Inhibition of Insulin-like Growth Factor I Receptor Expression in Neuroblastoma Cells Induces the Regression of Established Tumors in Mice

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ABSTRACT

Several lines of evidence now indicate that type 1 insulin-like growth factor receptor (IGFIR) function may be particularly important in the pathogenesis of the pediatric cancer neuroblastoma. Modulating the expression of specific genes involved in neuroblastoma tumorigenesis could provide a much needed alternative treatment strategy for poor prognosis disease. We now report construction of an antisense expression vector to the IGFIR that markedly reduces cellular IGFIR levels and inhibits the proliferation and clonogenicity of neuroblastoma cells in vitro but not that of IGFIR null cells. This antitumor activity is associated with the induction of apoptotic cell death in transfected cells, as measured by annexin V staining and flow cytometry. Direct injection of this vector into established tumors growing in syngeneic mice results in a marked inhibition of tumor growth with complete and durable tumor regression in one-half of the animals. This effect appears to be immunologically mediated in that vector injection of neuroblastoma tumors growing in severe combined immunodeficiency mice results in only modest delay of tumor growth. Our results suggest that inhibition of IGFIR expression by direct intratumoral delivery of an antisense construct could provide a novel therapeutic approach in the management of poor prognosis neuroblastoma.

INTRODUCTION

Disseminated neuroblastoma is a major therapeutic problem in pediatric oncology. Despite aggressive multimodality therapy and bone marrow transplantation, long-term survival rates remain poor (1). Signaling via the IGFIR is an important contributor to the malignant phenotype of many tumor types (2, 3) and may be particularly important in the pathogenesis of poor prognosis neuroblastoma (4). The IGFIR is activated by the ligands IGF-I and IGF-II at physiological concentrations, and IGF-II is known to be an important regulator of embryonic growth and differentiation (5). Because neuroblastoma is an embryonal tumor thought to arise from cells of neural crest origin, it is not surprising to find that many neuroblastoma cell lines both secrete and respond to IGF-II (6, 7). In addition, primary neuroblastoma tumor specimens have been shown to express mRNA for IGF-II and the IGFIR (8, 9). The monoclonal antibody a-IR3, which binds the IGFIR, blocks the mitogenic effects of exogenous IGF-I and IGF-II on neuroblastoma cells grown in vitro (6). Lastly, overexpression of IGFIR has been reported to confer resistance to apoptotic cell death in a subclone of the human neuroblastoma cell line SKNSh (10).

Reports from several laboratories have demonstrated that antisense-mediated inhibition of IGFIR expression can be used successfully to limit the proliferation of tumor cell lines in vitro including those derived from rat glioma (11, 12), human breast (13), and pancreatic (14) cancers, malignant melanoma (15), and sarcomas (16). Antisense inhibition of IGF-I (17, 18) and IGFIR (11, 15) expression has been shown to impair in vivo tumorigenicity in several animal models. However, it has been necessary to modify tumor cells in vitro by vector transfection or oligonucleotide treatment prior to inoculation into animals to demonstrate an antitumor effect. We now report construction of an antisense RNA expression vector to the IGFIR, which demonstrates antitumor activity after direct intratumoral injection in vivo using a well-established mouse model of neuroblastoma (19). This antineuroblastoma effect appears to be immunologically mediated, which raises the possibility that intratumoral injection could contribute to the control of both local and metastatic disease in this tumor.

MATERIALS AND METHODS

Vector Construction. A 1581-bp 5'-fragment of human IGFIR cDNA was excised from the plasmid pIGFlRSAL2 (a kind gift of S. Jacobs, Wellcome Research Labs, Research Triangle, NC) by double digestion with the enzymes KpnI and EcoRI. After gel purification, this fragment was ligated into the multiple cloning site of the the eukaryotic expression vector pcDNA 3.1(+) (Invitrogen, San Diego, CA). The expected antisense orientation of the CDNA fragment in the resultant construct relative to the strong cytomegalovirus promoter elements in the vector was confirmed by restriction mapping using double digestion with the enzymes NheI and EcoRI, which yielded 1.6- and 5.4-kb fragments as expected. Transfection grade DNA was prepared using Wizard Maxi Prep kits (Promega Corp., Madison, WI) per the manufacturer's recommendations.

Cell Culture and Transfection. The mouse neuroblastoma cell line neuro-2a (N2A) was generously provided by C. L. Fowler (University of Kentucky, Lexington, KY). The IGFIR-negative fibroblast cell line R+ was derived from a BALB/c IGFIR(-/-) mouse embryo (20) and kindly provided by R. Baserga (Jefferson Cancer Institute, Philadelphia, PA). The human neuroepithelioma cell line CHP100 was provided by L. Neckers (National Cancer Institute, Bethesda, MD). All cell lines were cultured in RPMI 1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA). Cultures were maintained in a humidified atmosphere of 6% CO2 in air at 37°C and passaged when 80% confluent. All cells were demonstrated to be free of Mycoplasma contamination by ELISA assay, and experiments were performed on cells within 15 serial passages. Cells were transfected by electroporation in serum-free medium using a Cell-Porator (BRL Life Technologies, Rockville, MD) and standard techniques. A field strength of 875 V/cm was used for all cell types. Pulse lengths were 13.2 ms for CHP100 cells and 9.0 ms for N2A and R+ cells. In some experiments, cells were transfected with the aid of a polycationic lipid (Lipofectamine; BRL Life Technologies) in serum-free medium per the manufacturer's recommendations.

Evaluation of Cellular IGFIR Levels. Cell surface expression of human IGFIR was analyzed by indirect immunofluorescent staining and flow cytometry as described previously (21). Cells were incubated with antibody 913R (Oncogene Science; Ab-1) or an equal concentration of isotype-matched mouse IgG (10 μg/ml) in PBS supplemented with 1% BSA and 0.1% sodium azide for 60 min at 4°C. Cells were analyzed on a Becton Dickinson FACStar flow cytometer. Total cellular IGFIR levels were analyzed by immunoblotting using peptide-derived antibody directed against amino acid residues 31–51 of the human IGFIR (N-20; Santa Cruz Biotechnology) as described previously (22). Total cellular protein (100 μg/lane) was fractionated by SDS-PAGE (7.5%) and transferred to nitrocellulose by electroblotting. Primary antibody was used at 1:1000 for 1 h at room temperature, followed by peroxidase-
conjugated secondary goat anti-rabbit (1:20,000). Immunoreactivity was detected using chemiluminescent substrate and exposure of membranes to Kodak XAR-5 film. Multiple exposure times were evaluated for each blot to ensure that the band intensities observed were within the dynamic response range of the film.

Proliferation, Clonogenicity, and Apoptosis Assays. After electroporation with control and antisense vectors, cells were diluted in complete culture medium and dispensed into six-well plates at 5 x 10⁵ cells/well. On days 1, 3, 5, and 7 after transfection, replicate wells were harvested by trypsinization, and viable cell numbers were quantitated by counting cells suspended in 0.2% trypan blue in a hemacytometer. The clonogenicity of electroporated cells was assessed by colony formation in soft agarose as described previously (21). The number of macroscopic colonies per well in replicate wells was counted 3 weeks after plating. In some experiments, the extent of apoptotic cell death occurring within transfected populations was assessed by staining cell suspensions with fluorescein-conjugated annexin V (Boehringer Mannheim, Indianapolis, IN) and propidium iodide. After transfection with either control or antisense vector, the adherent and floating cells within a well were collected, washed with PBS, and resuspended in staining buffer containing 1 µg/ml annexin V-FTTC, 5 µg/ml propidium iodide, 10 mM HEPES (pH 7.4), and 2.5 mM CaCl₂. After 10 min in staining buffer at room temperature, cells were analyzed using a Becton Dickinson FACStar flow cytometer, and the percentages of cells demonstrating no staining (live cells), annexin V-fluorescein staining only (apoptotic cells), or dual annexin V-fluorescein/propidium iodide staining (necrotic cells) were quantitated. Ethanol-fixed cells served as a positive control for dual staining in these assays.

Tumorigenicity Studies. N2A cells were harvested by trypsin/EDTA incubation and resuspended at 2 x 10⁶ cells/ml in PBS. Female A/J mice (Jackson Labs, Bar Harbor, ME) or scid mouse (Jackson Laboratories, Bar Harbor, ME) or seid mouse (CB17 seid/seid; University of California, San Francisco, CA) were inoculated s.c. with 10⁶ cells in the right inguinal area. When tumors had achieved an average volume of 40 mm³ (~10 days after cell inoculation), plasmid DNA was injected into tumors via a 30-gauge needle, followed by 20 µl of medium every other day for a total of five DNA doses (total 180 µg). Serial tumor measurements were performed in triplicate wells until tumors reached an average volume of 150 mm³. All experiments involving the use of animals were carried out under protocols approved by the Institutional Animal Care and Use Committee.

RESULTS

Vector-generated Antisense Reduces IGF-1R Levels. The human neuroepithelioma cell line CHP100 and the mouse neuroblastoma cell line N2A were electroporated with the control plasmid pcDNA3.1(+) or a recombinant modification of this plasmid encoding a 1581-bp 5′ fragment of human IGF1R cDNA in the antisense orientation. On the basis of experiments with reporter constructs encoding β-galactosidase or enhanced green fluorescent protein, the transfection conditions used resulted routinely in >50% transfected cells (data not shown). In CHP100, relative cell surface receptor levels were quantitated by indirect immunofluorescence staining and flow cytometry 48 h after transfection (Fig. 1A). Control vector transfectants displayed strong uniform staining for the IGF1R, whereas antisense transfection resulted in a majority population of cells that displayed receptor staining comparable with background control levels. The antibody used to stain human CHP100 cells does not recognize rodent IGF1R. We were able to obtain a rabbit polyclonal antibody to the α subunit of IGF1R, however, which performed well in Western blotting but not flow cytometry. We used this reagent to assess total cellular receptor levels in N2A cells. After electroporation using two different pulse lengths, a substantial decline in immunoreactive signal of the expected Mr 135,000 molecular size was observed. The antibody used to stain human CHP100 cells does not recognize rodent IGF1R. We were able to obtain a rabbit polyclonal antibody to the α subunit of IGF1R, however, which performed well in Western blotting but not flow cytometry. We used this reagent to assess total cellular receptor levels in N2A cells. After electroporation using two different pulse lengths, a substantial decline in immunoreactive signal of the expected Mr 135,000 molecular size was observed in antisense-transfected compared with control vector-transfected cells (Fig. 1B).

Selective Inhibition of Cell Growth/Survival in Vitro. To assess the biological consequences of inhibiting IGF1R expression in neuroblastoma cells, we next generated growth curves for N2A cells (in complete, serum-containing medium) after transient transfection with control or antisense plasmids (Fig. 2). Serial trypan blue cell counts revealed a marked decrease in the number of adherent, viable N2A cells present 5 days after electroporation with antisense vector com-
either cell line, indicating little or no spontaneous resistance to the encoded antibiotic resistance. Negligible cell survival was observed in cells, —2% of the number of G418-resistant clones per dish were cells were recovered after 14 days of G418 selection. Similar results encoding G418 resistance alone. As seen in the experiment depicted cultures compared with cultures transfected with the parental vector observed in N2A cells were mediated via the IGFIR.

This finding confirms that the antisense vector effects antisense vector did not result in decreased cell growth and/or survival at 5 days. This finding confirms that the antisense vector effects observed in N2A cells were mediated via the IGFIR.

In attempts to generate stable transfectants of the cell lines CHP100 and N2A, we consistently noted a marked decrease in the number of G418-resistant cells that could be recovered in antisense-transfected cultures compared with cultures transfected with the parental vector encoding G418 resistance alone. As seen in the experiment depicted in Fig. 3A, approximately one-tenth the number of antisense vector-transfected CHP100 cells as pcDNA 3.1 (Neo-R plasmid)-transfected cells were recovered after 14 days of G418 selection. Similar results were obtained with N2A. After antisense plasmid transfection of these cells, ~2% of the number of G418-resistant clones per dish were identified after 12 days of selection in antibiotic compared with transfection with control neo-resistance plasmid. The efficacy of G418 selection was monitored in these experiments by transfecting cultures with the vector pCMVß (control plasmid), which does not encode antibiotic resistance. Negligible cell survival was observed in either cell line, indicating little or no spontaneous resistance to the selection conditions used.

To better understand why some CHP100 cells were able to survive antisense vector transfection, we examined the level of IGF1R detectable on the surface of CHP100 clones that did grow out after 14 days of G418 selection. In nine independent clones analyzed, only one showed a decrease of at least 50% in receptor level as measured by flow cytometry (Table 1). In fact, four of nine clones demonstrated levels greater than or equal to that of wild-type control cells. The finding that most of the colonies surviving G418 selection had escaped from vector-mediated suppression of IGF1R expression is consistent with an essential role for IGF1R function in these cells.

In addition to effects on anchorage-dependent cell proliferation and survival, we also examined the effect of IGF1R inhibition on colony formation in soft agar. As depicted in Fig. 3B, transfection of both CHP 100 and N2A cells with antisense vector resulted in a marked decline in the number of macroscopic colonies detectable 3 weeks after plating in 0.3% agarose supplemented with 10% serum-contain-
Transfection with antisense vector induces apoptosis in neuroblastoma cells. N2A cells were transfected with control (A and C) or antisense (B and D) plasmids using cationic lipid and then analyzed by dual parameter flow cytometry after staining with annexin V-FITC and PI. In A and B, floating and adherent N2A cells were analyzed 36 h after transfection. The relative fluorescence intensity due to PI staining is plotted on the log scale y-axis, whereas fluorescence due to annexin V-FITC is plotted on the log scale x-axis. In C and D, N2A cells were analyzed at various times after transfection. The percentage of cells displaying necrotic or apoptotic staining characteristics was determined at 24, 36, and 48 h after transfection with either control (Fig. 4C) or antisense (Fig. 4D) plasmid. Very low levels of apoptosis were evident in control transfectants at any time point. Low levels of necrosis were evident, which rose somewhat by 48 h as cells began to overgrow. In contrast, transfection of cultures with antisense plasmid resulted in an increase in apoptotic cells detectable at 36 h, followed by a marked accumulation of necrotic cells at 48 h. These results are most consistent with antisense-induced apoptotic cell death, followed by necrotic degradation of the apoptotic cells. Nevertheless, the possibility that inhibition of IGF-1R stimulates both apoptotic and nonapoptotic death of N2A cells in culture cannot be excluded and may have important consequences for the antitumor effects in vivo discussed below. In either case, these results indicate that suppression of IGFIR expression, rather than exerting a simple cytostatic effect, actually stimulates neuroblastoma cell death, even in complete serum-containing medium.

Inhibition of Tumorigenicity. To determine whether the antineuroblastoma activity of 1GF-1R inhibition documented in vitro would impair in vivo tumorigenicity, we electroporated N2A cells with control or antisense plasmid, selected cells in G418 for 48 h, and then injected equal numbers of viable cells s.c. into syngeneic A/J mice. In four of four mice receiving antisense-transfected cells, no tumors were detectable for up to 24 days after cell inoculation. In contrast, four of four mice developed tumors of >1 cm\(^2\) by this time.

Encouraged by these results, we next examined the effect of direct injection of naked plasmid DNA into established N2A tumors. In control experiments using a luciferase reporter construct, we first verified that direct intratumoral injection yielded low but readily detectable levels of plasmid expression in N2A tumors (luciferase activity 24 h after DNA injection was 10–100-fold higher in tumors injected with luciferase construct compared with control construct). As depicted in Fig. 5A, we then examined the effect of antisense plasmid injection and found a marked inhibition of tumor growth compared with that observed in several relevant control treatment
groups. The controls indicate that this effect was specific for the antisense plasmid and did not result from general antitumor activity of bacterially produced DNA plasmids or the process of intratumoral injection itself. This effect persisted long after the period of active DNA administration. Closer examination of the antisense-treated group of mice revealed that in two of the four animals, complete tumor regression occurred that persisted for >40 days (Fig. 5B). In the two other antisense-treated mice, a substantial delay in tumor progression was evident, but subsequently tumors escaped and grew with kinetics comparable with that observed in control treatment groups. This experiment was subsequently repeated using larger numbers of mice per treatment group, and again the same effect was observed (Fig. 6A). Profound inhibition of average tumor growth was seen, and four of eight tumors demonstrated complete and durable regression.

When five mice from previous experiments in which complete tumor regression had occurred after antisense injections were rechallenged with a s.c. inoculation of \(0.5 \times 10^6\) unmodified N2A cells in the contralateral inguinal skin fold, no tumors formed in any of these animals up to 2 months later. This finding suggested a possible immune-mediated mechanism for the observed antitumor effect. To begin to explore this possibility, N2A tumors were established in scid mice, which lack B- and T-cell function. Tumors were injected with plasmid DNA using the same dose and schedule used in the previous experiments. In these scid mice, IGF1R antisense plasmid injections resulted in only a modest inhibition of average tumor growth during the period of DNA administration (Fig. 6B). Tumor regression was not observed in any of the treated mice, confirming a critical role for immune function in mediating the \textit{in vivo} antitumor activity associated with suppression of IGF1R expression in neuroblastoma cells.

**DISCUSSION**

Neuroblastoma appears to result from the arrested differentiation and clonal expansion of primitive sympathetic peripheral nervous system progenitors derived from cells of neural crest origin (24). The malignant phenotype of neuroblastoma results from both excessive cellular proliferation and impaired cell death, both biological processes that are highly dependent on the function of the IGF1R in developing embryonic tissues (25). Previous studies have demon-
stated that IGF-II, acting through IGF1R, can function as either an autocrine or paracrine growth factor for neuroblastoma tumors in vivo (8, 9). In addition, overexpression of the IGF1R in neuroblastoma cells appears to render them resistant to the induction of programmed cell death by serum deprivation and other stressors in vitro (10). In light of these findings and additional data in other cell types of neural derivation (26–28), we hypothesized that inhibition of IGF1R function could have profound antitumor activity against neuroblastoma.

A number of approaches to inhibiting IGF1R function in intact tumor cells have been reported including: the use of a blocking antibody (29, 30); antisense oligonucleotides (15, 31); triple helix-forming oligoribonucleotides (32); and antisense RNA expression vectors (11–13, 16). In addition, expression of dominant-negative receptor mutants (33) and COOH-terminus receptor fragments carrying a myristylation signal (34, 35) have also been shown to result in antitumor effects. To examine the role of IGF1R function in neuroblastoma, we adopted an antisense RNA expression vector strategy because this technique has been used extensively to modulate successfully the expression of a wide variety of genes (36) and is less prone to the technical artifacts that can be associated with oligonucleotides such as aptameric and sequence-independent effects (37). A number of constructs encoding varying lengths of IGF1R cDNA under the control of both inducible and constitutive promoters were tested (data not shown). We found that the most robust and reproducible inhibition of receptor expression at the protein level in neuroblastoma was achieved after transfection with a vector encoding ~1.6 kb of IGF1R antisense message under transcriptional control of the immediate-early-promoter elements of cytomegalovirus contained in the commercial eukaryotic expression vector pcDNA 3.1(+) (Fig. 1). As expected, high efficiency transient transfection with this construct resulted in a reduction in the number of neuroblastoma cells recoverable 5 days later but not that of IGF1R-negative fibroblasts.

Interestingly, the reduction in cell number did not appear to result primarily from inhibition of proliferation as reported previously for other primary pediatric neoplasms (16, 29, 38) but rather the induction of cell death in transfected cells. The IGF1R is well recognized for possessing an antiapoptotic function in certain cell types (39, 40). Disruption of this function typically results in cells death in vitro under conditions of serum/growth factor deprivation (41), c-myc overexpression (42), or exposure to chemotherapeutic agents (43). In contrast, the survival of the neuroectodermal tumor cells that we examined appears to display a distinct requirement for IGF1R function, even under basal culture conditions in vitro, and explains our difficulty in generating stable transfectants with >50% reduction in their cell surface receptor levels (Fig. 3 and Table 1). It has been proposed recently that the antiapoptotic signaling of IGF1R is mediated via activation of phosphatidylinositol 3-kinase and its putative effector Akt/PKB (40, 44). Our results suggest that neuroblastoma cells might display unique sensitivity to inhibitors of phosphatidylinositol 3-kinase, such as wortmannin (45) in vitro, and we are presently investigating this possibility.

In agreement with previous reports in other tumor models, we found that antisense-mediated suppression of IGF1R expression in neuroblastoma cells markedly inhibited their clonogenicity in soft agar (Fig. 3B). As a result, we examined the tumorigenicity of N2A cells that were transfected, briefly selected in G418 in vitro, and then injected s.c. into syngeneic mice. Complete inhibition of tumor formation was observed in four of four mice, which led us to evaluate the effect of direct injection of naked plasmid DNA into established tumors. Previous reports describing the effects of modifying IGF1R function on tumorigenicity have relied on modification of cells ex vivo before inoculation into mice. Direct gene transfer to mouse melanomas by intratumoral injection of free DNA, however, has been reported (46), and we were able to confirm successful transduction of a luciferase reporter construct into established N2A tumors using this approach (data not shown). Using dose and schedule parameters derived from a previous study demonstrating the antiangiogenic effects of intratumoral injections of antisense constructs targeting fibroblast growth factor signaling (47), we directly injected N2A tumors with control and antisense plasmid preparations. Unlike injection of two different control plasmids, antisense plasmid injection resulted in a marked antitumor effect, which was curative in 50% of the mice injected (Fig. 5 and Fig. 6A). Moreover, these mice were resistant to subsequent challenge with wild-type N2A cells in another anatomical location, suggesting the induction of a systemic, immune-mediated tumor rejection effect with similarities to those reported previously in experiments involving C6 glioma cells. In these experiments, however, cells were pretreated with oligonucleotides designed to inhibit IGF1R expression (11, 15) or stably transfected with antisense expression vectors to either the IGF1R (11) or IGF-I (17) before injection into syngeneic rats.

In terms of clinical application, direct injection of tumors in situ offers significant advantages in terms of ease and safety over ex vivo modification of tumors cells in culture (48). Direct injection into tumors of plasmids targeting IGF1R may prove feasible because stable transduction of tumor cells with our antisense construct was not required to elicit an antitumor effect. The reason that only 50% of tumors regressed in our experiments is unclear at this time but may be related to the low transfection efficiency of DNA plasmids in vivo. We are in the process of examining the use of polycationic lipids, which have been reported to increase the efficiency of gene transfer in vivo (49), and we have begun to explore the use of a neurotropic, replication-defective, herpes virus-based antisense vector (50) to see whether more consistent antitumor activity can be achieved in vivo.

The involvement of an immune-mediated component in the tumor regression we observed in syngeneic A/J mice was confirmed by using scid mice. In these immunocompromised animals, inhibition of tumor growth was evident only during the period of antisense plasmid injection, and all tumors subsequently progressed after completion of injections (Fig. 6). These findings are consistent with previous studies of C6 gliomas growing in immunocompetent rats. Trojan et al. (17) found that injection of tumor cells that had been transfected in vitro with an antisense construct to IGF-I resulted in the regression of established wild-type tumors. Marked infiltration of regressing tumors by CD8+ CTLs was demonstrated by immunostaining of tumor sections. Likewise, Resnicoff et al. (11) found that injection of C6 cells stably transfected with an antisense construct to the type 1 receptor also induced the regression of established tumors. Precedent clearly exists for the concept that inhibition of IGF signaling can render certain tumor cells immunogenic, but we have not yet determined what specific role T cells may play in mediating the regression of N2A tumors described in this report. Recently, it has become evident that the manner in which cells die can have profound consequences on their recognition by the host immune system. Devitt et al. (51) have reported that PS exposed on the outer surface of apoptotic cells appears to engage a specific receptor on phagocytic cells that activates a unique program of engulfment and processing of apoptotic cells. It is tempting to speculate that the exposure of PS that we documented on N2A cells by annexin V staining after antisense transfection in vitro (Fig. 4) could also result in altered recognition of N2A cells by antigen-processing cells in vivo, helping to break host tolerance for tumor antigens and eliciting an effective immune response. Alternatively, Melcher et al. (52) have proposed that tumor cells dying under stressful conditions activate heat shock protein expression and provide a unique stimulus for immune recognition and antigen processing, which serves to break tolerance to tumor antigens. We have prelim-
inary evidence that inhibition of IGF1R expression does induce heat shock protein expression in dying neuroblastoma cells, perhaps leading to their enhanced immunogenicity (data not shown).

It is interesting to note that neuroblastomas of low clinical stage in children under 1 year of age and neuroblastomas presenting as IVS disease often regress without cytotoxic therapy (53). It has been observed that inhibition of IGF1R expression does induce heat shock protein expression in dying neuroblastoma cells, perhaps leading to their enhanced immunogenicity (data not shown).

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