

Published in final edited form as:

Clin Cancer Res. 2008 October 1; 14(19): 6187–6192. doi:10.1158/1078-0432.CCR-08-0410.

Therapeutic potential of replication-selective oncolytic adenoviruses on cells from familial and sporadic desmoid tumors

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Abstract

Purpose: Constitutive activation of the Wnt signaling pathway is a hallmark of many cancers and has been associated with familial and sporadic desmoid tumors. The aim of the present study is to assess the therapeutic potential of oncolytic adenoviruses selectively replicating in cells in which the Wnt signaling pathway is active on primary cells from desmoid tumors.

Experimental design: Primary cells extracted from familial (n=3) or sporadic (n=3) desmoid tumors were cultured short term. Cancer cell survival and viral replication were measured *in vitro* upon infection with two different oncolytic adenoviruses targeting a constitutive activation of the Wnt signaling pathway. Adenoviral infectivity was also assessed.

Results: Although cells extracted from one sporadic desmoid tumor responded very well to the oncolytic action of the adenoviruses (less than 20% of viable cells upon infection at a multiplicity of infection of 10), cells from two tumor samples were totally resistant to the viral action. Cells from the remaining samples showed intermediate sensitivity to the oncolytic viruses. These effects were correlated to the level of infectivity of the cells. Finally, in responder cells, evidences of viral replication was observed.

Conclusions: Our experimental data suggest that the response of desmoid tumor cells to oncolytic adenovirus is neither correlated to the type of mutation activating the Wnt signaling pathway nor to the familial or sporadic nature of the tumor. In addition, they highlight the

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Statement of clinical relevance

This study investigates the potential of replication-selective oncolytic adenoviruses in the treatment of desmoid tumors. The results demonstrate that infectivity of desmoid tumor cells is variable and is the key factor in determining whether oncolytic adenoviruses are likely to be efficient against this type of tumor. Therefore, this factor should be assessed before embarking into a clinical trial.

variability of infectivity of individual tumors and predict a great variability in the response to oncolytic adenoviruses.

Keywords

desmoid tumors; gene therapy; oncolytic adenovirus; Wnt signaling; Na/I symporter

INTRODUCTION

Desmoid tumors are rare, benign, sometimes aggressive tumors resulting from unregulated proliferation of fibroblast-like cells (1, 2). Their natural history is one of slow growth with progressive infiltration of surrounding soft tissues leading in some cases to strangulation of nerves and blood vessels. Abdominal desmoid tumors are the most common and can be classified into sporadic and familial forms (2). In the latter case, inherited desmoid tumors are most frequently associated with Familial Adenomatous Polyposis (FAP). At the molecular level, the development of desmoid tumors has been associated with a constitutive activation of the Wnt signaling pathway (3-7). FAP-associated desmoid tumors are usually caused by germline *adenomatous polyposis coli* (*APC*) mutations followed by somatic inactivation of the wild-type *APC* allele (3-5) while sporadic desmoids are usually characterized by oncogenic mutations in the *β -catenin* gene, both identical molecular alterations to those found in the vast majority of colorectal cancers (3, 4).

The management of patients is difficult and these tumors are prone to local recurrence. Surgery is an obvious option but the development of 10 to 30% of sporadic abdominal wall desmoid tumors and 68 to 86% of FAP associated intra-abdominal tumors has been associated with surgical trauma (8). As a result, there is currently no satisfactory treatment for these tumors, especially in the context of FAP-related desmoid tumors for which they represent a major cause of morbidity and mortality (1, 9). Therefore, there is a need for alternative or complementary therapies that could delay surgery if not eradicate the tumor. In this context, gene therapy through direct injection of gene delivery vectors in the tumor mass has already been suggested through pre-clinical studies involving non-viral and replication-deficient adenoviral vectors (10, 11).

Oncolytic adenoviruses are a class of adenoviruses genetically modified to selectively replicate in cancer cells (12, 13). This selectivity can be achieved through deletions in the viral genome (14-16) or replacement of the viral endogenous promoters by cancer-selective promoters (17-19). The resulting viruses have the unique property of tumor-dependent self-amplification. In addition, initial clinical trials have demonstrated that these agents are very safe and well tolerated (20). Recently, adenoviruses with Tcf binding sites in the early promoters were generated and these oncolytic adenoviruses showed up to 100 000-fold selectivity for cells with activated Wnt signaling (17, 21). As a constitutive activation of the Wnt signaling pathway is a common molecular defect in sporadic or familial desmoid tumors, the aim of the present study is to assess the efficacy of Wnt-selective oncolytic adenoviruses in these cells. In addition and as molecular imaging of gene transfer using the Na/I symporter (NIS) as a reporter gene could provide crucial information on the extent of gene transfer *in vivo* through PET imaging (18, 22, 23), we have generated a Wnt-selective oncolytic adenoviruses encoding hNIS. The efficacy of these viruses was assessed *in vitro* on primary tumor cells extracted from familial (n=3) or sporadic (n=3) desmoid tumors.

MATERIALS AND METHODS

Establishment of primary cultures of desmoid cells, cell lines

Desmoid tumors samples were obtained following surgical resection. The protocol was used in conjunction with ethical approval from the Harrow or Leuven Research Ethics Committee. The tumors were macerated in small pieces with a scalpel and digested overnight at 37°C in RPMI containing 20% FBS (AutogenBioclear, Ltd, Calne, UK), 100 units/mL penicillin, 100 µg/mL streptomycin, 0.2 µg/mL Butyl-p-hydroxybenzoate supplemented with 1 mg/mL of Collagenase D (Roche Diagnostic GmbH, Lewes, UK). The digest was then filtered through a 100µm nylon cell strainer and centrifuged twice at 1000rpm. Cell pellets were washed twice with PBS, resuspended in RPMI 20%FBS containing antibiotics (see above), and seeded. Primary cultures were maintained in a 5% CO₂ atmosphere at 37°C. Erythrocytes and cell debris were washed away with PBS 24 hours later. Germline and somatic mutations were identified: Desmoid 1: β-catenin substitution TTT for TCT resulting is a Ser to Phe change at codon 45; B-Cat substitution TTT for TCT resulting is a Ser to Phe change at codon 45. Desmoid 3: Mutations in APC or β-catenin not detected; Desmoid 4: APC: exon 15 c.3927_3931delAAAGA (p.Glu1309AspfsX4); Desmoid 5: APC: exon 13 c.1495C>T (p.Arg499X); Desmoid 5.2: germline exon 6 c.646 C > T (p.R216X), somatic exon 15H: c.4385delA.

SKBR3, MCF7, HPAF, SUIT-2 and DLD-1 cells were cultured as previously described (11, 24, 25).

Generation and production of a Wnt-selective adenovirus encoding *NIS*

The plasmid vpKH1 encodes the genome of a Wnt-specific adenovirus in which TCF-4 binding sites are present in E1A, E1B and E4 of the viral genome (21). Insertion of the *NIS* cDNA in the gp19k of this adenovirus was performed as previously described (18), using homologous recombination in yeast (26). Virus were produced and titrated as previously described (27).

Adenovirus nomenclature

AdWt : Wild-type adenovirus type 5.

Ad-LacZ : Replication-deficient adenovirus type 5 encoding the β-galactosidase gene.

Ad-hNIS : Replication-deficient adenovirus type 5 encoding the *NIS* coding sequence (23).

AdKH1: Adenovirus selectively replicating in cells in which the Wnt signalling pathway is constitutively activated (21).

AdIP2: Adenovirus selectively replicating in cells in which the Wnt signalling pathway is constitutively activated and encoding *NIS*.

Infections with adenoviruses

Cells were seeded at a cell density of 10⁴ cells/cm². The following day they were infected with adenoviruses at different multiplicity of infection (MOI) in serum-free medium for 30 to 45 minutes. Culture medium was then added to reach a serum concentration up to 20%.

Biochemical assays

Cell survival assays, measurement of GFP-positive cells and assessment of *in vitro* viral replication and E1A expression were performed as previously described (28), (29), (19) and (18), respectively.

Coxsackievirus and Adenovirus Receptor (CAR) staining

Cells for staining were collected by trypsinisation, and washed. The pellet was re-suspended and incubated with the anti-CAR primary monoclonal mouse antibody (30) 1:500 in FACS buffer containing 5% normal goat serum (Dako Cytonation, Denmark) for 90min on ice. Cells were then washed three times and incubated for 30min at room temperature in the dark with secondary antibody goat anti-mouse FITC 1:30 in FACS buffer. The cells were washed three times and the final pellet was re-suspended in 400 μ l FACS buffer. Measurement of the number of CAR-positive cells was performed by FACS on at least 5 10^5 cells.

RESULTS

Effect of Wnt-selective oncolytic adenoviruses on desmoid tumor cells

To determine whether the insertion of the NIS cDNA affects the selectivity of the Wnt-selective virus, DLD-1 colon cancer cells carrying an *APC* mutation that results in constitutively high β -catenin signalling (31) and cell lines in which the Wnt signalling pathway is not activated in normal culture conditions (MCF7, SKBR3) were infected with AdIP2 and a wild-type adenovirus type 5 as positive control. Table 1 demonstrates that the EC50 of AdIP2 and AdWt are in the same range in DLD-1 target cells, while AdWt is more than two log potent than AdIP2 on non-target MCF7 or SKBR3 cells. These results demonstrate that AdIP2 can selectively kill cells in which the Wnt signalling pathway is constitutively activated.

To determine the effect of adenoviral infection on primary desmoid tumor cells, cells from sporadic (fig 1A) or FAP-related (fig 1B) desmoid tumors were infected at different multiplicity of infection (MOI) with AdKH1 or AdIP2. Different levels of sensitivity to oncolytic adenoviral treatment were observed with the following range of sensitivity: desmoid 2 (sporadic) > desmoid 5 (familial) ; desmoid 5.2 (familial) > desmoid 3 (sporadic) > desmoid 1 (sporadic); desmoid 4 (familial). Statistical analysis showed a highly significant difference ($P < 0.0001$) between uninfected cells and cell infected with AdKH1 and AdIP2 at MOI 100 for desmoids 2, 3, 5 and 5.2. These results demonstrated that the sensitivity to oncolytic adenoviral treatment is not related to the type of mutation in the Wnt signaling pathway or the sporadic or familial nature of desmoid tumors.

Correlation between infectivity and sensitivity to adenoviral vectors

The infectivity of primary cells extracted from the different desmoid tumors was determined by infection with a replication-deficient adenovirus (MOI 10) encoding the green fluorescent protein (GFP) and measurement of GFP-positive cells by FACS. Fig 2 shows that the range of infectivity is similar to that obtained for the sensitivity to adenoviral infection (fig 1). The coxsackievirus and adenovirus receptor (CAR) has been shown to act as a primary receptor to adenovirus and to play a critical role in viral entry in the cell (32). Surprisingly, CAR staining was negative in all desmoid cell samples (not shown). This is in sharp contrast to highly positive (>95%) A549 cells used as positive control (not shown) and known to express high levels of CAR (33). In an attempt to further demonstrate infection in the absence of CAR expression, E1A expression was monitored upon AdIP2 infection of Desmoid 3. Quantitative RT-PCR revealed that E1A expression was weak and hardly detectable twenty-four hours after infection but increased very significantly four days later (Supplementary data), demonstrating that incubation of Desmoid 3 with AdIP2 lead to E1A expression resulting from cell infection, independently of the presence or CAR. Altogether, these results suggest that sensitivity to adenoviral agents and infectivity are tightly correlated and that a CAR-independent mechanism is involved in adenoviral infection of primary cells from desmoid tumors 2, 3, 5 and 5.2.

Replication of adenovectors in primary desmoid tumor cells sensitive to the viruses

To determine whether the reduced cell viability observed upon adenoviral infection of cells from desmoid 2, 3, 5 and 5.2 was associated with viral replication, DNA from cells infected with AdIP2 was collected one day or four days after infection. This DNA was subjected to quantitative PCR to titrate the amount of adenoviral DNA per culture well. Fig 3 shows that in all cases, viral amplification of the adenoviral genome was observed between day 1 and 4, suggesting replication of the oncolytic virus in these cells.

Additional toxic effect of the *NIS* transgene

In addition, at MOI 100, AdIP2, the *NIS*-positive virus is statistically more efficient ($P < 0.0001$ for the three tumors) than AdKH1 (*NIS*-negative) in cells from desmoids 2, 5 and 5.2 (fig 1). To determine whether the presence of *NIS* in the viral genome and its expression is toxic for desmoid cells, independently from the oncolytic action of the virus, primary cells from the desmoid tumors 2, 5 and 5.2 were infected at a MOI of 100 with a replication-deficient recombinant adenovirus encoding either the β -galactosidase gene (Ad-LacZ) or *NIS* (Ad-hNIS) under the control of the immediate-early CMV promoter. Fig 4 demonstrate that the toxicity of Ad-hNIS is not observed with Ad-LacZ, suggesting a toxicity of the *NIS* gene product in desmoid tumor cells. By contrast, this hNIS cytotoxicity is not observed with established cell lines such as HPAF or SUIT-2 cells (Figure 4).

DISCUSSION

The Wnt signaling pathway is considered as the key molecular defect in sporadic or familial desmoid tumors (3-5) and therapeutic strategies targeting this pathway are expected to provide therapeutic benefits with minimal side-effects in patients affected with this pathology. In this context, an oncolytic adenovirus, replicating selectively in cells in which the Wnt signaling pathway is constitutively activated is particularly relevant.

Replication-selective oncolytic adenoviruses represent a novel class of therapeutic agents. Pre-clinical and clinical studies have demonstrated the safety and feasibility of the approach and their suitability for association with more classical cancer treatments such as chemotherapy has been demonstrated (34-36). However, a clear limitation of adenoviral vectors is that only a minimal proportion of the injected dose reaches the tumors after systemic administration. As a result, intratumoral injections are advised. Considering the lack of metastasis and the size of the tumors observed in patients with desmoids (1), local injection of oncolytic adenovirus represents a practical approach that could even be implemented in conjunction with chemotherapy. Therefore, desmoid tumors are a relevant indication for adenoviral vectors.

Our data clearly demonstrate that primary cells from some desmoid tumors allow replication and are sensitive to the action of oncolytic adenoviruses. However, in two cases, the cells were completely refractory and the key difference between responder- and non-responders-cells is the infectivity of the cells. Low or no CAR expression has already been reported in desmoids (37) and we confirm that CAR was absent from all the cellular samples tested, whether infectable with adenovirus or not. Taken together, these data suggest an alternative, CAR-independent mechanism of infection. Such mechanisms have been suggested (38) and may involve heparan sulfate proteoglycans (39) or coagulation factors (40, 41). Further studies will be required to address the mechanism of infection in these cells.

One of the particularity of desmoid tumors is their abundance in extracellular matrix that could prevent the diffusion of the virus in the tumour mass. In this context, pre-treatment or co-administration of the oncolytic virus with enzymes capable of dissociating this matrix, could enhance vector diffusion. A proof of principle has already been demonstrated using

hyaluronidase that was shown to improve adeno-associated virus-mediated gene transfer in the muscle (42) and this type of approach could be applied to the context of adenovirus gene therapy and desmoid tumors.

The fate of gene therapy vectors encoding *NIS* can be visualised after administration into a subject using positron emission tomography (PET) (22, 23) or single-photon emission computed tomography (SPECT) (18). Both imaging methodologies are potentially applicable to humans and can provide unique information on the anatomical distribution of gene transfer. In addition, *NIS* can also be used as a therapeutic transgene through the administration of ^{131}I and the accumulation of radioactivity in tumor deposits transduced by the gene therapy vector (43, 44). In this report, we demonstrate for the first time a direct effect of *NIS* expression on the viability cells. This effect is unique to primary desmoid cells as many different types of tumor cell lines can be transduced with the *NIS* gene without apparent toxicity and therapeutic doses of ^{131}I (45-47) or high doses of non radioactive iodide (48) are necessary to trigger cell death.

This report demonstrates that gene therapy for desmoid tumor is feasible and the efficacy of the treatment is highly dependent on the infectivity of the cells. In the clinical setting, direct injection of the oncolytic adenovirus into desmoids under radiological guidance could be envisaged. This gene therapy approach would probably aim at debulking the tumor before attempting surgical resection and in the case of a tumor susceptible to adenoviral infection, even a relatively modest effect could have an important clinical implication by making a previously unoperable intra-abdominal tumor amenable to surgery. Imaging of *NIS* gene transfer could also be performed to provide additional information on the extent of gene transfer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr Jhiang for providing the human *NIS* cDNA. This work is supported by grants from Cancer Research UK, the Medical Research Council, INSERM, la Ligue Nationale Contre le Cancer and by grant 0607 - 3D1615 - 66 / AO INSERM from the French National Cancer Institute (INCa). ST is a Senior Clinical Investigator of the Fund for Scientific Research-Flanders(Belgium) (Fonds Wetenschappelijk Onderzoek-Vlaanderen). This project was funded by the Belgian Federation against Cancer, and the Fund for Scientific Research Flanders (FWO). The publication of this article was possible thanks to financial support brought by Association Française pour l'Etude du Foie and BMS laboratories.

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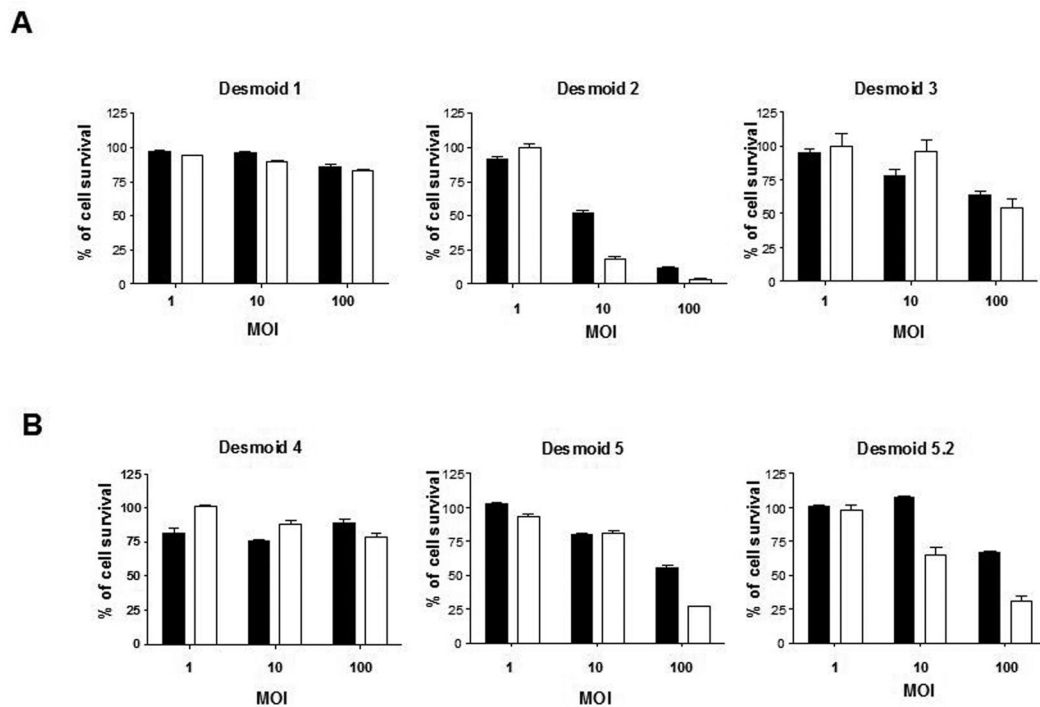


Figure 1.

Effect of Wnt-selective oncolytic adenoviruses on desmoid tumor cell survival. Primary cells from (A) sporadic or (B) familial desmoid tumors were infected at different multiplicities of infection (MOI) with AdKH1 (black bars) or AdIP2 (white bars). Six days later, the number of surviving cells was assessed using an MTT assay. 100% correspond to the number of cells in control, non-infected cells. Two-way ANOVA statistical analysis was performed. Results are mean \pm SEM of 6 experimental points. The data presented are representative of 3 independent experiments.

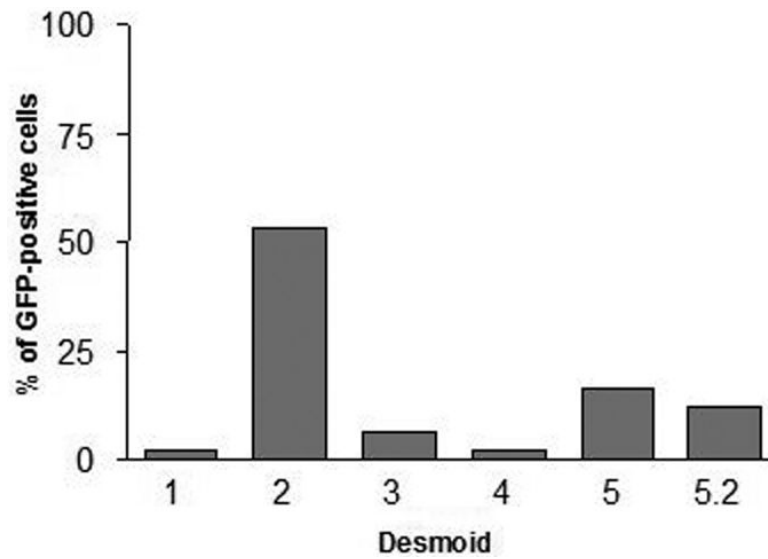


Figure 2. Infectivity of primary desmoid tumor cells. Adherent cells were infected with a replication-deficient recombinant adenovirus encoding GFP. Forty-eight hours later, the cells were trypsinized and the number of GFP-positive cells was determined by FACS. The data presented corresponds to an acquisition of at least 50 000 events and are representative of three independent experiments. Data were analyzed using the χ^2 test. In all conditions, comparisons of the uninfected versus the infected cells showed P values <0.0001 .

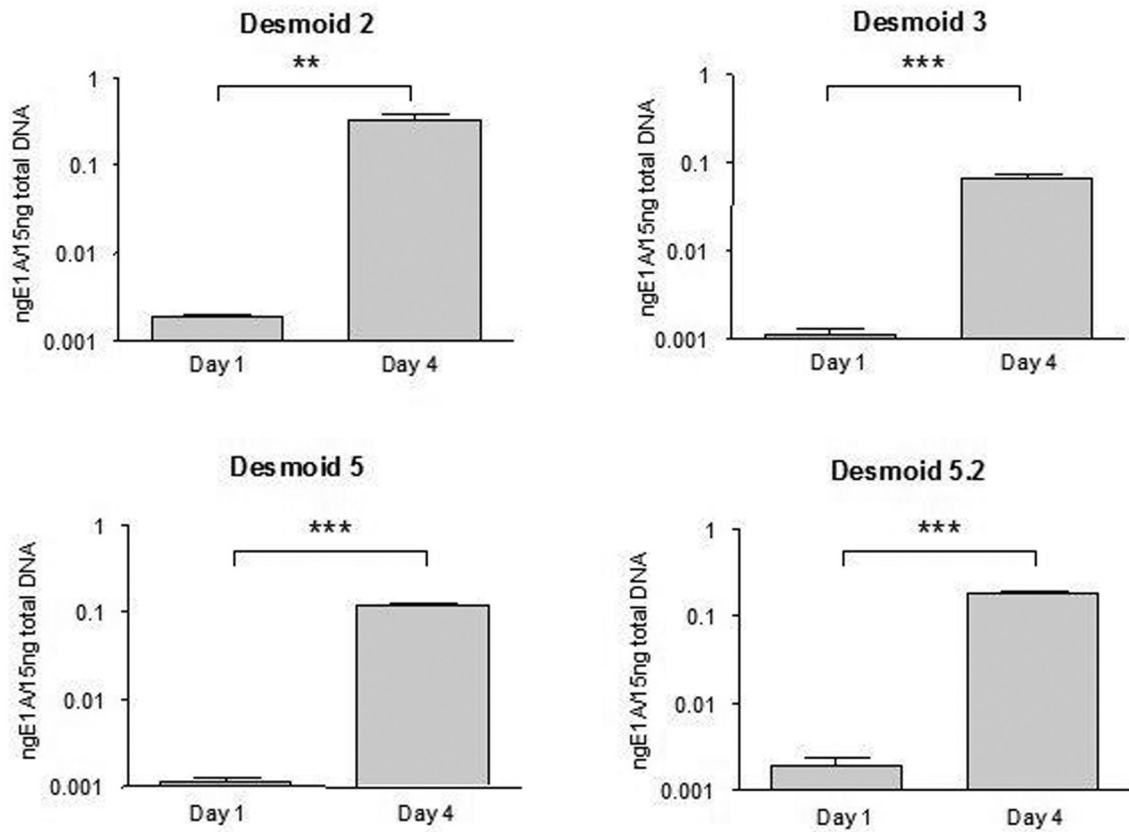


Figure 3.

Evidence of adenoviral replication in primary desmoid tumor cells infected with AdIP2. Cells were infected at low MOI (0.01) and total DNA was extracted on day one and four after infection. Adenoviral DNA was titrated by quantitative PCR. Statistical analysis was performed using a two-tailed student t test. P values are 0.0052 and 0.0008 for desmoids 2 and 3 respectively and < 0.0001 for desmoids 5 and 5.2. The data presented are the mean +/- SEM of triplicate and are representative of three experiments.

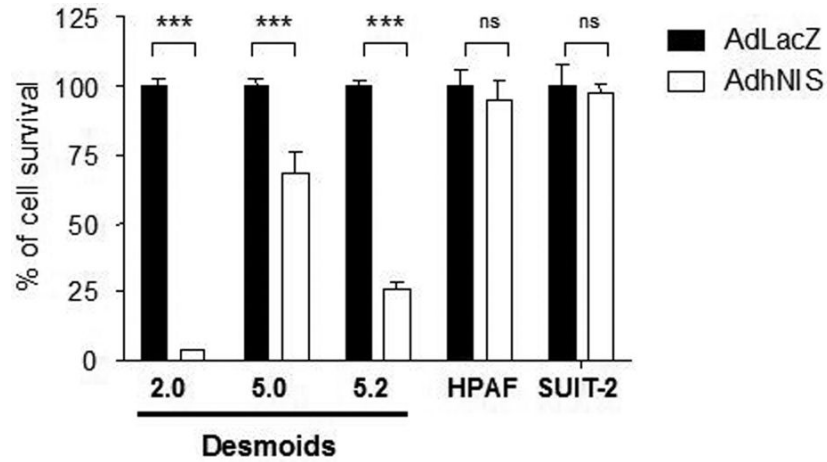


Figure 4.

Effect of replication incompetent adenoviruses encoding LacZ or NIS on desmoid tumor cell survival. Primary cells from desmoid tumors 2, 5 and 5.2 and cell lines HPAF and SUIT-2 were infected at MOI of 100 with a replication incompetent adenoviruses encoding LacZ (black bars) or NIS (white bars). Six days later, the number of surviving cells was assessed using an MTT assay. 100% correspond to the number of cells in control, non-infected cells. Statistical analysis was performed using a two-tailed student t test. P values are < 0.0001 in all the conditions compared involving the desmoid tumors and non-significant (ns, $P > 0.05$) with the cell lines HPAF and SUIT-2. The data presented are the mean \pm SEM of four experimental points and are representative of three independent experiments.

Table 1

	MCF7	SKBR3	DLD-1
AdWt	8	0.63	1.33
AdIP2	>100	>100	4.77

DLD-1 colon cancer cells carrying an *APC* mutation that results in constitutively high β -catenin signalling and cell lines in which the Wnt signalling pathway is not activated in normal culture conditions (MCF7, SKBR3) were infected with AdIP2 and a wild-type adenovirus type 5 as positive control at different multiplicity of infection. Six days later, the number of viable cells was determined using a MTT assay. Dose-response curves were generated and concentrations killing 50% of cells (EC50-values) were determined using untreated cells as reference and are presented in pfu.