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Functional identification of a novel transcript variant of INPP4B in human colon and breast cancer cells

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ABSTRACT

The 4-phosphatase Inositol polyphosphate 4-phosphatase II (INPP4B) is a regulator of the PI3K signalling pathway and functions to suppress or promote activation of downstream kinases depending on cell type and context. Here we report the identification of a novel small transcript variant of INPP4B (INPP4B-S) that has a role in promoting proliferation of colon and breast cancer cells. INPP4B-S differed from full length INPP4B (INPP4B-FL) by the insertion of a small exon between exons 15 and 16 and the deletion of exons 20–24. Nevertheless, INPP4B-S retained all the functional domains of INPP4B-FL and was similarly located to the cytoplasm. Overexpression of INPP4B-S increased, whereas selective knockdown of INPP4B-S reduced the rate of proliferation in HCT116 and MCF-7 cells. These results warrant further investigation of the role INPP4B-S in activation of downstream kinases and in regulation of cancer pathogenesis.

1. Introduction

Aberrant activation of the phosphatidylinositol 3-kinase (PI3K) pathway causes uncontrolled cell proliferation and resistance to apoptosis and plays an important role in the pathogenesis of cancer [1,2]. PI3K signalling is negatively regulated by three classes of inositol polyphosphate phosphatases. The inositol polyphosphate 3-phosphatase PTEN dephosphorylates the 3-position of $PI(3,4,5)P_3$ to generate $PI(4,5)P_2$, whereas 5-phosphatases, such as Src homology 2-containing inositol 5-phosphatase (SHIP) and phosphatidylinositol 4,5-bisphosphate 5-phosphatase (PIB5PA)/proline-rich inositol polyphosphate phosphatase (PIPP) dephosphorylate the 5-position to produce $PI(3,4)P_2$ [3,4]. The latter is in turn subjected to dephosphorylation by inositol polyphosphate 4-phosphatase type I (INPP4A) and type II (INPP4B) at the 4-position to generate PI(3)P, thus terminating PI3K signalling [5,6].

While PTEN is a well-established tumour suppressor and some 5-phosphatases such as SHIP2 and PIB5PA also function as tumour suppressive regulators [4,7,8], the function of INPP4B in cancer is controversial and appears to be dependent on cell type and context. Although INPP4B is tumour suppressive through inhibition of PI3K signalling in a variety of cancers [5,9,10], its oncogenic role has recently been reported in subsets of breast cancers, colon cancers and melanomas through INPP4B-dependent activation of SGK3. Moreover, INPP4B is associated with chemoresistance and poor outcome of patients with acute myeloid leukemia [11,12].

INPP4B was originally isolated from rat brain tissue and its amino acid sequence was found to be highly conserved in its human form (90%) [13]. It is expressed in a range of tissues including heart, skeletal muscle, pancreas, brain, liver, kidney and lung [13]. The INPP4B protein contains a C2-lipid binding domain at the *N*-terminus, a Nervy Homology 2 domain (NHR2) which may facilitate protein-protein interactions, and a dual phosphatase domain at the *C*-terminus [5,14].

There are 3 known transcript variants of INPP4B. Variants 1 and 2 (NCBI Reference Sequences NM_003866.3 and NM_001101669.2, respectively) differ only by an additional exon in the 5′ untranslated region (UTR) of transcript variant 1, with both variants encoding the most characterised INPP4B protein (Isoform 1, NCBI Reference Sequence NP_001095139.1). Variant 3 (NCBI Reference Sequence NM_001331040.1), which encodes the largely uncharacterised isoform 2 (NCBI Reference Sequence NP_001317969.1), possesses a

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different 5′ UTR and an alternate exon at the 3′ end, resulting in a 3′ coding and 3′ UTR sequence distinct from variants 1 and 2. Alternative splicing may give rise to additional transcript variants, in fact a Northern blot analysis revealed at least four transcripts of INPP4B in human tissues [13]. Furthermore, a recent study reported that an uncharacterised smaller isoform of the INPP4B protein was responsible for a tumour suppressive effect in human melanoma cells [15].

Here, we report the identification of a novel small INPP4B transcript variant, which we have termed INPP4B-S in cells of colon and breast epithelium origins. Moreover, we show that INPP4B-S is translated into a protein product that is located to the cytoplasm and promotes proliferation of colon and breast cancer cells.

2. Materials and methods

2.1. Cell culture and human tissues

Human colon cancer cell lines as described previously were provided by Professor Gordon Burns (Faculty of Health and Medicine, University of Newcastle, Australia) [16]. These cells were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, VIC, Australia). Human breast cancer cell lines MCF-7 and MDA-MB-231 were kindly provided by Professors Gordon Burns and Hubert Hondermarck respectively and were cultured in DMEM containing 10% FCS. The normal human colon epithelial cell line FHC (ATCC® CRL-1831™) was purchased from ATCC, and cultured in a 1:1 mixture of Ham's F12 and modified DMEM containing HEPES (25 mM), cholera toxin (10 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (100 ng/ml), 2% penicillin/streptomycin and 10% FCS. Human mammary epithelial (HMEC) cells were also a gift from Professor Hondermarck and were cultured in a 1:1 mixture of Ham's F12 and modified DMEM containing insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), epidermal growth factor (0.1 μg/ml), 2% penicillin/streptomycin and 10% FCS. Cancer cell line authentication was confirmed every 6 months using the AmpFISTR Identifiler PCR Amplification Kit from Applied Biosystems (Mulgrave, VIC, Australia) and GeneMarker V1.91 software (SoftGenetics LLC, State College, PA, USA). Resulting cell line STR profiles were cross-compared, where available, with the ATCC's and German Collection of Microorganisms and Cell cultures' online databases (Braunschweig, Germany). Cell lines were regularly tested for mycoplasma infection using Myco Alert according to the manufacturer's protocol (Lonza, Walkersville, MD, USA).

2.2. Antibodies and reagents

Antibodies against INPP4B, GFP and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat Anti-mouse IgG (H + L) Alexa-Fluor-488 secondary antibody was purchased from Invitrogen.

2.3. Construction of plasmids and stable cell lines

PCR products obtained using full length INPP4B primers (Supplementary Table 1) were cloned into pGEM-T-easy using the manufacturer's instructions (Promega). Sequencing of cloned inserts was carried out by the Australian Genome Research Facility (AGRF). INPP4B-S and INPP4B-FL were subcloned into pLV-eGFP (Addgene plasmid #36083), and packaged into lentiviral particles as described previously [4]. Parental cell lines were transduced with lentiviral particles 3 times to generate stable cell lines containing pLV-eGFP, pLV-eGFP-INPP4B-FL and pLV-eGFP-INPP4B-S. A GFP-positive population of greater than 90% was confirmed by flow cytometry.

2.4. RNA extraction, RT-PCR and qPCR

RNA extraction was carried out using RNeasy mini kit as described by the manufacturer (Qiagen). Reverse transcription was performed using qScript cDNA Supermix according to the manufacturer's instructions (QuantaBio). The Promega GoTaq Flexi system was used according to the instructions to carry out RT-PCR using INPP4B-S primers (Supplementary Table 1). qPCR was performed as described previously using SensiFAST LoRox reagent using qPCR primers for INPP4B-FL or INPP4B-S (Supplementary Table 1) [4]. The relative expression level of INPP4B-FL or INPP4B-S was normalised against β-actin mRNA.

2.5. Immunoblotting

Immunoblotting was carried out as described previously [4].

2.6. Immunofluorescent staining

Cells were seeded onto glass coverslips coated with 2% gelatin at 5×10^4 cells per well. After 48 h of culture, cells were fixed with 1% paraformaldehyde/PBS and blocked with 10% FCS/0.1% Saponin/PBS. Cells were incubated with primary antibody (GFP) for 45 min, followed by secondary antibody (Alexa-488) for 45 min, both diluted in 1% FCS/0.1% Saponin/PBS. Nuclei were stained with DAPI for 15 min. Coverslips were mounted onto slides with SlowFade Gold Antifade Reagent (Invitrogen). Slides were imaged using AxioImager.Z1 (Zeiss).

2.7. BrdU proliferation assays

BrdU cell proliferation assays were carried out using the BrdU Cell Proliferation Assay kit (Cell Signalling) as described before [4]. Briefly, cells were seeded at 5×10^3 cells per well in 96-well plates overnight before treatment as desired. BrdU (10 μM) was added and cells were incubated for 4 h before BrdU assays were carried out. Absorbance was read at 450 nm using a Synergy™ 2 multi-detection microplate reader (BioTek, VT).

2.8. Clonogenic assays

Clonogenic assays were carried out as described previously [4]. In brief, cells were seeded at 2000 cells/well onto 6-well culture plates. Cells were then allowed to grow for a further 12 days before fixation with methanol and staining with crystal violet (0.5% solution).

2.9. siRNA knockdown

siRNA targeting INPP4B-S (CCGCAAAUUGAUGCUCUCU) was synthesised by Genepharma (Shanghai GenePharma Co, Ltd, Shanghai, China). Briefly, siRNAs were transfected in Opti-MEM medium (Invitrogen) with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.10. Statistical analysis and data presentation

Statistical analysis was performed using JMP Statistics Made Visual™ software. Student's *t*-test was used to assess differences between different groups. A *P* value less than 0.05 was considered statistically significant.

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