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Hexokinase 2 is a determinant of neuroblastoma metastasis

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Background: Intersecting a genome-wide expression profile of metastatic and nonmetastatic human neuroblastoma xenograft variants with expression profiles of tumours from stage 1 and 4 neuroblastoma patients, we previously characterised hexokinase 2 (HK2) as a gene whose expression was upregulated in both metastatic neuroblastoma variants and tumours from stage 4 neuroblastoma patients.

Methods: Local and metastatic neuroblastoma cell variants as well as metastatic neuroblastoma cells genetically manipulated to downregulate the expression of HK2 were utilised for *in vitro* and *in vivo* examinations of the involvement of HK2 in neuroblastoma.

Results: Hexokinase 2 expression and its activity levels were increased in neuroblastoma metastatic variants as compared with the local variants. The upregulation of HK2 confers upon the metastatic cells high resistance to the antiproliferative effect of the HK2 inhibitor 3-BrPa and to the chemotherapy agent Deferoxamine. The inhibition of HK2 transcript lowered the proliferation and motility of sh-HK2 cells as compared with sh-control cells. Mice that were inoculated with sh-HK2 cells had a lower incidence of local tumours, smaller tumour volumes and a diminished load of lung metastasis compared with mice inoculated with sh-control cells.

Conclusions: Hexokinase 2 plays a significant role in shaping the malignant phenotype of neuroblastoma and influences the progression of this disease.

Although increased understanding of the metastatic process was achieved in recent years (Gupta and Massague, 2006; Mazzocca and Carloni, 2009; Sethi and Kang, 2011; Maman and Witz, 2013; Klein-Goldberg *et al*, 2014), the mechanisms underlying the progression of cancer cells to form metastasis are awaiting complete elucidation.

In neuroblastoma, the most common extracranial solid tumour in children, more than half of the patients present with metastatic disease at diagnosis (Maris *et al*, 2007; Mullassery *et al*, 2009; Modak and Cheung, 2010). Prevention strategies for neuroblastoma metastasis could be developed if molecules involved in this process are identified and their precise role deciphered.

For the identification of molecular pathways involved in neuroblastoma metastasis, we developed an orthotopic mouse model for human neuroblastoma metastasis (Nevo *et al*, 2008). An orthotopic implantation of two human neuroblastoma cell lines, MHH-NB11 (Pietsch *et al*, 1988) and SH-SY5Y (Biedler *et al*, 1978), into the adrenal gland of athymic nude mice yielded local adrenal tumours as well as lung metastases. After repeated cycles of *in vivo* passages, local adrenal and lung metastatic variants were generated. Originating in the same human neuroblastoma cell lines, these variants share a common genetic background. Proteomic and transcriptomic differences between these variants may thus be ascribed to their differential malignant phenotype.

Aiming to identify molecular correlates of neuroblastoma metastasis, the genetically identical local and lung metastasising human neuroblastoma variants were subjected to genome-wide expression profiling (Nevo *et al*, 2010) using a neuroblastoma-specific oligonucleotide microarray (Oberthuer *et al*, 2006). Genes that were found to be differentially expressed between local and

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metastatic neuroblastoma variants were intersected with genes differentially expressed in primary tumours of stage 1 and stage 4 neuroblastoma patients. A neuroblastoma metastatic gene expression signature was identified. This signature included several genes that could serve as therapeutic targets or prognosis markers in this disease (Nevo *et al*, 2010).

One of the genes that was found to be upregulated in the neuroblastoma metastatic variants compared with the local variants as well as in stage 4 neuroblastoma patients compared with stage 1 patients was hexokinase 2 (HK2) (Nevo *et al*, 2010). HK2 catalyses the essentially irreversible first step of the glycolytic pathway in which glucose is phosphorylated to glucose-6-phosphate (G6P) via phosphate transfer from ATP (Mathupala *et al*, 2009). In cancer, HK2 is a pivotal player in the 'Warburg effect', a high rate of aerobic glycolysis followed by lactic acid fermentation occurring in malignant tumours (Warburg, 1956).

Glycolytic enzymes including HK2 are overexpressed in at least 70% of human cancers (Altenberg and Greulich, 2004). Several systemic and cellular mechanisms promote the expression and transcriptional upregulation of HK2 in malignant tumours including epigenetic events (e.g., demethylation) and gene amplification (Mathupala *et al*, 2009). The proximal promoter of HK2 contains *cis*-elements for transcription initiation that are regulated by hypoxia-inducible factor-1α (HIF-1α) and by others (Mathupala *et al*, 1995, 1997; Marin-Hernandez *et al*, 2009).

The role of HK2 and glycolysis in the pathogenesis of neuroblastoma is not thoroughly investigated. Levy *et al* (2010) confirmed recently that treatment with the HK2 inhibitor, 3-bromo-2-oxopropionate-1-propyl ester (3-BrOP), decreased neuroblastoma cell viability by inducing apoptosis *in vitro* and generated smaller tumours *in vivo*.

In the present study we determine the role of HK2 in the formation of neuroblastoma metastasis and suggest that the inhibition of HK2 could potentially be used as future treatment for neuroblastoma.

MATERIALS AND METHODS

Cell culture. Neuroblastoma local adrenal tumour and lung metastatic variants were generated from the parental cell lines MHH-NB-11 (Pietsch *et al*, 1988) and SH-SY5Y (Biedler *et al*, 1978) using a mouse model for human neuroblastoma metastasis as detailed here (Nevo *et al*, 2008) and were maintained in culture as previously described (Nevo *et al*, 2008).

Authentication of local and metastatic neuroblastoma cells was performed as described here (Maman *et al*, 2013). All cultures were periodically examined for mycoplasma contamination.

Animals. Male athymic nude mice (BALB/c background) were purchased from Harlan Laboratories Limited (Jerusalem, Israel). Mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions at the animal quarters of Tel Aviv University (Tel Aviv, Israel) in accordance with current regulations and standards of the Tel Aviv University institutional animal care and use committee (IAUCUC). Mice were regularly checked by a certified veterinarian who is responsible for health monitoring, animal welfare supervision, experimental protocols and procedure revision. The 7–10-week-old mice were used in the *in vivo* experiments. The *in vivo* methodology used in these experiments has been reviewed and approved by the Tel Aviv University IAUCUC.

Antibodies. The following anti-human antibodies were used for western blot analysis: anti-HK2 at a dilution of 1 : 500, anti-ERK2 at 1 : 1000 and anti-AKT at 1 : 500 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-HIF-1α (R&D Systems, Inc., Minneapolis, MN, USA) at 1 : 1000 and anti-β-tubulin (Sigma-Aldrich, St Louis, MO, USA) at 1 : 2000. Horseradish peroxidase-conjugated goat anti-mouse and rabbit anti-goat antibodies were used according to the manufacturer's instructions (Jackson Immuno Research Laboratories, West Grove, PA, USA).

Western blotting. Neuroblastoma cells were lysed, resolved on SDS–PAGE and transferred onto nitrocellulose membrane as described previously (Edry Botzer *et al*, 2011). For the detection of target proteins, membranes were incubated with the relevant antibodies and bands were visualised by enhanced chemiluminescence reactions. The amount of the relevant protein in the lanes was estimated by densitometry using Scion Image software (Scion, Frederick, MD, USA).

HK activity assay. The activity of hexokinases (HKs) was determined by spectrophotometric measurement of the formation of β-nicotinamide adenine dinucleotide (NADH) that is coupled with the oxidation of G6P, the product of glucose phosphorylation by HK (Polakis and Wilson, 1982; Al jamal, 2005). The coupled assay produces 1 μmol of NADH per μmol of D-glucose phosphorylated.

Neuroblastoma cells were lysed as described before (Edry Botzer *et al*, 2011), diluted in lysis buffer to a volume of 70 μl and added in duplicates to a 96-well plate on ice. Reaction buffer (20 mM Hepes pH = 7.2, 5 mM $MgCl_2$, 5 mM KCl and 10 mM glucose, freshly supplemented with 1 U ml^{-1} PK, 1 U ml^{-1} LDH, 1 mM ATP, 1 mM PEP and 0.18 mg ml^{-1} NADH) was added to the wells in a volume of 200 μl and the plate was immediately read using an automated microplate reader (SpectraMax 190; Molecular Devices Corp., Sunnyvale, CA, USA) at 25 °C, OD_{340}. Measurements were taken every 15 s for 25 min. The HK activity was calculated as follows: ΔOD/min (Vmax) − ΔOD/min blank × 1000 (for 1000 μl) × dilution factor/6.22 (mM extinction coefficient of NADH).

HK2 inhibition. 3-Bromopyruvate (3-BrPa, Sigma-Aldrich) was reconstituted freshly in PBS before use. Neuroblastoma cells were cultured in growth media containing 0, 25, 50 or 100 μM of 3-BrPa for 2 h. Cell viability was monitored using XTT-based viability assays.

***In vitro* viability assays.** Neuroblastoma cells were seeded (1×10^5 per well) in triplicate in a 96-well tissue culture plate. Viability was monitored using XTT-based viability assay according to the manufacturer's instructions (Biological Industries, Beit Haemek, Israel).

Inducible knockdown of HK2 by sh-RNA. Lentiviral pTRIPz vectors encoding Tet-inducible sh-HK2 (RHS4696) and nonsilencing control (RHS4743) were purchased from Open Biosystems (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). These plasmids contain RFP cassette downstream of the Tet/ON promoter.

To produce the infectious viruses, 293T packaging cell line was co-transfected using calcium phosphate with the following retroviral backbone plasmids: the TRIPz-RFP/sh-HK2 vectors (a pool of three siRNA sequences), the packaging plasmid pCMVΔ8.2 and the envelope plasmid pVSV-G.

After 48 h of incubation, the virus particles in the medium were collected and filtrated (0.45 μm filter). Neuroblastoma lung metastatic cells were plated (2×10^6) 24 h before infection. The cells were infected in the presence of 1 μg ml^{-1} Polybrene overnight and the virus-containing medium was replaced with fresh medium. After 72 h, 1 μg ml^{-1} Puromycin (InvivoGen, San Diego, CA, USA) was added for 7 days in order to stably select infected cell population. After selection, Puromycin was regularly added to the cultures.

The Tet-On mode of sh-HK2 infected cells was activated by the addition of 1 μg ml^{-1} Doxycycline (Sigma-Aldrich). Efficiency of infection was examined by visualising RFP in Leica DMRB Fluorescence Microscope (Leica Biosystems, Nussloch GmbH,

Germany). Efficacy of sh-HK2 inhibition was verified by western blotting.

Orthotopic inoculation of tumour cells. The Tet-On mode of sh-HK2 infected cells was activated by the addition of 1 $\mu g\,ml^{-1}$ Doxycycline to neuroblastoma cell cultures for 1 week. An orthotopic inoculation to the adrenal gland of athymic nude mice was performed with either sh-HK2 or sh-control cells (1×10^6/50 μl) as detailed here (Nevo *et al*, 2008). We monitored and verified the development of local tumours in the adrenal gland using an ultrasound imaging *in vivo* as previously described (Edry Botzer *et al*, 2011).

For the *in vivo* activation of sh-HK2 Tet-On cells, 5 $mg\,ml^{-1}$ Dox and 1.25 $mg\,ml^{-1}$ sucrose were added to the drinking water (replaced every 72 h). Two weeks after local tumours were detected by ultrasound, the mice were moribund and killed. Mice inoculated with sh-control cells were killed at the same time point for comparison. Lungs were harvested, immediately put on dry ice and kept in $-80\,^{\circ}C$ until use.

Determination of the presence of human cells in mouse tissues by qRT–PCR. The PCR oligonucleotide primers were designed according to sequences derived from the National Center for Biotechnology Information (NCBI) database to react specifically with the human plasminogen activator receptor urokinase type (PLAUR) sequence and have no crossreactivity with the mouse PLAUR sequence (forward: 5′-GTCACCTATTCCCGAAGCCG-3′, reverse: 5′CGGTACTGGACATCCAGGTCT-3′).

Amplification reactions were performed with SYBR Green I (Thermo Fisher Scientific, ABgene, Epsom, UK) in triplicate in Rotor-gene 6000TM (Qiagen, Valencia, CA, USA) as previously described (Edry Botzer *et al*, 2011).

Wound healing assay. The Tet-On mode of sh-HK2-infected cells was activated by the addition of 1 $\mu g\,ml^{-1}$ Doxycycline to neuroblastoma cell cultures for 1 week. Cells were then seeded in 6-well plates coated with 10 $\mu g\,ml^{-1}$ fibronectin (Biological Industries). Upon confluence, the cell monolayer was wounded with a plastic tip, then washed twice with RPMI medium and replaced with fresh growth medium. Closure of the denuded area was monitored using an inverted microscope (Eclipse TE 2000-S; Nikon, Enfield, CT, USA) fitted with a digital camera (DXM1200F; Nikon). Photos were taken at days 0, 1, 2 and 3 after the wounding. Analysis of wound closure was performed using WIMASIS image analysis software (Wimasis GmbH, Munich, Germany).

Hypoxia assays. Neuroblastoma cells (2×10^6 per well) were seeded in tissue culture plates. After an overnight incubation, the plates were transferred into hypoxia chamber (1% oxygen) for 4 h. Control cells were left in normoxia conditions. Hypoxia-mimetic conditions were induced by the addition of 25, and 50 μM Deferoxamine mesylate salt (Sigma-Aldrich) to culture plates for 24 h. No Deferoxamine was added to control cells. Nonadherent and adherent cells were lysed in RIPA buffer on ice. The expression of HK2 and HIF-1α was analysed using western blot analysis.

Statistical analysis. Paired and unpaired Student's *t*-test and Wilcoxon rank test were used to compare results.

RESULTS

HK2 is highly expressed and activated in metastatic neuroblastoma variants. A gene expression analysis of local (adrenal) and lung metastasising human neuroblastoma cells using a neuroblastoma-specific oligonucleotide microarray (Oberthuer *et al*, 2006) revealed that HK2, the enzyme that is responsible for the first step in most glucose metabolism pathways, was upregulated in neuroblastoma lung metastasising variants, as well as in stage 4 neuroblastoma patients as compared with local adrenal variants and stage 1 patients (Nevo *et al*, 2010). The upregulation of the expression of HK2 in metastatic neuroblastoma variants compared with its expression in the local (adrenal) variants was observed at the protein level as well (Nevo *et al*, 2010). An examination of the enzymatic activity of HK isoforms (Polakis and Wilson, 1982) in local and metastatic neuroblastoma variants indicated that the activity was significantly higher in the metastatic variants than in the corresponding local variants (Figure 1).

Hypoxia induces high levels of HK2 in metastatic neuroblastoma variants. The following experiments were aimed to identify the mechanism that promotes the upregulated expression and the high enzymatic activity of HK2 in neuroblastoma metastases. Hypoxia-inducible factor-1α (HIF-1α) promotes the transcription of a broad range of genes involved in cancer progression and metastasis (Haase, 2010). One of these genes is HK2, harbouring consensus motifs for HIF-1α in its promoter (Mathupala *et al*, 2001). We previously demonstrated that hypoxia-mimetic conditions (Deferoxamine treatment) upregulate HIF-1α expression in metastatic neuroblastoma variants to significantly higher levels than in local neuroblastoma variants exposed to the same conditions (Nevo *et al*, 2008).

We asked whether the high HK2 expression in the metastatic variants is because of the high activation of HIF-1α in these cells, and whether HK2 is regulated by hypoxic conditions. To answer these questions we examined the expression levels of these molecules under normoxia and hypoxia as well as by mimicking hypoxic conditions using Deferoxamine.

Cells were treated with 0, 25 and 50 μM Deferoxamine or kept under hypoxic conditions (1% oxygen) in a hypoxia chamber. The expression levels of HIF-1α and HK2 were measured by western blot analysis. As demonstrated in Figure 2, under normoxia HIF-1α expression was undetectable in the local and metastatic variants. Hypoxia and Deferoxamine treatment induced the expression of HIF-1α in all neuroblastoma variants. An upregulation of HK2 expression correlated with the upregulation of HIF-1α under hypoxic and hypoxia-mimetic conditions and was higher in the metastatic variants than in the corresponding local variants (Figure 2).

We suggest that the high expression and enzymatic activity level of HK2 in the metastatic variants are a result of the selective pressure exerted by hypoxic conditions that enhance the malignant and metastatic phenotype of these cells.

Differential sensitivity of neuroblastoma variants to the HK2 inhibitor 3-BrPa. The pyruvate analogue 3-BrPa is an inhibitor of HK2 that reduces the metabolism of cancer cell energy and triggers cell death (Chen *et al*, 2009). We asked whether the higher

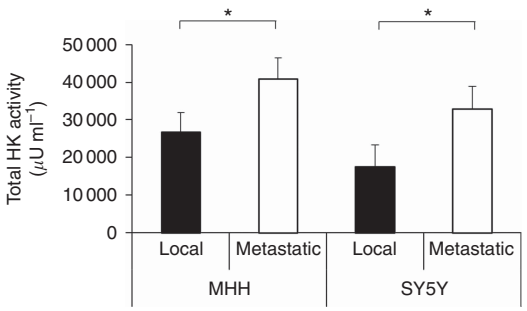

Figure 1. Activity levels of HK2 are higher in neuroblastoma metastatic variants than in local variants. Total HK activity in neuroblastoma variants was determined by measuring microunit enzyme per ml ($\mu U\,ml^{-1}$). Data are means of three independent experiments + s.d. Significance was evaluated using Student's *t*-test. *$P<0.05$.

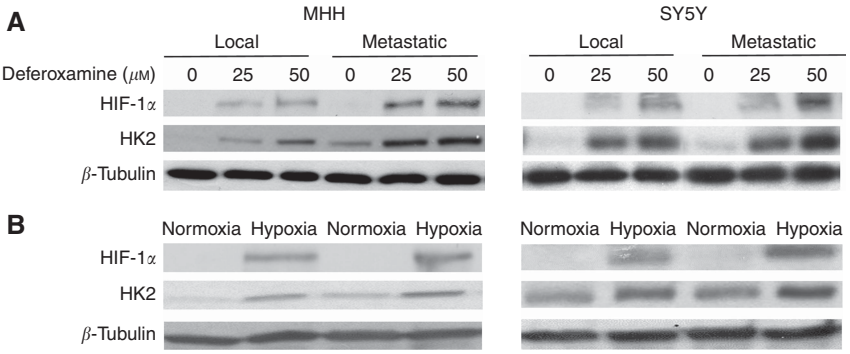

Figure 2. Hypoxic conditions induce high levels of HK2 expression in neuroblastoma metastatic variants. Local and metastatic neuroblastoma variants were treated with 0, 25 and 50 μM Deferoxamine (**A**) or incubated in a hypoxia chamber (1% oxygen) (**B**). Following treatment, the expression level of HIF-1α and HK2 was measured by western blot analysis. Shown are the results of one representative blot out of four independent experiments.

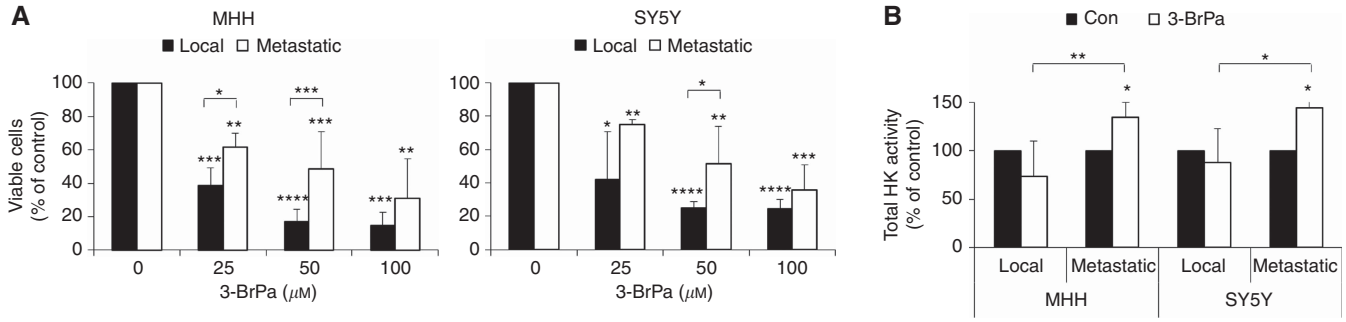

Figure 3. Metastatic neuroblastoma variants are more resistant than local variants to growth inhibition mediated by the HK2 inhibitor 3-BrPa. (**A**) Viability of local and metastatic neuroblastoma variants was determined by XTT-based viability assays following treatment with 25, 50 and 100 μM 3-BrPa. (**B**) The activity of all HK isoforms in neuroblastoma variants was determined following treatment with 50 μM 3-BrPa by measuring microunit enzyme per ml ($\mu U\,ml^{-1}$) normalised to control untreated cells. Data are means of three independent experiments + s.d. Significance was evaluated using Student's *t*-test. *$P<0.05$, **$P<0.01$, ***$P<0.005$ and ****$P<0.001$.

expression and activity levels of HK2 in the metastatic variants than in the local variants affects the sensitivity of these cells to 3-BrPa.

Neuroblastoma cells were treated with 0, 25, 50 and 100 μM 3-BrPa for 2 h. Cell viability was then measured using XTT-based proliferation assays. Figure 3A demonstrates that the viability of all 3-BrPa-treated neuroblastoma variants was impaired. However, the metastatic variants of both neuroblastoma tumours (MHH and SY5Y) were more resistant to the viability restraining effects of 3-BrPa than the local variants, possibly because of higher reservoir levels of HK2 in the metastatic cells. To establish whether 3-BrPa treatment inhibited HK2 activity, we measured the enzymatic activity of all HK isoforms in cells treated with 50 μM 3-BrPa. Figure 3B demonstrates that 3-BrPa treatment did not influence HK activity in the local neuroblastoma variants, possibly because of the fact that these variants express low levels of HK2 and therefore blockage with 3-BrPa does not significantly influence total HK activity in these cells. In the treated metastatic variants, total HK activity was increased. It is possible that the inhibition of HK2 in the metastatic cells triggered a compensatory mechanism of other HK isoforms that eventually raised total HK activity in these cells.

Inducible downregulation of HK2 expression in metastatic neuroblastoma variants. In order to examine the possibility that high expression levels and high enzymatic activity of HK2 play a role in conferring a metastatic phenotype upon neuroblastoma cells, we downregulated the expression of HK2 by Tet-On Tetracycline-controlled transcriptional activation of sh-RNA plasmids specific to human HK2 (sh-HK2) and examined the influence of this downregulation on metastatic neuroblastoma variants. Control cells were infected with scrambled sequence (sh-control). The sh-HK2 and control plasmids contained a red fluorescent protein (RFP) expression cassette downstream of the Tet-On promoter. The infected cells in each experiment were activated with Doxycycline and visualised by fluorescence microscopy to confirm the transcriptional state of the integrated plasmid. In addition, the expression level of HK2 was analysed using immunoblotting.

At 1 week following plasmid activation, the expression level of HK2 in sh-HK2 cells was reduced to 60% of its expression in sh-control cells (Figure 4A). At 2 weeks post activation, HK2 expression dropped to 10% of its expression in sh-control cells (Figure 4A). Total HK activity was decreased by 50% in sh-HK2 cells compared with sh-control cells (Figure 4B). HK activity assays measure the activity of all HK isoforms (Polakis and Wilson, 1982). Therefore, downregulating HK2 in these cells does not completely block total HK activity or the metabolic flux in the cells.

High expression levels of HK2 account for the resistance of metastatic neuroblastoma cells to 3-BrPa. Based on the results reported above that metastatic neuroblastoma cells expressing high levels of HK2 are more resistant to the toxic effects of the HK2 inhibitor 3-BrPa than the local variants expressing lower levels of HK2 enzyme, we hypothesised that resistance to the toxic effects of the inhibitor is because of the high level of endogenous expression of HK2.

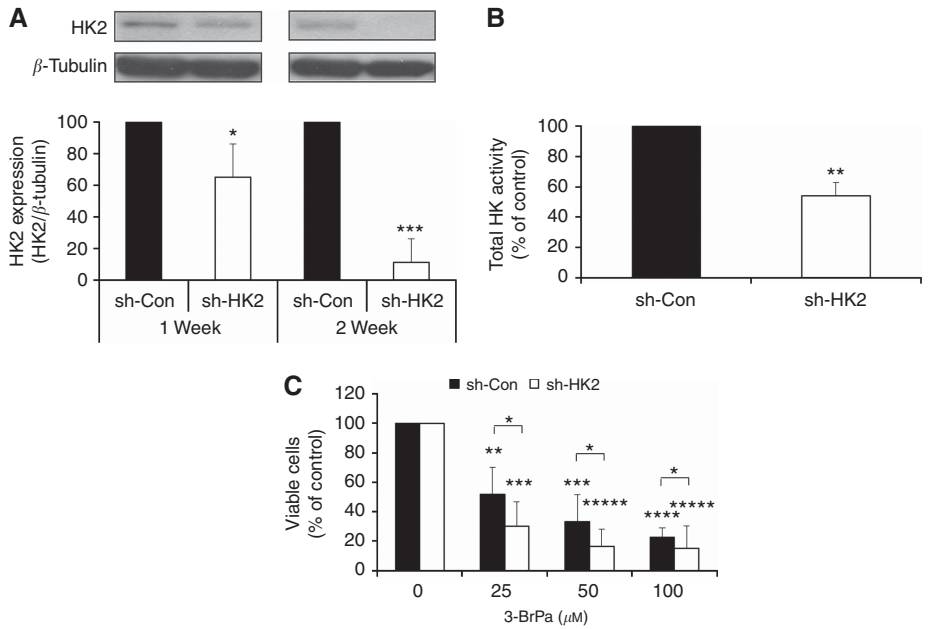

Figure 4. Sh-HK2 reduces HK2 expression level and total HK activity and induces sensitivity to the HK2 inhibitor 3-BrPa. (**A**) Whole-cell lysates of neuroblastoma sh-HK2 or sh-Con cells were subjected to western blot analysis and immunostaining. The expression level was measured 1 week or 2 weeks following plasmid activation. (**B**) Total HK activity in neuroblastoma sh-Con or sh-HK2 cells was determined by measuring microunit enzyme per ml ($\mu U\,ml^{-1}$). (**C**) Viability of sh-HK2 and sh-Con cells was determined by XTT-based viability assay following treatment with 25, 50 and 100 μM 3-BrPa. Data are means of three independent experiments + s.d. The blots shown are of a representative experiment out of three. Significance was evaluated using Student's *t*-test. *$P<0.05$, **$P<0.01$, ***$P<0.005$, ****$P<0.001$ and *****$P<0.0005$.

Figure 4C shows that indeed the sensitivity of sh-HK2 cells to 3-BrPa is significantly higher than that of sh-control cells. These results support the hypothesis that the low sensitivity of the metastatic variants to 3-BrPa is because of high expression levels of HK2 in these variants.

Knocking down HK2 expression ameliorates the malignant phenotype of metastatic neuroblastoma cells. Metastatic and local human neuroblastoma variants exhibit a differential malignant phenotype (Nevo *et al*, 2008). The metastatic variants are highly tumourigenic in xenografted nude mice, yield a higher metastatic load in these mice, possess a higher migratory capacity and are relatively more resistant to chemotherapy than the corresponding local variants (Nevo *et al*, 2008).

If high levels of HK2 are related to a high malignancy phenotype, it is to be expected that downregulating the levels of this enzyme would ameliorate the malignancy phenotype of the cells. In the *in vitro* and *in vivo* experiments described below we examined this hypothesis.

Proliferation and morphology. HK2 promotes tumour cell growth and survival (Pedersen *et al*, 2002). In order to evaluate the effects of silencing HK2 expression on neuroblastoma cell proliferation, we measured the viability of the infected cells after plasmid activation using XTT-based viability assays. No influence on cell viability was apparent in sh-control and sh-HK2 cells at 96 h (Figure 5A) and up to 7 days (Figure 5B) after plasmid activation. However, the viability of sh-HK2 cells cultured for 14 and 17 days after plasmid activation was dramatically decreased (Figure 5B).

Another apparent difference between sh-control and sh-HK2 cells was their morphology. sh-HK2 cells grew as crowded clumps whereas sh-control cells presented with an elongated shape and a higher degree of spreading associated with the morphology of the lung metastatic variants from which they originated (Figure 5B). Thus, downregulating the expression of HK2 resulted in a changed morphology of the metastatic variant to resemble that of the less malignant local tumour variant (Nevo *et al*, 2008).

Migratory capacity. Little is known about the involvement of HK2 in the migration of tumour cells. Migratory capacity of sh-HK2 neuroblastoma cells was examined in wound healing assays and analysed by WIMASIS software. Figure 5C shows that downregulation of HK2 reduces motility. The sh-control cells closed the wound more rapidly than sh-HK2 cells (57% and 25% closure, respectively, 24 h after wounding). At the end of the experiment, sh-control cells closed 100% of the wound, whereas sh-hk2 cells closed only 30% of it.

These results suggest that HK2 is indeed involved in the metastatic process, of which migratory capacity of cancer cells is a key factor. This suggestion is supported by our previous observation that neuroblastoma metastatic variants expressing high levels of HK2 migrate faster than nonmetastatic local variants that express low levels of HK2 (Nevo *et al*, 2008).

Tumourigenicity and metastasis formation. The aim of the next set of experiments was to determine the capacity of sh-HK2 and sh-control cells to form local tumours in the adrenal gland and to generate distant metastases following an orthotopic xenograft of the cells into nude mice.

The sh-RNA plasmids were activated *in vitro* a week before the inoculation and maintained activated *in vivo* by adding Doxycycline to the drinking water of the mice. Local tumour incidence and volume was determined using ultrasound (US) 6 weeks post inoculation. The presence of metastatic cells in the lungs was determined by measuring the amount of human RNA in the lungs of the xenografted mice using qRT–PCR.

Mice inoculated with sh-HK2 cells had a lower incidence of local adrenal tumours (Figure 5D) and smaller tumour volumes (Figure 5E). The load of lung metastasis was notably lower in mice inoculated with sh-HK2 cells than in mice inoculated with sh-control cells (Figure 5F). These results were statistically significant as determined by Wilcoxon rank test ($P<0.05$). These results

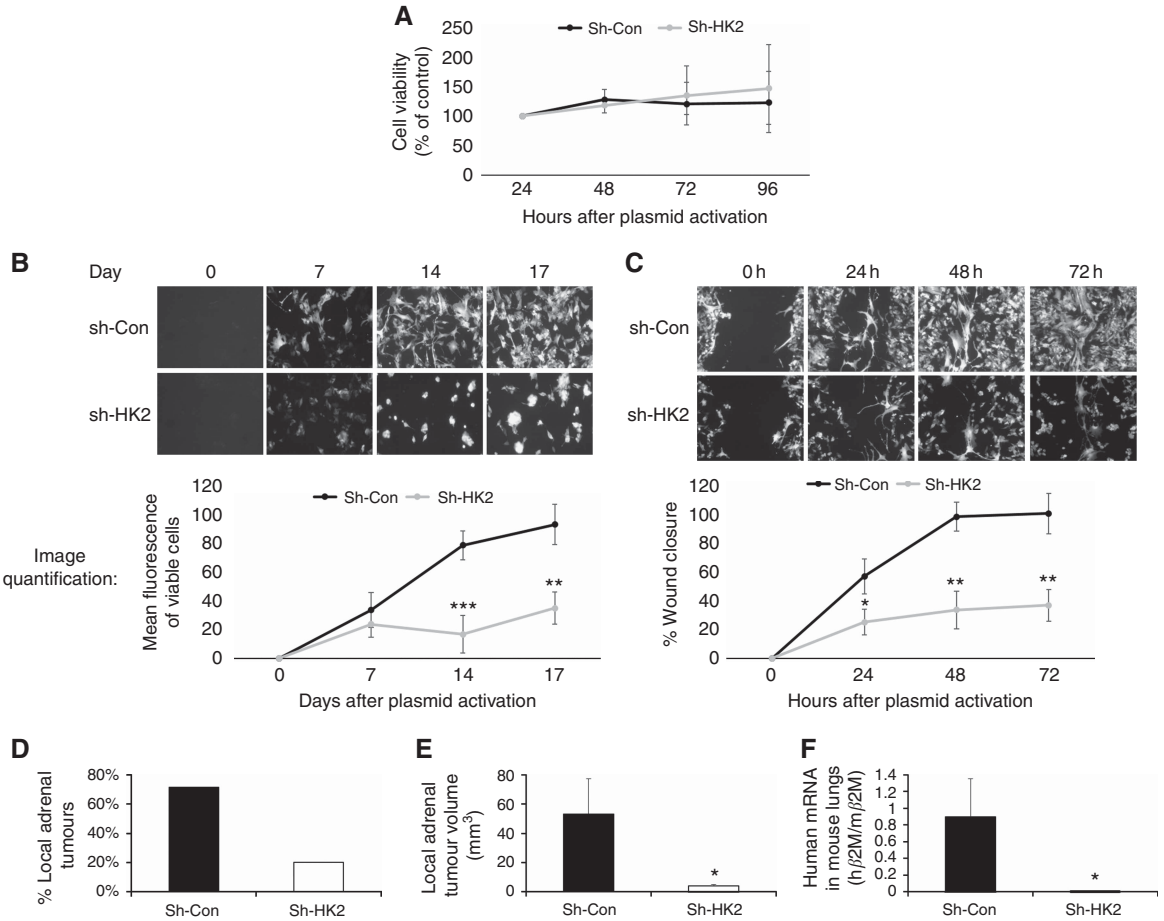

Figure 5. Downregulation of HK2 expression reduces the malignant phenotype of neuroblastoma cells. (**A**) Sh-Con and Sh-HK2 cell viability was examined using XTT-based viability assays 24, 48 and 72 h post plasmid activation. (**B**) Sh-Con and sh-HK2 cells were activated (day 0) and fluorescent pictures demonstrating cell proliferation and morphology were taken on days 0, 7, 14 and 17 post plasmid activation. (**C**) Fluorescence photomicrographs of scratched monolayers in a wound healing assay of sh-Con and sh-HK2 cells. The scratches were performed 1 week after plasmid activation (0 h). Pictures were taken at 0, 24, 48 and 72 h after the scratch was performed. (**D**) Percentage of the development of local adrenal tumours in mice inoculated with sh-Con and sh-HK2 cells. (**E**) Volume measurements of local adrenal tumours in mice inoculated with sh-Con and sh-HK2 cells. (**F**) Quantification of human mRNA in mouse lungs using qRT–PCR reactions indicating lung metastasis of human origin in mice inoculated with sh-Con and sh-HK2 cells. Data are means of three independent experiments + s.d. (**A**–**C**) or of mice in each group ($n = 14$, 7 mice in each group) + s.d. (**D**–**F**). *$P < 0.05$, **$P > 0.01$ and ***$P < 0.005$.

clearly indicate that HK2 is functionally involved in tumourigenesis and metastasis of neuroblastoma cells.

DISCUSSION

This study provides information on molecular mechanisms in human neuroblastoma that links three cancer hallmarks (Hanahan and Weinberg, 2011): increased viability (proliferative capacity and/or cell death resistance), deregulated cellular metabolism and metastasis.

Several genes that regulate cancer spread and dissemination and those that contribute to organ-specific metastasis have been identified in different cancer types such as lung and bone metastasis in breast cancer (Chambers *et al*, 2002; Nguyen *et al*, 2009). However, little is known about the molecules involved in neuroblastoma metastasis.

In a previous study, we described the development of an *in vivo* model for neuroblastoma metastasis (Nevo *et al*, 2008). Using a neuroblastoma-specific oligonucleotide microarray (Oberthuer *et al*, 2006), we identified genes that were differentially expressed in local and metastatic variants originating in the same neuroblastoma tumours (Nevo *et al*, 2010). Comparing the transcriptomes of local and metastatic variants and intersecting them with the transcriptomes of cells from local tumours of phase 1 and 4 neuroblastoma patients, we were able to identify a small, clinically relevant, gene set associated with neuroblastoma metastatic progression (Nevo *et al*, 2010).

Hexokinase 2, the enzyme that catalyses the first committed step of glucose metabolism, was identified as a member of this gene set, exhibiting a significantly higher expression level in metastatic neuroblastoma variants as well as in tumours derived from stage 4 neuroblastoma patients than in local neuroblastoma variants and in tumours derived from stage 1 patients. In the present study, we confirmed this differential expression and showed that high HK2 expression levels confer a more malignant, tumourigenic and metastatic phenotype on neuroblastoma cells.

Hexokinase 2 plays a pivotal and direct role in the Warburg effect, a hallmark of many malignant tumours. Hexokinase 2 is usually expressed in very low levels in normal cells (Pedersen *et al*, 2002), whereas in many tumours, for example, liver (Stubbs *et al*, 2003; Marin-Hernandez *et al*, 2006), breast (Balinsky *et al*, 1984) and cervical cancer (Marin-Hernandez *et al*, 2006), as well as

pancreatic and colon carcinomas (Peng *et al*, 2008a, 2009), HK2 expression is extensively increased, consistent with enhanced glycolytic activity. Additional findings support the possible involvement of HK2 in the metastatic cascade. For example, HK2 overexpression contributes to progression of hepatocellular carcinoma (Peng *et al*, 2008b) and of cholangiocellular carcinoma (Paudyal *et al*, 2008). Despite these reports and despite the fact that HK2 is used for the detection of metastasis by positron emission tomography (PET), there are only a few studies examining its role in metastasis formation.

Hypoxia is a common characteristic of the solid tumour microenvironment (Fardin *et al*, 2010) including rapidly expanding neuroblastoma tumours. The HIF-1α is a metastasis-regulating gene strongly linked to invasion, tumourigenesis and angiogenesis (Subarsky and Hill, 2003; Brahimi-Horn and Pouyssegur, 2006). The observed correlation between HK2 expression and HIF-1α expression implies that one of the mechanisms that promote the upregulated expression activity levels of HK2 in neuroblastoma metastases is coupled with low oxygen levels associated with solid tumours. Interestingly, the upregulation of HIF-1α and HK2 expression under hypoxic and hypoxia-mimetic conditions was more significant in the metastatic variants than in the local ones. This suggests that the metastatic variants cope more efficiently than local variants with stressful hypoxic microenvironments.

Glycolytic inhibitions have been proposed as a therapeutic strategy for cancer treatment (Sheng *et al*, 2009; Pradelli *et al*, 2010). 3-BrPa is a small-molecule analogue of pyruvate that inhibits HK2 and prevents glucose from entering the glycolytic pathway (Ko *et al*, 2001). In HCC (Kim *et al*, 2007; Vali *et al*, 2007), breast cancer (Buijs *et al*, 2009), melanoma (Qin *et al*, 2010) and colon cancer (Sanchez-Arago and Cuezva, 2011), 3-BrPa exhibits promising anticancer activities.

Treatment with 3-BrPa reduced the HK activity and the viability of both local and metastatic neuroblastoma variants. However, the metastatic variants, expressing higher levels of HK2, were significantly less influenced by this treatment – their viability was only slightly impaired and their total HK activity even increased under this treatment.

Two nonmutually exclusive mechanisms may account for the differential sensitivity of the local and metastatic variants to 3-BrPa. The first is resistance of the metastatic cells to 3-BrPa that results from high reserves of active HK2 enzyme that could allow these cells to maintain a high HK2 activity even under the inhibitory influence of 3-BrPa. The second mechanism that could explain this phenomenon is that the metastatic variants upregulate other isoforms of HK as a compensation mechanism in order to maintain a high glycolytic rate when HK2 activity is inhibited by 3-BrPa. If indeed such a compensatory mechanism exists, a treatment for neuroblastoma should inhibit the activity of all HK isoforms.

The efficient inhibition of HK2 by sh-RNA in neuroblastoma metastatic variants lowered the malignant phenotype of the cells. The sh-HK2 cells exhibited a lower proliferation rate and migratory capacity and generated a lower incidence of local adrenal tumours, a smaller tumour volume and a significantly lower lung metastasis rate.

Based on the results of this study we propose that HK2, playing a key role in the progression of neuroblastoma, may serve as a therapeutic target for this disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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