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High metastatic efficiency of human sarcoma cells in Rag2/γc double knockout mice provides a powerful test system for antimetastatic targeted therapy

Patrizia Nanni [a,b], *Giordano Nicoletti* [c], *Lorena Landuzzi* [c], *Stefania Croci* [a], *Annalisa Murgo* [a], *Arianna Palladini* [a], *Agnese Antognoli* [a], *Marianna L. Ianzano* [a], *Valeria Stivani* [a], *Valentina Grosso* [a], *Sauveur-Michel Maira* [d], *Carlos García-Echeverría* [d], *Katia Scotlandi* [c], *Carla De Giovanni* [a,b,*], *Pier-Luigi Lollini* [b,e]

[a] *Cancer Research Section, Department of Experimental Pathology, Viale Filopanti 22, University of Bologna, 40126 Bologna, Italy*
[b] *Interdepartmental Centre for Cancer Research "Giorgio Prodi", University of Bologna, Bologna, Italy*
[c] *Laboratory of Experimental Oncology, Rizzoli Orthopedic Institute, Bologna, Italy*
[d] *Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel, Switzerland*
[e] *Department of Hematology and Oncologic Sciences "L. e A. Seragnoli", University of Bologna, Bologna, Italy*

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ABSTRACT

Immunodeficient animal models are invaluable tools to investigate the metastatic propensity of human tumours. However residual immune responses, in particular natural killer (NK) cells, severely hamper the traffic and growth of human tumour cells. We studied whether a genetically modified mouse host lacking T, B and NK immunity allowed an improved expression of the metastatic phenotype of malignant human tumours. Metastatic spread of a panel of human sarcoma cell lines was studied in double knockout Rag2$^{-/-}$;γc$^{-/-}$ mice in comparison with NK-depleted *nude* mice. Rag2$^{-/-}$;γc$^{-/-}$ mice receiving intravenous (i.v.) or subcutaneous (s.c.) human sarcoma cell lines developed extensive multiorgan metastases. Metastatic efficiency in Rag2$^{-/-}$;γc$^{-/-}$ was superior than in *nude* mice in terms of both metastatic sites and metastasis number. Metastatic growth in Rag2$^{-/-}$;γc$^{-/-}$ mice was faster than that in *nude* mice, thus allowing an earlier metastasis evaluation. Most human sarcomas metastasised in the liver of Rag2$^{-/-}$;γc$^{-/-}$ mice, a kind of organ preference undetectable in *nude* mice and specific of sarcomas, as several carcinoma cell lines failed to colonise the liver of Rag2$^{-/-}$;γc$^{-/-}$ mice, independently of their metastatic spread to other sites. *In vitro* analysis of the molecular mechanisms of liver metastasis of sarcomas implicated liver-produced growth and motility factors, in particular the insulin-like growth factor (IGF) axis. NVP-BEZ235, a specific inhibitor of downstream signal transduction targeting PI3K and mTOR, strongly inhibited liver metastasis of human sarcoma cells. In conclusion, the Rag2$^{-/-}$;γc$^{-/-}$ mouse model allowed the expression of human metastatic phenotypes inapparent in conventional immunodeficient mice and the preclinical testing of appropriate targeted therapies.

* *Corresponding author:* Address: Cancer Research Section, Department of Experimental Pathology, Viale Filopanti 22, University of Bologna, 40126 Bologna, Italy. Tel.: +39 051 209 4792; fax: +39 051 242 169.
E-mail address: carla.degiovanni@unibo.it (C. De Giovanni).
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1. Introduction

The major threat of malignant neoplasms is metastatic spread, rather than local tumour growth.[1] The only way to perform preclinical *in vivo* studies of metastatic mechanisms and of antimetastatic strategies of human tumours is to implant human cells in immunodepressed animals.[2,3] Unfortunately, xenogeneic growth of human neoplastic cells in most currently available animal systems fails to reproduce the malignancy observed in the original patient. Most human tumours grow in mice as local masses, but fail to invade surrounding tissues and to disseminate to distant organs. This state of things severely hampers the development of metastasis research and therapy.

The causes of metastatic inefficiency of human malignant cells in immunodepressed animal hosts are manifold. Some barriers relate to defective interaction of cognate, but xenogeneic, molecules, and could be solved only by genetic engineering of the host. Further obstacles are the residual immune responses of the immunodepressed host, which can kill human cells during the metastatic spread.[2]

Athymic *nude* (*Foxn1*$^{nu/nu}$) mice, probably the most widely used animal model in human oncology, provide a clear example of how the residual immune response of an immunocompromised host can differentially affect local or metastatic growth. *Nude* mice have a strong natural killer (NK) cell activity, which plays a minor role in hampering tumour growth in most primary sites. However NK cells, unlike most circulating leukocytes, are highly functional in the peripheral blood, actively killing human cells injected intravenously or entering the bloodstream from local tumours, thus severely hampering the metastatic spread.[4,5]

Early attempts toward the development of more permissive hosts allowing the study of human metastatic potential made use of different immunodepressed mouse mutants,[6] however such models provided only marginal advantages over *nude* mice, and did not reach widespread use. Significant improvements were obtained through the treatment of *nude* mice with depleting strategies directed against NK cells.[5,7–13] However such treatments are costly, cumbersome and are of transient efficacy. Genetically engineered mice with more pronounced combined immunodeficiency could constitute new metastatic models.[14,15]

Here we report that Rag2$^{-/-}$;γc$^{-/-}$ mice, which constitutively lack T, B and NK immunity, represent a highly permissive host for metastasis of human tumours, particularly for sarcoma cells.

2. Materials and methods

2.1. Mice

Rag2$^{-/-}$;γc$^{-/-}$ breeders were kindly given by Drs. T. Nomura and M. Ito of the Central Institute for Experimental Animals (Kawasaki, Japan)[16]; mice were then bred in our animal facilities under sterile conditions. Athymic Crl:CD-1-*Foxn1*$^{nu/nu}$ mice (referred to as *nude* mice) were purchased from Charles River Italy and kept under sterile conditions. Experiments were authorised by the institutional review board of the University of Bologna and done according to Italian and European guidelines.

2.2. Cell lines

The panel of human sarcoma cell lines consisted in Saos-2 osteosarcoma, U2OS osteosarcoma, TC-71 and 6647 Ewing's sarcoma cell lines, gift from T. Triche, SJ-Rh4 alveolar rhabdomyosarcoma and RD/18 and RD/12, two clonal derivatives of the RD embryonal rhabdomyosarcoma cell line.[17–19] TC-71 cells were transfected with pEGFP-N1 plasmid (Clontech, Mountain View, CA) and stably expressed Enhanced Green Fluorescent Protein (EGFP). Human carcinoma cell lines were: HepG2 hepatocellular carcinoma (ATCC, USA), Caco-2 colo-rectal carcinoma (ATCC), HT-29 colon adenocarcinoma (ATCC), MCF7 mammary carcinoma and SK-OV-3 ovarian carcinoma.[20] Cells were routinely cultured in Iscove's modified Dulbecco's medium (IMDM) or in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and were maintained at 37 °C in a humidified 7% CO_2 atmosphere. All medium constituents were purchased from Invitrogen, Milan, Italy.

2.3. Metastasis induction and therapy

Rag2$^{-/-}$;γc$^{-/-}$ mice (9–20 weeks-old) received the intravenous (i.v.) injection of viable human tumour cells (see tables for cell doses) in 0.4 ml phosphate-buffered saline (PBS). *Nude* mice (5–6 weeks-old) were i.v. treated with anti-asialo GM1 antiserum (Wako, Dusseldorf, Germany), 0.4 ml of a 1:30 dilution in PBS, to deplete NK activity,[11,12] 24 h prior to the i.v. injection of human tumour cells as mentioned above. Pilot experiments were performed to assess for each cell line the time at which experimental metastases could be detected. Mice were sacrificed at various times (see tables) and were subjected to an accurate necropsy. Lungs were stained with black India ink to better outline metastases and fixed in Fekete's solution. Lung and liver metastases were counted using a dissection microscope. In some experiments human sarcoma cells were subcutaneously (s.c.) injected (see Table 2 for cell doses) and spontaneous metastases were evaluated as reported above. NVP-BEZ235 (Novartis Institutes for BioMedical Research-Oncology, Basel, Switzerland) was formulated in 1-methyl-2-pyrrolidone (NMP)/polyethylene glycol 300 (PEG300) (Fluka) (10/90, v/v).[21] Solutions (5 mg/ml) were prepared fresh each day of treatment as follows: the powder was dissolved in NMP, warmed in hot water (100 °C) for 1–2 min, the remaining volume of PEG 300 was then added. A dose of 50 mg/kg was given *per os* daily starting from the day after cell injection. Mice received three drug administrations in the first week, and five drug administrations in the following weeks, for a total amount of 18 treatments. Mice were sacrificed 5 d after the last treatment and subjected to an accurate necropsy.

2.4. Growth and migration in conditioned media

To obtain conditioned medium, 4×10^5 human liver-derived HepG2 cells were seeded in RPMI+ 10% FCS in 6-well plates. Cells were cultured for 48 h, and then the medium was switched to serum-free DMEM or IMDM and incubation of the

nearly confluent monolayer continued. Conditioned medium was collected 24 h later, and cells conditioning the medium were harvested and counted. HepG2-conditioned medium was centrifuged at 2000*g* for 20 min at 4 °C and stored at −20 °C. The whole procedure was applied to aliquots of DMEM or IMDM serum-free medium to be used as control medium. For the growth assay in conditioned medium, human sarcoma and carcinoma cell lines were seeded in triplicate in 96-well plates, 10^4 cells/well, in 100 μl of HepG2-conditioned medium or control medium and cultured at 37 °C in a 7% CO_2 incubator. Proliferation was evaluated after 24 and 48 h of culture, by an assay employing sulfonated tetrazolium salt WST-1 (4-[3-(4-iodo-phenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche Diagnostic, Mannheim, Germany). The measurement is based on the ability of viable cells to cleave tetrazolium salts by mitochondrial dehydrogenases. Briefly, 10 μl per well WST-1 reagent was added and after 2 h incubation, absorbance of the samples was measured using a microplate reader (Sunrise TECAN, Switzerland) at 450 nm with a reference wavelength of 620 nm with control medium alone as the background level. Migration assay was performed using Transwell chambers (Costar, Cambridge, MA) with 8 μm pore size, polyvinylpirrolidone-free polycarbonate filters. Conditioned or fresh medium were put in the lower compartments. Human tumour cultures were harvested and counted, 5×10^5 cells were washed and seeded in serum-free medium in the upper compartment of the Transwell chambers, and incubated for 18 h at 37 °C in a 7% CO_2 incubator. Cells migrated through the filter to reach the lower chamber were counted using an inverted microscope or harvested and counted in a Neubauer hemocytometer. Type 1 IGF receptor (IGF1R) neutralisation was performed by incubation of the human tumour cells with 5 μg/ml of the anti-human IGF1R monoclonal antibody clone αIR3 (Calbiochem, Oncogene Research Products).

3. Results

3.1. *Multiorgan metastatic ability of human sarcomas in Rag2$^{-/-}$;γc$^{-/-}$ mice*

The metastatic potential of human sarcomas is expressed poorly in *nude* mice, even after the intravenous injection in NK-depleted hosts. Rag2$^{-/-}$;γc$^{-/-}$ double knockout mice, which constitutively lack T, B and NK immunity, could represent a more permissive host for metastasis studies. We compared the metastatic ability of a panel of human musculo-skeletal sarcomas (rhabdomyosarcomas, osteosarcomas and Ewing's sarcomas) in Rag2$^{-/-}$;γc$^{-/-}$ and in *nude* mice (Table 1A). All cell lines produced more metastases in Rag2$^{-/-}$;γc$^{-/-}$ than in *nude* mice, thus indicating that the former was a better host for studies of human metastatic behaviour. Metastatic growth in Rag2$^{-/-}$;γc$^{-/-}$ mice was faster than that in *nude* mice, thus allowing an earlier metastasis evaluation. The most striking result was the very high metastatic burden found in the liver of Rag2$^{-/-}$;γc$^{-/-}$ mice, as the same cells completely failed to colonise the liver of *nude* mice (Table 1A). Liver metastasis was the result of organ-selective homing of individual tumour cell lines, independent of the ability to colonise the lungs or other organs (Table 1A). Fig. 1A shows the extent of liver and lung colonisation of three sarcoma cell lines with divergent organ-specific metastatic propensities. Metastatic spread to organs other than liver and lungs was also enhanced in Rag2$^{-/-}$;γc$^{-/-}$ mice (Table 1A and Fig. 1B). Metastases were found in Rag2$^{-/-}$;γc$^{-/-}$ mice i.v. injected with as few as 1×10^5 cells (Table 1B). Rag2$^{-/-}$;γc$^{-/-}$ mice can also allow multiorgan metastasis after s.c. tumour cell growth (Table 2), in fact s.c. injected U2-OS osteosarcoma cells gave origin to local tumours and to spontaneous massive metastasis to lungs and kidneys at about 2 months after cell injection.

To investigate whether Rag2$^{-/-}$;γc$^{-/-}$ mice were equally prone to metastatic colonisation by all types of human tumours, we tested a panel of carcinomas of diverse histologic origin (liver, colorectal, breast or ovary). All human carcinomas were completely unable to metastasise to the liver of Rag2$^{-/-}$;γc$^{-/-}$ mice (Table 3), irrespective of their ability to colonise the lungs (HT-29, SK-OV-3), or other mouse organs (HepG2). An increased level of metastasis was evident in Rag2$^{-/-}$;γc$^{-/-}$ mice as shown by the number of lung metastases observed one month after the i.v. injection of SK-OV-3 cells (with a median of >200), which by far exceeds the number obtained two months after SK-OV-3 i.v. injection in NK-depleted nude mice (with a median of 32) (Table 3).

3.2. *In vitro growth and migration in liver-conditioned media*

To investigate whether the hepatic tropism observed *in vivo* was a cell-autonomous property of human tumours, we exposed *in vitro* tumour cells to the supernatant of human liver-derived HepG2 cells. Fig. 2 shows that the growth of sarcoma cell lines (with the exception of U2-OS) was stimulated by HepG2-conditioned medium, whereas carcinomas grew equally well in normal or conditioned medium.

Chemotaxis (Fig. 3A) is a further cell response relevant for metastatic spread that was stimulated by HepG2-conditioned medium in some sarcoma cell lines (Saos-2 and RD/12), whereas the migratory propensity of carcinomas was not further stimulated by conditioned medium. The liver produces a wealth of factors that could attract tumour cells and make them grow, which is one of the reasons why it is a frequent site of metastatic spread. One logical link between the liver and sarcomas is the insulin-like growth factor (IGF) axis. The liver is the major producer of IGF in the body,[22,23] and studies performed by many Laboratories, including our own, have shown that IGFs and their receptors can stimulate human sarcoma cells, in particular rhabdomyosarcomas and Ewing's sarcomas, in a paracrine and/or autocrine manner.[23,24] To evaluate the involvement of IGF in the chemotactic behaviour induced by HepG2-conditioned medium, we used a blocking monoclonal antibody against type I IGF receptor (IGF1R) which mediates responses to both IGF1 and IGF2. Fig. 3B shows that blocking of IGF1R reduced by half the chemotactic activity of HepG2-conditioned medium, thus demonstrating a significant contribution of hepatocyte-derived IGF.

3.3. *Therapy of liver metastases by dual PI3K/mTOR kinase inhibitor*

The involvement of the IGF axis in the liver tropism of human sarcoma cells suggests that therapeutic agents targeting

Table 1A – Metastatic capacity of human sarcoma cells (2×10^6 i.v.) in immunodepressed mice.

Cell line	i.v. cell dose	Mice	Median time to sacrifice (d)	Lung metastases				Liver metastases				Other metastatic sites			
				Incidence	%	Median	Range	Incidence	%	Median	Range	Incidence	%	Median	Range
Saos-2	2×10^6	Rag2/γc-KO	68	4/4	100	>200**	>200–>200	4/4**	100	>200**	>200–>200	4/4**	100	3**	1–3[b]
	2×10^6	Rag2/γc-KO	48	5/5*	100	>200**	>200–>200	5/5**	100	7**	2–17	0/5	0	0	0–0
	2×10^6	nude[a]	89	8/19	42	0	0–161	0/19	0	0	0–0	0/19	0	0	0–0
U2-OS	2×10^6	Rag2/γc-KO	36	9/9	100	>200**	>200–>200	0/9	0	0	0–0	0/9	0	0	0–0
	2×10^6	nude[a]	63	17/20	85	41	0–>200	0/20	0	0	0–0	0/20	0	0	0–0
TC-71	2×10^6	Rag2/γc-KO	30	12/12**	100	37**	1–105	12/12**	100	>200**	>200–>200	8/12	67	1	0–6[b]
	2×10^6	nude[a]	78	8/24	33	0	0–10	0/24	0	0	0–0	16/24	67	1	0–3[b]
6647	2×10^6	Rag2/γc-KO	21	5/5	100	114**	18–157	5/5**	100	>200**	>200–>200	5/5	100	21**	13–29[b]
	2×10^6	nude[a]	54	13/20	65	5	0–67	0/20	0	0	0–0	18/20	90	3	0–16[b]
SJ-RH4	2×10^6	Rag2/γc-KO	59	1/5	20	0	0–4	5/5	100	>200	>200–>200	2/5	40	0	0–2[b]
	2×10^6	Rag2/γc-KO	35	0/11	0	0	0–0	11/11	100	40	14–113	4/11	36	0	0–2[b]
	2×10^6	nude[a]		*n.d.*				*n.d.*				*n.d.*			
RD/12	2×10^6	Rag2/γc-KO	79	11/14**	79	3**	0–17	14/14**	100	75**	25–218	10/14**	71	2**	0–3[b]
	2×10^6	nude[a]	97	6/19	32	0	0–8	0/19	0	0	0–0	0/19	0	0	0–0
RD/18	2×10^6	Rag2/γc-KO	36	15/15	100	>200**	112–>200	15/15**	100	>200**	>200–>200	15/15	100	6**	4–57[b]
	2×10^6	nude[a]	58	14/15	93	26	0–327	0/15	0	0	0–0	15/15	100	2	1–8[b]

$Rag2^{-/-};\gamma_c^{-/-}$ indicated as Rag2/γc-KO. *n.d.*=not done.

a Pretreated with anti-NK antibodies (see Section 2).

b Metastatic sites: Saos-2: kidneys, adrenals, lymphoid organs; TC-71: kidneys, adrenals, bone, ovary; 6647: kidneys, adrenals, ovary, brown fat, lymphoid organs; SJ-Rh4: kidneys; RD/12: kidneys, adrenals, ovary; RD/18: kidneys, adrenals, ovary, lymphoid organs.

* Significance of difference versus *nude* mice is $p < 0.05$ (χ^2 test for frequency, non-parametric Mann–Whitney rank sum test for metastasis number).

** Significance of difference versus *nude* mice is $p < 0.01$ (χ^2 test for frequency, non-parametric Mann–Whitney rank sum test for metastasis number).

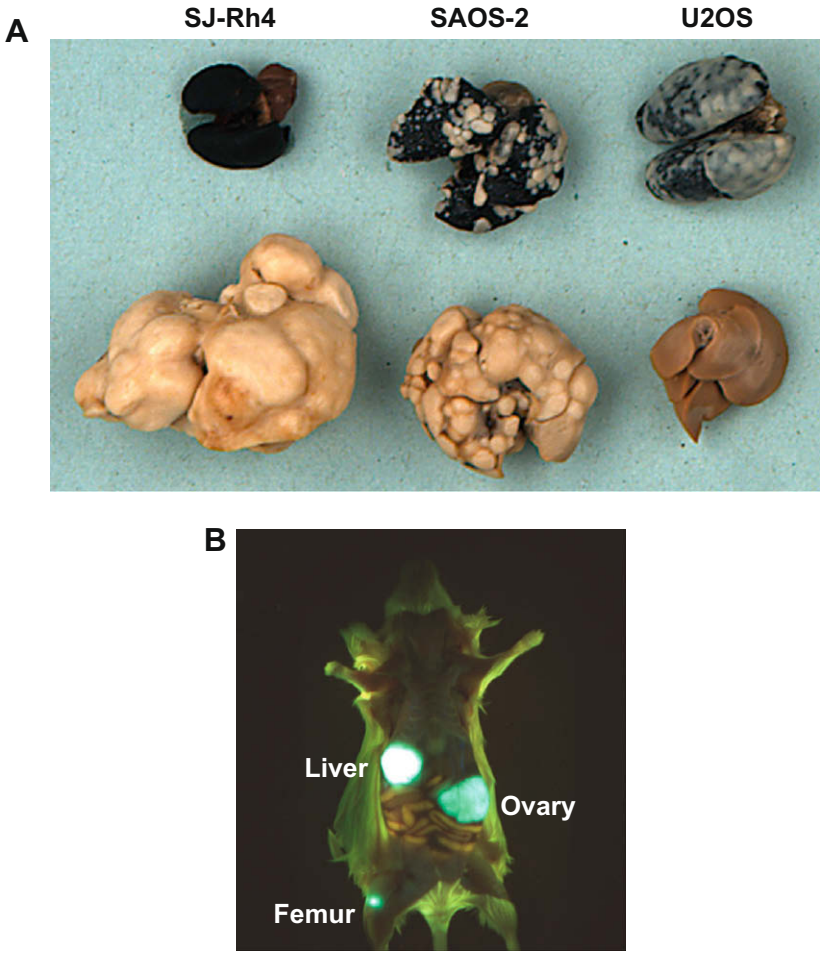

Fig. 1 – Metastatic capacity of human sarcoma cells injected i.v. in Rag2$^{-/-}$;$\gamma_c^{-/-}$ mice. (A) Lungs (above, filled with black India ink) and liver (below) of mice which had received the i.v. injection of indicated human sarcoma cells. (B) Metastases to different sites induced by the i.v. injection of EGFP-expressing TC-71 human Ewing's sarcoma cells.

IGF1R or downstream signal transducers could have a specific antimetastatic effect in this system. To test this hypothesis we treated Rag2$^{-/-}$;γc$^{-/-}$ mice bearing RD/18 liver micrometastases (i.e. 1 d after tumour cell i.v. injection) with the dual PI3K/mTOR kinase inhibitor NVP-BEZ235. We obtained a striking reduction in liver metastatic burden, which could be clearly appreciated upon visual inspection (Fig. 4A), and was statistically significant (Fig. 4B). The numbers of metastases in lungs and other sites were also significantly decreased (Fig. 4B). This result also illustrates the translational potential of the Rag2$^{-/-}$;γc$^{-/-}$ mouse model of metastatic spread.

4. Discussion

The metastatic potential of human sarcomas was expressed better in Rag2$^{-/-}$;γc$^{-/-}$ mice than in *nude* mice in terms of both metastatic sites and metastasis number: in Rag2$^{-/-}$;γc$^{-/-}$ mice we observed a strong increase of metastatic ability to lungs, liver and other sites. Metastatic growth in Rag2$^{-/-}$;γc$^{-/-}$ mice was faster than that in *nude* mice, thus allowing an earlier metastasis evaluation. The relative inefficiency of *nude* mice as hosts for metastatic studies was attributed mainly to their NK activity, which efficiently kills circulating tumour cells and contributes also to metastatic control in various organs.[4,5] Routine pretreatment of *nude* mice with anti-NK antibodies, as was done here, improved metastatic spread of human tumours, but had distinct limitations (the effect is time-limited, it is unclear to which degree diverse parenchymatous NK populations were depleted) and disadvantages (cost, additional treatments to be administered before tumour cells). Moreover it is worth remembering that the mutation harboured by *nude* mice affects thymic epithelium, not lymphocytes, hence some degree of T cell immunity and autoimmunity develops over time as a consequence of extrathymic T cell maturation[25]; B cells are affected even more indirectly, due to the lack of T cell help. Severely combined immunodepressed mice could provide an environment more favourable to reveal metastatic propensity.[14,15,26,27] Here we found that the stable deficiency of T and B cells caused by Rag2 knockout, coupled with the NK deficit mediated by the absence of the γ_c interleukin receptor chain, provides a superior host for metastasis studies with an impressive multiorgan metastasisation after both i.v. and s.c. injections.

The study of human tumours in Rag2$^{-/-}$;γc$^{-/-}$ mice uncovered a strong liver tropism. This phenomenon was clearly tumour-specific, because it was restricted to human sarcomas,

Table 1B – Metastatic capacity of i.v. injected human sarcoma cells (5 or 1×10^5 i.v.) in immunodepressed mice.

Cell line	i.v. cell dose	Mice	Median time to sacrifice (d)	Lung metastases				Liver metastases				Other metastatic sites			
				Incidence	%	Median	Range	Incidence	%	Median	Range	Incidence	%	Median	Range
Saos-2	5×10^5	Rag2/γc-KO	71	3/3	100	88	24–103	3/3	100	16	5–33	0/3	0	0	0–0
	1×10^5	Rag2/γc-KO	71	1/3	33	0	0–5	3/3	33	7	2–15	0/3	0	0	0–0
U2-OS	5×10^5	Rag2/γc-KO	23	4/4	100	>200	>200–>200	0/4	0	0	0–0	0/4	0	0	0–0
	1×10^5	Rag2/γc-KO	29	11/11	100	35	13–>200	0/11	0	0	0–0	0/11	0	0	0–0
TC-71	5×10^5	Rag2/γc-KO	26	8/8	100	8	1–35	8/8	100	18	4–>200	8/8	100	2	1–4
	1×10^5	Rag2/γc-KO	35	5/7	71	10	0–28	7/7	100	13	5–>200	4/7	57	1	0–2
RD/18	5×10^5	Rag2/γc-KO	42	8/8	100	81	32–172	8/8	100	>200	>200–>200	8/8	100	15	2–27
	1×10^5	Rag2/γc-KO	42	8/8	100	22	9–27	8/8	100	187	115–>200	8/8	100	3	1–8

$Rag2^{-/-};\gamma_c^{-/-}$ mice are indicated as Rag2/γc-KO.

Table 2 – Tumourigenicity and spontaneous metastasis of human sarcoma cells.

Cell line	s.c. cell dose	Mice	Median time to sacrifice (d)	Tumourigenicity			Lung metastases				Liver metastases				Other metastatic sites			
				Incidence	%	Median latency (d)	Incidence	%	Median	Range	Incidence	%	Median	Range	Incidence	%	Median	Range
U2-OS	10×10^6	Rag2/γc-KO	78	5/5	100	31	5/5	100	>200**	>200–>200	2/5	40	0	0–1	4/5*	80	3*	0–4[a]
	30×10^6	nude	118	4/5	80	33	2/5	40	0	0–45	0/5	0	0	0–0	0/5	0	0	0–0
TC-71	2×10^6	Rag2/γc-KO	27	5/5	100	10	3/5	60	3	0–14	0/5	0	0	0–0	3/5	60	1	0–1[a]
	3×10^6	nude	42	5/5	100	8	0/5	0	0	0–0	0/5	0	0	0–0	0/5	0	0	0–0

a Metastatic sites: U2-OS: kidneys, adrenals, peritoneum; TC-71: lymphnodes.

* Significance of difference versus *nude* mice is $p < 0.05$ (χ^2 test for frequency, non-parametric Mann–Whitney rank sum test for metastasis number).

** Significance of difference versus *nude* mice is $p < 0.01$ (χ^2 test for frequency, non-parametric Mann–Whitney rank sum test for metastasis number).

Table 3 – Metastatic capacity of human carcinoma cells (2×10^6 i.v.) in $Rag2^{-/-};\gamma_c^{-/-}$ mice.

Cell line	i.v. cell dose	Mice	Median time to sacrifice (d)	Lung metastases				Liver metastases			Other metastatic sites			
				Incidence	%	Median	Range	Incidence	%	Median	Range	Incidence	%	Median	Range
HepG2	2×10^6	Rag2/γc-KO	43	0/5	0	0	0–0	0/5	0	0	0–0	5/5	100	6	4–11[b]
Caco-2	2×10^6	Rag2/γc-KO	97	0/4	0	0	0–0	0/4	0	0	0–0	0/4	0	0	0–0
HT-29	2×10^6	Rag2/γc-KO	19	5/5	100	>200	>200–>200	0/5	0	0	0–0	0/5	0	0	0–0
MCF7	2×10^6	Rag2/γc-KO	97	1/4	25	0	0–1	0/4	0	0	0–0	0/4	0	0	0–0
SK-OV-3	2×10^6	Rag2/γc-KO	37	3/3	100	>200	>200–>200	0/3	0	0	0–0	0/3	0	0	0–0
	2×10^6	nude[a]	61	4/4	100	32	5–>200	0/4	0	0	0–0	0/4	0	0	0–0

$Rag2^{-/-};\gamma_c^{-/-}$ mice are indicated as Rag2/γc-KO.
a Pretreated with anti-NK antibodies (see Section 2).
b Metastatic sites: kidneys, adrenals, urogenital, lymphoid organs.

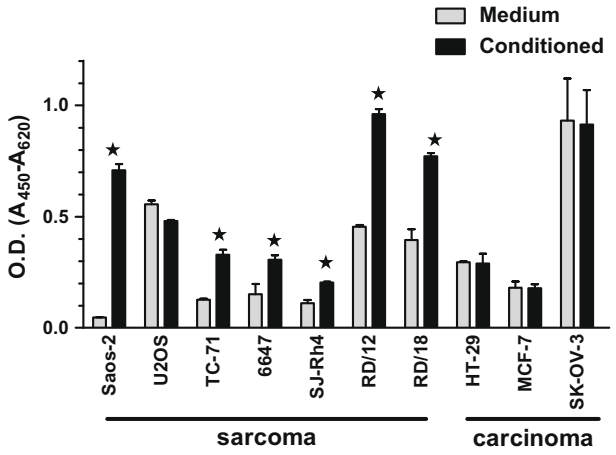

Fig. 2 – *In vitro* growth of human sarcoma cells stimulated by medium conditioned by human liver-derived cells (HepG2). Star indicates a significant difference between normal and conditioned medium ($p < 0.05$ at least, Student's *t* test).

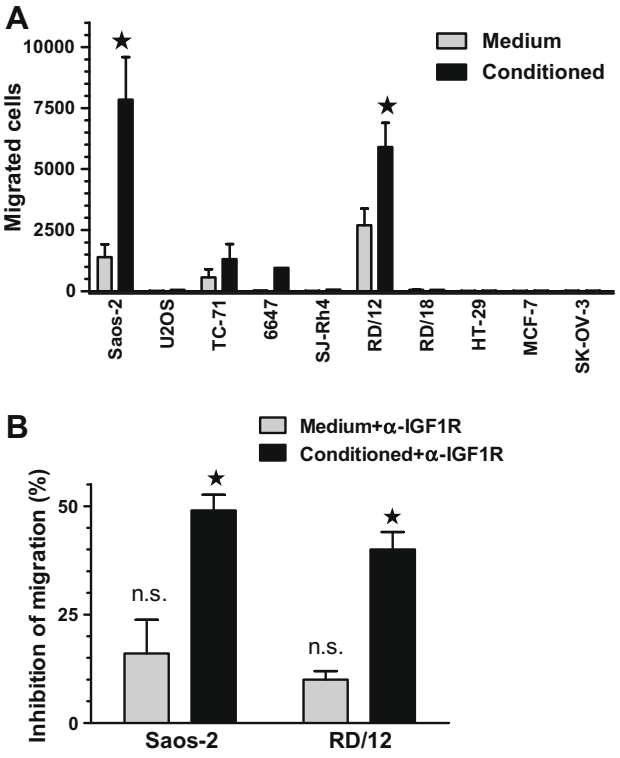

Fig. 3 – Migration of human sarcoma cells in medium conditioned by human liver-derived cells (HepG2). (A) Migration assay. Star indicates a significant difference between fresh and conditioned medium ($p < 0.05$ at least, Student's *t* test). (B) Percentage of reduction of liver-stimulated or basal migration caused by IGF1R neutralisation. Star, n.s.: significance or non-significance versus cell migration in the absence of anti-IGF1R (star = $p < 0.05$ at least, Student's *t* test; n.s. = not significant).

whereas carcinomas did not colonise the liver of $Rag2^{-/-};\gamma c^{-/-}$ mice. In humans the liver tropism of sarcomas depends on the anatomical location of the primary tumour.[28,29] In gen-

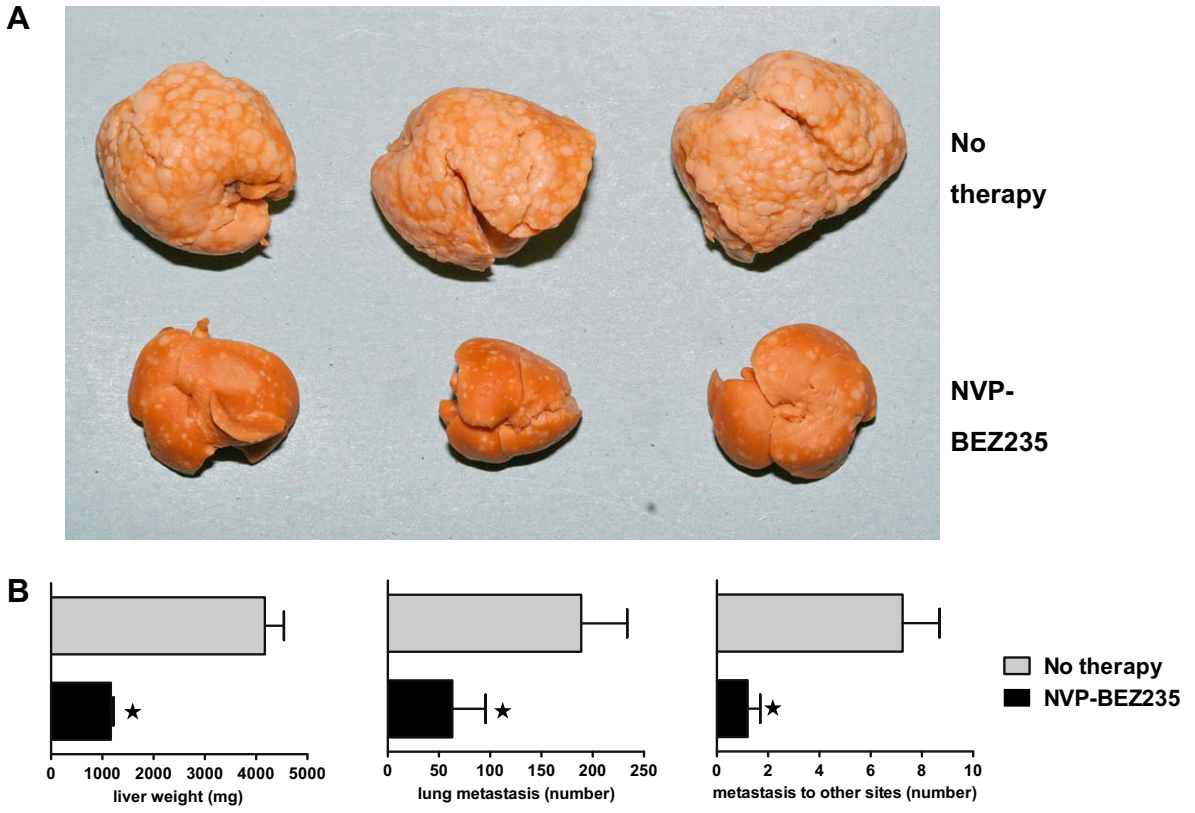

Fig. 4 – Therapy by NVP-BEZ235 of metastases induced by the i.v. injection of human rhabdomyosarcoma RD/18 cells in Rag2$^{-/-}$;$\gamma_c^{-/-}$ mice. (A) Liver metastatic burden in untreated mice (above) or in mice treated with NVP-BEZ235 (below). (B) Quantitative evaluation of metastatic load to liver (left), lungs (middle), and other sites (right). Mean and SEM from five mice is shown for each group. Star indicates a statistically significant difference ($p < 0.05$, Student's t test). Liver weight in normal Rag2$^{-/-}$;$\gamma_c^{-/-}$ mice was 1133 ± 30 mg.

eral, visceral neoplasms metastasise efficiently to the liver, whereas tumours arising in the limbs do so more rarely. Our results suggest that this phenotype depends more on NK control of circulating tumour cells than on cell-intrinsic ability of limb sarcomas to colonise the liver.

The ability of visceral sarcomas to reach the liver without undergoing NK control could be construed as a novel immune escape mechanism, which could in turn lead to immunotherapeutic strategies based on the route of metastatic dissemination of tumour cells ('highwayman immunotherapy'), for example by specifically potentiating local NK activity in visceral sarcomas. Musculo-skeletal sarcomas frequently arise in peripheral locations, and are normally thought to metastasise only rarely to the liver. It must be considered however that improvements in survival brought about by therapeutic advancements result also in modified metastatic patterns.[30] Indeed it was found that the relative proportion of patients with liver secondaries was increased in recent patient cohorts as compared, for example, with pre-adjuvant therapy patients.[30] Therefore highwayman immunotherapy might in the future find application also in the field of musculo-skeletal sarcomas.

One issue left open by our study is why carcinomas, which are known to metastasise the liver of cancer patients, fail to do so in Rag2$^{-/-}$;$\gamma c^{-/-}$ mice. We can offer some working hypotheses that could be explored experimentally in the future. First, carcinomas could be more sensitive than sarcomas to residual immune components of Rag2$^{-/-}$;$\gamma c^{-/-}$ mice, in particular liver Kupffer cells. If this is the case, then treatments that selectively impair liver phagocytosis[31] could enhance liver metastasis of human carcinomas, and eventually foster the development of a further generation of genetically modified hosts combining a macrophage deficit with those of Rag2$^{-/-}$;$\gamma c^{-/-}$ mice. A specular hypothesis would be that Rag2$^{-/-}$;$\gamma c^{-/-}$ mice lack immune components actually favouring liver metastasisation of human carcinomas. For example a major factor in liver metastatic propensity of colorectal carcinomas is IL-10,[32] a cytokine physiologically produced by various cell types missing in Rag2$^{-/-}$;$\gamma c^{-/-}$ mice, like activated T helper, B and NK cells. A final possibility involves species-specific receptor/counter-receptor pairs required for tumour cell adhesion, migration or proliferation. For instance the human carcinoembryonic antigen (CEA) system is evolutionarily different from the murine equivalent,[33] and it is known that CEA expression can enhance the metastatic potential of human colorectal carcinomas.[32] Also in this case genetic manipulation of the host could be used to introduce expression of the required human molecules in Rag2$^{-/-}$;$\gamma c^{-/-}$ mice.

To investigate the molecular determinants of liver metastatic propensity of human sarcomas we analysed the behaviour of tumour cells exposed *in vitro* to media conditioned by HepG2 cells. Two important determinants of metastatic pro-

cess were studied: cell growth and migration. HepG2-conditioned medium induced cell growth and chemotaxis only among the subset of sarcoma cells that are able to colonise the liver of Rag2$^{-/-}$;γc$^{-/-}$ mice. The fact that liver metastatic and non-metastatic cells could be discriminated *in vitro* confirmed that the different behaviour observed *in vivo* is a cell-autonomous phenotype.

In the search of molecules mediating the observed effects, IGF1 appeared as a logical candidate, because the liver is the major bodily producer of the growth factor,[22,23] and sarcomas express IGF1R inducing cell proliferation and chemotaxis.[23] Blocking monoclonal antibody experiments indeed confirmed the importance of the IGF1R axis in our system. It should be noted that the effect of liver supernatant was not completely abolished by IGF1R blocking antibody, thus indicating the involvement of additional mediators, as could be expected given the complex metabolic role of the liver.[34] One such mediator worth of further analysis is hepatocyte growth factor/scatter factor (HGF/SF), which is also a paracrine and autocrine growth and migration factor for human musculo-skeletal sarcomas.[35,36] In particular, we previously found that RD/18 cells, which did not show migratory response to HepG2-conditioned medium, were chemoattracted by HGF.[36]

In conclusion, lack of NK cells can cooperate with IGFs, and eventually other microenvironment signals, in determining the metastatic potential of human sarcoma cells.

The identification of IGF as an important factor of metastasisation of human sarcomas suggested that agents specifically targeting the IGF axis or downstream signal transducers[24] could be effective therapeutic antimetastatic approaches. One such agent, the dual PI3K/mTOR kinase inhibitor NVP-BEZ235,[21] strongly decreased metastatic burden to liver, lung and other metastatic sites in Rag2$^{-/-}$;γc$^{-/-}$ mice, indicating that this targeted therapy could be effective in the treatment of metastatic human sarcoma patients, who still face a dire prognosis and are in need of novel, effective therapeutic approaches.

Conflict of interest statement

C. Garcia-Echeverria and S.-M. Maira are employees and shareholders of Novartis Pharma.

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