Establishment and Characterization of New Orthotopic and Metastatic Neuroblastoma Models

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Abstract. Background/Aim: Treatment of metastatic neuroblastoma remains a challenge in pediatric oncology. Relevant preclinical models may improve exploration of oncogenesis and new therapies. We developed new orthotopic and metastatic models derived from stage 4 neuroblastoma. Material and Methods: Orthotopic and systemic models were established in BalbC Rag2–/–gammaC–/– mice following adrenal and intravenous injection of luciferase-transfected IMR-32 and IGR-N91 cells, respectively. Results: All four models exhibited 100% tumor take rate. Metastatic spread of orthotopic IMR-32-Luc cells was observed mainly to the lung, liver and bone; that of IGR-N91-Luc cells to liver, spleen and adrenals. Interestingly, systemic IMR-32-Luc cells metastasized rather to the lung, liver and bone, and IGR-N91-Luc to liver, lung, spleen and adrenals. Feasibility of non-invasive, real-time antitumor response evaluation was validated in the systemic models. Conclusion: These neuroblastoma models with distinct patterns of metastatic spread represent relevant tools for exploring local and metastatic tumor cell tropism, mechanisms of spread and evaluating new cancer therapeutics.

In the context of the rarity that characterizes childhood cancer, therapeutic research in experimental models is essential in order to select new anticancer agents before clinical exploration in children. The development of preclinical models mimicking human disease, such as orthotopic and metastatic models may thus better-predict antitumor activity than subcutaneous, non-metastatic models and permit exploration of the disease in its microenvironment and metastatic behavior.

Neuroblastoma is the most common solid extracranial childhood malignancy arising from the sympathetic ganglion. Neuroblastomas metastatic at diagnosis, or with molecular and genetic abnormalities, still represent a clinical challenge having survival rates of 40 to 50% (1, 2). Most preclinical in vivo models are subcutaneous models of established cell lines, rarely derived from primary tumors, and maintained in vivo (e.g. IGR-N91, IGR-NB8, IGR-NB3, IGR-N835) (3, 4). Subcutaneous models neither have the capacity to develop metastatic spread nor possess the microenvironment of the sympathetic ganglion. The development and exploration of relevant models may help to identify mechanisms involved in metastatic spread of this disease, as well as potential effective treatments for advanced neuroblastoma. Few orthotopic and metastatic models have been described for neuroblastoma (5, 6), although these models are increasingly used to study therapeutic efficacy. Bioluminescence is used to allow location and follow-up of early-stage tumors in real-time metastases, and assessment of efficacy of treatments in vivo. Current bioluminescence imaging techniques achieve a detection sensitivity in vivo of as few as 1,000 luciferase (luc)-transfected cells (7, 8).

We developed and characterized additional orthotopic and metastatic bioluminescent models of human neuroblastoma, IMR-32-Luc and IGR-N91-Luc, derived from primary stage 4 neuroblastoma with MYCN gene amplification. These have
distinct patterns in growth behavior and metastatic spread, and thus represent relevant tools for evaluating anticancer agents in a metastatic disease setting, as well as in a sympathetic microenvironment.

Materials and Methods

Neuroblastoma cell lines. The IGR-N91 cell line was established from a bone marrow metastasis of a neuroblastoma with amplified MYCN in an 8-year-old child (9). IMR-32 is derived from a stage 4 neuroblastoma in a 13-month-old child (10). Cells were grown in Dulbecco’s minimum essential medium (DMEM) with Glutamax and Roswell Park Memorial Institute (RPMI) medium, respectively, supplemented with 10% fetal calf serum (all Invitrogen, Saint Aubin, France).

Luciferase construct and transfection. The pcDNA3-Luc2 vector was constructed from two plasmids: one expressing firefly luciferase (Luc2; pGL4.17; Promega, Charbonnieres, France) and the second carrying a strong cytomegalovirus (CMV) promoter and a eukaryotic selective gene (pcDNA; Invitrogen, Saint Aubin, France). Luc2 gene was excised from plasmid pGL4.17 then inserted into the multiple cloning site of plasmid pcDNA3. Cells were transfected using lipofectamine (Lipofectamine 2000) and selected for neomycin resistance (both from Invitrogen, Saint Aubin, France). Luciferase expression and activity were measured by western blot (luciferase antibody) and bioluminescence (Luciferase Assay System; Promega, Charbonnieres, France). Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assay.

In vivo models of neuroblastoma. Animal experiments were validated by the CEEA26 Ethics Committee (approval number: 2010-02) and carried-out under the conditions established by the European Community (Directive 2010/63/UE). Balb –/–γC–/– mice were bred at Gustave Roussy Institute (Villejuif, France) and housed in ventilated cages under standard conditions of controlled temperature and humidity, and exposed to a 12-hour light/dark cycle. They were provided with standard diet and water ad libitum. Orthotopic models were established in 6- to 8-week-old mice by a left subcostal incision under 3% isoflurane anesthesia; Echo-Doppler (E1605; Promega, Charbonnieres, France) intraperitoneally, were anesthetized with 3% isoflurane, and imaged after 5 min, which is the peak of maximum bioluminescence, with an IVIS50 system (Perkin Elmer, Courtaboeuf, France). For orthotopic models, the whole body was initially imaged in three sections (back, left side, front). For chest imaging and detection of metastatic spread, the distance between camera and subject was shortened and the primary tumor covered to exclude its signal. Systemic models were imaged for two minutes each, back and front. Bioluminescence intensity is expressed as photons per second (p/s).

Ex vivo protocol: Organs were sampled after in vivo imaging and immersed in 150 μg/ml D-luciferin after sacrifice. Luciferase activity was measured with the IVIS50 system. Data for all groups of organs were normalized with acquisition parameters at each examination.

Histology. Organs were fixed in Finefix (Milestone Medical, Italy), and paraffin-embedded. Sections were stained with hematoxilin-eosin-saffranin (HES) and examined using light microscopy (Zeiss, Marly-le-Roy, France).

Antitumor evaluation in vivo. Animals carrying IGR-N91-Luc disseminated neuroblastoma were divided into groups of five and seven mice which received irinotecan (CPT-11; purchased from Pfizer, Paris, France) intravenously at 27 mg/kg for five days or saline vehicle (3). Animals were measured for bioluminescence twice a week. At occurrence of clinical symptoms, mice were euthanized; organs were harvested, examined by bioluminescence and fixed for histological analysis.

Statistical analysis. In vivo bioluminescence intensity and tumor volumes are reported as the mean±standard error of mean (SEM) using Graphpad Prism® software version 4.00 (Graphpad software Inc, La Jolla, CA, USA). Pearson test was used to determine correlations between signal intensity and tumor volumes. Statistical significance was determined using the two-tailed non-parametric Mann–Whitney or Kruskal–Wallis test. Cell doubling time was defined as the doubling time of each cell line in the exponential phase of growth; tumor doubling time in vivo was defined as the time a 200 mm3 tumor reached 400 mm3.

Results

Characterization of luciferase-transfected neuroblastoma cells. IMR-32 and IGR-N91 cell lines were chosen because they exhibit MYCN amplification, the hallmark of aggressive neuroblastoma, and were reported having the ability to form tumors when injected orthotopically into the supra-renal area (11, 12). Lipoctectamine transfection of the pcDNA3-Luc2 plasmid resulted in 20% luciferase-positive IMR-32 and 10% positive IGR-N91 clones, and the strongest luciferase-expressing stable clones were selected for further experiments (Figure 1A). Bioluminescence >104 p/s was detected in 1,000 cells, with IMR-32-Luc exhibiting higher intensity than IGR-N91-Luc, but bioluminescence increased proportionally to the number of of plated cells (Figure 1B). Growth and proliferation behavior of cells transfected with plasmid pcDNA3-Luc was assessed using the MTS assay (Figure 1C and D). Luciferase-transfected cells exhibited higher proliferation rates than their parental cells. The doubling time of IMR-32-Luc cells was 42
h versus 57 h for parental IMR-32 ($p=0.0043$, Mann–Whitney test), and 50 h and 66 h for IGR-N91-Luc and IGR-N91 cells, respectively ($p=0.0159$). Cell morphology and expression of characteristic markers such as disialogangliosid-2 remained unchanged (data not shown).

**Characterization of orthotopic neuroblastoma models.** In order to facilitate metastatic take, we chose Balb Rag2$^{-/-}$γC$^{-/-}$ mice that are deficient in natural killer (NK) cells, and B- and T-lymphocytes. In our hands, this gave improved take rates compared to prior experiments using athymic Swiss/nude mice (11).

**Orthotopic adrenal IMR-32-Luc model.** All mice (n=7) injected orthotopically with 1×10$^6$ IMR-32-Luc cells into the left adrenal presented bioluminescent signal at the injection site at day 3 after injection, with a constant increase until the experiment end at day 94 (Figure 2A and C). Using ultrasound (Figure 2B), injected glands were detectable at first imaging on day 8, however, with a size equal to that of the controlateral non-injected side (2 mm$^3$). In our experience, an increase in adrenal mass greater than approximately 10 mm$^3$ allowed prediction of tumor growth. Tumors greater in size than 10 mm$^3$ were first detectable at day 21 and growth of tumor volumes up to approximately 1,000 mm$^3$ correlated with increase in bioluminescent signals ($p<0.0001$; Pearson test). The median tumor doubling time was 4.1 days; 50% reached 1,000 mm$^3$ at day 76 after injection. Adrenal IMR-32-Luc tumors were found on ultrasound with heterogeneous and less echogenic areas, particularly large tumors. Neo-angiogenesis in adrenal tumors with vessels of large size (>40 μm) was detected by Doppler ultrasonography at day 15; numbers increased over time from one in the periphery to a mean of 18 vessels per tumor (range 14-29), with a slow increase from day 27 and an exponential one after day 47 (data not shown).

After coverage of the primary tumor mass, bioluminescence imaging further allowed detection of metastatic spread from day 65 post-injection (Figure 2D). New signals *in vivo* occurred in the chest, localized in the lung region in three animals; one of them presented with a signal near the liver. Autopsy in five animals (two died prior to study end) revealed large adrenal tumors but none had macroscopically-visible metastases. In addition to primary left adrenal tumors, *ex vivo*
Figure 2. Orthotopic IMR-32-Luc neuroblastoma in Balb Rag2<sup>−/−</sup>γC<sup>−/−</sup> mice. 1×10<sup>6</sup> IMR32-Luc cells were injected into the left adrenal gland and Balb Rag2<sup>−/−</sup>γC<sup>−/−</sup> mice imaged for bioluminescence with the IVIS50 system (A) and tumor growth by ultrasound (B) weekly for 12 weeks. C: Bioluminescent measurements (BLI) are plotted compared to tumor volume measured by ultrasonography. Graph shows means of seven animals; error bars represent the standard error of mean. Increase in bioluminescent signals of primary tumors was correlated with tumor volume growth (p<0.0001; Pearson test). Tumor doubling time in ultrasound measurements was 4.1 days. D: When covering the primary tumor site, additional bioluminescence signals in the thoracic area were detected from day 65 after injection of IMR32-Luc cells. E: Ex vivo bioluminescence in various organs of five animals bearing IMR-32-Luc adrenal tumors incubated in 150 μg/ml luciferin solution and detected with the IVIS50 system (left panel). Bioluminescence signals in adrenal tumor, lungs, bone and liver organ groups were considered statistically different compared to luciferin-injected control organs (right panel; *p<0.05; **p<0.01; ***p<0.001; Kruskal-Wallis test). Plots represent median +range. F: Histological Hematoxylin Eosin Saffron stainings of adrenal tumor and liver metastasis at ×50 magnification.
Figure 3. Orthotopic IGR-N91-Luc tumors in Balb Rag2−/−γC−/− mice. A total of 1×10^6 IGR-N91-Luc cells were injected into the left adrenal loge and mice imaged for bioluminescence with the IVIS50 system (A) and adrenal tumor growth by ultrasound (B) weekly for five weeks. C: Bioluminescent measurements are plotted compared to tumor volume measured by ultrasonography. Graph shows means of seven animals; error bars represent standard error of mean. Increase in bioluminescent signals of primary tumors was correlated with tumor volume growth (p=0.0417; Pearson test). Tumor doubling time in ultrasound measurements was 1.6 days. D: Coverage of orthotopic tumors allowed detection of additional bioluminescence signals in vivo from day 18 post-injection. E: Ex vivo bioluminescence signals in organs are shown per animal (left panel); signals in adrenal tumors, liver, spleen and contralateral adrenal/kidney organ groups (right panel) were considered statistically different compared to negative control (*p<0.05; **p<0.01; ***p<0.001; Kruskal-Wallis test). Plots represent median ± range. F: Hematoxylin Eosin Saffron stainings show adrenal tumor and liver metastases at ×50 magnification.
analysis detected bioluminescence ($>10^5$ p/s) in most organs in all five animals, suggesting the presence of luciferase-positive neuroblastoma cells in lung (5/5), liver (4/5), bone (5/5), and the contralateral adrenal/kidney (2/5) (Figure 2E). HES staining (Figure 2F) showed adrenal neuroblastoma in all five animals. Infiltrates were also observed in the animal’s liver, which had in vivo bioluminescence signal in the right abdominal region. Ex vivo bioluminescence in this organ was $1.6 \times 10^5$ p/s, which was less than that of several other organs including lungs in all five animals ($2.4 \times 10^6$ to $1.4 \times 10^7$ p/s), where histology did not detect metastases.

Thus, orthotopic IMR-32-Luc neuroblastoma developed adrenal tumors and metastases in 100% of animals, mainly in the lung ($p<0.001$; Kruskal–Wallis test), liver and bone ($p<0.05$ each), as compared to control organs of luciferin-injected animals ($3,000-10,000$ p/s).

Orthotopic adrenal IGR-N91-Luc model. All IGR-N91-Luc injected mice (n=7) presented with bioluminescent signals at the left adrenal gland on day 4 after inoculation (Figure 3A and C). Tumors greater in size than $10 \text{ mm}^3$ were first detected by ultrasound on day 22 (Figure 3B) and tumor growth became evident by days 28-42 (Figure 3A, C). Bioluminescence ex vivo in bone, liver and lung organ groups were considered statistically different compared to negative control for IMR-32-Luc and those in liver, spleen and adrenal/kidney organ groups in IGR-N91-Luc ($p<0.05$; **$p<0.01$; ***$p<0.001$; Kruskal-Wallis test). Plots represent median±range. C: Hematoxylin Eosin Saffron staining shows metastasis in bone marrow and lung respectively at $\times50$ magnification.
volumes increased in all animals except one. The median tumor doubling time was 1.6 days and tumor growth was paralleled by exponential increase in bioluminescence intensity during four weeks ($p<0.0417$; Pearson test). Similar to IMR-32-Luc, echopoor areas were observed in larger tumors and the first large vessels spreading from the periphery in the third week, increasing in numbers until the experiment end (mean=18; range=6-29) (data not shown).

Metastatic spread to the chest or liver region in the form of bioluminescence signals *in vivo* was detected at day 29 in four mice (Figure 3D). Autopsy detected large left adrenal neuroblastomas in five out of six animals. The animal which had no growing orthotopic mass presented with normal adrenal gland. However, corresponding to the bioluminescent signals in the left adrenal gland *in vivo*, histology detected neuroblastoma cells in the gland. None of the mice had macroscopical visible metastasis. Bioluminescence was detected *ex vivo* in all analyzed organs of all animals ($>10^5$ p/s; Figure 3E). Significant involvement was found in the spleen, contralateral adrenal/kidney (both $p<0.01$) and liver ($p<0.05$). Neuroblastoma metastases were detected by histology in the liver in two out of six animals, the spleen in one, and the left kidney by extension from adrenal tumor in two cases (Figure 3F).

Thus, IGR-N91-Luc neuroblastoma is a second reliable orthotopic model with rapid tumor doubling time and metastasis to most organs but mainly spleen, adrenal/kidney or liver.

**Characterization of systemic neuroblastoma models.** In order to develop models with increased capacity for metastatic spread, we established additional systemic models by intravenous administration of luciferase-transfected neuroblastoma cells.

**Systemic IMR-32-Luc model.** Following injection of $1\times10^6$ IMR-32-Luc cells into the tail vein, all four Balb $Rag2^{-/-}\gammaC^{-/-}$ mice developed disseminated bioluminescence signals at week 3 in the liver region and extremities, which increased with time (Figure 4A). Additional signals occurred subsequently in the spinal region in two animals and the abdominal region (4/4); all signals increased until the onset of disease symptoms at week 12, when animals were sacrificed.

Autopsy revealed macroscopic metastases in the liver in three animals. *Ex vivo* bioluminescence $>10^5$ p/s was detected in lungs (4/4), liver (3/4), spleen (2/4), kidney/adrenal (3/4), brain (1/4), spine (3/4) and bones (4/4) (Figure 4B). One animal with limited spread had bone and...
lungs and metastasizes. Overall mean luciferase expression in lungs, bone (both \( p<0.001 \)) and liver (\( p<0.05 \)) was significantly different from control organs. Histological analysis revealed neuroblastoma metastases in lungs (2/4), liver (3/4), spine (2/4) and adrenal/kidney (2/4), including one with only \( 10^4 \) p/s, being at negative controls level.

**Systemic IGR-N91-Luc model.** All four IGR-N91-Luc-injected animals exhibited initial bioluminescence signals in the liver region at day 8 after injection, subsequently in the left kidney and spleen which increased over time until week 7 when clinical symptoms appeared (Figure 4A).

Autopsy showed metastases in liver and spleen in all animals. Ex vivo bioluminescence identified luciferase-positive cells in all organs with \( >10^6 \) p/s: most intensively in liver \(( p<0.001 )\), lungs, spleen and adrenals/kidney (all \( p<0.01 \)) but also in heart, brain and bone (Figure 4B). HES staining detected metastatic infiltrations of neuroblastoma cells in liver (4/4), spleen (4/4), lung (1/4) and adrenal/kidneys (2/4).

Thus, intraoperative injection of both types of neuroblastoma cells resulted in disseminated metastatic models, with main spread to lung, bone and liver for IMR-32-Luc, and liver, spleen, adrenal/kidney and lung for IGR-N91-Luc.

**Irinotecan exhibits antitumor activity against systemic neuroblastoma xenografts in vivo.** We finally intended to validate our metastatic neuroblastoma model for exploring the efficacy of anticancer agents. We chose irinotecan which had shown activity against subcutaneous IGR-N91-Luc xenografts (3).

Animals were randomized 22 days after intravenous injection and treatment started at day 27 when bioluminescence signals rose (mean±SD: \( 6.0 \times 10^7 ± 2.4 \times 10^7 \) p/s and \( 9.6 \times 10^7 ± 5.1 \times 10^7 \) p/s, respectively for control and treatment groups). Irinotecan at 27 mg/kg for five consecutive days resulted in significant reduction of bioluminescence signals at days 8 and 10 \(( 1.1 \times 10^7 ± 7.0 \times 10^6 \) p/s for irinotecan-treated animals versus \( 3.3 \times 10^9 ± 4.0 \times 10^8 \) p/s in controls; \( p<0.001 \); Kruskal–Wallis; Figure 5A). Ex vivo analysis showed significant bioluminescence reduction in all samples (brain, spine, bones, liver, lung, spleen and kidney/adrenal) with a mean bioluminescence of \( 7.0 \times 10^8 ± 3.5 \times 10^8 \) p/s for treated animals and \( 3.7 \times 10^7 \) p/s±\( 1.6 \times 10^7 \) p/s for controls \(( p<0.001 \); Figure 5B). Histological analysis showed reduced metastatic infiltration as shown in Figure 5C for liver and adrenal glands.

Thus, bioluminescence imaging can be used successfully to evaluate treatment response in real time in systemic or metastatic models.

**Discussion**

We established two new orthotopic and two systemic models of MYCN-amplified childhood neuroblastoma, with a potential for distinct metastatic distribution. The inserted luciferase gene allowed early detection, in real time measuring of disease distribution, and response to treatment by bioluminescence imaging prior to conventional imaging techniques and clinical evaluations.

The first step to achieve in vivo cancer models detectable by bioluminescence is to produce cell lines that stably express the luciferase enzyme. The firefly luciferase enzyme exhibits light emission properties that are consistent with detection in deep tissue in vivo in contrast to green fluorescent protein that has an emission wavelength close to the auto-fluorescence of tissues and thereby masking the fluorescence signal (7, 13). The CMV promoter-containing plasmid allowed activation of both genes of interest, the luciferase gene and a eukaryotic selection to isolate bioluminescent clones. Ten to 20% of cells were successfully transfected by chemical method; luciferase expression remained stable in both cell lines throughout the entire in vitro and in vivo experiments.

Bioluminescence for equal numbers of IMR-32-Luc cells in vitro was higher than IGR-N91-Luc but was measurable at as low as 1,000 cells. The detection threshold of cells in vivo is assumed to be higher than in vitro due to the partial absorption of light by tissues depending on the depth of transmitted signal and staining of tissue traversed. Several authors have shown that a minimum of 1,000 cells was required for bioluminescence emission to be detected by CCD cameras (7). Ex vivo measurement of various organs of luciferin-only-injected animals showed a mean bioluminescence of \( <10^4 \) p/s which we considered as the background noise level and we set a threshold of \( 10^5 \) p/s for positive detection of tumor cells ex vivo.

To establish orthotopic and systemic models, we chose BalbC Rag2\(^{-/-}\)γC\(^{-/-}\) mice. Lack of B-lymphocytes and cytotoxic NK cells in addition to T-lymphocytes (14) proved to be beneficial for the development of orthotopic neuroblastoma, with 100% take rates and metastatic spread for both cell lines, as compared to our prior experience in athymic Swiss/nude mice (11). Our findings are consistent with those of Sartelet et al., who reported superior take rates of the SK-N-SH and SK-N-DZ neuroblastoma cell lines following subcutaneous and intravenous injection in B-, T-, and NK cells and interleukin-2-deficient NSG mice as compared to nude mice (6).

Bioluminescence imaging in vivo allowed to detect emergent orthotopic adrenal IMR-32-Luc and IGR-N91-Luc tumors approximately two to three weeks prior to ultrasound imaging. Metastatic spread of neuroblastoma cells was also assessed well ahead of the onset of palpable mass or clinical signs. These findings are in agreement with prior reports on NB1691 neuroblastoma, (5) and models of other cancer types using luciferase-transfected cells (15, 16). Quantification of bioluminescence photons correlated with measures of tumor volume by conventional techniques, albeit a plateau of bioluminescence intensity between \( 10^9 \) and \( 10^{10} \).
p/s was observed in both models. Camera settings were modified to exclude a technical limit of bioluminescence saturation. Dickson et al. suggested this effect as being attributed to hypoxia in large necrotic tumors (5). Although ultrasound showed less echogenic areas in larger tumors, histological evaluation did reveal only small areas of necrosis that should not explain such signal decrease in both neuroblastomas. Another explanation for this phenomenon could be intra-tumor hypoxia due to reduced perfusion and oxygenation in the center of the tumor, thus inhibiting the enzyme reaction luciferin/luciferase (17).

In vivo bioluminescence imaging allowed global tumor load to be followed in animals, whereas it appeared less sensitive at detecting metastatic spread, particularly micrometastases, than ex vivo bioluminescence evaluation of post-mortem harvested organs. On one hand, in vivo bioluminescence quantification is hampered when the number of light-emitting cells is small, and the signal is not detected or will be associated with background noise. The minimum detection of the IVIS50 system is determined by the manufacturer to be 60 pixels counted per image. On the other hand, intense signals at the orthotopic injection site hid weaker signals nearby and made detection of metastases difficult, even after its coverage. Furthermore, the IVIS50 system is unable to precisely determine the anatomical location of the signals as compared to other imaging techniques, such as computed tomography, and magnetic resonance imaging, even if the animals are imaged in different positions (dorsal, lateral, ventral). Our Institution will soon be equipped with the latest bioluminescence imaging system that will allow 3D reconstruction and location, which will improve the evaluation of our models.

Finally, as to be expected, ex vivo evaluation of organs seemed the most sensitive in detecting tumor cells in tissues. Similarly to in vivo imaging, histological analysis of randomly cut sections did not reveal smaller cell infiltrates. Serial sections of organs may need to be performed to increase the sensitivity of this technique.

Based on these observations, the characterization in metastatic dissemination behavior of our models was guided by ex vivo bioluminescence detection of luciferase-positive neuroblastoma cells. The models exhibited 100% take rates, with promptly growing tumors. IGR-N91-Luc is a more aggressive model exhibiting rapid growth behavior, and higher levels of in vivo and ex vivo bioluminescence, despite the fact that IMR-32-Luc cells in vitro have higher bioluminescence than IGR-N91-Luc.

In orthotopic models, distant bioluminescence signals appeared in the second half of experiments in the thoracic or right abdominal regions in several animals. Ex vivo analysis confirmed luciferin-positive neuroblastoma cells in multiple organs, mainly in lung, bone and liver for IMR-32-Luc, and spleen, right adrenal/kidney and liver for IGR-N91-Luc. Systemic administration of IMR-32-Luc and IGR-N91-Luc cells allowed more favorable detection of metastatic dissemination in vivo. Interestingly the two neuroblastoma models exhibited distinct distributions of metastases. IMR-32-Luc cells preferably homed to bone marrow, lungs and liver, as observed for NB1691 (5). IGR-N91-Luc cells were mainly found in liver, spleen, adrenal, and miliary infiltrations in lungs. In children, metastases of neuroblastoma frequently occur in bone marrow, bones and lymph nodes, occasionally in other sites such as the liver, but very rarely in lungs (18). The reason for the distinct tropism of these cell lines is as yet unclear. Metastases occur as a consequence of multiple cellular-biological processes which involve dissemination and invasion of cancer cells to anatomically distant organ sites and their subsequent adaptation to foreign tissue microenvironments.

The cancer environment comprises of tumor cells, as well as a multitude of stromal and vascular cells, participating in the cellular and molecular events necessary for invasion and metastasis (19). Each of the events is driven by the acquisition of genetic and epigenetic alterations within tumor cells and the co-option of nonepithelial stromal cells, which together endow incipient metastatic cells with traits needed to generate macroscopic metastases (19-21). The distinct metastatic pattern of our neuroblastoma models may allow selection of the best preclinical model according to the planned exploration of metastatic niches and microenvironment.

We finally intended to validate our models in regard to their usefulness for evaluating anticancer treatments. We chose the systemic IGRN-91-Luc model given the known sensitivity of IGR-N91 as subcutaneous model to the topoisomerase I inhibitor irinotecan (3). Bioluminescence imaging allowed stratification of animals into homogeneous treatment groups and evaluation of treatment response in vivo. Significant reduction of bioluminescent signals, almost disappearance in some, one week after treatment started resembled the regression pattern observed in subcutaneous IGR-N91 tumors. Reduced metastatic spread was confirmed ex vivo by bioluminescence analysis, as well as histology, highlighting the potential of our luciferase models for therapeutic use.

Explorations of local orthotopic tumors and distant metastases may improve our understanding of mechanisms involved in metastatic spread and further allow exploration of agents targeting the tumor microenvironment and metastatic niche. Orthotopic and metastatic models may be a step further in improving preclinical models mimicking residual and metastatic disease, which remains the biggest challenge in neuroblastoma.

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