in vivo *26*: 19-30 (2012)

Description of a New Xenograft Model of Metastatic Neuroblastoma Using NOD/SCID/*Il2rg* Null (NSG) Mice

HERVÉ SARTELET[1,2], LUDOVIC DURRIEU[1], FRANÇOIS FONTAINE[1], CARINE NYALENDO[1,2] and ÉLIE HADDAD[1,3,4]

[1]CHU Sainte-Justine Research Center, Center de Cancérologie Charles-Bruneau, Montréal, QC, Canada; Departments of [1]Pathology and [3]Peadiatrics, CHU Sainte-Justine, Montréal, QC, Canada; [4]Department of Microbiology and Immunology, Université de Montréal, Montréal, QC, Canada

Abstract. *In order to develop a relevant xenogenic animal model of neuroblastoma (NB), we compared the tumorigenicity and metastatic potential of SK-N-SH and SK-N-DZ NB cell lines in nude mice and NOD/SCID Il2rg null (NSG) mice. Subcutaneous injection of cell lines induced tumor formation only in NSG mice and was accompanied by metastasis to the liver, adrenal glands, skull and bone marrow. NSG mice injected intravenously showed a profile of distant metastatis that was not observed in nude mice. In addition, tumor growth rates and organ infiltration patterns associated with injected NB cell lines correlated with the in vitro proliferation properties and genetic markers of poor prognosis in NB patients. We also showed that cisplatin chemotherapy was able to inhibit tumor growth. These results clearly demonstrate the higher tumorigenic and metastatic potential of NB cells in NSG mice. Therefore, this xenograft NB model should prove useful in testing the efficacy of new therapeutic approaches for NB.*

Neuroblastoma (NB) is the most common and deadly extracranial solid tumor of childhood as it accounts for 6-8% of all childhood cases of cancer (1). It is also responsible for approximately 15% of all paediatric oncology deaths (2). The main clinical problem in treating NB is metastasis, specifically metastatic stages III and IV according to the International Neuroblastoma Staging System (1, 2), because metastatic lesions are often resistant to current therapies. About half of all patients with NB present metastasis at the time of diagnosis (2). The incidence of metastasis to various sites at diagnosis is as follows: 70.5% to bone marrow, 55.7% to bone, 30.9% to lymph nodes, 29.6% to liver, 18.2% to the cranium, 3.3% to lung, and 0.6% to the CNS (3). Altogether, fewer than 30 to 40% of patients with NB survive despite aggressive treatment combinations that involve multiagent chemotherapy, surgery, autologous bone marrow transplantation, and radiation (3-5). Therefore, stage-specific and patient-specific innovative therapies are urgently needed. The development of such therapies and their translation into the clinic relies on the use of animal models that mimic features of the human disease. In this regard, xenograft mouse models represent an attractive strategy.

Immunodeficient mice that cannot reject xenotransplanted cells have been shown to be the best living recipient for developing xenograft models of human cancer (6-8). To date, the most extensively used mouse strain for developing NB xenograft models are SCID mice (9-12), SCID/beige mice (13, 14) and nude mice (5, 15-19) that are all T-cell deficient hosts. However, all these mouse strains display residual immune functions, in particular, natural killer (NK) cells that can inhibit tumor formation and metastatic spread of cancer cells (20, 21). Therefore, the recently described NOD/SCID/interleukin-2 receptor γ (*Il2rg*) null (NSG) mice have been shown to be one of the most immunodeficient mouse hosts to date since these mice are deficient in both T and B lymphocytes, do not have any NK cells, and display impaired dendritic cell functions (22). This improved immunodeficient profile has established NSG mice as the new 'gold standard' for human haematopoietic stem cell (23-25) and cancer research. Indeed, NSG mice have been shown to be better recipients than NOD/SCID, nude or other immunodeficient mouse strains for developing and studying various xenograft models of human cancer, such as melanoma (20, 26, 27), leukemia (28), pancreatic (29), cervical (30) and breast cancer (31).

Recently, NSG mice have been used in an NB xenograft model for studying tumor-infiltrating human lymphocytes in subcutaneous (*s.c.*) tumors of NB cells embedded in Matrigel® (32). However, such an artificial basement membrane-like matrix is known to enhance tumor growth

Correspondence to: Dr. Élie Haddad, Service d'immunologie et rhumatologie pédiatriques, CHU Sainte-Justine, 3175 Côte Sainte-Catherine, Montréal, Québec H3T 1C5, Canada. Tel: +1 5143454931 ext. 6217, Fax: +1 5143454897, e-mail: elie.haddad@umontreal.ca

Key Words: Neuroblastoma, mouse model, xenograft, metastasis, cisplatin, NSG.

and metastasis (33, 34), hence modifying the intrinsic tumorigenic properties of cancer cells and the pathogenesis of the related disease itself. Another report described the intravenous (*i.v.*) injection of NB cell lines into NSG, SCID-beige, and nude mice, and demonstrated that the use of NSG mice was advantageous in therapeutic and survival studies (35). However, the metastatic potential of free NB cells injected into NSG mice needs to be investigated further in order to validate the use of these mice as a relevant xenograft model of human NB and as a preclinical model for the development of new therapeutic strategies.

Therefore, we report here the study of the potential tumorigenicity of two NB cell lines, by evaluating proliferation rates and metastatic spreading capacity of these cell lines injected *i.v.* or *s.c.* without the use of an artificial matrix into NSG mice as compared to nude mice, which are still the most frequently used mouse strain for xenograft models of NB. We also studied the efficacy of cisplatin chemotherapy on tumor growth and metastatic potential in NSG mice.

Materials and Methods

Cell lines. SK-N-SH and SK-N-DZ NB cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). NB cell lines were maintained in culture in Dubelcco's Modified Eagle's Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS) (Life Technologies) and gentamycin 10 μg/ml, and incubated at 37˚C in 5% CO_2. Cells at 80-90% confluency were passaged using 0.25% trypsin-EDTA (Life Technologies).

Cell proliferation assay. SK-N-SH and SK-N-DZ NB cells were seeded in triplicate into 96-well plates and incubated for 24 h at 37˚C. Cells were then incubated with methylthiazole tetrazolium (MTT) solution for 4 hours (Promega – Fisher Scientific, Nepean, ON, Canada). Absorbance at 570 nm of the formazan product (indicative of cell viability) was measured in a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Each experiment was repeated at least three times.

DNA extraction and single nucleotide polymorphism (SNP) genotyping. DNA was extracted from SK-N-SH and SK-N-DZ cell lines using QIAamp® DNA Mini Kit (Qiagen, Mississauga, ON, Canada). SNP genotyping was performed with the Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA), containing more than 906,600 SNPs and more than 946,000 probes for the detection of copy number variation, according to the manufacturer's recommendations. Briefly, 500 ng of DNA was digested with restriction enzymes (NspI and StyI) followed by ligation to *Nsp*I and *Sty*I adaptors. Ligated DNA was amplified by PCR and purified using a filter plate. The DNA was subsequently fragmented and end-labelled prior to being hybridized to the SNP array. Hybridized chips were then washed, incubated with streptavidin-phycoerythrin (SAPE), and stained with a biotinylated anti-streptavidin antibody. The chips were scanned with a GeneChip Scanner 3000 7G (Bedford, UK) according to the manufacturer's instructions. Data was processed with Genotyping Console software (Affymetrix) and analyzed with Chromosome Analysis Suite software (Affymetrix).

Animal experiments. NSG mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and B6.Cg-*Foxn1nu*/J (nude) mice were purchased from Taconic (Germantown, NY, USA). The protocol of the present study was approved by our Good Laboratory Practices for Animal Research (GLPAR) Institutional Committee (Protocol #C328). All mice were housed under pathogen-free conditions in the animal facility and received autoclaved water and food. Seven-week-old NSG and nude mice were used in the study. SK-N-SH and SK-N-DZ cells were suspended in phosphate-buffered saline (PBS) at 5×10^7 cells/ml. A total of 5×10^6 cells (100 μl) were injected either *s.c.* into the left flank (n=31) or *i.v.* (n=29) into the lateral tail vein using a 28-gauge needle. Mice injected *s.c.* were monitored three times a week for tumor growth. Tumor size was measured with a Vernier caliper and tumor volume was calculated using the ellipsoid formula (length × width × height × 0.52). Blood was collected weekly and evaluated for the presence of circulating NB cells. Mice were sacrificed when limit points were reached as requested by our GLPAR committee. Mice injected *s.c.* were sacrificed when the tumor size reached 2,500 mm^3 or greater. At the time of sacrifice, subcutaneous tumor (if present), liver, adrenal glands, blood, spleen, lungs, femurs, and brain were harvested and evaluated for tumor cell infiltrate by histopathology and flow cytometry. Mice injected *i.v.* were sacrificed 6 weeks post-injection of NB cells to evaluate cell infiltrate in target organs. To test the efficacy of chemotherapy in our mouse model, mice injected *s.c.* with NB cells were treated with cisplatin (Mayne Pharma, Kirkland, QC, Canada) (n=6) delivered *i.p.* at 8 mg/kg from day 10 to 13 post-cell injection, as reported by others (36, 37). Mice receiving PBS at the same time were used as negative controls (n=6).

Flow cytometry. Detection of NB cells in the blood by flow cytometry was conducted following red cell lysis with ammonium chloride. Organ single-cell suspensions were obtained by crushing one half of the organ in PBS through a 70 μm strainer. Femurs were flushed with PBS using a 28-gauge needle to obtain cell suspensions from bone marrow. Detection of NB cell lines by flow cytometry was performed as described by Nagai *et al.* (38). Briefly, cells were stained with monoclonal antibodies against human CD81 (Fluorescein isothiocyanate(FITC)-conjugated) and human CD56 (Phycoerythrin(PE)-conjugated) with 7-amino actinomycin D (7-AAD) added for exclusion of nonviable cells from the assay. All flow cytometry reagents were purchased from BD Biosciences (Mississauga, ON, Canada). Acquisition of samples was performed on a FACSAria (BD Biosciences) and data were analysed using Diva software (BD Biosciences).

Histology and immunohistochemistry. Formalin-fixed, paraffin-embedded (FFPE) sections, 4 μm thickness, were haematoxylin, eosin and saffron (HES) stained and examined using an optical microscope (Zeiss, Germany). Immunohistochemistry (IHC) was performed on 4 μm FFPE sections using Ultraview Universal DAB detection kit (Ventana Medical Systems, Tucson, AR, USA). Mouse monoclonal anti-human neuron-specific enolase (NSE) (E27 Ventana; Ventana Medical Systems) and mouse monoclonal anti-CD56 (1B6 1:20; Vector Laboratories, Burlingame, CA, USA) were applied for 16 min, followed by application of the Ultraview Universal DAB detection kit. Metastatic sites composed of NB cells in the target organs were assessed by qualitative analysis under optical microscopy. Normal mouse or rabbit IgG, at same concentration as the primary antibody served as a negative control.

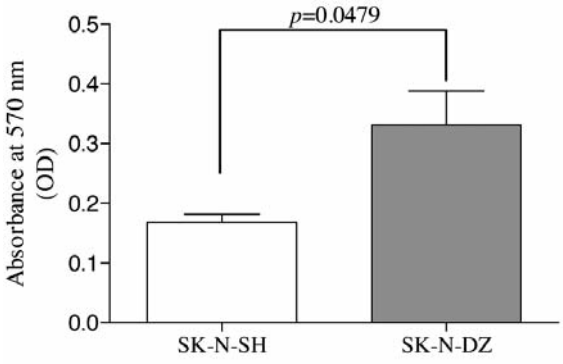

Figure 1. *In vitro proliferation of SK-N-SH and SK-N-DZ NB cell lines. NB cells were cultured for 24 h and then incubated with MTT for 4 hours. The figure shows the results of three independant experiments each performed in triplicate.*

Statistical analysis. The statistical significance of the differences in tumor growth was assessed using the Mann-Whitney test and the differences in metastatic incidence were assessed using the Fisher's exact test. *P*-values <0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 5™ software (GraphPad Software, La Jolla, CA, USA).

Results

Proliferation rate and genotypic analysis of SK-N-DZ and SK-N-SH cell lines. We performed an *in vitro* MTT proliferation assay to compare the proliferation rates of SK-N-DZ and SK-N-SH cell lines. We observed that the proliferation rate was higher for SK-N-DZ cells than for SK-N-SH cells (p=0.0479) (Figure 1). We also performed genetic analysis of the cell lines to assess myc myelocytomatosis viral-related oncogene, neuroblastoma-derived (*MYCN*) amplification, loss of heterozygosity/deletion of chromosomes 1p, 11q, and 14q, and gain of chromosome 17q, which are all known to be associated with a worse prognosis in NB patients (39-42). We detected *MYCN* amplification only in SK-N-DZ cells and we observed 1p, 11q, and 14q deletions in SK-N-DZ cells but not in SK-N-SH cells (Table I). Neither of the cell lines showed a 17q gain.

Subcutaneous injection of NB cells in NSG mice. Primary tumor: The *s.c*. injection of free SK-N-SH and SK-N-DZ cells gave rise to the rapid growth of subcutaneous tumors at the site of injection for all NSG mice (Figure 2A and Table II), while no tumors were observed in any of the nude mice (n=6) for up to 7 months following injection (data not shown). The absence of NB cells in nude mice was confirmed by immuno-histological analysis of the skin at the site of injection (data not shown). Tumors were detected earlier in NSG mice injected with SK-N-DZ cells than in those injected with SK-N-SH cells, as shown by the linear regression of the tumor size with the day of observation (p<0.0001) (Figure 2B). Indeed, detection of tumors ranged from day 32 to day 63 (mean=45.1±3.2) for mice injected with SK-N-SH cells, while it ranged from day 16 to day 41 (mean=30.1±2.7) for mice injected with SK-N-DZ cells (p=0.0002) (Figure 2). Tumor growth continued to increase rapidly thereafter, with doubling times of 3.21±0.22 days and 2.78±0.17 days (although not significant, p=0.14) for SK-N-SH- and SK-N-DZ-injected mice, respectively. Mice injected *s.c*. were sacrificed when their tumors reached 2,500 mm^3, as established by our GLPAR institutional committee. At the same time, we also sacrificed three nude mice injected *s.c*. with either SK-N-SH or SK-N-DZ cells and evaluated them for the presence of NB cells in the target organs. At necropsy, a large subcutaneous tumor was observed in all NSG mice injected with these cell lines (Figure 3A and Table II) while no tumors were observed in nude mice. Microscopic analysis showed that all tumors were comprised of sheets and cords of small undifferentiated cells typical of NB (Figure 3B). Tumor cells displayed membrane expression of CD56 and cytoplasmic expression of NSE by IHC, as did the target organs with large numbers of apparent mitotic figures (data not shown). There were no obvious differences in the morphology of tumors generated from the different cell lines and there were no signs of differentiation (*e.g*. detection of neuropils or ganglion cells). The tumor stroma was well vascularized, possibly contributing to the rapid growth of the tumor, and haemorrhage was also observed in the periphery of the tumor, typical of haemorrhagic human NB (43) (Figure 3A). The tumors, however, did not contain cells of schwannian stroma.

Metastasis: Strikingly, after *s.c*. injection of either cell line, we observed distant metastatic sites of free NB cells in some NSG mice (Table II). Indeed, liver metastasis was detected in 4 out of 13 NSG mice injected with SK-N-SH cells and in 2 out of 15 NSG mice injected with SK-N-DZ cells, while intracranial metastasis was detected only in NSG mice injected with SK-N-DZ cells (4 out of the 15; p=0.10, Table II). This intracranial metastasis was detected as tumor arising from the dura and in the epidural space, without invasion of the leptomeninges or brain parenchyma. Furthermore, metastasis to the adrenal glands was observed in one NSG mouse

Table I. *Genetic alterations of NB cell lines.*

Cell line	*MYCN* status	1p del	11q del	14q del	17q gain
SK-N-DZ	Amplified	+	+	+	–
SK-N-SH	2 Copies	–	–	–	–

MYCN status, loss of heterozygosity of the 1p, 11q and 14q chromosomes and gain of the 17q chromosome were performed by SNP genotyping of SH and DZ NB cells cultured *in vitro*. del: Deletion.

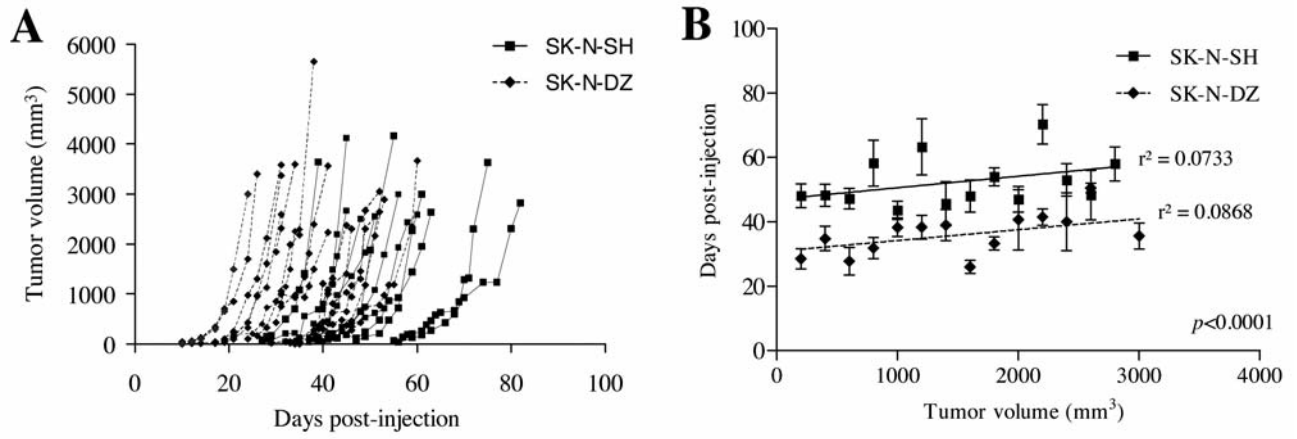

Figure 2. *Subcutaneous tumors are detected earlier in NSG mice injected with SK-N-DZ cells than these injected with SK-N-SH cells. A: Tumor volume was measured three times a week after s.c. injection of SK-N-SH (dotted line) or SK-N-DZ (solid line) cells into NSG mice. Each curve represents the tumor volume in a single mouse. B: Linear regression of tumor volume measured over time calculated for NSG mice injected with SK-N-SH cells (n=13) or SK-N-DZ cells (n=15).*

Table II. *Tumor growth and detection of metastasis† in mice injected s.c. with NB cell lines.*

Mouse strain	Cells	Day of sacrifice	Hepatic metastasis	Adrenal glands metastasis	Intracranial metastasis	Circulating cells	Lungs metastasis	Splenic metastasis	Bone marrow metastasis
NSG	SK-N-SH	58±3	4/13	1/13	0/13	0/13	0/13	0/13	0/6
NSG	SK-N-DZ	39±3	2/15	0/15	4/15	0/15	0/15	0/15	1/6

†Defined as detection of NB cells by immunohistology. Circulating cells were detected in blood by flow cytometry.

Table III. *Tumor growth and detection of metastasis† in mice injected i.v. with NB cell lines.*

Mouse strain	Cells	Hepatic metastasis	Adrenal glands metastasis	Intracranial metastasis	Circulating cells	Lungs metastasis	Splenic metastasis	Bone marrow metastasis
NSG	SK-N-SH	10/10	5/10	1/10	1/10	0/10	1/10	3/4
NSG	SK-N-DZ	15/16	2/16	6/16	4/16	2/16	0/16	3/4

†Defined as detection of NB cells by immunohistology. Circulating cells were detected in blood by flow cytometry.

injected with SK-N-SH cells, as well as in the bone marrow of another NSG mouse injected with SK-N-DZ cells. Histologically, these metastases corresponded either to isolated cells or to small nests of less than ten cells. No metastases were found in the spleen, the lungs or the circulation. Flow cytometric analysis of the tumor cell population revealed that the cells consisted primarily of CD81 $^{+}$/CD56 $^{+}$ SK-N-DZ or SK-N-SH cells (Figure 4). In most cases, CD81 $^{+}$/CD56 $^{+}$ NB cells were detected by flow cytometry when a metastasis was found by immunohistology. On the other hand, no tumor cells were detected in the organs of any of the nude mice injected *s.c.* with either cell line. Furthermore, nude mice sacrificed 7 months following the injection of NB cells with these cell lines did not have detectable metastasis to any of the organs examined (data not shown).

Intravenous injection of NB cells in NSG mice. Mice injected *i.v.* with NB cells were sacrificed at day 42. Widespread metastasis profile was detected in NSG mice injected with either cell line (Table III), while in nude mice the presence of NB cells in the target organs, whether by IHC or by flow cytometry (n=6), was never observed. More precisely, we

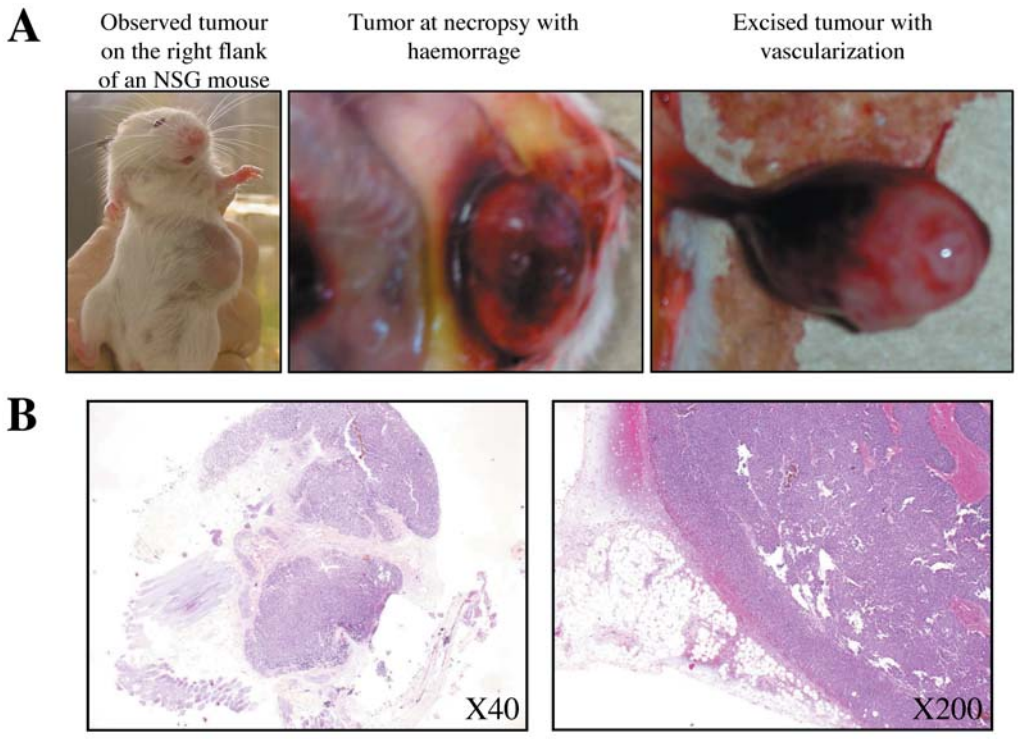

Figure 3. *Representative gross and light microscopy images of subcutaneous tumors in SK-N-DZ- or SK-N-SH-injected NSG mice. A: A subcutaneous tumor in an NSG mouse injected with SK-N-SH cells showing tumor vascularization and the presence of haemorrhage in the periphery of the tumor. B: Haematoxylin, eosin, and saffron stained sections of a subcutanenous tumor from an NSG mouse injected with SK-N-SH cells showing the presence of sheets and cords of small undifferentiated cells typical of NB.*

found liver metastasis in all NSG mice injected with SK-N-SH cells (n=10) and in 15 out of 16 mice injected with SK-N-DZ cells (Table III). The hepatic colonization by NB cells after *i.v.* injection in NSG mice was characterized macroscopically at necropsy as small white tumor masses. Immunohistological analysis of the liver of NSG mice showed multiple metastases composed of small blue cells with high membranous expression of CD56 and high cytoplasmic expression of NSE, corresponding to the hepatic location of the NB cells (Figure 5). Metastasis to the adrenal glands was also observed in two SK-N-SH- and five SK-N-DZ-injected mice.

Furthermore, we detected intracranial metastasis in 1 mouse out of 10 injected with SK-N-SH cells and in 6 mice out of 16 injected with SK-N-DZ cells. Metastasis to the skull was significantly more pronounced with SK-N-DZ cells than with SK-N-SH cells, when taking into account both administration routes (*s.c.* and *i.v.*) (p=0.0159). In addition, circulating NB cells in mice at the time of sacrifice were detected in the blood by flow cytometry in 1 out of 10 mice injected with SK-N-SH and in 4 out of 16 mice injected with SK-N-DZ cells (Table III). With *i.v.* injection, as opposed to *s.c.* injection, we detected a low level of metastasis to the spleen (1 out of 10 with SK-N-SH) and to the lungs (2 out of 16 with SK-N-DZ). Finally, both SK-N-SH and SK-N-DZ cells were observed in the bone marrow of most of the studied NSG mice (3 out of 4 in both cases).

Cisplatin treatment in NSG mice injected s.c. with NB cells. NSG mice injected *s.c.* with either SK-N-SH or SK-N-DZ cells were treated with cisplatin (8 mg/kg) from day 10 to 13 after cell injection. Cisplatin-treated mice showed a significant delay in the appearance of tumors compared to PBS-treated mice (n=3 in each group, p<0.0001 for SK-N-SH and SK-N-DZ cells) (Figure 6). Although metastasis was not observed in mice treated with cisplatin when compared to untreated mice, the difference was not significant (p=0.14).

Discussion

We investigated whether the NSG mouse strain was a better xenograft mouse model of human NB compared to the well-established nude mouse strain. Our results clearly show that the NSG mouse is a much better tool than the nude mouse to develop a relevant xenograft model of NB. Indeed, we

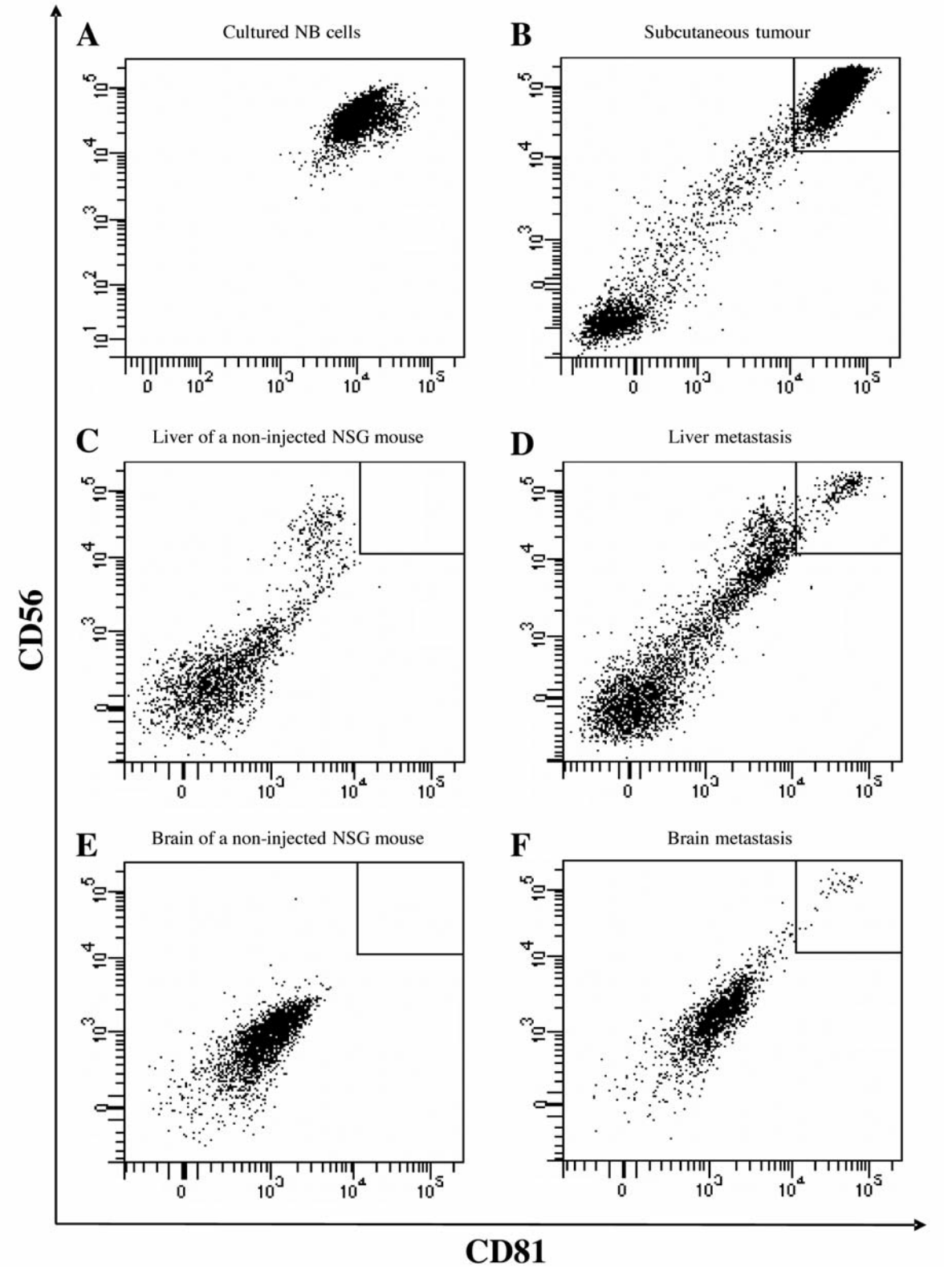

Figure 4. *Metastases consist mainly of CD56- and CD81-expressing cells as measured by flow cytometry. A: Surface expression of CD56 and CD81 in cultured SK-N-SH cells. B: Representative analysis of a subcutaneous tumor harvested at time of sacrifice from an NSG mouse injected with SK-N-SH cells. C: Representative analysis of a liver from a non-injected NSG mouse. D: Liver metastasis from an NSG mouse injected with SK-N-SH cells. E: Representative analysis of a brain from a non-injected NSG mouse. F: Brain metastasis from an NSG mouse injected with SK-N-SH cells.*

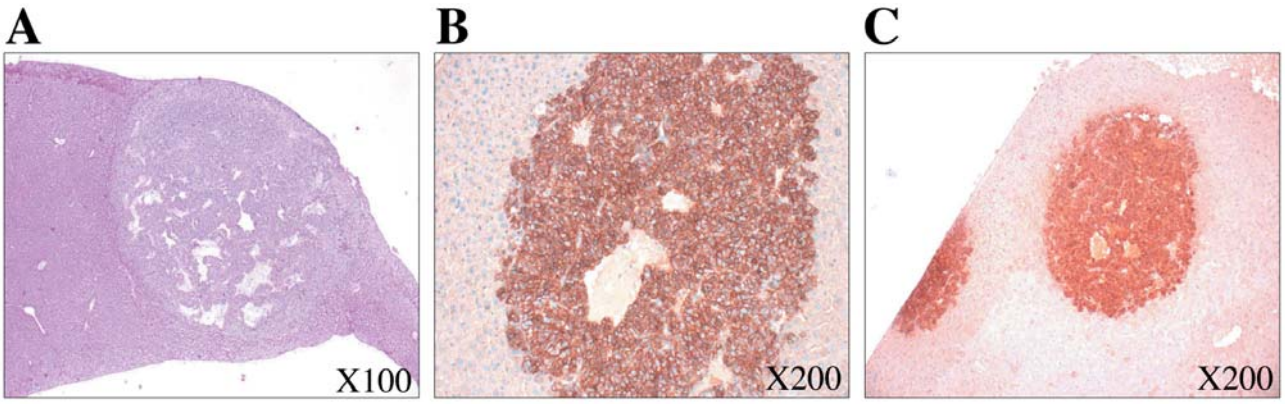

Figure 5. *Detection of high level expression of CD56 and NSE in a liver metastasis. A: A haematoxylin, eosin, and saffron stained section of a liver from an NSG mouse injected i.v. with SK-N-SH cells. B: Membranous expression of CD56 by IHC. C: Cytoplasmic expression of NSE in metastatic nodules.*

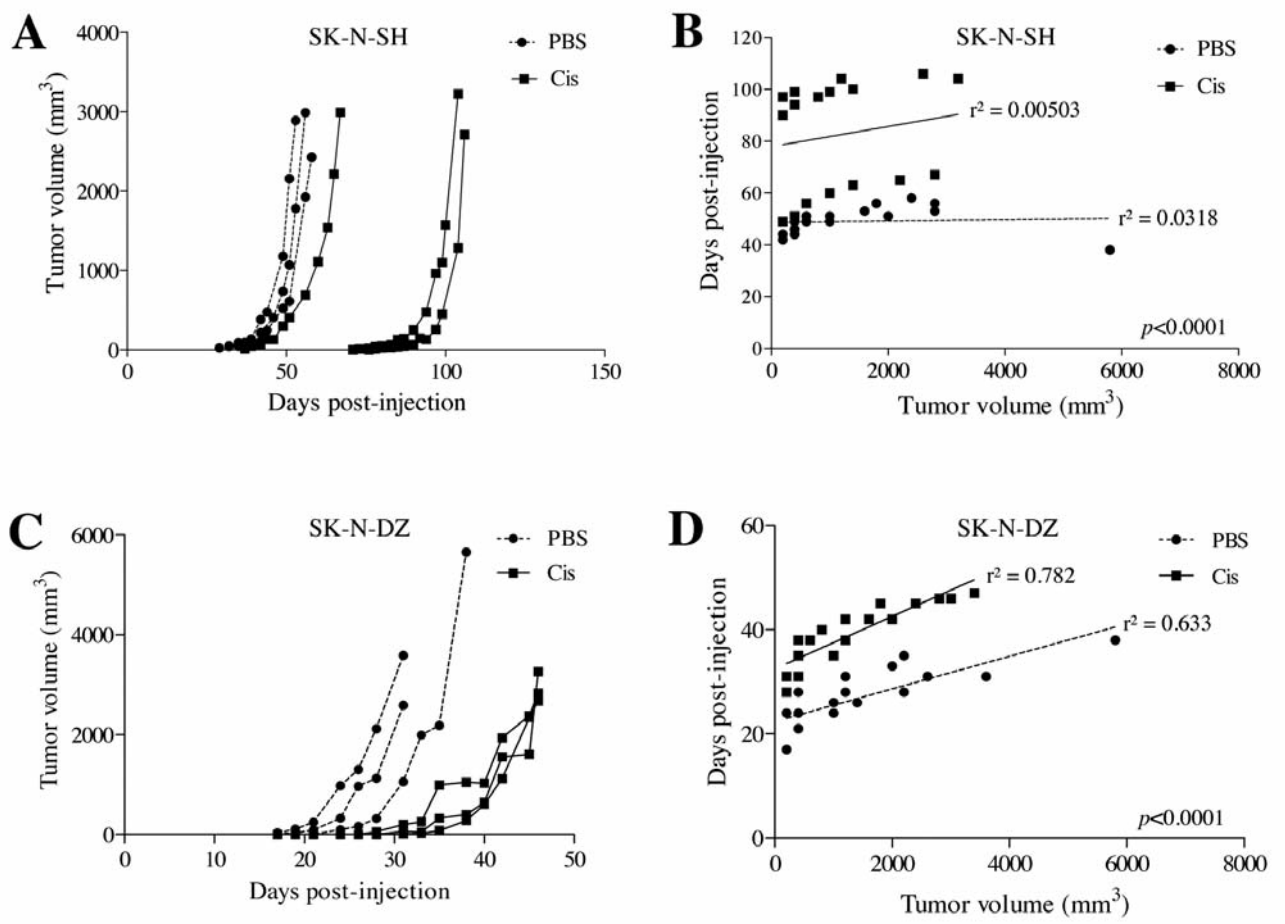

Figure 6. *Cisplatin treatment significantly delays the appearance of tumors. NSG mice were injected s.c. with SK-N-SH or SK-N-DZ cells and subsequently treated from day 10 to 13 with cisplatin at 8 mg/kg (n=3, solid line), or PBS (n=3, dotted line). A and C: Tumor volume was measured three times a week after injection. Each curve represents tumor growth in a single mouse. B and D: Linear regression of tumor volume with time. P<0.0001 for both cell lines.*

showed that unlike nude mice, NSG mice injected *s.c.* with SK-N-SH or SK-N-DZ NB cell lines displayed rapid tumor formation at the injection site. Previous studies had shown that following *s.c.* injection, NB tumor formation was more likely to occur when using cells suspended in a basement membrane-like matrix extract in nude (5, 44), SCID/beige (14), and NSG mice (32). In the present study, the induction of tumor growth in NSG mice was observed after *s.c.* injection of NB cell lines without the need for any basement membrane-like matrix. These basement membrane-like matrix extracts (*e.g.* Matrigel®) are composed of murine or bovine proteins and can contain growth factors, all of which can influence the immunopathology of NB (34, 45).

Flickinger *et al.* (16) and Kang *et al.* (46) reported that subcutaneous tumor growth occurred in nude mice inoculated with free NB or SK-N-SH cells 3 to 6 weeks post-injection, as described by Yang *et al.* (19) using 1×10^7 NBL-W-S cells; however, tumor growth kinetic data was not reported. In our study, we never detected any evidence of tumor growth in the nude mice under similar conditions for up to 7 months following cell injection. The tumor growth rate obtained in NSG mice was approximately twice as rapid when compared with other *s.c.* human NB xenograft mouse models, the best reported tumor doubling time being 5 days, as has been observed in nude mice injected *s.c.* with tumor fragments obtained by *in vivo* passage with SK-N-SH cells (17).

Our results also suggest that the NSG xenogenic model of NB can be used to assess the tumorigenicity and the aggressivity of tumor cells *in vivo*. Indeed, we found that SK-N-DZ cells had a higher invasive capacity since the kinetics of tumor appearance was significantly faster with SK-N-DZ than SK-N-SH cells following *s.c.* injection. Moreover, intracranial metastasis was observed with significantly greater frequency with SK-N-DZ than SK-N-SH cells. These *in vivo* results are in accordance with our *in vitro* findings that SK-N-DZ cells have a higher proliferation rate than SK-N-SH cells and display genotypic alterations, such as *MYCN* amplification and 1q, 11q and 14q deletions, known to be associated with poor prognosis in NB patients (39-42). The amplification of the human *MYCN* oncogene is the most powerful predictor of treatment failure in childhood (47). Furthermore, *MYCN* amplification has been shown to increase the invasive potential of NB cells (48). These observations suggest that specific tumorigenicity kinetics of NB cells lines *in vivo* in NSG mice parallel their *in vitro* and oncogenic properties.

Subcutaneous tumor growth after injection of free cancer cells in NSG mice has been shown previously with cervical cancer cells (30), but metastatic potential was not investigated. The *s.c.* injection of free NB cells along with their metastatic profile in NSG mice has never been systematically investigated previously. In our study, *s.c.* NB tumor formation was accompanied, in some NSG mice, by spontaneous distant metastasis to the liver, adrenal glands, cranium and bone marrow. In most of the reported xenograft models of NB, metastasis was observed only when NB cells were injected *i.v.* (5, 15, 43) or into orthotopic sites (13, 49, 50). Recently, the presence of metastasis after *s.c.* injection of free NB cell lines has been reported under specific conditions, with metastasis restricted to certain organs. Indeed, some reports described pulmonary metastasis after *s.c.* injection of SK-N-SH cells with or without Matrigel® in SCID mice (9, 12, 51), while no pulmonary metastasis was observed in our model, in NSG mice after *s.c.* injection. One possible explanation for this could be that free NB cells injected *s.c.* subsequently invade the blood circulation and are then trapped in the liver preferentially over the lungs in the NSG strain compared to other immunodeficient strains. In another recent study, micrometastasis was detected by PCR in bone marrow, spleen and liver after 2-3 months following *s.c.* injection of small numbers of SK-N-SH cells embedded in Matrigel® in NOD/SCID mice preconditioned with the alkylating agent, busulfan (52). By comparison, in our model, we detected metastasis without the need for PCR amplification or ablative conditions in bone marrow, spleen and liver of NSG mice in a shorter time period using higher cell numbers.

When NB cells were injected *i.v.*, NSG mice formed tumor nodules in the liver, while no tumors were observed in nude mice, thereby affirming the efficient cell dissemination and metastatic potential in NSG mice compared to nude mice. In addition, NSG mice exhibited infiltrating NB cells in adrenal glands, cranium, bone marrow, lungs and spleen. A previous study evaluated the metastatic profile of three NB cell lines injected *i.v.* into NOD/SCID mice, and at time of sacrifice, NB cells were detected in bone marrow and kidney as the major infiltrated organs; in contrast with the present study, however, they did not detect NB cells in liver or brain (53). The only study that reported the *i.v.* injection of NB cell lines into NSG mice was published by Brignole *et al.* (35), but the metastatic potential in target organs was not investigated.

Interestingly, the metastatic profile observed in mice in the present study is very similar to the characteristic pattern of metastatic sites found in human NB. Indeed, the frequency of metastasis to the liver and cranium in humans and in our mouse model is not that different (30% and 20%, respectively) (3). Metastasis to the CNS and lungs is rare in humans, as in our model, and is typically considered a terminal event (50, 53, 54). Metastasis to the bone marrow is, however, less frequent in our model. Lymph nodes could not be studied in our mouse model since NSG mice do not have lymph nodes. Finally, the high frequency of metastasis to the adrenal glands (7 out of 26) is very relevant since half of all primary NB tumors in humans arise in the adrenal medulla (1, 3). This suggests that the adrenal tissues of NSG mice are a favourable niche for the growth of injected human NB cells.

We also showed that cisplatin treatment inhibited tumor growth in NSG mice, as reported for other xenograft models (36, 37, 55), thereby demonstrating that the NSG xenograft model of NB could be used as a preclinical model to test the efficacy of novel chemotherapeutic agents prior to their validation in clinical trials.

In summary, we have described the tumor growth kinetics and the metastatic dissemination of free NB cell lines after *s.c.* and *i.v.* injection in NSG mice in order to generate a straightforward and relevant xenograft model of human NB. The simplicity of *s.c.* injection of cell lines described herein overcomes all the technical drawbacks involved in the use of more complex injection procedures such as orthotopic or secondary engraftment of metastatic tumors. Moreover, since NSG mice display very severe immunodeficiency, devoid of T-, B-, and NK-cells (22), these mice should prove very useful as a preclinical model to test new immunotherapeutic strategies. Indeed, it is very likely that immunotherapeutic effects observed, when NB cells are injected into NSG mice, will be strictly related to the effect on the injected cells and not on the residual immune system of the mice. Moreover, NSG mice have been described as a very relevant model for graft-*versus*-host disease that can be initiated simply by injecting human peripheral blood mononuclear cells (56, 57). Therefore, by using NSG mice, it should be possible to test whether the immunotherapeutic strategy can induce graft-*versus*-host disease, thereby making it possible to evaluate the efficacy and safety of a given immunotherapeutic strategy in the same mouse model, a strategy that is less appropriate in nude mice.

Altogether, our data clearly demonstrate that NSG mice are much more useful than nude mice for the establishment of a relevant xenograft model of human NB. This model will give us the opportunity not only to study the pathogenesis of human NB but also to test the efficacy and safety of new therapeutic approaches, and has thus the potential to greatly facilitate their clinical application.

Acknowledgments

The Authors would like to thank Leucan and La Fondation Charles-Bruneau for financial support. LD received a scholarship from La Fondation des étoiles and La Fondation CHU Sainte-Justine. The Authors also thank the staff of the animal facility for housing and maintaining the mice and for their excellent technical assistance and advice.

References

1 Brodeur GM, Seeger RC, Barrett A, Berthold F, Castleberry RP, D'Angio G, De Bernardi B, Evans AE, Favrot M and Freeman AI: International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. J Clin Oncol *6*: 1874-1881, 1988.

2 Maris JM, Hogarty MD, Bagatell R and Cohn SL: Neuroblastoma. Lancet *369*: 2106-2120, 2007.

3 DuBois SG, Kalika Y, Lukens JN, Brodeur GM, Seeger RC, Atkinson JB, Haase GM, Black CT, Perez C, Shimada H, Gerbing R, Stram DO and Matthay KK: Metastatic sites in stage IV and IVS neuroblastoma correlate with age, tumor biology, and survival. J Pediatr Hematol Oncol *21*: 181-189, 1999.

4 Saulnier Sholler GL, Brard L, Straub JA, Dorf L, Illeyne S, Koto K, Kalkunte S, Bosenberg M, Ashikaga T and Nishi R: Nifurtimox induces apoptosis of neuroblastoma cells *in vitro* and *in vivo*. J Pediatr Hematol Oncol *31*: 187-193, 2009.

5 Beltinger C and Debatin KM: Murine models for experimental therapy of pediatric solid tumors with poor prognosis. Int J Cancer *92*: 313-318, 2001.

6 von Kalle C, Wolf J, Becker A, Sckaer A, Munck M, Engert A, Kapp U, Fonatsch C, Komitowski D, Feaux de Lacroix W and Diehl V: Growth of Hodgkin cell lines in severely combined immunodeficient mice. Int J Cancer *52*: 887-891, 1992.

7 Lunardi-Iskandar Y, Bryant JL, Zeman RA, Lam VH, Samaniego F, Besnier JM, Hermans P, Thierry AR, Gill P and Gallo RC: Tumorigenesis and metastasis of neoplastic Kaposi's sarcoma cell line in immunodeficient mice blocked by a human pregnancy hormone. Nature *375*: 64-68, 1995.

8 Hudson WA, Li Q, Le C and Kersey JH: Xenotransplantation of human lymphoid malignancies is optimized in mice with multiple immunologic defects. Leukemia *12*: 2029-2033, 1998.

9 Krieger-Hinck N, Schumacher U, Muller A and Valentiner U: The effect of the PPAR-gamma agonist rosiglitazone on neuroblastoma SK-N-SH cells in a metastatic xenograft mouse model. Oncol Res *18*: 387-393, 2010.

10 Hamner JB, Dickson PV, Sims TL, Zhou J, Spence Y, Ng CY and Davidoff AM: Bortezomib inhibits angiogenesis and reduces tumor burden in a murine model of neuroblastoma. Surgery *142*: 185-191, 2007.

11 Baker J, Rogers K and Thomas A: A patient pathway to renal replacement therapy: implementation in the progressive renal insufficiency population. CANNT J *11*: 18-22, 2001.

12 Valentiner U, Valentiner FU and Schumacher U: Expression of CD44 is associated with a metastatic pattern of human neuroblastoma cells in a SCID mouse xenograft model. Tumor Biol *29*: 152-160, 2008.

13 Khanna C, Jaboin JJ, Drakos E, Tsokos M and Thiele CJ: Biologically relevant orthotopic neuroblastoma xenograft models: primary adrenal tumor growth and spontaneous distant metastasis. In Vivo *16*: 77-85, 2002.

14 Hansford LM, McKee AE, Zhang L, George RE, Gerstle JT, Thorner PS, Smith KM, Look AT, Yeger H, Miller FD, Irwin MS, Thiele CJ and Kaplan DR: Neuroblastoma cells isolated from bone marrow metastases contain a naturally enriched tumor-initiating cell. Cancer Res *67*: 11234-11243, 2007.

15 Turner WJ, Chatten J and Lampson LA: Human neuroblastoma cell growth in xenogeneic hosts: comparison of T cell-deficient and NK-deficient hosts, and subcutaneous or intravenous injection routes. J Neurooncol *8*: 121-132, 1990.

16 Flickinger KS, Judware R, Lechner R, Carter WG and Culp LA: Integrin expression in human neuroblastoma cells with or without *N-MYC* amplification and in ectopic/orthotopic nude mouse tumors. Exp Cell Res *213*: 156-163, 1994.

17 Rutgers M, Buitenhuis CK, Hoefnagel CA, Voute PA and Smets LA: Targeting of meta-iodobenzylguanidine to SK-N-SH human neuroblastoma xenografts: tissue distribution, metabolism and therapeutic efficacy. Int J Cancer *87*: 412-422, 2000.

18 Raffaghello L, Pagnan G, Pastorino F, Cosimo E, Brignole C, Marimpietri D, Montaldo PG, Gambini C, Allen TM, Bogenmann E and Ponzoni M: *In vitro* and *in vivo* antitumor activity of liposomal Fenretinide targeted to human neuroblastoma. Int J Cancer *104*: 559-567, 2003.

19 Yang Q, Liu S, Tian Y, Hasan C, Kersey D, Salwen HR, Chlenski A, Perlman EJ and Cohn SL: Methylation-associated silencing of the heat-shock protein 47 gene in human neuroblastoma. Cancer Res *64*: 4531-4538, 2004.

20 Carreno BM, Garbow JR, Kolar GR, Jackson EN, Engelbach JA, Becker-Hapak M, Carayannopoulos LN, Piwnica-Worms D and Linette GP: Immunodeficient mouse strains display marked variability in growth of human melanoma lung metastases. Clin Cancer Res *15*: 3277-3286, 2009.

21 Trinchieri G: Biology of natural killer cells. Adv Immunol *47*: 187-376, 1989.

22 Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, Ueyama Y, Koyanagi Y, Sugamura K, Tsuji K, Heike T and Nakahata T: NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood *100*: 3175-3182, 2002.

23 Hiramatsu H, Nishikomori R, Heike T, Ito M, Kobayashi K, Katamura K and Nakahata T: Complete reconstitution of human lymphocytes from cord blood CD34 $^{+}$ cells using the NOD/SCID/gammacnull mice model. Blood *102*: 873-880, 2003.

24 Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, Greiner DL and Handgretinger R: Human lymphoid and myeloid cell development in NOD/LtSz-SCID *Il2r* gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol *174*: 6477-6489, 2005.

25 McDermott SP, Eppert K, Lechman ER, Doedens M and Dick JE: Comparison of human cord blood engraftment between immunocompromised mouse strains. Blood *116*: 193-200, 2010.

26 Ma J, Lin JY, Alloo A, Wilson BJ, Schatton T, Zhan Q, Murphy GF, Waaga-Gasser AM, Gasser M, Stephen Hodi F, Frank NY and Frank MH: Isolation of tumorigenic circulating melanoma cells. Biochem Biophys Res Commun *402*: 711-717, 2010.

27 Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM and Morrison SJ: Efficient tumor formation by single human melanoma cells. Nature *456*: 593-598, 2008.

28 Agliano A, Martin-Padura I, Mancuso P, Marighetti P, Rabascio C, Pruneri G, Shultz LD and Bertolini F: Human acute leukemia cells injected in NOD/LtSz-SCID/*Il2r*gamma null mice generate a faster and more efficient disease compared to other NOD/SCID-related strains. Int J Cancer *123*: 2222-2227, 2008.

29 Suemizu H, Monnai M, Ohnishi Y, Ito M, Tamaoki N and Nakamura M: Identification of a key molecular regulator of liver metastasis in human pancreatic carcinoma using a novel quantitative model of metastasis in NOD/SCID/gammacnull (NOG) mice. Int J Oncol *31*: 741-751, 2007.

30 Machida K, Suemizu H, Kawai K, Ishikawa T, Sawada R, Ohnishi Y and Tsuchiya T: Higher susceptibility of NOG mice to xenotransplanted tumors. J Toxicol Sci *34*: 123-127, 2009.

31 Wege AK, Ernst W, Eckl J, Frankenberger B, Vollmann-Zwerenz A, Mannel DN, Ortmann O, Kroemer A and Brockhoff G: Humanized tumor mice – a new model to study and manipulate the immune response in advanced cancer therapy. Int J Cancer, 2011.

32 Reid GS, Shan X, Coughlin CM, Lassoued W, Pawel BR, Wexler LH, Thiele CJ, Tsokos M, Pinkus JL, Pinkus GS, Grupp SA and Vonderheide RH: Interferon-gamma-dependent infiltration of human T cells into neuroblastoma tumors *in vivo*. Clin Cancer Res *15*: 6602-6608, 2009.

33 Akbasak A, Toevs CC and Laske DW: Reconstituted basement membrane (matrigel) enhances the growth of human glioma cell lines in nude mice. J Neurooncol *27*: 23-30, 1996.

34 Noel A, De Pauw-Gillet MC, Purnell G, Nusgens B, Lapiere CM and Foidart JM: Enhancement of tumorigenicity of human breast adenocarcinoma cells in nude mice by matrigel and fibroblasts. Br J Cancer *68*: 909-915, 1993.

35 Brignole C, Marimpietri D, Di Paolo D, Perri P, Morandi F, Pastorino F, Zorzoli A, Pagnan G, Loi M, Caffa I, Erminio G, Haupt R, Gambini C, Pistoia V and Ponzoni M: Therapeutic targeting of TLR9 inhibits cell growth and induces apoptosis in neuroblastoma. Cancer Res *70*: 9816-9826, 2010.

36 Harned TM, Kalous O, Neuwelt A, Loera J, Ji L, Iovine P, Sposto R, Neuwelt EA and Reynolds CP: Sodium thiosulfate administered six hours after cisplatin does not compromise antineuroblastoma activity. Clin Cancer Res *14*: 533-540, 2008.

37 Das B, Antoon R, Tsuchida R, Lotfi S, Morozova O, Farhat W, Malkin D, Koren G, Yeger H and Baruchel S: Squalene selectively protects mouse bone marrow progenitors against cisplatin and carboplatin-induced cytotoxicity *in vivo* without protecting tumor growth. Neoplasia *10*: 1105-1119, 2008.

38 Nagai J, Ishida Y, Koga N, Tanaka Y, Ohnuma K, Toyoda Y, Katoh A, Hayabuchi Y and Kigasawa H: A new sensitive and specific combination of CD81/CD56/CD45 monoclonal antibodies for detecting circulating neuroblastoma cells in peripheral blood using flow cytometry. J Pediatr Hematol Oncol *22*: 20-26, 2000.

39 Attiyeh EF, London WB, Mosse YP, Wang Q, Winter C, Khazi D, McGrady PW, Seeger RC, Look AT, Shimada H, Brodeur GM, Cohn SL, Matthay KK and Maris JM: Chromosome 1p and 11q deletions and outcome in neuroblastoma. N Engl J Med *353*: 2243-2253, 2005.

40 Suzuki T, Yokota J, Mugishima H, Okabe I, Ookuni M, Sugimura T and Terada M: Frequent loss of heterozygosity on chromosome 14q in neuroblastoma. Cancer Res *49*: 1095-1098, 1989.

41 Lastowska M, Cullinane C, Variend S, Cotterill S, Bown N, O'Neill S, Mazzocco K, Roberts P, Nicholson J, Ellershaw C, Pearson AD and Jackson MS: Comprehensive genetic and histopathologic study reveals three types of neuroblastoma tumors. J Clin Oncol *19*: 3080-3090, 2001.

42 Bown N, Cotterill S, Lastowska M, O'Neill S, Pearson AD, Plantaz D, Meddeb M, Danglot G, Brinkschmidt C, Christiansen H, Laureys G, Speleman F, Nicholson J, Bernheim A, Betts DR, Vandesompele J and Van Roy N: Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. N Engl J Med *340*: 1954-1961, 1999.

43 Bogenmann E: A metastatic neuroblastoma model in SCID mice. Int J Cancer *67*: 379-385, 1996.

44 Mahller YY, Williams JP, Baird WH, Mitton B, Grossheim J, Saeki Y, Cancelas JA, Ratner N and Cripe TP: Neuroblastoma cell lines contain pluripotent tumor initiating cells that are susceptible to a targeted oncolytic virus. PLoS One *4*: e4235, 2009.

45 Fridman R, Kibbey MC, Royce LS, Zain M, Sweeney M, Jicha DL, Yannelli JR, Martin GR and Kleinman HK: Enhanced tumor growth of both primary and established human and murine tumor cells in athymic mice after coinjection with Matrigel. J Natl Cancer Inst *83*: 769-774, 1991.

46 Kang J, Kamal A, Burrows FJ, Evers BM and Chung DH: Inhibition of neuroblastoma xenograft growth by Hsp90 inhibitors. Anticancer Res *26*: 1903-1908, 2006.

47 Burkhart CA, Watt F, Murray J, Pajic M, Prokvolit A, Xue C, Flemming C, Smith J, Purmal A, Isachenko N, Komarov PG, Gurova KV, Sartorelli AC, Marshall GM, Norris MD, Gudkov AV and Haber M: Small-molecule multidrug resistance-associated protein 1 inhibitor reversan increases the therapeutic index of chemotherapy in mouse models of neuroblastoma. Cancer Res *69*: 6573-6580, 2009.

48 Zaizen Y, Taniguchi S, Noguchi S and Suita S: The effect of *N-MYC* amplification and expression on invasiveness of neuroblastoma cells. J Pediatr Surg *28*: 766-769, 1993.

49 Joseph JM, Gross N, Lassau N, Rouffiac V, Opolon P, Laudani L, Auderset K, Geay JF, Muhlethaler-Mottet A and Vassal G: In vivo echographic evidence of tumoral vascularization and microenvironment interactions in metastatic orthotopic human neuroblastoma xenografts. Int J Cancer *113*: 881-890, 2005.

50 Nevo I, Sagi-Assif O, Edry Botzer L, Amar D, Maman S, Kariv N, Leider-Trejo LE, Savelyeva L, Schwab M, Yron I and Witz IP: Generation and characterization of novel local and metastatic human neuroblastoma variants. Neoplasia *10*: 816-827, 2008.

51 Valentiner U, Haane C, Nehmann N and Schumacher U: Effects of bortezomib on human neuroblastoma cells *in vitro* and in a metastatic xenograft model. Anticancer Res *29*: 1219-1225, 2009.

52 Siapati EK, Rouka E, Kyriakou D and Vassilopoulos G: Neuroblastoma cells negative for CD44 possess tumor-initiating properties. Cell Oncol (Dordr) *34*: 189-197, 2011.

53 Castriconi R, Dondero A, Cilli M, Ognio E, Pezzolo A, De Giovanni B, Gambini C, Pistoia V, Moretta L, Moretta A and Corrias MV: Human NK cell infusions prolong survival of metastatic human neuroblastoma-bearing NOD/SCID mice. Cancer Immunol Immunother *56*: 1733-1742, 2007.

54 Ziegler MM, Ishizu H, Nagabuchi E, Takada N and Arya G: A comparative review of the immunobiology of murine neuroblastoma and human neuroblastoma. Cancer *79*: 1757-1766, 1997.

55 Teitz T, Stanke JJ, Federico S, Bradley CL, Brennan R, Zhang J, Johnson MD, Sedlacik J, Inoue M, Zhang ZM, Frase S, Rehg JE, Hillenbrand CM, Finkelstein D, Calabrese C, Dyer MA and Lahti JM: Preclinical models for neuroblastoma: establishing a baseline for treatment. PLoS One *6*: e19133, 2011.

56 Ito R, Katano I, Kawai K, Hirata H, Ogura T, Kamisako T, Eto T and Ito M: Highly sensitive model for xenogenic GVHD using severe immunodeficient NOG mice. Transplantation *87*: 1654-1658, 2009.

57 Gregoire-Gauthier J, Durrieu L, Duval A, Fontaine F, Dieng MM, Bourgey M, Patey-Mariaud de Serre N, Louis I and Haddad E: Use of immunoglobulins in the prevention of GvHD in a xenogeneic NOD/SCID/gammac- mouse model. Bone Marrow Transplant, May 16, 2011.

Received August 26, 2011
Revised October 20, 2011
Accepted October 21, 2011