Loss of WNK2 expression by promoter gene methylation occurs in adult gliomas and triggers Rac1-mediated tumour cell invasiveness

Sónia Moniz1,2,†, Olga Martinho4,5,6,†, Filipe Pinto4,5, Bárbara Sousa7, Cláudia Loureiro1,2, Maria José Oliveira8, Luis Ferreira Moita3, Minalini Honavar9, Célia Pinheiro10, Manuel Pires10, José Manuel Lopes7, Chris Jones11, Joseph F. Costello12, Joana Paredes7, Rui Manuel Reis4,5,6,∗,† and Peter Jordan1,2,∗,†

1Department of Human Genetics, Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisbon, Portugal, 2BioFig – Centre for Biodiversity, Functional and Integrative Genomics and 3Institute of Molecular Medicine, University of Lisbon, Lisbon, Portugal, 4Life and Health Sciences Research Institute (ICVS), Health Sciences School, University of Minho, Braga, Portugal, 5ICVS/3B’s – PT Government Associate Laboratory, Braga/Guimarães, Portugal, 6Molecular Oncology Research Center, Barretos Cancer Hospital, S. Paulo, Brazil, 7Cancer Genetics, IPATIMUP, Porto, Portugal, 8Institute for Biomedical Engineering (INEB), University of Porto, Porto, Portugal, 9Hospital Pedro Hispano, Matosinhos, Portugal, 10Hospital Santo António, Porto, Portugal, 11Paediatric Oncology, The Institute of Cancer Research, Sutton, Surrey, UK and 12Department of Neurological Surgery and Brain Tumor Research Center, University of California, San Francisco, CA, USA

Received July 11, 2012; Revised and Accepted September 21, 2012

The gene encoding protein kinase WNK2 was recently identified to be silenced by promoter hypermethylation in gliomas and meningiomas, suggesting a tumour-suppressor role in these brain tumours. Following experimental depletion in cell lines, WNK2 was further found to control GTP-loading of Rac1, a signalling guanosine triphosphatase involved in cell migration and motility. Here we show that WNK2 promoter methylation also occurs in 17.5% (29 out of 166) of adult gliomas, whereas it is infrequent in its paediatric forms (1.6%; 1 out of 66). Re-expression of WNK2 in glioblastoma cells presenting WNK2 gene silencing reduced cell proliferation in vitro, tumour growth in vivo and also cell migration and invasion, an effect correlated with reduced activation of Rac1. In contrast, when endogenous WNK2 was depleted from glioblastoma cells with unmethylated WNK2 promoter, changes in cell morphology, an increase in invasion and activation of Rac1 were observed. Together, these results validate the WNK2 gene as a recurrent target for epigenetic silencing in glia-derived brain tumours and provide first mechanistic evidence for a tumour-suppressing role of WNK2 that is related to Rac1 signalling and tumour cell invasion and proliferation.

INTRODUCTION

Gliomas are the most common primary adult and paediatric brain tumours, and are composed by distinct histological subtypes and World Health Organization (WHO) malignancy grades (1,2). Astrocytic tumours are the most frequent histological subtype and can be divided into pilocytic astrocytomas (WHO grade I), diffuse astrocytomas (WHO grade II), anaplastic astrocytomas (WHO grade III) and glioblastomas (WHO grade IV) (1). Oligodendrogliomas are the second most frequent subtype, and can be subdivided into low-grade oligodendrogliomas (WHO grade II) and anaplastic...
oligodendrogliomas (WHO grade III). In adults, glioblastoma (WHO grade IV) is not only the most aggressive type, with a mean survival of ~16 months, but also the most frequent, accounting for ~50% of all gliomas and ~15% of all primary brain tumours (1). In contrast to adults, in children, the most common form is pilocytic astrocytomas. Despite the rarity of malignant gliomas in the paediatric context, they are one of the leading causes of cancer-related deaths in children. A growing body of evidence demonstrates that paediatric malignant gliomas not only exhibit distinct clinicopathological features but also harbour a different genetic profile (3). One of the major causes of the high mortality of glioblastoma is their infiltrative and invasive property that hampers complete surgical excision, with consequent tumour regrowth and recurrence (2,4).

Cell invasion is known to be regulated by Rho family guanosine triphosphatases (GTPases), which control the dynamics of the actin cytoskeleton (5). Meanwhile, two different types of tumour cell motility have been identified in 3D matrices (6,7). A RhoA-dependent bleb-associated motility (amoeboid migration mode) exists that does not require pericellular proteolysis and generates hydrostatic pressure, which squeezes tumour cells through pre-existing gaps in the matrix. In contrast, an elongated mechanism of cell motility (mesenchymal migration mode) is associated with Rac1-dependent polymerization of actin filaments and induces branching of existing actin filaments that results in cell-surface structures, known as lamellipodia or membrane ruffles.

WNK2 is a member of the WNK (with-no-lysine [K]) subfamily of protein kinases (8,9), which is predominantly expressed in the brain, heart muscle, small intestine and colon (10). Functional analysis has revealed that reducing WNK2 expression by RNA interference in epithelial cell lines resulted in increased GTP-loading of Rac1 and concomitant stimulation of the Rac1-effector kinase PAK1 (p21-activated kinase). This effect activates a signalling cross-talk, in which PAK1 phosphorylates MAPK/extracellular signal-regulated kinase (ERK) 1 (MEK1) at serine 298, thereby increasing the efficiency with which MEK1 activates ERK1/2 upon growth factor stimulation (11).

Recently, WNK2 has been proposed to act as a specific tumour-suppressor gene for brain tumours. First, an unbiased genome-wide approach to mapping non-random and tumour type-specific epigenetic gene silencing identified the WNK2 gene in 29 of 31 infiltrative gliomas (12), the underlying mechanism being promoter methylation. Second, WNK2 was reported to be aberrantly methylated in 83 and 71% of grade II and III meningiomas, respectively, but rarely in a total of 209 tumours from 13 other tumour types (13).

In this study, we detected WNK2 silencing in ~17.5% of adult gliomas and describe that experimental manipulation of WNK2 expression levels affected tumorigenic growth properties of glioblastoma cell lines and conferred increased invasiveness in a Rac1-dependent manner.

RESULTS

Methylation of the human WNK2 gene promoter in glioblastoma

Based on previous reports showing that the WNK2 gene is a recurrent target for epigenetic silencing in oligodendrogliomas and meningiomas (12,13), we developed a methylation-specific polymerase chain reaction (PCR) (MSP) to test for the prevalence of WNK2 promoter methylation in adult and paediatric malignant glioma cases. Among 61 childhood high-grade gliomas, only one case (1.6%) was identified with WNK2 promoter methylation, corresponding to an 18-year-old patient. In contrast, we found that 29 out of 166 adult glioma samples (17.5%) showed WNK2 methylation (Fig. 1A). The majority of samples were glioblastoma ($n = 115$; 18.3% methylated). In order to further confirm the MSP results in six adult glioblastoma cases (three methylated and three unmethylated), we performed bisulphite sequencing.
of their WNK2 promoter region and were able to fully validate the observed methylation status at nucleotide-level resolution (Fig. 1B and Supplementary Material, Fig. S1).

No significant association was observed between WNK2 promoter methylation and clinicopathological data such as age, gender, cellular lineage, histological subtype, malignancy grade or patient survival (Supplementary Material, Table S1). Additionally, we made use of a glioblastoma data set from The Cancer Genome Atlas (TCGA) containing data for both methylation and mRNA expression of WNK2. A significant correlation between the downregulation of WNK2 expression and the presence of promoter methylation was found; however, similar to our data, there was no correlation between WNK2 methylation and clinicopathological data (Supplementary Material, Table S2). Interestingly, when only mRNA expression was considered, a significant correlation was found between WNK2 downregulation and higher age or lower survival time (Supplementary Material, Table S2 and Fig. S2).

These results suggested that other mechanisms may exist besides the described promoter methylation, which lead to the downregulation of WNK2 expression. In fact, a genome-wide sequencing of 22 glioblastoma identified one case with the missense mutation A1267T (14). In order to test the hypothesis that this mutation could be an alternative genetic event, we determined the frequency of this mutation in 48 primary glioblastomas (12 methylated and 36 unmethylated). None of the 48 glioblastomas exhibited the A1267T WNK2 mutation (data not shown).

In order to select appropriate models to assess the cellular effects of WNK2 silencing, eight glioblastoma cell lines (SNB-19, U87-MG, U251, U373, SW1783, SW1088, A172, GAMG) and a cell line derived from normal human astrocytes (NHA) were also characterized by MSP for their WNK2 promoter methylation status. Promoter methylation was observed in cell lines SNB19, U87-MG, U251, U373 and A172 (Fig. 1C), the latter presenting the strongest methylation signal, confirming previously described data (12).

WNK2 expression in different glioblastoma cell lines

Next, we characterized the above-mentioned cell lines for their expression levels of WNK2 transcript (Fig. 2A) and protein (Fig. 2B). In general, we found that WNK2 expression was absent or low in cell lines with promoter methylation, including A172 cells in which we showed previously that WNK2 expression could be reactivated after treatment with demethylation drugs (12). In contrast, WNK2 expression was clearly detectable in all non-methylated cell lines, among which SW1088 cells expressed the highest levels of WNK2 transcript and protein. We thus selected two cell lines as models for the subsequent experimental manipulation of WNK2 expression levels: SW1088 cells that express endogenous WNK2 from an unmethylated promoter and A172 cells that express no WNK2 due to promoter methylation.

Depletion of endogenous WNK2 increases growth and invasion of SW1088 cells

Expression of endogenous WNK2 in SW1088 cells was inhibited by RNA interference. First, stable cell lines expressing either a control short hairpin RNA (shRNA) or one of two distinct WNK2-specific shRNAs (shW2.2 and shW2.3) were selected (Fig. 3A). Clones shW2.2 and shW2.3 were selected, which revealed distinct degrees of WNK2 depletion. In addition, transient transfection of parental SW1088 cells with small interfering RNAs (siRNAs) allowed downregulation of WNK2 expression in ~50% of the cells (Fig. 3B).

The soft agar colony formation assay indicates anchorage-independent growth and demonstrated a significant (P < 0.05) increase in the number of the colonies formed by the stable shW2-transfected cells (Fig. 4A).

During the selection of the shW2 clones, we also observed differences in morphology and actin cytoskeleton organization (Fig. 4B). Unlike parental SW1088 and shCtrl cells, shWNK2.2 cells and especially shWNK2.3 cells showed a more flat spread-out appearance and displayed pronounced cytoplasmic protrusions consistent with lamellipodia formation. Immunofluorescence staining for actin and endogenous Rac1 showed increased accumulation at the plasma membrane (Fig. 4C).

These observations prompted us to analyse cell migration properties in wound-healing assays. First, parental SW1088 cells were transiently transfected with control or siWNK2 oligonucleotides. Approximately 48 h after siRNA transfection, scratch wounds were made across the cell monolayer and monitored by time-lapse photography for 24 h (Fig. 5A). Under these conditions, the depletion of WNK2 reached ~50% (Fig. 3B), and an increase in wound closure was observed.

Cell invasion and migration are known to be regulated by Rho family GTPases, which control the dynamics of the actin cytoskeleton (5), and our previous functional analysis of WNK2 in epithelial cells has demonstrated that depletion of WNK2 expression resulted in increased GTP-loading...
molecule of Rac1 and concomitant reduction in active RhoA (11). We thus confirmed the occurrence of an increase in Rac1 activity and a concomitant reduction in RhoA active levels in siWNK2-transfected SW1088 cells (Fig. 5B and C). These data indicated increased cell migration capacity upon the downregulation of WNK2 expression.

In order to study the motile and invasive cell properties in a more homogenous cell population, the stable shW2 clones were first investigated by in vivo time-lapse microscopy. Migration experiments confirmed that the shW2.2 and shW2.3 clones exhibited increased ability in closing scratch wounds (Fig. 6A and Supplementary Material, Movies M1 and M2). The in vivo live-cell observations further revealed that shWNK2.2 and shWNK2.3 cells presented higher cell motility and formed larger lamellipodia protrusions (Fig. 6B and Supplementary Material, Movies M3 and M4).

Based on these findings, the clones were analysed in Matrigel invasion assays. An increase in the number of cells that passed the membrane was observed comparing control with shW2.2 and shW2.3 cells (Fig. 6C), and this increase was proportional to the levels of active Rac1 measured in these cells (Fig. 6D). Thus, clones expressing lower WNK2 levels showed higher invasiveness compared with parental and shCtrl SW1088 cells.

Together, these data demonstrate that the downregulation of WNK2 expression in glioblastoma cells increased their cell migration and invasion capacity with a concomitant increase in Rac1 activity.

**Re-expression of ectopic WNK2 inhibits growth and migration of A172 cells**

Considering that our data have so far supported a role for WNK2 as a tumour-suppressor gene in gliomas, we wondered whether restoration of WNK2 expression would inhibit the major cell properties described above. Promoter-methylated A172 cells that lost endogenous WNK2 expression were therefore transfected with a WNK2 cDNA construct. Owing to the inefficiency of transient plasmid transfection into A172 cells, we established pools of stable transfected cells expressing either the WNK2 coding sequence (A172-HW2) or the corresponding empty vector (A172-HEv), and documented the corresponding WNK2 transcript and protein levels (Fig. 7A and 7B). These pools were analysed for their growth and migration properties.

The soft agar colony formation assay demonstrated a significant (P < 0.05) decrease in colony numbers formed by A172HW2 cells, in comparison with A172HEv (Fig. 7C).

To examine the effect of WNK2 re-expression on tumour growth in vivo, we performed the chick embryo chorioallantoic membrane (CAM) assay (Fig. 7D). The stable A172 cell pools were implanted into the embryo CAM in ovo (A172HEv cells, n = 10; A172HW2 cells, n = 15), and 7 days later the chicken embryos were sacrificed to evaluate tumour growth ex ovo, as described (15). The mean perimeter values of the tumours formed by the control A172HEv and the A172HW2 cells were 15405.82 ± 3853.42 µm and 5502.97 ± 2804.83 µm, respectively, being the difference statistically significant (P < 0.05) (Fig. 7E). The CAM assay further permits to evaluate angiogenesis modulation. When the number of vessels around the tumours was counted, no statistically significant difference was found between A172HEv and A172HW2 cells (73 ± 10 and 66 ± 17 vessels, respectively) (Fig. 7E).

Furthermore, control A172HEv cells were compared with the stable A172-HW2 cells in wound closure assays. A clear delay in wound closure was observed for A172-HW2 cells due to re-expression of WNK2 (Fig. 8A). Because our previous data found that the loss of WNK2 expression promoted the activation of the small GTPase Rac1, we compared these cells in CRIB (Cdc42/Rac1 interactive binding)-domain pull-down assays. We found lower Rac1 activity in A172HW2 cells than in A172HEv or parental cells (Fig. 8B). In addition, treatment of A172HEv cells with the Rac1 inhibitor NSC23766 also delayed wound closure (data not shown). Together, these data are consistent with a decreased cell growth and migration capacity upon re-expression of WNK2 in promoter-methylated glioblastoma cells, indicating it as a therapeutic target.

**DISCUSSION**

The main novel findings described in this article are that the loss of WNK2 expression (i) occurs in a subset of 17.5% of adult but not paediatric malignant gliomas, and (ii) leads to increased Rac1 activity and invasion of glioblastoma cells.

Initially, we started by confirming whether epigenetic silencing of the WNK2 gene through promoter methylation was a
specific target in malignant gliomas. First, our results showed that methylation occurred less frequently in adult gliomas (17.5%; \( n = 166 \)) than in previously described limited series of mainly oligodendroglomas (94%; \( n = 31 \)) (12) or in meningiomas (83% in grade II, \( n = 6 \); 71% in grade III, \( n = 7 \)) (13). These differences may in part be overestimated due to the varying sample sizes analysed in the three studies. Nevertheless, our results clearly confirm that \( WNK2 \) gene silencing occurs in gliomas and support the hypothesis of a brain-specific tumour-suppressor gene function (13,16); however, it can formally not be excluded that these \( WNK2 \)-silenced tumours arose from a glia stem cell type in which the \( WNK2 \) gene is normally methylated.

Second, we found that paediatric malignant gliomas did not share \( WNK2 \) promoter methylation as a common epigenetic event. This underlines our and other authors’ reports demonstrating that adult and paediatric gliomas are genetically distinct entities (17–19).

Third, we analysed a presumably alternative \( WNK2 \)-compromising mutation, the p.A1267T point mutation previously identified by whole-genome sequencing in one glioblastoma sample (14). In 48 of the glioblastoma samples, this mutation could not be detected, indicating that it does not significantly contribute to \( WNK2 \) dysfunction, similar to what has been reported in oligodendroglioma samples (12).

Subsequently, we tested the effect of experimental reduction of \( WNK2 \) expression by siRNAs in SW1088 cells, which have no promoter methylation and thus express endogenous \( WNK2 \). We found that anchorage-independent cell growth increased under conditions of reduced \( WNK2 \) expression and that changes in cell morphology occurred, including increased cell spreading and formation of lamellipodia. Consistently, an increase in Rac1 activation was detected in \( WNK2 \)-depleted cells, in agreement with the changes in activity that we previously described to occur in HeLa cells (11).

Several recent studies have suggested a predominant role for Rac1 in glioma cell motility and invasiveness, based on the identification of changes in expression or activity of either Rac1 (20–29), or individual Rac-specific guanine nucleotide exchange factors (GEFs) (30–33), or Rac effector proteins (34,35). It is well known that Rac1 drives a mesenchymal type of cell migration, suggesting that this type allows the infiltration of glia-derived tumour cells within the neuronal matrix of the brain (29). Consistent with this role of Rac1, experimental \( WNK2 \) suppression in SW1088 cells
led to increased cell migration in wound-healing assays and increased invasion in Matrigel assays. Our findings indicate that distinct mechanisms can contribute to the activation of Rac1 during glioma progression, including the loss of WNK2 expression described in this article. Our previous analysis of WNK2 indicated that its direct target could be a Rho-GEF so that the loss of WNK2 leads to a decrease in RhoA and a reciprocal increase in Rac1 activity (11).

Finally, the tumour-suppressing role of WNK2 in gliomas implies that its re-expression should be able to revert the aggressive phenotype of tumour cells. We clearly found that restoration of WNK2 in the promoter-methylated cell line A172 inhibited colony formation in soft agar and the cell migration capacity, including the endogenous levels of GTP-bound active Rac1. Moreover, we found that tumour growth was reduced using the *in vivo* CAM assay.

Together our data support an important role for WNK2 in the control of glia-derived cell migration and tumorigenic growth and provide mechanistic insights into the pathways involved.

**MATERIALS AND METHODS**

**Patient samples and cell lines**

Representative formalin-fixed paraffin-embedded blocks from 227 glioma tissues (166 were from adult and 61 were from paediatric patients) were retrospectively retrieved from pathology archives of the Department of Pathology at three hospitals in northern Portugal—Hospital São João, Porto, Hospital Santo António, Porto, and Hospital Pedro Hispano, Matosinhos, and from the UK at the King’s College Hospital, London, and St George’s Hospital, London (Supplementary Material, Table S1). The cohort includes astrocytic and oligodendrogial tumours, of diverse malignant grades including 115 adult glioblastoma, which were all classified according to the WHO criteria (1). Follow-up data were available in 133 adult patients (range: 0–64 months, mean: 12.8 ± 1.0 months) and only in 18 paediatric patients (range: 0–80 months, mean: 21.7 ± 5.2 months). The procedures for this study were approved by local and multicenter Ethical Review Committees, and, in accordance with institutional ethical standards, the biological samples were unlinked and unidentified from their donors.

Eight glioblastoma cell lines were used in this study and include SW1088, SW1783, U87-MG and A172 (obtained from ATCC—American Type Culture Collection), SNB-19 and GAMG (obtained from DSMZ—German Collection of Microorganisms and Cell Cultures) and U251 and U373.

**Figure 5.** Effect of transient WNK2 depletion in SW1088 cells. (A) Cells were transiently transfected with control (siCtrl) or WNK2-specific (siWNK2) siRNAs under the conditions documented in Figure 3 and analysed. (A) Representative images from wound-healing migration assays, in which scratch wounds were made with a pipette tip across a confluent cell monolayer, and distances between the wound edges measured after 8, 12 and 24 h. In the graph below, the migration distances determined in seven independent assays are expressed as fold differences compared with parental cells and show the distances migrated in relation to time 0. (B) Activation levels of endogenous Rac1 as determined under the same transfection conditions by CRIB-domain pull-down assays and analysed by western blot comparing total and GTP-loaded Rac1 fractions (*n* = 7). (C) Control or WNK2-depleted SW1088 cells were lysed and Rho A activation levels determined in a G-Lisa assay (*n* = 4). Note the increase in cell migration and Rac1 activation accompanied by a decrease in active RhoA following transient WNK2 depletion in parental SW1088 cells. Statistically significant differences are indicated as *P* < 0.05 or as *P* < 0.005.

Human Molecular Genetics, 2013, Vol. 22, No. 1
Their mutation profile of major cancer-associated genes is shown in Supplementary Material, Table S3. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) medium as previously described (15) and regularly checked for the absence of mycoplasm infection. Authentication of cell lines was performed by IdentiCell Laboratories [Department of Molecular Medicine (MOMA) at Aarhus University Hospital Skejby in Århus, Denmark] in August 2011. Genotyping confirmed the complete identity of all cell lines, with the exception of U373, which was shown to be a subclone of U251 cell line.

**MSP, WNK2 promoter sequencing and mutation analysis**

For the isolation of genomic DNA, selected areas from the formalin-fixed paraffin-embedded blocks containing at least 85% of tumour tissue were macro-dissected into a microfuge tube using a sterile needle (Neolus, 25G, 0.5 mm) and DNA isolated using QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany), as previously described (37). DNA from the cell lines was isolated using TRIzol® Reagent (Invitrogen S.A., Barcelona, Spain) as recommended by the manufacturer.

For MSP, 500 ng of DNA were bisulphite-treated using EZ DNA Methylation Golf Kit (Zymo Research Corporation, Irvine, CA, USA), as previously described (37). Specific primers to distinguish methylated (131 bp PCR product 1, Fw: 5′-CGTTCGTTTTGTGAGTGTC; Rv: 5′-ACGACGACTCCACAAAAA) from unmethylated DNA (131 bp PCR product 2; Fw: 5′-GTTTGTTTGTTTTGTGAGTGTT and Rv: 5′-CCAACAACAACTCCACAAAAA) were designed using the Methyl Primer Express Software v1.0. CpGenome Universal Methylated DNA (Chemicon Millipore, Billerica, MA, USA) was used as methylated control and blood DNA of a young healthy individual was used as unmethylated control. Bisulphite sequencing of the WNK2 promoter covered 73 contiguous CpGs in the WNK2 CpG island and was analysed by sequencing 10 subclones of PCR products from bisulphite-treated DNA, as previously described (12,13).

The prevalence of the WNK2-A1267T missense mutation was determined following PCR amplification of exon 16 with primers WNK2-A1267T-F (5′-CGA GCA GAT GAA GGA TGT CA) and WNK2-A1267T-R (5′-GAA TGA GGT GGA GGG TCA GA) and direct sequencing of the obtained product.

**Cell culture, transfections and RNA interference**

A172 and SW1088 cell lines were maintained in DMEM, supplemented with 10% fetal bovine serum (Invitrogen) and
regularly checked for the absence of mycoplasm infection. Cells were transfected using a reverse transfection protocol in which $1 \times 10^6$ cells were trypsinized and seeded together with the premixed plasmid DNA/LipofectAMINE 2000 (Invitrogen) complex, according to the manufacturer’s instructions. Cells were analysed after 22 h, and transfection efficiencies found to be $\approx 40\%$. Total amounts of transfected plasmid DNA were kept constant at 4 mg per 60 mm dish or at 2 mg per 35 mm dish and adjusted with empty vector if required. siRNAs were obtained from Eurofins MWG-Biotech AG (Ebersberg, Germany) and reverse-transfected using $5 \times 10^5$ trypsinized cells and siRNA/LipofectAMINE 2000 complexes containing 300 pmol of siRNA per 35 mm dish or 600 pmol per 60 mm dish. siRNA sequences were siWNK2-a (5'-GCU CGA GGA UGC UGA CAU ATT), siWNK2-b (5'-GGA CGC ACC CGA UGA AAU UTT) and as control siGFP (5'-GGC UAC GUC CAG GAG CGC ACC TT). Cells transfected with siRNAs were analysed after 48 h, and transfection efficiencies found to be $\approx 60\%$ using an FITC-coupled siRNA (Qiagen). The achieved reduction in target gene expression was determined in each experiment by removing a 30 µl of aliquot from the cell lysate for the extraction of total RNA, as described below.

For the selection of stable cell lines with reconstituted WNK2 expression in A172 cells, the WNK2 cDNA was subcloned as an EcoRI/EcoRV fragment into pcDNA3-Hygro (Invitrogen). After cell transfection (see above), stable cell pools were obtained following treatment with 200 µg/ml of Hygromycin B (Sigma-Aldrich). A control stable cell pool transfected with the corresponding empty vector was also selected. For the selection of stable SW1088 cell lines with depleted endogenous WNK2 expression, shRNA encoding plasmids [part of the LKO.1 shRNA constructs obtained from the RNAi Consortium (TRC) (Broad Institute, Cambridge, MA, USA)] containing either a non-specific shRNA sequence (shCtrl) or two previously validated shRNAs sequences targeting WNK2 were transfected. Cell clones were obtained following the treatment of transfected cells with 2.5 µg/ml of puromycin (Sigma-Aldrich).
Soft agar colony formation assay

For soft agar colony formation, 1 ml underlayer (base agar layers) consisting of 0.6% agar medium was prepared in six-well plates by combining equal volumes of 1.2% Noble agar with 2× DMEM medium containing 20% FBS. Cells were trypsinized, centrifuged and resuspended in 0.35% agar medium (top agar layer; equal volumes of 0.7% Noble agar and 2× DMEM with 20% FBS) before 5×10³ cells were plated onto the previously prepared base agar layers. The cells were incubated at 37°C in a humidified 5% CO₂ atmosphere for 3 weeks and the colonies formed stained with 0.05% violet crystal for 15 min. Stained colonies were photographed in a stereomicroscope (Olympus Z2×16) using a digital camera (Olympus DP71) (Olympus, Hamburg, Germany) and counted with the Image J software. Results are expressed as the mean number of colonies per field. The assay was done in triplicate and repeated at least three times.

Cell lysis and immunoprecipitation, Rac1-CRIB pull-down or Rho G-Lisa assays

Cells in 60 mm dishes were lysed on ice in 250 μl of lysis buffer (20 mM Tris–HCl, pH 7.5, 1% NP-40, 130 mM NaCl, 10% glycerol, 10 mM MgCl₂ containing 10 mM NaF, 0.1 mM Na₃VO₄, 1 mM DTT and a protease inhibitor cocktail composed of 1 mM PMSF, 1 mM 1,10-phenanthroline, 1 mM EGTA, 10 mM E64 and 10 μg/ml of each aprotinin, leupeptin and pepstatin A (all from Sigma-Aldrich). For RNA extraction, a 30 μl of aliquot was removed and processed as described below. For protein analysis, a 40 μl of aliquot was added to 10 μl of 5×Laemmli sample buffer, boiled for 10 min, centrifuged at 2500g for 30 s and analysed as given below. To document WNK2 protein levels in A172HW2 cells, a confluent 100 mm dish was lysed and immunoprecipitated with 15 μg of a rabbit polyclonal WNK2 antibody, SPT81 (10). For comparison, an equivalent immunoprecipitation was performed in SW1088 and in colorectal HT29 cell lysates as a previously described positive control (11). The Rac1 CRIB-domain pull-down assay and the activation assays for RhoA were performed as described (11,38), the latter using the G-Lisa RhoA luminescence-based Biochem kit (Cytoskeleton, Denver, CO, USA).

Transcript expression analysis and semiquantitative PCR

Total RNA was extracted from cell lines or lysates with the RNAeasy kit (Qiagen) and 1 μg reverse-transcribed using random primers (Invitrogen) and Ready-to-Go You-Prime beads (GE Healthcare, Buckinghamshire, UK). Primers and reaction conditions for specific amplification of WNK2 and for RNA polymerase II (Pol II) were as described (11). These semi-quantitative amplification conditions were experimentally controlled by co-amplification of serial dilutions of a cDNA sample. Products were separated on 1.5% agarose gels and band intensities quantified using the Image J software followed by normalization to Pol II. No amplification was obtained when RNA was mock-transcribed without adding reverse transcriptase.

Antibodies and western blot procedures

Protein samples were separated in a 10% SDS–PAGE Mini-Protean III gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred onto a PVDF membrane (Bio-Rad) using...
a Mini Trans-Blot cell (Bio-Rad) at 100 v for 60 min and Coomassie-stained to check for equal transfer. Membranes were blocked in TBS, 0.1% Triton X-100, 5% milk powder, probed using the indicated antibodies, then incubated with a secondary peroxidase-conjugated antibody (Bio-Rad) and specific binding detected in a chemiluminescence reaction. For densitometric estimation of protein quantities, the luminescence film exposures from at least three independent experiments were digitalized and analysed using the ImageJ software (NIH). The antibodies used were anti-α-tubulin (clone B5-1-2) from Sigma-Aldrich, monoclonal anti-Rac1 (clone 23A8) from Upstate Millipore, Billerica, MA, USA, and rabbit anti-WNK2 (10).

**Immunofluorescence microscopy**

Parental and selected shRNA-expressing SW1088 cells were grown on cover slips and fixed with 3.7% paraformaldehyde in PBS followed by permeabilization with 0.1% Triton X-100 in PBS. Cells were washed 3 × 5 min in PBS/0.01% Tween 20 (PBS-T), then labelled for 1 h with primary anti-Rac1 antibody, washed 3 × 5 min in PBS-T and incubated again for 1 h with a mixture of secondary anti-mouse Alexa-488 antibody (Invitrogen-Molecular Probes) and 0.2 µg/ml phalloidin-TRITC (Sigma-Aldrich). Cover slips were washed again 3 × 5 min in PBS-T, post-fixed for 15 min in 4% (v/v) formaldehyde and mounted on microscope slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Digital images were recorded on a Leica TCS SPE (Leica Microsystems, Wetzlar, Germany) confocal microscope and processed with the Adobe Photoshop software.

**Cell migration and invasion assays**

For wound-healing assays, either transfected cells or stable cell lines were allowed to grow to a confluent monolayer in 6-well or 12-well plates before a wound was carefully made with a pipette tip so that the neighbouring cells were only minimally disturbed. The medium was replaced by fresh complete medium and wound closure monitored by time-lapse photography for 12 h, using a phase contrast microscope and a 10× magnification. Migration was measured on digital images by determining the mean remaining distance between the wound edges. In some experiments, cells were treated with 200 µM of the inhibitor NSC23766 (Calbiochem) for 16 h prior to wounding.

Matrigel invasion assays were performed using 8 µm-pore size BD BioCoat™ Matrigel Invasion Chambers (BD Biosciences, San Jose, CA, USA). The upper compartment of the chamber received 2.5 × 10^5 cells, whereas the lower compartment contained only fresh medium supplemented with 10% FBS. After 24 h incubation at 37°C, the upper surface of the filter was cleared from residual cells with a cotton swab, the filter washed with PBS, then fixed with cold methanol and invasive cells attached to the lower filter surface stained with DAPI. Images were recorded on a Leica SPE confocal microscope at 10× magnification and invasive cells counted.

**Time-lapse microscopy for cell motility and wound-healing**

For motility and migration assays of shRNA-transfected clones, cells were either seeded in 6-well or 12-well dishes and left to adhere for 24 h at 37°C in a temperature- and 5% CO₂-controlled micro-chamber module, as described (39). For each time-lapse experiment, bright-field images were acquired in intervals of 5 min during 12 h using a 20× objective of an Axiovert 200M microscope and the AxioVision software (Carl Zeiss, Jena, Germany). For the wound-healing assays, distance measurements were done between the wound edges at several time points. For the cell motility assay, the positions of 80–95 individual cells seeded at low density were marked (based on the centre of their nuclei) for each experimental condition and followed in the sequential image series. The moved distances were determined manually and expressed as cell speed (µm/h).

**Chick CAM assay**

To assess in vivo tumour proliferation and angiogenesis, we used the CAM assay as previously described (15,40). Briefly, fertilized chicken eggs were incubated at 37°C and 70% humidity, and on day 4 of development, a window was made into the shell, which was sealed with tape, and the eggs were returned to the incubator. On day 9 of development, small plastic rings were placed on the CAM, and on day 10 of development, 3 × 10^5 cells, resuspended in 20 µl of DMEM medium, were injected in the rings over the CAM. On day 17 of development, the tumour formed was photographed in ovo using a stereomicroscope (Olympus S2 ×16). The chicken embryos were sacrificed at −80°C for 10 min, and the CAM and tumours were fixed with formaldehyde at 4% and photographed ex ovo. The perimeter of the tumours was measured using the Cell B software (Olympus), and blood vessels were manually counted.

**Statistical analysis**

Correlations between WNK2 methylation, expression and clinical data of the patients were performed using the chi-square test. Cumulative survival probabilities were calculated using the Kaplan–Meier method. Differences between survival rates were tested using the log-rank test. The statistical analysis was performed using the SPSS software for Windows, version 17.0. For in vitro assays, simple comparison between two different conditions were analysed using Student’s t-test. The level of significance in the statistical analyses is indicated as *P < 0.05 or as **P < 0.005.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**Conflict of Interest statement.** None declared.

**FUNDING**

This work was supported by the Portuguese Fundação para a Ciência e Tecnologia (grant PTDC/SAU-OBD/100079/2008

REFERENCES


