Novel role for insulin as an autocrine growth factor for malignant brain tumour cells

Alexandre ARCARO^{*1}, Kathrin T. DOEPFNER^{*}, Danielle BOLLER^{*}, Ana S. GUERREIRO^{*}, Tarek SHALABY[†], Shaun P. JACKSON[‡], Simone M. SCHOENWAELEDER[‡], Olivier DELATTRE^{§||}, Michael A. GROTZER[†] and Barbara FISCHER^{*}

^{*}Division of Clinical Chemistry and Biochemistry, University Children's Hospital Zurich, CH-8032 Zurich, Switzerland, [†]Department of Oncology, University Children's Hospital Zurich, CH-8032 Zurich, Switzerland, [‡]Australian Centre for Blood Diseases, Monash University, 6th Floor, Burnet Building, Alfred Medical Research and Education Precinct, Prahran, VIC 3181, Australia, [§]Institut Curie, Laboratoire de Pathologie Moléculaire des Cancers, 26 rue d'Ulm, Paris 75248, France, and ^{||}INSERM U509, Paris 75248, France

AT/RTs (atypical teratoid/rhabdoid tumours) of the CNS (central nervous system) are childhood malignancies associated with poor survival rates due to resistance to conventional treatments such as chemotherapy. We characterized a panel of human AT/RT and MRT (malignant rhabdoid tumour) cell lines for expression of RTKs (receptor tyrosine kinases) and their involvement in tumour growth and survival. When compared with normal brain tissue, AT/RT cell lines overexpressed the IR (insulin receptor) and the IGFIR (insulin-like growth factor-I receptor). Moreover, insulin was secreted by AT/RT cells grown in serum-free medium. Insulin potentially activated Akt (also called protein kinase B) in AT/RT cells, as compared with other growth factors, such as epidermal growth factor. Pharmacological inhibitors, neutralizing antibodies, or RNAi (RNA interference) targeting

the IR impaired the growth of AT/RT cell lines and induced apoptosis. Inhibitors of the PI3K (phosphoinositide 3-kinase)/Akt pathway also impaired basal and insulin-stimulated AT/RT cell proliferation. Experiments using RNAi and isoform-specific pharmacological inhibitors established a key role for the class I_A PI3K p110 α isoform in AT/RT cell growth and insulin signalling. Taken together, our results reveal a novel role for autocrine signalling by insulin and the IR in growth and survival of malignant human CNS tumour cells via the PI3K/Akt pathway.

Key words: Akt, atypical teratoid/rhabdoid tumour (AT/RT), cell proliferation, central nervous system, insulin-like growth factor (IGF), phosphoinositide 3-kinase (PI3K).

INTRODUCTION

The AT/RT (atypical teratoid/rhabdoid tumour) is a highly malignant paediatric tumour of the CNS (central nervous system), which is characterized by unique clinical, biological and histological features [1–4]. Patients with CNS AT/RT respond very poorly to chemotherapy and radiotherapy and thus the prognosis for this particular malignancy is very poor [4–6]. AT/RT is related to other rhabdoid tumours of the kidney [MRT (malignant rhabdoid tumour)] and soft tissues. Most of the rhabdoid tumours harbour inactivating biallelic alterations in the *hSNF5* (Human *SNF5*)/*INI1* gene, a tumour suppressor gene on chromosome 22 [7–9]. *hSNF5/INI1* is a component of the ATP-dependent chromatin remodelling SWI/SNF complex [10]. There are multiple sets of mammalian SWI/SNF complexes, with varying subunit compositions, which play important roles in transcriptional regulation, through both activation and repression of gene transcription [10–12]. Mice with a targeted disruption of the *INI1* gene developed tumours at a high frequency and the resulting tumours displayed loss of expression of the *hSNF5/INI1* protein [13,14]. Deletion of *hSNF5/INI1* was recently reported to cooperate with p53 loss in oncogenic transformation in murine models [15,16]. One of the mechanisms by which *hSNF5/INI1* exerts its tumour suppressor function was shown to involve repression of cyclin D1 gene expression [17]. Targeting cyclin

D1 gene expression was thus suggested to represent a novel therapeutic strategy for AT/RT [17,18].

The insulin/IGF (insulin-like growth factor) family of growth factors are part of an evolutionarily conserved signalling system with a critical role in the growth and development of many tissues as well as the regulation of overall growth and metabolism. This signalling system is characterized by a high complexity and involves multiple proteins including three receptors [IR (insulin receptor), IGFIR (IGF-I receptor) and IGF-II/M-6-PR (mannose 6-phosphate receptor)], three ligands (insulin, IGF-I and IGF-II) and six known types of circulating binding proteins [IGFBP1 (IGF-binding protein 1)–IGFBP6] [19,20]. Both IGF-I and IGF-II bind to the IGFIR, although IGF-I shows a higher affinity than IGF-II [20]. Insulin, the main ligand for the IR, has an IGFIR-binding affinity that is much lower than that of IGF-I [19,20]. The specific receptor for IGF-II, the M-6-PR, differs significantly from the IGFIR, possesses no tyrosine kinase activity and was reported to target IGF-II for lysosomal degradation [21,22]. Signalling by the IGFIR plays a fundamental role in cell growth and malignant transformation and is an important inhibitor of apoptosis [23,24]. The IGFIR is overexpressed in a variety of human tumours including malignant brain tumours [25]. Decreased receptor expression or impaired function was reported to induce a reversal of the transformed phenotype, apoptosis and a decrease in cellular radioresistance and chemoresistance [26]. The IGFIR has thus

Abbreviations used: AT/RT, atypical teratoid/rhabdoid tumour; CNS, central nervous system; Cy3, indocarbocyanine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; EGFR, EGF receptor; ERK1/2, extracellular-signal-regulated kinase 1/2; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; *hSNF5*, human *SNF5*; IGF, insulin-like growth factor; IGFBP1, IGF-binding protein 1; IGFIR, IGF-I receptor; IR, insulin receptor; M-6-PR, mannose 6-phosphate receptor; MRT, malignant rhabdoid tumour; mTOR, mammalian target of rapamycin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PDX-1, pancreatic duodenal homeobox-1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RNAi, RNA interference; RTKs, receptor tyrosine kinases; RT, reverse transcriptase; SCF, stem cell factor; shRNA, small-hairpin RNA; siRNAs, small interfering RNAs; S6K, S6 kinase; Tos-Lys-CH₂Cl, tosyl-lysylchloromethane.

¹ To whom correspondence should be addressed (email Alexandre.Arcaro@kispi.unizh.ch).

been proposed to be a target for the development of novel anti-cancer therapies [26–29]. In medulloblastoma, targeting of the IGFIR with the inhibitor NVP-AEW541 [30] was recently shown to impair cell growth and survival [31]. In AT/RT cells, a recent report has shown that the IGFIR is involved in anti-apoptotic signalling and contributes to chemoresistance [32]. Less is known about the potential involvement of the related IR in human cancer, although its role has been described in the pathogenesis of certain malignancies [33].

A critical intracellular signalling mediator of the IGFIR is the PI3K (phosphoinositide 3-kinase)/Akt [also called PKB (protein kinase B)] pathway [27,34,35]. Indeed, PI3K signalling is implicated in the control of cell proliferation, survival and motility/metastasis downstream of many different growth factor receptors [35,36]. The importance of PI3K signalling in human cancer is highlighted by the fact that mutations in the tumour suppressor gene *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) occur frequently in human tumours [36,37]. *PTEN* is a phosphatase that antagonizes the action of PI3K by dephosphorylating the D-3 position of polyphosphoinositides [38]. Moreover, previous reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110 α isoform of class I_A PI3K in a variety of human cancers, including, breast, colon and ovarian cancers, as well as medulloblastoma [39,40].

In the present study, we have investigated the expression pattern and biological functions of components of the IR and IGFIR signalling system in human AT/RT and MRT cell lines. Moreover, we have evaluated the potential of targeting the IR or the IGFIR using RNAi (RNA interference), neutralizing antibodies or the inhibitor NVP-AEW541 [30] as an antiproliferative approach in AT/RT cells. Finally, we have investigated whether targeting downstream signalling mediators of the IR could suppress growth and induce apoptosis in AT/RT cell lines. Our findings describe for the first time a role for autocrine signalling by insulin and the IR in growth and survival of AT/RT cells, which involves the PI3K/Akt pathway.

MATERIALS AND METHODS

Reagents and antibodies

Antibodies against Akt/PKB, caspase 3, HA (haemagglutinin) epitope tag, ERK1/2 (extracellular-signal-regulated kinase 1/2), IGFIR β , INI1, IR β , lamin B, p110 β and p110 δ were obtained from Santa Cruz Biotechnology. Antibodies against activated ERK1/2 (Thr²⁰²/Tyr²⁰⁴), activated Akt (Ser⁴⁷³), and Thr³⁸⁹-phosphorylated S6K (S6 kinase) were from Cell Signaling Technology. The Akt inhibitor [1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate], IGF-I, the IGFIR neutralizing antibody (clone α IR3), LY294002, rapamycin and PD98059 were from Calbiochem. The anti- β -tubulin antibody and insulin were from Sigma–Aldrich. The IR neutralizing antibody (clone 47-9) was from Biosource. The anti-p85 α antibody was from Upstate Biotechnology. The antibody against p110 α (clone U3A) was a generous gift from Dr A. Klippel (atugen, Berlin, Germany). NVP-AEW541 (Novartis Pharma), YM024, TGX-221 and IC87114 (ICOS Corporation) were dissolved in DMSO at 10 mM and diluted into cell culture medium just prior to use.

Cell lines and cell culture

The human MRT cell lines AS, LP, MON and STM [9] have been described previously. The DAOY medulloblastoma cell line was purchased from the A.T.C.C. (Rockville, MD, U.S.A.). The BT-12 and BT-16 human CNS AT/RT cell lines [32,41] were gifts from

Dr Jaclyn Biegel (The Children's Hospital of Philadelphia, PA, U.S.A.). These cell lines have been established from two infants with CNS AT/RT (BT-12 from a 6-week-old female; BT-16 from a 2-year-old male). They have been analysed for *INI1* mutations by Dr Jaclyn Biegel and both contain *INI1* mutations. Human AT/RT cell lines were grown in DMEM (Dulbecco's modified Eagle's medium; Life Technologies/Invitrogen) with 10% (v/v) FCS (foetal calf serum) and penicillin/streptomycin/L-glutamine, and passaged every 3–5 days by trypsinization. Human MRT cell lines were grown in RPMI 1640 (Life Technologies/Invitrogen) with 10% FCS and penicillin/streptomycin/L-glutamine. For serum-starving, the cells were incubated for 16 h in DMEM containing 0.5% FCS. All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Cell proliferation and apoptosis

AT/RT cell lines (10⁵/ml) were grown in 96-well plates for 3 days in serum (1 or 10%)-containing medium in the presence or absence of inhibitors. For growth factor stimulations, cells were incubated in medium containing 1% FCS. Cell proliferation was analysed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay using the CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (Promega).

For detection of apoptosis, AT/RT cells [1 × 10⁶ cells/well in 6-well plates (35 mm)] were incubated for 24 h in the presence or absence of inhibitors. The cells were then washed in 1 × PBS and lysed in 1 × gel-loading buffer [50 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 100 mM DTT (dithiothreitol), 0.1% (w/v) Bromophenol Blue and 10% (w/v) glycerol]. The samples were denatured for 3 min at 100°C and analysed by SDS/PAGE and Western blot with anti-caspase 3 antibodies.

ELISAs

AT/RT cells were seeded at 5 × 10⁴ cells/well in 6-well plates (35 mm). After 3 h, the cells were washed three times in serum-free medium and incubated in 2 ml of DMEM containing penicillin/streptomycin/L-glutamine. After 5 days, the supernatants were collected, centrifuged to remove cellular contaminants and human insulin was quantified by using the AIA-PACK IRI immunoassay (Tosoh Corporation). The background signal caused by the cell culture medium in the absence of cells was subtracted from the measured values.

Immunofluorescence

AT/RT cells were grown on coverslips and serum-starved for 24 h. The cells were then fixed with 4% (w/v) paraformaldehyde in 1 × PBS and permeabilized with methanol for 10 min at –20°C. The coverslips were blocked with 0.5% (w/v) non-fat dry milk in 1 × PBS for 1 h and incubated with anti-insulin antibody (1:50; Dako) or anti-IR β (1:100; Santa Cruz Biotechnology) for 1 h. The detection was performed using secondary anti-guinea-pig antibody coupled with FITC, or anti-rabbit antibody coupled with Cy3 (indocarbocyanine). Pictures were taken at ×100 magnification with a Zeiss Axioskop fluorescence microscope.

Immunoprecipitations

AT/RT cells grown to confluence on 10 cm dishes were serum-starved and stimulated with growth factors for 10 min at 37°C. After washing with ice-cold 1 × PBS, the cells were lysed for 20 min on ice in 1 ml of lysis buffer [20 mM Hepes/NaOH,

pH 7.4, 150 mM NaCl, 1 % (w/v) Triton X-100, 2 mM EDTA, 10 mM sodium fluoride, 10 % (w/v) glycerol, 1 mM PMSF, 5 mM benzamidine, 1 mM Tos-Lys-CH₂Cl (tosyl-lysylchloromethane), 10 μ M leupeptin, 10 μ M pepstatin A, 2 mM Na₃VO₄ (sodium orthovanadate), 10 mM 2-glycerophosphate and 10 mM NaF]. Immunoprecipitation was performed for 2 h at 4 °C with primary antibodies (diluted according to the manufacturer's instructions). Protein G–Sepharose CL-4B (Amersham Biosciences) was then added, and the incubation was continued for 1 h at 4 °C. The immunoprecipitates were washed three times in lysis buffer and resuspended in 1× gel-loading buffer. The samples were denatured for 3 min at 100 °C and analysed by SDS/PAGE and Western blot.

MS analysis

Samples (10 μ l) were desalted using Millipore C18 ZipTips. An aliquot of 0.5 μ l was applied directly to a prespotted AnchorChip™ target. After 3 min of incubation, 7 μ l of washing solution (0.1 % trifluoroacetic acid) was added on the analyte solution and the whole droplet was removed after a few seconds. MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) mass spectra were acquired in linear mode using a Bruker Daltonics Autoflex™ mass spectrometer.

RT (reverse transcriptase)–PCR

Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For each RT–PCR, 2 μ g of total RNA was used with the Qiagen One-Step RT–PCR kit. The following primers were used: insulin primer, sense 5'-CGTTCCCCGCACACTAGGTT-3' and antisense 5'-GCAGCCTTTGTGAACCAACAC-3'; IGF-I primer, sense 5'-GTGCTGCTTTTGTGATTTCTT-3' and antisense 5'-GTCTTGGGCATGTCCGGTGTGG-3'; IGF-II primer, sense 5'-ATGGGGAAGTCGATGCTGGTG-3' and antisense 5'-ACGGGTATCTGGGGAAGTTG-3'; PDX-1 (pancreatic duodenal homeobox-1) primer, sense 5'-CTGCGGAGCCGGAGGAGAC-3' and antisense 5'-TCTAGAACTACACAGAGAGC-3'; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primer, sense 5'-GAAGGTGAAGGTCGGAGTC-3' and antisense 5'-GAAGATGGTGATGGGATTTC-3'. The reaction conditions were as follows: reverse transcription at 50 °C for 30 min, initial PCR activation at 95 °C for 15 min followed by 40 cycles at 95 °C for 15 s, 55 °C for 20 s and 72 °C for 40 s. The PCR products were analysed in 3 % (w/v) agarose gel.

SDS/PAGE and Western-blot analysis

Cellular lysates were prepared with lysis buffer [50 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 % Triton X-100, 1 % (w/v) deoxycholic acid, 0.1 % SDS, 10 % glycerol, 10 μ M leupeptin, 10 μ M pepstatin A, 1 mM PMSF, 5 mM iodoacetamide, 5 mM benzamidine, 1 mM Tos-Lys-CH₂Cl, 2 mM Na₃VO₄, 10 mM 2-glycerophosphate and 10 mM NaF]. The cells were lysed for 20 min on ice, scraped and lysates were centrifuged at 12000 g for 20 min at 4 °C. The supernatants were collected and normalized for protein content by using the BCA (bicinchoninic acid) protein assay kit (Pierce). One volume of 2× gel-loading buffer [100 mM Tris/HCl, pH 6.8, 4 % SDS, 200 mM DTT, 0.2 % (w/v) Bromophenol Blue and 20 % (w/v) glycerol] was then added to the samples, followed by denaturation for 3 min at 100 °C and analysed by SDS/PAGE. The gels were transferred on to a hydrophobic PVDF membrane (Hybond-

Figure 1 Expression of the IR and its downstream signalling components in MRT and AT/RT cells

(A) Equal amounts of lysates from adult brain (Ad), foetal brain (Fe), foetal cerebellum (Ce), or the cell lines indicated were analysed by Western blot for expression of the proteins indicated. The cell lines analysed were AS, LP, MON, STM (MRT), BT-12, BT-16 (AT/RT) and DAOY (medulloblastoma). (B) Equal amounts of lysates from the cell lines indicated were analysed by Western blot for expression of INI1.

P; Amersham Biosciences) by electrophoresis. The membranes were then blocked in 1× PBS, 5 % (w/v) BSA (phospho-specific antibodies) or 1× PBS/3 % non-fat dry milk (all other antibodies) for 16 h at 4 °C. The membranes were incubated with primary antibodies (diluted according to the manufacturer's protocol) for 16 h at 4 °C. After washing in 1× PBS and 0.1 % (w/v) Tween 20, the immunoblots were incubated with donkey anti-rabbit IgG or sheep anti-mouse IgG secondary antibodies (1:10 000 dilution) coupled with horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature. After washing of the immunoblots, chemiluminescence was used for detection, using the ECL® (enhanced chemiluminescence) Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's protocol.

Transient and stable transfections in AT/RT cells

Human AT/RT cells were transiently transfected with Akt/PKB constructs in pUSE (Upstate Biotechnology), or with PI3K shRNA (small-hairpin RNA) constructs in pRETRO-SUPER [42] by using Lipofectamine™ 2000 (Invitrogen). The transfections

Figure 2 Human AT/RT cells secrete insulin under serum-free conditions

(**A**) Serum-free supernatants from AT/RT cells were analysed after 5 days in culture by ELISA to detect insulin production. Results are the means with S.D. from a representative experiment (out of three) performed in triplicate. (**B**) Serum-free supernatants from BT-16 cells (upper panel) or purified recombinant human insulin (lower panel) were analysed by MS. (**C**) Expression of insulin (left) and the IR β (centre) in BT-16 cells was investigated by immunofluorescence ($\times 100$ magnification). The detection was performed using secondary antibodies coupled with FITC (insulin) or Cy3 (IR β). The right panel shows the merged pictures and the cell nucleus stained with DAPI (4',6-diamidino-2-phenylindole). (**D**) The expression of PDX-1 was detected in MRT and AT/RT cells using RT-PCR. GAPDH expression was analysed in parallel as a loading control.

were performed in 96-well plates (MTS assays) or 6-well plates (Western-blot analysis), using the amounts of DNA and LipofectamineTM 2000 recommended by the manufacturer's protocol. Opti-MEM I medium (Invitrogen) was used for the transfection and replaced by growth medium after 24 h.

The siRNAs (small interfering RNAs) targeting the IR β and IGFIR β and non-targeting control siRNA were purchased from Dharmacon. The siRNAs were transfected into BT-12 or BT-16 cells using LipofectamineTM 2000 (Invitrogen). The transfections were performed in 96-well plates (MTS assays) or 6-well plates (Western-blot analysis), using the amounts of siRNA and LipofectamineTM 2000 recommended by the manufacturer's protocol. Opti-MEM I medium (Invitrogen) was used for the transfection and replaced by growth medium after 24 h. Cell responses were assessed 72 h post-transfection.

For stable expression BT-16 cells (in 10 cm dishes) were transfected with an HA-tagged INI1 construct in pcDNA3 (Invitrogen) or with empty vector using LipofectamineTM (Invitrogen). The amounts of DNA and LipofectamineTM used were as recommended by the manufacturer's protocol. After 48 h, the cells were diluted 1:10 in growth medium containing G418 (1.0 mg/ml). A population of resistant cells was expanded and analysed after

selection. Western-blot analysis was used to confirm expression of HA-tagged INI1.

RESULTS

Expression of components of the IR and IGFIR signalling pathways in AT/RT cells

We characterized a panel of established human AT/RT cell lines (BT-12 and BT-16) [32,41], MRT lines (AS, LP, MON and STM) [9] and medulloblastoma (DAOY) for expression of RTKs (receptor tyrosine kinases) to study further their involvement in tumour cell proliferation and survival. Lysates from normal adult brain, foetal brain and foetal cerebellum were analysed in parallel as controls. Western-blot analysis revealed that MRT and AT/RT cell lines overexpressed the IR and IGFIR, as compared with the normal brain samples (Figure 1A). Expression of the EGFR [EGF (epidermal growth factor) receptor], ErbB2 and c-Kit were also detected in the panel of cell lines (Figure 1A and results not shown). In contrast, the PDGFR (platelet-derived growth factor receptor) was not detected in the cell lines under study (results not shown). The expression of components of the PI3K/Akt signalling pathway in the panel of MRT and AT/RT cell lines was then

Figure 3 A pharmacological inhibitor or a neutralizing antibody targeting the IR impairs AT/RT cell proliferation

(A) AT/RT cells were incubated in the presence of increasing concentrations of the inhibitor NVP-AEW541 in a medium containing 10% (squares) or 0.5% (diamonds) FCS. Cell proliferation was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (B) BT-16 cells were incubated in serum-free medium in the presence of neutralizing antibodies targeting either the IGFIR or the IR (10 μg/ml) and cell proliferation measured after 72 h. Results are the means with S.D. from three experiments performed in quadruplicate (*, $P < 0.05$ by ANOVA test). (C) BT-16 cells were incubated with increasing concentrations of insulin in serum-free medium in the presence of LY294002 (10 μM, diamonds) or vehicle (squares). Cell proliferation was measured after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (D) BT-12 cells were incubated for 20 h in the presence of increasing concentrations of the inhibitor NVP-AEW541 and the induction of apoptosis assessed by Western blot for the active fragments of caspase 3 (20 and 17 kDa, arrows). The blots were reprobed for lamin B as a loading control.

investigated. All cell lines expressed the PI3K regulatory subunit p85α and the p110α and p110β catalytic subunits (Figure 1A). In addition, both AT/RT cell lines overexpressed the p110δ isoform (Figure 1A). MRT and AT/RT cell lines displayed comparable levels of IRS-1 (IR substrate-1), mTOR (mammalian target of rapamycin) and ERK1/2 (Figure 1A and results not shown). The AT/RT and MRT cell lines did not express INI1, in contrast with DAOY medulloblastoma cells, as expected (Figure 1B). Indeed, AT/RT and MRT cell lines and tumours harbour inactivating biallelic alterations in the *hSNF5/INI1* gene, leading to loss of expression of the INI1 protein [1,9]. In contrast, these mutations are not found in medulloblastoma [43].

Human AT/RT secrete insulin in an autocrine manner

We next investigated whether AT/RT cells produced growth factors in an autocrine fashion. An ELISA revealed that both BT-12 and BT-16 cell lines secreted insulin into the growth medium, when cultivated under serum-free conditions (Figure 2A). The BT-16 cell line appeared to produce higher amounts of insulin than

BT-12 (2628 pmol/l versus 867 pmol/l). IGF-I or IGF-II secretion could not be detected in either BT-12 or BT-16 cells grown in serum-free medium (results not shown). However, an RT-PCR analysis revealed that IGF-I mRNA was expressed in BT-16 cells, while IGF-II expression was detectable in BT-12 cells (see Supplementary Figure 1 at <http://www.BiochemJ.org/bj/406/bj4060057add.htm>). To confirm that the growth factor secreted by BT-16 cells corresponded to human insulin, supernatants of BT-16 cells were analysed by MS. MS analysis revealed the presence of a protein of 5807 Da, corresponding to human insulin in serum-free supernatants obtained from BT-16 cells (Figure 2B). Immunofluorescence analysis revealed that individual BT-16 cells co-expressed insulin and the IR, indicating the presence of an autocrine signalling loop in these cells (Figure 2C). We next performed RT-PCR analysis to investigate the expression of the transcription factor PDX-1 in the panel of cell lines. PDX-1 is a master regulator of pancreas development and β-cell function, which participates in the transcription of several genes, including *insulin* [44]. This analysis revealed strong expression of PDX-1 mRNA in the BT-12, BT-16 and MRT cell lines, confirming their

insulin-producing phenotype (Figure 2D). We next investigated whether other human cancer cell lines also secrete insulin under similar culture conditions. However, an analysis of a panel of human medulloblastoma and neuroblastoma cell lines failed to document insulin secretion under serum-free culture conditions (results not shown), indicating that the response is selective for CNS AT/RT cells.

Inhibition of AT/RT cell proliferation by targeting the IR

The specific IGFIR/IR kinase inhibitor NVP-AEW541 [30] inhibited proliferation of BT-12 and BT-16 cell lines (Figure 3A), in a dose-dependent manner with IC_{50} values of 2 μ M in BT-12 and 5 μ M in BT-16 cells (Figure 3A). In BT-12 cells, NVP-AEW541 was effective at slightly lower concentrations, when cells were incubated in medium containing low serum, as compared with high serum (Figure 3A). In contrast, no differences in sensitivities were observed in the BT-16 cell line (Figure 3A). To confirm these observations, cell proliferation of BT-12 or BT-16 cells was assessed in the presence of neutralizing antibodies specific either for the IGFIR or for the IR. Proliferation of BT-16 cells in serum-free medium was significantly inhibited by the anti-IR neutralizing antibody (Figure 3B). A similar effect was observed with the anti-IGFIR neutralizing antibody (Figure 3B). The anti-IR neutralizing antibody also significantly inhibited proliferation of BT-12 and BT-16 cells in serum-containing medium (results not shown). Increasing concentrations of insulin stimulated proliferation of BT-16 cells in serum-free medium (Figure 3C) and the response was completely inhibited by a pharmacological PI3K inhibitor (LY294002), indicating that PI3K is essential for insulin-stimulated cellular responses in AT/RT cells. The maximal increase in cell proliferation was observed at an insulin concentration of 50 ng/ml (8.6 nM) and concentrations up to 1000 ng/ml had no significantly higher effect (Figure 3C). Inhibiting IGFIR/IR function with NVP-AEW541 also induced apoptosis in AT/RT cells, as assessed by caspase 3 activation (Figure 3D), indicating that the autocrine signalling loop involving the IR contributes to cell survival.

An RNAi approach was used to confirm the critical role of the IR and IGFIR in supporting AT/RT cell growth and survival. Transfection of BT-12 and BT-16 cells with siRNA targeting either the IR β or the IGFIR β resulted in a significant decrease in cell viability (Figures 4A and 4B and results not shown). To verify the specificity of the effect of the siRNA targeting the IR β , protein down-regulation was assessed by Western-blot analysis in BT-12 and BT-16 cells (Figure 4C and results not shown).

Activation of the PI3K/Akt signalling pathway by insulin in AT/RT cells

The ability of polypeptide growth factors to activate PI3K/Akt, ERK1/2 and ribosomal protein S6K was then investigated in BT-12 and BT-16 cells. Insulin most potently activated Akt, as compared with EGF and SCF (stem cell factor), whereas activation of ERK1/2 was comparable between all growth factors (Figure 5A). The IGFIR/IR kinase inhibitor NVP-AEW541 (2.5 μ M) completely inhibited IGF-I- and insulin-activated responses (Figure 5B). Autophosphorylation of the IR was also detected in insulin-stimulated AT/RT cells, confirming the specificity of the responses observed (Figure 5C).

Our data so far had revealed activation of the PI3K/Akt, S6K and ERK1/2 pathways in AT/RT cells stimulated by polypeptide growth factors. Therefore we next investigated the contributions of these pathways to proliferation in BT-12 and BT-16 cells.

Figure 4 Down-regulation of IR by siRNA treatment impairs AT/RT cell proliferation

(A, B) BT-12 or BT-16 AT/RT cells were transiently transfected with non-targeting siRNA (ctr), or siRNA targeting the IGFIR or the IR, alone or in combination, where indicated. Cell proliferation was measured after 72 h. Results are the means with S.D. from three experiments performed in quadruplicate (*, $P < 0.05$ by ANOVA test). (C) BT-12 AT/RT cells were transiently transfected with siRNAs as indicated and expression of the IR β and IGFIR β was analysed by Western blot after 72 h.

Cell proliferation was assessed in the presence of the PI3K inhibitor LY294002, rapamycin, the MEK [MAPK (mitogen-activated protein kinase)/ERK kinase] inhibitor PD98059, or a pharmacological Akt/PKB inhibitor. AT/RT cell proliferation was significantly impaired by LY294002 or rapamycin, in both BT-12 and BT-16 cells grown in low (1%) or high (10%) serum (Figure 5D). The Akt inhibitor completely inhibited proliferation of the BT-12 cell line in low, but not high serum, while having a lesser effect in the BT-16 cell line (Figure 5D). To confirm the involvement of PI3K/Akt signalling in AT/RT cell proliferation, a dominant-negative catalytically inactive Akt (AktDN) construct was transiently transfected into BT-12 and BT-16 cells. Western-blot analysis confirmed expression of the construct in AT/RT cells (results not shown). The AktDN construct significantly inhibited proliferation in both cell lines (Figure 5E). In contrast, transfection of an activated Akt mutant (myrAktCA) had no significant effect on AT/RT cell proliferation.

The class I α PI3K isoform p110 α controls AT/RT cell proliferation and insulin-stimulated Akt activation

To confirm the involvement of PI3K signalling in AT/RT cell responses to insulin, a panel of isoform-specific class I α PI3K inhibitors was tested in AT/RT cells. The pharmacological p110 α inhibitor YM024 [45] inhibited proliferation of AT/RT cells in a dose-dependent manner with an IC_{50} of 1.5 μ M (Figure 6A). In contrast, the p110 β inhibitor TGX-221 [46] and the p110 δ

Figure 5 Insulin activates the PI3K/Akt pathway in AT/RT cells

(A) Serum-starved AT/RT cells were stimulated with growth factors where indicated (40 ng/ml EGF; 50 ng/ml Ins; 20 ng/ml SCF) for 10 min, and activation of Akt and ERK was determined by Western blot with phospho-specific antibodies. Abbreviations: p-Akt, Ser⁴⁷³-phosphorylated Akt; p-ERK, Thr²⁰²/Tyr²⁰⁴-phosphorylated ERK. (B) Serum-starved BT-16 cells were pretreated with NVP-AEW541 (2.5 μ M), stimulated with the growth factors indicated (50 ng/ml) for 10 min and activation of Akt and S6K1 was determined as above. Abbreviation: p-S6K1, Thr³⁸⁹-phosphorylated S6K. (C) Serum-starved AT/RT cells were stimulated with the growth factors indicated (10 min) and anti-phosphotyrosine (pY) immunoprecipitates were analysed by Western blots for the relevant receptors. (D) BT-12 (left panel) or BT-16 (right panel) cells were incubated in a medium containing 10% (closed bars) or 0.5% (open bars) FCS in the presence of the inhibitors indicated [LY294002 10 μ M (LY); rapamycin 20 ng/ml (Rapa); PD98059 25 μ M (PD); Akt inhibitor 20 μ M (Akt Inh)], or vehicle (DMSO). Cell proliferation was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (E) BT-12 (left panel) or BT-16 (right panel) cells were transiently transfected with empty vector, or constructs encoding activated (myrAktCA) or dominant-negative Akt (AktDN). Cell proliferation was determined after 72 h. Results are the means with S.D. from three experiments performed in quadruplicate (*, $P < 0.05$ by ANOVA test).

inhibitor IC87114 [47] had a less pronounced effect on AT/RT cell proliferation (Figure 6A). The pharmacological p110 α inhibitor YM024 also inhibited insulin-stimulated Akt activation, whereas TGX-221 and IC87114 did not impair the response (Figure 6B and results not shown). shRNA constructs targeting the catalytic subunits of class I α PI3Ks were then transiently transfected into BT-16 cells to investigate their impact on cell proliferation. Western-blot analysis confirmed down-regulation of the expression of the target genes by the relevant shRNA construct

(Figure 6D). The shRNA constructs targeting *PIK3CA* (encoding p110 α) strongly (> 80%) inhibited BT-16 cell proliferation (Figure 6C). In contrast, RNAi targeting of *PIK3CB* resulted in no significant effect on AT/RT cell proliferation (Figure 6C). Moreover, insulin-stimulated Akt activation was abrogated by transfection of BT-16 cells with the shRNA construct targeting *PIK3CA* (results not shown). Thus the p110 α isoform appears to play a crucial role in controlling AT/RT cell proliferation and insulin-stimulated Akt activation.

Figure 6 The PI3K p110 α isoform controls AT/RT cell proliferation and insulin signalling

(A) BT-16 cells were incubated with increasing concentrations of isoform-specific PI3K inhibitors [0.01 μ M (light grey bars), 0.1 μ M (medium grey bars), 1 μ M (dark grey bars), 10 μ M (closed bars) or vehicle (open bars)]. Cell proliferation was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed in quadruplicate. (B) Serum-starved BT-16 cells were pre-incubated with the p110 α inhibitor YM024 (micromolar concentrations) and stimulated with growth factors (50 ng/ml insulin; 50 ng/ml IGF-I) where indicated for 10 min. Akt activation was determined by Western blot with phospho-specific antibodies. Abbreviation: p-Akt, Ser⁴⁷³-phosphorylated Akt. (C, D) BT-16 cells were transiently transfected with shRNA constructs targeting PI3K isoforms (PIK3CA: p110 α ; PIK3CB: p110 β) or empty vector (pRS). (C) Cell proliferation was assessed after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (D) Protein down-regulation was determined by Western-blot analysis.

Ectopic expression of INI1 does not affect the IR signalling pathway in AT/RT cells

Since AT/RT cells frequently display mutations in the *INI1* gene, we investigated the impact of ectopic re-expression of the protein in BT-16 cells. BT-16 stably transfected with an *INI1* expression vector displayed reduced proliferation under either low- or high-serum culture conditions (Figure 7A). However, BT-16 transfected with *INI1* still displayed detectable insulin mRNA expression (Figure 7C). Moreover, the expression levels of the IR and IGFIR were not affected, as assessed by Western-blot analysis (Figure 7D). These results indicate that the establishment of the autocrine loop involving insulin and its receptor is independent of *INI1* loss in AT/RT cells.

DISCUSSION

Molecular abnormalities linked with human AT/RT include germline and somatic mutations in the *INI1* gene that encodes a chromatin remodelling factor and has been suggested to function as a tumour suppressor [7–9]. A recent report has also documented epigenetic repression of the *RASSF1* (Ras association domain family 1) gene in AT/RT cell lines and primary tumours [41]. RTK signalling has not yet been studied in detail in AT/RT cells. Expression of the IGFIR and IGF-II was reported in a limited number of cases of AT/RT tumours [25,48]. Targeting the IGFIR with antisense oligonucleotides resulted in increased apoptosis and sensitivity to the chemotherapeutic agents cisplatin and doxorubicin in AT/RT cells [32]. In the case of the MRTs, the EGFR inhibitor gefitinib was reported to have anti-tumour effects *in vitro* and *in vivo* [49]. Moreover, the PI3K/Akt pathway was reported to be involved in the resistance of MRT cells to chemotherapy and radiotherapy [50].

We have characterized a panel of human MRT and AT/RT cell lines for expression of RTKs and their downstream signalling mediators, in order to study the potential of targeting these molecules to inhibit cell proliferation and survival. The IR and

Figure 7 Ectopic expression of *INI1* reduces the proliferation of AT/RT cells, without effect on IR expression

(A) BT-16 cells were stably transfected with empty vector (pcDNA) or HA-tagged *INI1*. Cell proliferation in a medium containing 0.5% (open bars) or 10% (closed bars) FCS was determined after 72 h. Results are the means with S.D. from a representative experiment (out of three) performed with eight repetitions. (B) Expression of HA-tagged *INI1* in the transfected BT-16 cells was verified by Western-blot analysis. (C) Insulin mRNA expression in BT-16 cells transfected with empty vector (pcDNA) or HA-tagged *INI1* was determined by RT-PCR. GAPDH was analysed in parallel as a control. (D) The expression of the IR β and the IGFIR β in BT-16 cells transfected with empty vector (pcDNA) or HA-tagged *INI1* was determined by Western blot.

IGFIR were found overexpressed in both AT/RT and MRT cell lines, as compared with normal brain tissue. Moreover and surprisingly, AT/RT cell lines secreted insulin when grown under serum-free conditions, indicating autocrine signalling events. To our knowledge these AT/RT cell lines are the first example of human CNS tumour cell lines secreting insulin. Moreover, the

only other examples of insulin secretion by cells other than pancreatic β -cells are sperm cells [51]. The concentration of insulin measured in supernatants from BT-16 cells was 2.5 nM, which is in the biological range. Maximal biological responsiveness to insulin tends to occur at concentrations in the range 10–20 nM [52–54] and insulin had maximal effects on BT-16 cell proliferation at a concentration of 8.6 nM. It is conceivable that AT/RT cells also produce other growth factors in an autocrine fashion. Indeed, IGF-I and IGF-II were detected at the mRNA level in BT-16 and BT-12 cells respectively, as recently reported [32]. We were unable to detect free IGF-I and IGF-II in supernatants from AT/RT cells by ELISA, but this could be due to the fact that IGFs were bound to IGF-binding proteins. Autocrine signalling by IGF-I via the IGFIR in BT-16 cells would explain the inhibitory effects of IGFIR-neutralizing antibodies or siRNA on the proliferation of these cells.

Insulin activated PI3K/Akt, S6K and ERK signalling, whereas other growth factors such as EGF or SCF selectively activated some, but not all of the pathways. Targeting the IGFIR kinase activity with NVP-AEW541 resulted in inhibition of AT/RT proliferation, although at higher IC₅₀ values than reported in other tumour cells [30]. These increased IC₅₀ values possibly reflect co-expression of the IR and IGFIR in AT/RT cell lines, since the IC₅₀ of NVP-AEW541 for the IR was reported to be 2.3 μ M in cells [30,55]. This model was supported by the observations that neutralizing antibodies or siRNA targeting the IR β inhibited the proliferation of AT/RT cells. Consequently, our results demonstrate that insulin secreted by AT/RT cells in an autocrine fashion activates the IR and thus contributes to AT/RT cell proliferation and survival. This mechanism of IR activation appears to be different from the previously reported activation loop involving IGF-II and the IR isoform A in human cancer cells [56–58]. The molecular mechanisms underlying the establishment of the autocrine insulin signalling loop in AT/RT cells are currently unclear. Loss of *hSNF5/INI1* did not appear to be a major cause of the expression of insulin and the IR by AT/RT cells, since transfection of INI1 did not substantially alter the expression of these molecules. Intriguingly, the pancreatic transcription factor PDX-1 was detected in AT/RT and MRT cells, indicating that aberrant expression of transcription factors may drive these cancer cells towards an insulin-producing phenotype. A recent survey of mutations in human tumour genomes has revealed frequent mutations in transcription factors, emphasizing the contribution of abnormalities in transcription to the development of human cancer [59].

To investigate whether targeting downstream signalling mediators of the IR could provide a means of inhibiting proliferation of AT/RT cells, we used a combination of pharmacological and RNAi approaches. This revealed that the PI3K/Akt/mTOR pathway was crucial in the control of AT/RT cell proliferation. Moreover, a selective role for the PI3K isoform p110 α in transducing signals from the IR was uncovered in AT/RT cells, in good agreement with recent reports documenting the crucial role of p110 α in insulin signalling [60,61]. Thus pharmacological inhibitors targeting the PI3K p110 α isoform may have antiproliferative potential in human cancer cells where this isoform is activated by the IR.

Insulin has various biological effects in a broad range of tissues, and its functions in the brain include neuronal survival and regulation of energy homeostasis [62]. Although a large body of evidence exists implicating IGF-I and IGF-II as autocrine growth factors in a broad range of human malignancies [24,27], such a function has not yet been described for insulin in CNS tumours. In the light of the novel function for insulin as an autocrine growth factor for CNS AT/RT cells, further studies are warranted to uncover its potential role in other human cancers.

This work was supported by grants from the Oncosuisse (OCS-01501-02-2004), the Novartis Stiftung für Medizinisch-Biologische Forschung, the Forschungskredit der Universität Zürich, the Krebsliga Zürich and the Stiftung zur Krebsbekämpfung (A.A.). We thank Dr F. Hofmann and Dr M. A. Pearson (Novartis Institutes for BioMedical Research, Novartis Pharma, Basel, Switzerland) for providing NVP-AEW541. We thank Dr J. S. Hayflick (ICOS Corporation, Bothell, WA, U.S.A.) for providing IC87114. We thank Dr J. Biegel (Division of Human Genetics and Molecular Biology, The Children's Hospital of Philadelphia, Philadelphia, PA, U.S.A.), Dr O. E. Pardo and Dr J. Downward (Signal Transduction Laboratory, Cancer Research UK London Research Institute, London, U.K.), Dr A. Kiiptel (atugen, Berlin, Germany) and Dr U. Zangemeister-Wittke (Institute of Pharmacology, University of Bern, Bern, Switzerland) for providing reagents and cell lines. We thank Dr A. Lauber-Biason and E. Allig-Schuster (Division of Endocrinology and Diabetology, University Children's Hospital Zurich, Zurich, Switzerland) for performing insulin determinations, and Dr H. Troxler and P. Kleinert (Division of Chemistry and Biochemistry, University Children's Hospital Zurich, Zurich, Switzerland) for performing MS analysis.

REFERENCES

- Biegel, J. A., Tan, L., Zhang, F., Wainwright, L., Russo, P. and Rorke, L. B. (2002) Alterations of the *hSNF5/INI1* gene in central nervous system atypical teratoid/rhabdoid tumors and renal and extrarenal rhabdoid tumors. *Clin. Cancer Res.* **8**, 3461–3467
- Judkins, A. R., Mauger, J., Ht, A., Rorke, L. B. and Biegel, J. A. (2004) Immunohistochemical analysis of *hSNF5/INI1* in pediatric CNS neoplasms. *Am. J. Surg. Pathol.* **28**, 644–650
- Pomeroy, S. L., Tamayo, P., Gaasenbeek, M., Sturla, L. M., Angelo, M., McLaughlin, M. E., Kim, J. Y., Goumnerova, L. C., Black, P. M., Lau, C. et al. (2002) Prediction of central nervous system embryonal tumour outcome based on gene expression. *Nature* **415**, 436–442
- Reddy, A. T. (2005) A typical teratoid/rhabdoid tumors of the central nervous system. *J. Neurooncol.* **75**, 309–313
- Hilden, J. M., Meerbaum, S., Burger, P., Finlay, J., Janss, A., Scheithauer, B. W., Walter, A. W., Rorke, L. B. and Biegel, J. A. (2004) Central nervous system atypical teratoid/rhabdoid tumor: results of therapy in children enrolled in a registry. *J. Clin. Oncol.* **22**, 2877–2884
- Tekautz, T. M., Fuller, C. E., Blaney, S., Fouladi, M., Broniscer, A., Merchant, T. E., Krasin, M., Dalton, J., Hale, G., Kun, L. E. et al. (2005) A typical teratoid/rhabdoid tumors (ATRT): improved survival in children 3 years of age and older with radiation therapy and high-dose alkylator-based chemotherapy. *J. Clin. Oncol.* **23**, 1491–1499
- Biegel, J. A., Zhou, J. Y., Rorke, L. B., Stenstrom, C., Wainwright, L. M. and Fogelgren, B. (1999) Germ-line and acquired mutations of *INI1* in atypical teratoid and rhabdoid tumors. *Cancer Res.* **59**, 74–79
- Sevenet, N., Lellouch-Tubiana, A., Schofield, D., Hoang-Xuan, K., Gessler, M., Birnbaum, D., Jeanpierre, C., Juvet, A. and Delattre, O. (1999) Spectrum of *hSNF5/INI1* somatic mutations in human cancer and genotype–phenotype correlations. *Hum. Mol. Genet.* **8**, 2359–2368
- Versteeg, I., Sevenet, N., Lange, J., Rousseau-Merck, M. F., Ambros, P., Handgretinger, R., Aurias, A. and Delattre, O. (1998) Truncating mutations of *hSNF5/INI1* in aggressive paediatric cancer. *Nature* **394**, 203–206
- Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P. A., Biggar, S. R., Muchardt, C., Kalpana, G. V., Goff, S. P., Yaniv, M. et al. (1996) Purification and biochemical heterogeneity of the mammalian SWI–SNF complex. *EMBO J.* **15**, 5370–5382
- Wang, W., Xue, Y., Zhou, S., Kuo, A., Cairns, B. R. and Crabtree, G. R. (1996) Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev.* **10**, 2117–2130
- Roberts, C. W. and Orkin, S. H. (2004) The SWI/SNF complex – chromatin and cancer. *Nat. Rev. Cancer* **4**, 133–142
- Guidi, C. J., Sands, A. T., Zambrowicz, B. P., Turner, T. K., Demers, D. A., Webster, W., Smith, T. W., Imbalzano, A. N. and Jones, S. N. (2001) Disruption of *Ini1* leads to peri-implantation lethality and tumorigenesis in mice. *Mol. Cell. Biol.* **21**, 3598–3603
- Roberts, C. W., Galusha, S. A., McMenamin, M. E., Fletcher, C. D. and Orkin, S. H. (2000) Haploinsufficiency of *Snf5* (integrator interactor 1) predisposes to malignant rhabdoid tumors in mice. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 13796–13800
- Isakoff, M. S., Sansam, C. G., Tamayo, P., Subramanian, A., Evans, J. A., Fillmore, C. M., Wang, X., Biegel, J. A., Pomeroy, S. L., Mesirov, J. P. and Roberts, C. W. (2005) Inactivation of the *Snf5* tumor suppressor stimulates cell cycle progression and cooperates with p53 loss in oncogenic transformation. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 17745–17750
- Klochender-Yeivin, A., Picarsky, E. and Yaniv, M. (2006) Increased DNA damage sensitivity and apoptosis in cells lacking the *Snf5/Ini1* subunit of the SWI/SNF chromatin remodeling complex. *Mol. Cell. Biol.* **26**, 2661–2674
- Zhang, Z. K., Davies, K. P., Allen, J., Zhu, L., Pestell, R. G., Zagzag, D. and Kalpana, G. V. (2002) Cell cycle arrest and repression of cyclin D1 transcription by *INI1/hSNF5*. *Mol. Cell. Biol.* **22**, 5975–5988

- 18 Alarcon-Vargas, D., Zhang, Z., Agarwal, B., Challagulla, K., Mani, S. and Kalpana, G. V. (2006) Targeting cyclin D1, a downstream effector of IN1/hSNF5, in rhabdoid tumors. *Oncogene* **25**, 722–734
- 19 Jones, J. I. and Clemmons, D. R. (1995) Insulin-like growth factors and their binding proteins: biological actions. *Endocr. Rev.* **16**, 3–34
- 20 Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C. and Forbes, B. E. (2005) Molecular interactions of the IGF system. *Cytokine Growth Factor Rev.* **16**, 421–439
- 21 Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate/insulin like growth factor II receptors. *Annu. Rev. Biochem.* **61**, 307–330
- 22 Hebert, E. (2006) Mannose-6-phosphate/insulin-like growth factor II receptor expression and tumor development. *Biosci. Rep.* **26**, 7–17
- 23 Foulstone, E., Prince, S., Zaccheo, O., Burns, J. L., Harper, J., Jacobs, C., Church, D. and Hassan, A. B. (2005) Insulin-like growth factor ligands, receptors, and binding proteins in cancer. *J. Pathol.* **205**, 145–153
- 24 Pollak, M. N., Schernhammer, E. S. and Hankinson, S. E. (2004) Insulin-like growth factors and neoplasia. *Nat. Rev. Cancer* **4**, 505–518
- 25 Ogino, S., Kubo, S., Abdul-Karim, F. W. and Cohen, M. L. (2001) Comparative immunohistochemical study of insulin-like growth factor II and insulin-like growth factor receptor type 1 in pediatric brain tumors. *Pediatr. Dev. Pathol.* **4**, 23–31
- 26 Surmacz, E. (2003) Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor. *Oncogene* **22**, 6589–6597
- 27 Guerreiro, A. S., Boller, D., Doepfner, K. T. and Arcaro, A. (2006) IGF-IR: potential role in antitumor agents. *Drug News Perspect.* **19**, 261–272
- 28 Yee, D. (2006) Targeting insulin-like growth factor pathways. *Br. J. Cancer* **94**, 465–468
- 29 Miller, B. S. and Yee, D. (2005) Type I insulin-like growth factor receptor as a therapeutic target in cancer. *Cancer Res.* **65**, 10123–10127
- 30 Garcia-Echeverria, C., Pearson, M. A., Marti, A., Meyer, T., Mestan, J., Zimmermann, J., Gao, J., Brueggen, J., Capraro, H. G., Cozens, R. et al. (2004) *In vivo* antitumor activity of NVP-AEW541 – a novel, potent, and selective inhibitor of the IGF-IR kinase. *Cancer Cell* **5**, 231–239
- 31 Urbanska, K., Trojanek, J., Del Valle, L., Eldeen, M. B., Hofmann, F., Garcia-Echeverria, C., Khalili, K. and Reiss, K. (2007) Inhibition of IGF-I receptor in anchorage-independence attenuates GSK-3 β constitutive phosphorylation and compromises growth and survival of medulloblastoma cell lines. *Oncogene* **26**, 2308–2317
- 32 D'Cunja, J., Shalaby, T., Rivera, P., von Buren, A., Patti, R., Heppner, F. L., Arcaro, A., Rorke-Adams, L. B., Phillips, P. C. and Grotzer, M. A. (2007) Antisense treatment of IGF-IR induces apoptosis and enhances chemosensitivity in central nervous system atypical teratoid/rhabdoid tumours cells. *Eur. J. Cancer* **43**, 1581–1589
- 33 Denley, A., Wallace, J. C., Cosgrove, L. J. and Forbes, B. E. (2003) The insulin receptor isoform exon 11 – (IR-A) in cancer and other diseases: a review. *Horm. Metab. Res.* **35**, 778–785
- 34 Vanhaesebroeck, B., Leeyers, S. J., Panayotou, G. and Waterfield, M. D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem. Sci.* **22**, 267–272
- 35 Vanhaesebroeck, B., Leeyers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J. and Waterfield, M. D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70**, 535–602
- 36 Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J. and Waterfield, M. D. (2001) Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu. Rev. Cell Dev. Biol.* **17**, 615–675
- 37 Cantley, L. C. and Neel, B. G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 4240–4245
- 38 Maehama, T., Taylor, G. S. and Dixon, J. E. (2001) PTEN and myotubularin: novel phosphoinositide phosphatases. *Annu. Rev. Biochem.* **70**, 247–279
- 39 Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S. M., Riggins, G. J. et al. (2004) High frequency of mutations of the PIK3CA gene in human cancers. *Science* **304**, 554
- 40 Broderick, D. K., Di, C., Parrett, T. J., Samuels, Y. R., Cummins, J. M., McLendon, R. E., Fu, D. W., Velculescu, V. E., Bigner, D. D. and Yan, H. (2004) Mutations of PIK3CA in anaplastic oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. *Cancer Res.* **64**, 5048–5050
- 41 Muhlis, J., Schwering, A., Grotzer, M., Vince, G. H., Roggendorf, W., Hagemann, C., Sorensen, N., Rickert, C. H., Osada, N., Jurgens, H. and Fruhwald, M. C. (2006) Epigenetic repression of RASSF1A but not CASP8 in supratentorial PNET (sPNET) and atypical teratoid/rhabdoid tumors (AT/RT) of childhood. *Oncogene* **25**, 1111–1117
- 42 Paddison, P. J., Silva, J. M., Conklin, D. S., Schlachbach, M., Li, M., Aruleba, S., Balijs, V., O'Shaughnessy, A., Gnoj, L., Scobie, K. et al. (2004) A resource for large-scale RNA-interference-based screens in mammals. *Nature* **428**, 427–431
- 43 Biegel, J. A., Fogelgren, B., Zhou, J. Y., James, C. D., Janss, A. J., Allen, J. C., Zagzag, D., Raffel, C. and Rorke, L. B. (2000) Mutations of the IN1 rhabdoid tumor suppressor gene in medulloblastomas and primitive neuroectodermal tumors of the central nervous system. *Clin. Cancer Res.* **6**, 2759–2763
- 44 Ohlsson, H., Karlsson, K. and Edlund, T. (1993) IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO J.* **12**, 4251–4259
- 45 Condliffe, A. M., Davidson, K., Anderson, K. E., Ellson, C. D., Crabbe, T., Okkenhaug, K., Vanhaesebroeck, B., Turner, M., Webb, L., Wymann, M. P. et al. (2005) Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils. *Blood* **106**, 1432–1440
- 46 Jackson, S. P., Schoenwaelder, S. M., Goncalves, I., Nesbitt, W. S., Yap, C. L., Wright, C. E., Kenche, V., Anderson, K. E., Doppeide, S. M., Yuan, Y. et al. (2005) PI 3-kinase p110 β : a new target for antithrombotic therapy. *Nat. Med.* **11**, 507–514
- 47 Sadhu, C., Masinovsky, B., Dick, K., Sowell, C. G. and Staunton, D. E. (2003) Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J. Immunol.* **170**, 2647–2654
- 48 Ogino, S., Cohen, M. L. and Abdul-Karim, F. W. (1999) Atypical teratoid/rhabdoid tumor of the CNS: cytopathology and immunohistochemistry of insulin-like growth factor-II, insulin-like growth factor receptor type 1, cathepsin D, and Ki-67. *Mod. Pathol.* **12**, 379–385
- 49 Kuwahara, Y., Hosoi, H., Ozone, S., Kita, M., Iehara, T., Kuroda, H. and Sugimoto, T. (2004) Antitumor activity of gefitinib in malignant rhabdoid tumor cells *in vitro* and *in vivo*. *Clin. Cancer Res.* **10**, 5940–5948
- 50 Nocentini, S. (2003) Apoptotic response of malignant rhabdoid tumor cells. *Cancer Cell Int.* **3**, 11
- 51 Aquila, S., Gentile, M., Middea, E., Catalano, S. and Ando, S. (2005) Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* **146**, 552–557
- 52 Kern, M., Wells, J. A., Stephens, J. M., Elton, C. W., Friedman, J. E., Tapscott, E. B., Pekala, P. H. and Dohm, G. L. (1990) Insulin responsiveness in skeletal muscle is determined by glucose transporter (Glut4) protein level. *Biochem. J.* **270**, 397–400
- 53 Desbois, C., Capeau, J., Hainault, I., Wicke, D., Reynet, C., Veissiere, D., Caron, M., Picard, J., Guerre-Millo, M. and Cherqui, G. (1992) Differential role of insulin receptor autophosphorylation sites 1162 and 1163 in the long-term insulin stimulation of glucose transport, glycogenesis, and protein synthesis. *J. Biol. Chem.* **267**, 13488–13497
- 54 Wong, E. H., Tan, C. H., Khoo, H. E., Ng, F. H., Lim, K. L. and Ciaraldi, T. P. (1995) Rat fibroblast cells overexpressing kinase-inactive human insulin receptors are insulin responsive: influence of growth conditions. *Endocrinology* **136**, 1459–1467
- 55 Guerreiro, A. S., Boller, D., Shalaby, T., Grotzer, M. A. and Arcaro, A. (2006) Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor receptor inhibition. *Int. J. Cancer* **119**, 2527–2538
- 56 Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I. D., Belfiore, A. and Vigneri, R. (1999) Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol. Cell. Biol.* **19**, 3278–3288
- 57 Sciacca, L., Costantino, A., Pandini, G., Mineo, R., Frasca, F., Scalia, P., Sbraccia, P., Goldfine, I. D., Vigneri, R. and Belfiore, A. (1999) Insulin receptor activation by IGF-II in breast cancers: evidence for a new autocrine/paracrine mechanism. *Oncogene* **18**, 2471–2479
- 58 Morriene, A., Valentini, B., Xu, S. Q., Yumet, G., Louvi, A., Efstratiadis, A. and Baserga, R. (1997) Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3777–3782
- 59 Sjoblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., Mandelker, D., Leary, R. J., Ptak, J., Silliman, N. et al. (2006) The consensus coding sequences of human breast and colorectal cancers. *Science* **314**, 268–274
- 60 Foukas, L. C., Claret, M., Pearce, W., Okkenhaug, K., Meek, S., Peskett, E., Sancho, S., Smith, A. J., Withers, D. J. and Vanhaesebroeck, B. (2006) Critical role for the p110 α phosphoinositide 3-OH kinase in growth and metabolic regulation. *Nature* **441**, 366–370
- 61 Knight, Z. A., Gonzalez, B., Feldman, M. E., Zunder, E. R., Goldenberg, D. D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B. et al. (2006) A pharmacological map of the PI3-K family defines a role for p110 α in insulin signaling. *Cell* **125**, 733–747
- 62 Plum, L., Belgardt, B. F. and Bruning, J. C. (2006) Central insulin action in energy and glucose homeostasis. *J. Clin. Invest.* **116**, 1761–1766

Received 2 March 2007/8 May 2007; accepted 16 May 2007

Published as BJ Immediate Publication 16 May 2007, doi:10.1042/BJ20070309