Ad (92). Studies were performed to determine whether this phenomenon could also be observed in mice upon i.p. administration of Ad. The results showed that Ad5 liver uptake was not changed in i.p.-infected mice upon pre-treatment with warfarin, suggesting that warfarin does not mitigate hepatic sequestration of Ad administered via the i.p. route (unpublished data).

Furthermore, the ability to 'wash' the peritoneal cavity with a fluid containing oncolytic virotherapy agents provides an important option of '*in situ*' treatment of ovarian cancer patients, 60% of whom develop metastatic lesions throughout the peritoneum. The efficacy with which Ad-mediated gene transfer can occur *in vivo* via systemic administration is greatly mitigated by the presence of inhibitory antibodies in patient serum. Early reports suggested that antibodies present in malignant ascites could also result in clearance of therapeutic virus and inhibit efficient Ad infection of ovarian cancer cells (93,94), thereby revealing a problem that needs to be overcome for the effective targeting of ovarian cancer *in situ*.

Elkas *et al* hypothesized that the addition of the RGD motif to the fiber knob would confer an immune privilege to the virus, since RGD moiety is present in a large number of proteins expressed on various human tissues (94). Experiments have shown that gene transfer efficiency was not inhibited when an Ad5RGD vector that had been exposed to ascites was used to infect ovarian cancer cells, as opposed to an unmodified vector that was also exposed to ascites (29,93). This indicated that an immunological tolerance was conferred upon the modified Ad by addition of the RGD-ligand to the viral capsid. However, preliminary studies using Western blot analysis and ELISA on a limited number of patient samples did not demonstrate any qualitative or quantitative differences in the antibody binding to Ad5RGD as compared with the conventional Ad5 vector. This suggested that differences in the kinetics of Ad5RGD binding and entry into cells may favor the virus-cell interaction as compared with the virus-antibody reaction. Alternatively, differences in the density of receptor targets, or increased affinity of the modified virus for the integrin molecules, may contribute to the relative efficiency of entry of the modified virus into the target cells in the presence of neutralizing antibodies. Regardless of its molecular mechanism, the ability of a modified Ad vector to avoid immune clearance when introduced into the peritoneum (95) suggests a possible role of the capsid RGD modification in overcoming the problem of pre-existing neutralizing antibodies in patients.

10. Clinical use of oncolytic adenoviral agents

Oncolytic viral therapy has been developed as an alternative treatment strategy for cancer for several decades (96). With the advancement of oncolytic vectors in pre-clinical studies throughout the US, virotherapy moved rapidly from bench-to-beside in the 1990's. Clinical gene therapy trials utilizing viral vectors were halted in the late 1990's due to the safety and toxicity concerns raised after the death of an 18-year-old patient, Jesse Gelsinger (97-101). Since this tragic event, there have been tremendous advancements in pre-clinical research, leading to more stringent animal modeling as well as heightened diligent federal regulations, which have led to the safer use of viral vectors in the clinical setting.

To date, gene therapy is becoming a more accepted treatment strategy, as evidenced by the increased number of patients that have been treated with Ad-based agents (102). Oncolytic Ad vectors have been used for a variety of cancer therapies, including ovarian cancer (103). Advanced CRAd agents were tested in a variety of ovarian cancer studies. One of the earliest ovarian cancer clinical trials with Ads was based on a concept that has subsequently been proven to be false (104). This trial involved use of an Ad, dl1520 (i.e., H101, Onyx-015). The replication of this Ad was reportedly dependent upon p53, but was subsequently determined to be regulated by viral mRNA transport mechanisms (105). On one hand, this error tarnished the public perception of gene therapy (104). On the other hand, the 'negative' ovarian clinical trial data have been informative with regard to dosing and toxicological information. Early ovarian cancer studies also revealed the need for improvement of Ad5 viral uptake by tumors (104) and reduction of its liver sequestration. Various animal studies illustrate that hepatic macrophages or Kupffer cells (KCs) play a major role in trapping viral particles within the liver (106-108).

Minimizing Ad sequestration by the liver would offer benefits that are two-pronged: i) reduction in the treatment hepatotoxicity that resulted in the tragic death of Jesse Gelsinger (101), and ii) allowance of a widespread dissemination of viral particles for uptake into the target cells. It was initially assumed that CAR-ablating fiber-modifications could detarget Ads from hepatocytes or KCs and eliminate liver toxicity. However, CAR-independent mechanisms of hepatocyte infection *in vivo* have recently been discovered (109,110). Additional research has led to the observation that a plasma protein factor, factor IX (FIX), and the complement binding protein-4 (CBP-4) act

as a bridge, binding the Ad fiber knob to heptatocyte receptors and KCs, thereby bypassing CAR (111). The Ad5 hexon hypervariable regions (HVRs) have been thought to be major binding sites for blood factors such as FIX and factor X (FX) (112,113). FX binding affinity varies between Ad serotypes. In line with this notion, a chimeric Ad5/48 containing hexon HVRs of Ad48 (AdHVR48) showed a dramatically reduced liver tropism. The FX binding to chimeric Ad5HVR48 was substantially reduced, as was binding of Ad5 vectors with hexon modifications. Transgene expression in the liver was substantially diminished after i.v. delivery of these vectors (112). These modifications may potentially be used in the next generation of CRAd agents to make them more effective by providing resistance to liver sequestration.

Several clinical trials have used Ad vectors as ovarian cancer therapy in the context of i.p. administration (114-116). As earlier ovarian cancer studies illustrated the need for better viral uptake into the cancer cells, the vast body of work has focused on developing CAR-independent methods of virus entry (86). In this regard, serotype chimerism strategy has been implemented for fiber modification of agents utilized in pre-clinical and clinical ovarian cancer trials (83,95). The employed therapeutic strategies used in conjunction with Ad vectors include: delivery and intratumoral production of a cytotoxic prodrug enzyme herpes simplex virus thymidine kinase (HSV-tk) (115,116) for the selective killing of ovarian tumor cells, an anti-ErbB2 single-chain antibody to target the cancer-specific marker (114,117), or the wild-type p53 gene for replacement of its mutated version in ovarian cancer cell genomes (118,119).

Furthermore, RGD-modified CRAds have shown infectivity enhancement and antitumor efficacy with less toxicity than wild-type Ad5 in i.p. models of ovarian cancer (120). In this regard, an ovarian cancer Phase I clinical trial, using a novel infectivity-enhanced CRAd, 'AdΔ24-RGD', has recently been completed at the University of Alabama at Birmingham. This accomplishment highlights that these CRAd agents are currently being explored as a single or a combinational therapy for ovarian cancer. The scientific community is presently awaiting the results of the first human trial involving the treatment of ovarian cancer patients with an infectivity enhanced oncolytic agent.

11. Future perspectives of ovarian cancer targeted virotherapy

Incorporation of the RGD-4C peptide into the Ad5 fiber knob domain resulted in augmentation of transduction of various cancer types. However, the relative infectivity enhancement observed for ovarian cancer cells was not as robust as that for vectors bearing the Ad5/3 fiber chimera, pK7-modified fiber, or a combination of pK7 with the RGD modification in the same fiber (80). Therefore, the use of alternate modifications in the context of CRAd agents for ovarian cancer virotherapy applications is a promising possibility.

Although engrafting the RGD-containing peptide into the surface of the knob domains derived from fibers of various Ad serotypes proved to be technically feasible and effective in improving Ad infectivity for many cancer cells, the number of additional interactions of such modified fiber molecules with cell surface integrins is limited by the number of fiber molecules that can be housed in one adenovirus particle (36 copies). In this regard, it can be reasoned that incorporation of additional RGD moieties into other capsid locales, such as hexon, penton base, or pIX, could further improve integrin targeting of Ad5-based virotherapy vectors. Among Ad structural proteins, the minor capsid protein IX (pIX) may be of particular interest owing to: an extremely high tolerance of the Ad capsid to large C-terminal modifications of pIX, the relative abundance of pIX on the capsid (240 copies), and its partial compatibility with Ad transductional targeting applications (121,122). Experiments aimed at testing the utility of alternate Ad5 capsid locales for incorporation of additional targeting RGD ligands as a means to improve vector infectivity of ovarian cancer cells *in vitro* and *in vivo* are currently underway in our laboratories. Considering the observed resistance of the vector with an RGD-modified fiber to the Ad-specific neutralizing antibodies in the peritoneal cavity of patients with pre-existing anti-Ad immunity (93), it is tempting to suggest that incorporation of extra RGD ligands could further improve the ability of the virus to evade anti-adenoviral immunity, and may even render it less immunogenic. Thus, the potential benefit of integrin targeting strategy could be 2-fold: an improved transduction of CRAds in tumors, and resistance to pre-existing anti-Ad immunity in patients.

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