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Author/s:

He, H;Baldwin, GS

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P21-activated kinase 1 promotes colorectal cancer survival by up-regulation of hypoxia-inducible factor-1 .

Kevin H Liu, Nhi Huynh, Oneel Patel, Arthur Shulkes, Graham Baldwin, Hong He*

Department of Surgery, University of Melbourne, Austin Health, Heidelberg, Victoria 3084, Australia

Running title: PAK1 is critical for colorectal cancer survival

*To whom correspondence should be addressed: Dept. of Surgery, University of Melbourne, Austin Health, Studley Rd., Heidelberg, Victoria 3084, Australia.

Tel.: 613 9496 5593; Fax: 613 9458 1650; Email: hong.he@unimelb.edu.au

Abstract

P21 activated kinase 1 (PAK1) enhances colorectal cancer (CRC) progression by stimulating Wnt/ β -catenin and Ras oncogene, which promote CRC survival via stimulation of hypoxia-inducible factor 1 (HIF-1). The aim of this study was to assess the mechanism involved in the stimulation by PAK1 of CRC survival. PAK1 promoted CRC cell survival by up-regulation of HIF-1. PAK1 was over-expressed and hyper-activated in tumors of $Apc^{\Delta14/+}$ mice, which was correlated with over-expression of HIF-1 and β -catenin. Inhibition of PAK1 decreased tumor growth and the expression of HIF-1 and β -catenin in tumors of $Apc^{\Delta14/+}$ mice, and suppressed xenograft tumor survival in SCID mice. These findings indicate that PAK1 stimulates CRC survival by up-regulation of HIF-1.

Key words: PAK1, HIF-1, β -catenin, CRC

1. Introduction

Mutations of Ras and components of the Wnt/ β -catenin pathway occur in 50% and 90%, respectively, of human colorectal cancers (CRC) [1,2,3]. Mutations of the adenomatous polyposis coli (Apc) gene, a component of the Wnt/ β -catenin pathway, synergise with oncogenic Ras to enhance intestinal tumor formation and progression by stimulating the translocation of β -catenin to the nucleus [4,5]. Ras activates the P21-activated kinase 1 (PAK1), which is over-expressed in human CRCs [6]. The expression of PAK1 increases with progression through the adenoma to carcinoma sequence, with the most dramatic increases in invasive and metastatic CRC [6]. We have reported previously that PAK1 knockdown inhibits growth, survival and migration of CRC cell lines by inactivation of ERK and AKT, the downstream targets of Ras [7], and abrogates growth and metastasis of CRC cell lines in xenograft and liver metastasis models in mice [8]. PAK1 associates with and phosphorylates β -catenin in CRC cells, and PAK1 knockdown inhibits β -catenin expression and suppresses β -catenin/TCF4 transcriptional activity [8,9]. Together these findings indicate that PAK1 play a key role in mediating the cross-talk between Ras and Wnt/ β -catenin signalling in CRC progression (for review see [10]).

Hypoxia-inducible factor 1 (HIF-1) regulates oxygen delivery (via angiogenesis) and metabolic adaptation to hypoxia (via glycolysis). HIF-1 consists of a constitutively expressed HIF-1 β subunit and an oxygen- and growth-factor-regulated HIF-1 α subunit. Intratumoral hypoxia induces and stabilizes the expression of HIF-1, which in turn transactivates the gene products that mediate tumor angiogenesis and glycolytic metabolism [11]. Overexpression of HIF-1 has been found in primary and metastatic human cancers and the level of expression correlates with tumor angiogenesis and patient mortality [11]. The Wnt/ β -catenin signalling pathway is also activated in response to the hypoxia associated with tumour expansion [12]. Under hypoxia, β -catenin switches from binding with TCF4 and promoting cell proliferation to binding with HIF-1 and enhancing HIF-1-mediated transcription, thereby promoting cell survival and adaptation to hypoxia [13]. In addition to hypoxia, HIF-1 can also be regulated by oncogenic pathways. For example Ras mutants have been shown to stimulate HIF-1 in CRC cells [14]. These findings suggest that HIF-1 plays an important role in the stimulation of CRC survival that results from activation of Ras and the Wnt/ β -catenin pathway.

As mentioned above, PAK1 acts as a convergence point in CRC progression, by mediating the signals from Ras and the Wnt/ β -catenin pathway [10]. In turn, activation of both Ras and the Wnt/ β -catenin pathway stimulates CRC survival through regulation of HIF-1. However, it is not known whether PAK1 promotes CRC survival by stimulation of HIF-1. In order to test this hypothesis, the effect of PAK1 on HIF-1 expression, VEGF secretion and CRC cell survival *in vitro* and *in vivo* was assessed. The *in vitro* PAK1 expression was either decreased by transfection with plasmids encoding shRNAs or increased by over-expression of constitutively active PAK1. HIF-1 and β -catenin expression were measured by Western blotting and immunohistochemistry, VEGF secretion by ELISA, survival by cell counting in CRC cell lines. The *in vivo* studies used APC ^{Δ 14/+} C57BL/6 mice and human CRC cell xenografts in SCID mice. The *in vivo* PAK1 expression was reduced by treatment with siRNAs. The number and size of tumors were measured and the characteristics of the tumours determined by haematoxylin and eosin staining and by immunohistochemistry for PAK1, pPAK1, β -catenin and HIF-1.

2. Material and methods

2.1. Cell culture and transfection

The human colorectal cancer (CRC) cell lines DLD1, HCT116, HT29 and SW480 were obtained from the ATCC and cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS). The derivation of PAK1 knockdown (KD) clones of DLD1 cells by transfection with plasmid DNAs encoding either shRNA sequences (SABioscience, Frederick, MD, USA) to silence the PAK1 gene specifically or with a scrambled sequence as a negative control (NC) using Lipofectin Reagent (Invitrogen, Melbourne, Australia) according to the manufacturer's instructions has been described previously [7]. Stable clones were selected with G418 (1mg/ml), and PAK1 protein expression was detected by Western blot.

The constitutively active (CA, PAK1^{T423E}) and kinase inactive (KI, PAK1^{K299A}) PAK1 constructs (generously provided by Dr. Gary Bokoch, The Scripps Research Institute, La Jolla, CA) were sub-cloned into the pCDNA3.1 vector (Invitrogen, Melbourne, Australia). CA and KI PAK1 plasmid DNAs were transfected into SW480 cells using Lipofectin Reagent (Invitrogen) according to the manufacturer's instructions. Stable clones were selected with G418 (1mg/ml), and the CA or KI PAK1 proteins were detected by Western blot using anti-myc-tag antibody (Genesearch, Melbourne, Australia).

2.2. Cell survival assay

Cell survival was measured as previously described [7]. Cells were seeded in a 96-well plate at $3-5 \times 10^3$ cells/well in DMEM containing 5% FBS and 10 Ci/ml [methyl-³H]-thymidine and cultured for 20h. The medium containing free unincorporated ³H-thymidine was then removed, and cells were cultured with or without serum for a further 24h in the absence or presence of CoCl₂ (Sigma, St. Louis, MO), before harvest with a NUNC cell harvester. The ³H-thymidine remaining from that incorporated in the first 20h was measured with a β -counter (Packard, Meriden, CT). Cell survival was measured as the ratio of the ³H-thymidine incorporated after different treatments compared to the corresponding value for the serum control.

2.3. VEGF assay

Cells were cultured in 6-well plates under normoxia (N, 5% O₂) or hypoxia (H, 1% O₂) for 24h (SW480) or 48h (DLD1) with serum. The media were then collected, cleared by

centrifugation, and the VEGF concentrations determined using a VEGF ELISA kit (R&D systems, Minneapolis, MN, USA) following the manufacturer's instruction. The concentrations of VEGF from the culture media were assayed in duplicate at a 1:4 dilution and corrected for total cell numbers.

2.4. Sequences of siRNAs

siRNAs were designed as duplexes of 21-base sense and anti-sense oligonucleotides [15,16,17]. For the CRC cell xenograft study, human PAK1 siRNA (Supplementary Table 1) was used for the treatment and Lamin A/C as control [15]. For the APC^{Δ14/+} mouse study, mouse PAK1 siRNA (Supplementary Table1) was used for the treatment, and Luciferase GL2 siRNA [15] was used as the negative control. All double-stranded siRNAs were synthesized by Sigma-Aldrich (St. Louis, MO, USA).

2.5. Mouse xenograft study

The mouse experiments were conducted at the Austin Bioresource Centre, Austin Health (Melbourne, Australia) with ethics approval from the Austin Health Animal Ethics Committee. SCID mice and wild type C57BL/6 mice were purchased from the Animal Resource Centre (Perth, Australia). APC^{Δ14/+} mice on the C57BL/6 background were generously provided by Dr. Julie Pannequin (National Centre for Scientific Research, Institute of Functional Genomics, Montpellier, France).

HCT116 cells (5×10^6 cells/100 l/site) were injected subcutaneously into opposite flanks of 6 week old SCID mice. From the second day after the injection of CRC cells, siRNAs (250 µg/Kg) were given by intraperitoneal injection every other day for 3 weeks. There were eight mice in both PAK1 siRNA-treated and lamin A/C siRNA (control) groups. Tumor volume was measured from day 7 to 21, when mice were sacrificed. Tumors were excised, weighed and fixed in formalin for histological analysis.

For tumor viability analysis, images were taken from haematoxylin and eosin (H&E) stained slides using a Coolscope (Nikon Eclipsnet VSL, Japan) at 2x magnification, and then analyzed using Image J software (National Institutes of Health, Bethesda, MD, US). The necrotic and total areas on each image were measured, and the area of viable tumor was calculated by subtraction of the necrotic area from the total area. The ratio of viable to total area for each tumor was then calculated.

2.6. *APC^{Δ14/+} mouse study*

APC^{Δ14/+} C57BL/6 mice were treated with either PAK1 siRNA or Luciferase siRNA (control). The mice were weighed at 10 weeks old and injected intraperitoneally 3 times for the first week and once weekly thereafter until 23 weeks old with siRNAs (100μl, 250μg/Kg). Mice were weighed through 13 weeks and culled at 23 weeks.

Samples of small intestine, colon and rectum were dissected out from APC^{Δ14/+} mice, everted on wooden skewers, removed from the skewer, rinsed with 70% ethanol, and opened lengthwise. Polyps were counted under a dissecting microscope at 15x magnification. The whole intestine was then rolled as a Swiss Roll for immunohistochemistry. Swiss Roll samples of small intestine, and colon and rectum of APC^{Δ14/+} mouse were sectioned for H&E staining. The images of H&E stained Swiss Roll samples were taken using a Coolscope at 4x magnification. The number of tumors from each mouse intestine was counted.

2.7. *Western blot*

Cells were lysed in SDS sample buffer. The proteins in cell lysates were resolved by SDS-polyacrylamide gel (SDS-PAGE), and detected with antibodies against HIF-1 (BD Biosciences, San Jose, California, USA), PAK1, phospho-PAK1 (Thr423) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Cell Signaling). Bound antibodies were visualized using ECL reagents (GE Healthcare, Buckinghamshire, UK), and the density of each band was analysed using Multi-gauge computer software (Berthold, Bundoora, Australia).

Colorectal tumors and adjacent normal tissues were dissected from 20-week-old APC^{Δ14/+} mice. 50 mg of frozen tissue was homogenised using an ultra Turrax T25 homogeniser (Janke and Kunkel, Staufen, Germany) in 500 μl tissue lysis buffer (150mM NaCl, pH 7.5, 50mM HEPES, 10mM EDTA, 10mM Na₄P₂O₇, 100mM NaF, protease inhibitor cocktail (Roche)). The lysates were then centrifuged at 13,000 rpm for 10 minutes at 4°C, and supernatants were collected. Proteins were separated by SDS-PAGE. Protein expression was determined with antibodies against PAK1, pPAK1, β-catenin (Cell Signalling) and HIF-1α.

2.8. *Immunohistochemistry staining*

For xenograft study, sample slides were incubated with DAKO peroxidase blocker (DAKOPATTS, Copenhagen, Denmark) to block endogenous peroxidases. Antigens were

retrieved by incubation in citrate buffer (10 mM Na₃citrate) in a 100°C water bath (Thermo Fisher Scientific, Waltham, MA) for 30 minutes. After blocking with ultra V block (Thermo Fisher Scientific), the sample slides were incubated with antibodies against pPAK1, HIF-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit IgG at a 1:100 dilution overnight at 4°C. After washing with TBST (20 mM Tris, 0.8% NaCl, pH7.6, 0.05% Tween 20), slides were incubated with horse radish peroxidase-labeled goat anti-rabbit IgG (Dakopatts) for 1 h, followed by diaminobenzidine (Dakopatts) staining. The slides were then dipped in haematoxylin and Scott's tap water to stain cellular components. Images were taken with a Coolscope (Nikon) and analysed using the Image Pro-Plus 6.0 image analysis program (Media Cybernetics Inc., Silver Spring, MD) or Multi-gauge software.

For APC^{Δ14/+} mouse study, Paraffin-embedded sections of colon and rectum were stained with antibodies against PAK1, pPAK1, β-catenin and HIF-1 as described above. The antibody dilution was 1:100 for PAK1, pPAK1 and HIF-1, and 1:500 for β-catenin. Images were taken with a Coolscope (Nikon), and the intensity of positive staining was measured and analyzed using Image J and Multi-gauge software.

2.9. Statistical analysis

All values are expressed as means ± standard error. Results were analyzed by one-way analysis of variance or t-test as appropriate with the program SigmaStat (SPSS, Chicago, IL). Differences between two means with $p < 0.05$ were considered significant.

3. Results

3.1. *PAK1 knockdown inhibited HIF-1 expression and CRC cell survival*

Both negative control (NC) and PAK1 knockdown (KD) DLD1 cells were cultured under normoxia or hypoxia (1% O₂) for 48h. The expressions of HIF-1, total PAK1 and active phosphorylated PAK1 (pPAK1) were determined by Western blots, and VEGF secretion was measured by ELISA. The induction of HIF-1 expression by hypoxia was significantly lower in PAK1 KD cells than in NC cells (Fig. 1A). The reduced HIF-1 expression in PAK1 KD cells was associated with lower concentrations of total PAK1 and of phosphorylated and active PAK1. Consistent with our previous report [7], PAK1 KD cells produced less VEGF under normoxia (5% O₂) (Fig. 1B). Importantly, hypoxia triggered significantly more secretion of VEGF from NC cells than from PAK1 KD cells (Fig. 1B). VEGF secretion by PAK1 KD cells in response to hypoxia was only 25% of VEGF secretion by NC cells. These results showed that PAK1 knockdown reduced hypoxia-induced VEGF secretion and HIF-1 expression, which has previously been shown to be important for cell survival under hypoxia [18].

Treatment of cells with CoCl₂ induces cell death, which in turn triggers HIF-1 expression leading to cell survival [19]. After 24h incubation with CoCl₂ (300 μM) in the absence of serum, PAK1 KD cells expressed significantly less HIF-1 than NC cells (Fig. 1C). Serum withdrawal induced significant cell death in PAK1 KD, but not in NC cells (Fig. 1D). CoCl₂ at concentrations of 75 μM and above further enhanced the death of PAK1 KD cells, but death of NC cells was only induced at CoCl₂ concentrations of 150 μM and above (Fig. 1D). To summarise, compared to NC cells, PAK1 knockdown decreased CoCl₂-induced HIF-1 expression, and the reduction was associated with reduced cell survival under CoCl₂ treatment. These results indicated that PAK1 knockdown inhibited cell survival by down-regulation of HIF-1.

3.2. *Constitutively active PAK1 stimulated HIF-1 expression and CRC cell survival*

The effects of activation of PAK1 on HIF-1 expression and cell survival were determined by over-expression of the constitutively active (CA) or kinase inactive (KI) forms of PAK1 in CRC cells. SW480 cells, which had the lowest PAK1 expression among four CRC cell lines tested (Supplementary Fig.1), were transfected with either CA- or KI-PAK1,

or vector only (VO). The transfected SW480 cells were cultured under normoxia or hypoxia (1% O₂) for 24h. The concentrations of HIF-1, PAK1 and active phosphorylated PAK1 were determined by Western blots, and VEGF secretion was measured by ELISA. Hypoxia induced higher levels of HIF-1 expression in CA-PAK1 cells than in VO or KI-PAK1 cells (Fig. 2A). Similarly, the VEGF secretion by CA-PAK1 cells was significantly higher than VO cells under both normoxia and hypoxia (Fig. 2B). Although the concentration of pPAK1 in KI-PAK1 cells was decreased, the concentrations of HIF-1 and of secreted VEGF were not significantly lower than in VO cells. The results indicated that constitutive activation of PAK1 stimulated HIF-1 expression and VEGF secretion under hypoxia.

To determine the effect of constitutive activation of PAK1 on cell survival, the transfected SW480 cells were treated with CoCl₂. HIF-1 expression and cell survival were measured as for PAK1 KD DLD1 cells. In the absence of serum, CoCl₂ (300 μM) induced higher levels of HIF-1 expression in CA-PAK1 cells than in VO cells (Fig. 2C). CoCl₂ induced cell death in VO cells at concentrations of 75 μM and above, and in KI-PAK1 cells at 300 μM (Fig. 2D). In contrast, 300 μM CoCl₂ did not induce cell death in CA-PAK1 cells. These results showed that constitutive activation of PAK1 enhanced CoCl₂-induced HIF-1 expression and promoted cell survival.

3.3. Overexpression and hyperactivation of PAK1 was correlated with up-regulation of HIF-1 and -catenin in intestinal tumors in APC^{Δ14/+} mice

To determine whether or not PAK1 also promoted CRC cell survival by stimulation of HIF-1 *in vivo*, the interaction between PAK1 and HIF-1 or -catenin *in vivo* was measured in APC^{Δ14/+} mice in which constitutive activation of -catenin is associated with tumorigenesis in small intestine, colon and rectum [20]. The concentrations of HIF-1, -catenin, PAK1 and pPAK1 were determined by Western blots and compared between tumor and adjacent normal tissue from the small intestine, colon and rectum of 20-week-old APC^{Δ14/+} mice. In colon and rectum, the concentrations of HIF-1, -catenin, PAK1 and pPAK1 (Fig. 3A) were significantly higher in tumors than in adjacent normal tissue (Fig. 3B: male mice; Fig. 3C: female mice). More importantly, the concentrations of over-expressed HIF-1 (Fig. 4A&B) or -catenin (Fig. 4C&D) were correlated with the concentrations of active pPAK1 in tumors but

not in normal tissues (Supplementary Table 2). Likewise the concentration of HIF-1 (Supplementary Fig. 2A&B) was also correlated with the total concentration of PAK1 expression in tumors but not in normal tissues (Supplementary Table 2). Although the concentration of β -catenin (Supplementary Fig. 2C&D) was correlated with the total concentration of PAK1 expression in both tumors and normal tissues, the correlation between PAK1 and β -catenin was higher in tumors (Supplementary Table 2). Similar results were obtained with tumors from the small intestine (Supplementary Table 2 & Supplementary Figs. 3-5). These results indicated that the up-regulation of HIF-1 and β -catenin was significantly correlated with expression and activation of PAK1 in tumors from the colon, rectum and small intestine in APC $^{\Delta14/+}$ mice. The significant correlation between activation of PAK1 and up-regulation of HIF-1 or β -catenin in tumors suggested that the interaction between these proteins was important for tumor growth and survival.

3.4. Inhibition of PAK1 suppressed tumor growth and survival, and decreased the expression of HIF-1 and β -catenin in vivo

To determine whether or not inhibition of PAK1 would down-regulate HIF-1 and β -catenin and suppress tumor growth and survival, 10-week-old APC $^{\Delta14/+}$ mice were treated with PAK1 siRNA for 13 weeks. At 23 weeks, the mice were killed, the small intestine, colon and rectum dissected out, and protein expression and tumor growth were analysed as described in Material and methods. A small but significant reduction was observed in the expression of total PAK1 (20%) and pPAK1 (14%) in the colorectal tumors of the mice treated with PAK1 siRNA (Fig. 5A). Moreover the expression of β -catenin and HIF-1 in the colorectal tumors of the mice treated with PAK1 siRNA was significantly decreased to 90% and 79%, respectively, of the values in control siRNA treated mice (Fig. 5A). Importantly, inhibition of PAK1 by siRNA treatment significantly reduced the numbers of small intestinal tumors (Fig. 5B). A similar trend was observed in the numbers of colorectal tumors after siRNA treatment, although more mice would be needed to reach statistical significance because of the small number of colorectal tumors available (2-5 per mouse). These results indicated that inhibition of PAK1 by siRNA treatment suppressed HIF-1 and β -catenin, and decreased tumor growth *in vivo*.

To determine whether or not inhibition of PAK1 would reduce the growth and survival of CRC xenografts, the human CRC cell line HCT116 was injected into the flank of

SCID mice (6-8 each group), and the mice were treated with PAK1 siRNA for 3 weeks. A small but significant reduction was observed in the expression of pPAK1 (24%) and HIF-1 (18%) in the xenografted tumors in the mice treated with PAK1 siRNA compared to the values in control siRNA-treated mice (Fig. 6A, 6B). Although the PAK1 siRNA treatment decreased tumor size only at 7 days (Fig. 6C), tumor viability as determined by the extent of necrosis was reduced to less than 80% of the control value after treatment for 3 weeks (Fig. 6D). The results indicated that inhibition of PAK1 down-regulated HIF-1 and decreased tumor survival *in vivo*.

4. Discussion

In current study, we have demonstrated for the first time that PAK1 plays a role in the regulation of HIF-1 in CRC progression. In CRC cells *in vitro*, knocking down PAK1 inhibited HIF-1 expression and VEGF secretion in response to hypoxia. Conversely, constitutively active PAK1 stimulated HIF-1 expression and VEGF secretion. Likewise HIF-1 expression induced by CoCl₂, a stimulus that mimics hypoxia, was inhibited in PAK1 knockdown cells, and the inhibition was associated with reduced cell survival under CoCl₂ treatment. In contrast, CoCl₂-induced HIF-1 expression was stimulated by constitutive activation of PAK1, and the stimulation was associated with increased cell survival. These results imply that PAK1 stimulates CRC cell survival via up-regulation of HIF-1. Our previous data indicated that PAK1 promotes CRC cell growth, survival and migration via activation of both AKT- and ERK-dependent pathways [7]. Since HIF-1 expression can be stimulated by the activation of PI3K/AKT [21,22] and Ras/Raf/MEK/ERK pathways [23,24,25], PAK1 may up-regulate HIF-1 through activation of ERK and/or AKT.

In agreement with the *in vitro* results, we observed that PAK1 was over-expressed and hyper-activated in tumors from small intestine, colon and rectum in the Apc^{Δ14/+} mouse model of intestinal cancer. β -catenin and HIF-1 were also significantly higher in the tumors from Apc^{Δ14/+} mice. More importantly we discovered that the overexpression and hyperactivation of PAK1 was positively correlated with both β -catenin and HIF-1 in the tumors from Apc^{Δ14/+} mice. The dysplastic lesions in Apc^{Δ14/+} mice have previously been reported to over-express β -catenin [20]. Ras mutation increases the tumor growth of Apc mutant mice via stimulation of Wnt/ β -catenin signalling [4]. In CRC cells, PAK1, activated downstream of Ras, associates with β -catenin and stimulates its transcriptional activity by phosphorylation and stabilisation of β -catenin [8]. In agreement with these previous reports, our observation that higher activity and expression of PAK1 was closely correlated with higher expression of β -catenin in colorectal tumors of Apc^{Δ14/+} mice indicates the importance of PAK1 in β -catenin activation and CRC progression.

Furthermore our observation that higher activity and expression of PAK1 was also closely correlated with higher HIF-1 expression in the tumors from Apc^{Δ14/+} mice indicates that PAK1 likely regulates HIF-1 in CRC *in vivo*. HIF-1 is often deregulated in tumors

located in hypoxic microenvironments and plays an important role in angiogenesis, tumor progression, and resistance to chemo- and radiotherapies [26]. In human CRC patients, high HIF-1 expression is associated with increased concentrations of VEGF and a decreased survival rate [27]. Compared to the adjacent normal tissues, the higher expression and activity of PAK1 and HIF-1 and the close correlation between PAK1 and HIF-1 in the tumors from $Apc^{\Delta14/+}$ mice imply an interaction between PAK1 and HIF-1 in these tumors.

More importantly, our finding further indicates that the interaction between PAK1 and HIF-1 is important in tumor growth and survival. For example, inhibition of PAK1 by treatment with PAK1 siRNA decreased the expression of β -catenin and HIF-1 in the tumors from $Apc^{\Delta14/+}$ mice, and reduced tumor numbers in the small intestine (Fig 5). Similarly inhibition of PAK1 by siRNA treatment also reduced PAK1 activity and HIF-1 expression in tumors grown from xenografted human CRC cells in SCID mice, and the reduction was associated with suppression of tumor survival (Fig 6). These results demonstrate that PAK1 stimulates CRC growth and survival by up-regulation of β -catenin and/or HIF-1 *in vivo*.

The connection between PAK1 and HIF-1 further exemplifies the complex signalling networks that underlie tumorigenesis. HIF-1 plays a key role in tumor survival in a hypoxic environment through induction of survival factors and angiogenic growth factors such as VEGF [28]. Indeed HIF-1 gene transcription has been reported to directly regulate VEGF expression in xenografted tumors [29]. Increased expression of HIF-1 and VEGF is correlated with a more advanced tumor stage and a poorer prognosis in CRC patients [30]. Although numerous efforts have been made to identify molecules that inhibit HIF-1 and/or target the HIF-1 pathway in tumor progression, the currently available inhibitors suffer from lack of specificity [31]. The stimulatory effects of PAK1 on HIF-1 expression and VEGF secretion by CRC cells in response to hypoxia provide a molecular basis for the stimulation by PAK1 of CRC survival. The link established here between PAK1 and HIF-1, a major player in cancer survival and metastasis, implies that small molecule inhibitors of PAK1 may be an effective treatment for CRC in humans.

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Conflict of Interest Statement

The authors of this paper have no conflict of interest to declare.

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Figure legends**Fig. 1. PAK1 knockdown inhibited HIF-1 expression and cell survival.**

DLD1 cells were cultured under normoxia (N, 5% O₂) or hypoxia (H, 1% O₂) for 48h. Hypoxia induced significantly higher HIF-1 protein expression in control (NC1) cells than in two independent clones of PAK1 knockdown (KD) cells (A). Correspondingly hypoxia triggered increased VEGF secretion from control cells, but not PAK1 KD cells (B). In the absence of serum, the hypoxia-mimetic CoCl₂ induced significantly higher HIF-1 (C) and lower cell death (D) in NC cells than in PAK1 KD1 cells by 24h. Since there was no significant difference in HIF-1 expression with or without serum, expression after CoCl₂ treatment without serum was compared with expression with serum. Data is averaged from three independent sets of experiments, and similar data to those in panels C and D were obtained for PAK1 KD2 cells. * and #, p<0.05; **, p<0.01; *** and ####, p<0.001; compared to the values from NC cells or as indicated. For clarity P values are shown only for the lowest concentration of CoCl₂ at which the difference was significant.

Fig. 2. Constitutively active PAK1 promoted HIF-1 expression and cell survival.

SW480 cells were cultured under normoxia (N, 5% O₂) or hypoxia (H, 1% O₂) for 24h. Under hypoxia, HIF-1 was increased in all cell lines compared to the same cell lines under normoxia, with a significantly higher increment in the two clones of cell transfected with constitutively active PAK1 (A). Correspondingly, VEGF secretion was increased in all cell lines under hypoxia compared to the same cell lines under normoxia, with significantly greater increments in CA cells than in control cells transfected with vector only (VO) (B). The kinase inactive PAK1 did not affect either HIF-1 or VEGF secretion. Similarly, in the absence of serum, CoCl₂ (300 M) induced significantly higher concentrations of HIF-1 in CA cells than in VO or KI cells (C). CoCl₂ induced significant cell death in VO cells at 75 M, and in KI cells at 300 M, but did not alter cell death in CA cells at any concentration tested

(D). Data is averaged from three independent sets of experiments. * and # $p < 0.05$, ** $p < 0.01$ compared to the values obtained from VO cells (A, B), or to the values obtained in the presence of serum (D). In panels A and C the arrow head shows the position of the transfected CA- or KI- PAK1, and the arrow the position of the slightly smaller endogenous PAK1. The single major band for pPAK1 is attributed to endogenous wild-type pPAK1, since CA-PAK1 will not be recognised by the anti-pPAK1 antibody (Santa Cruz) because the phosphorylation site has been mutated. Similarly the KI-PAK1 will not be recognised because it is unable to phosphorylate itself. CA13 and CA16 represented clone numbers 13 and 16 after transfection by CA-PAK1, and KI23 clone number 23 after transfection by KI-PAK1. Anti-PAK1 antibody from Santa Cruz was used for the blots.

Fig. 3. Activation of PAK1 and expression of HIF-1 and β -catenin were increased in the colorectal tumors of $Apc^{\Delta14/+}$ mice.

Tumors and adjacent normal tissues were dissected from the colon and rectum of $Apc^{\Delta14/+}$ mice. The protein concentrations of total PAK1, phosphorylated and active PAK1 (pPAK1), HIF-1 and β -catenin were determined by Western blot, and representative blots from 3 mice (m1-m3) are presented (A). Protein expression was normalised to GAPDH, averaged over 3 replicate blots, and compared between normal and tumour tissues obtained from 5 male (B, 20 samples) and 4 female (C, 18 samples) mice. -cat and b-cat: β -catenin, N: normal tissue, T: tumour tissue. *, $p < 0.05$, **, $p < 0.01$ compared to the values obtained from normal tissues.

Fig. 4. Increased activity of PAK1 was correlated with β -catenin and HIF-1 expression in the colorectal tumors of $Apc^{\Delta14/+}$ mice.

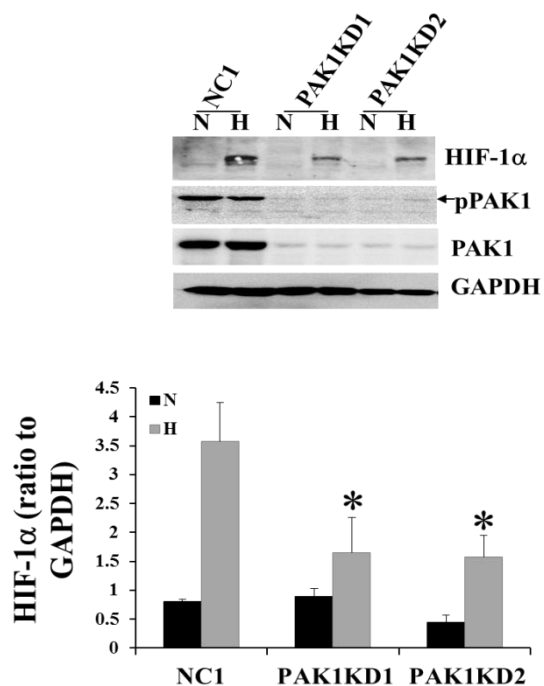
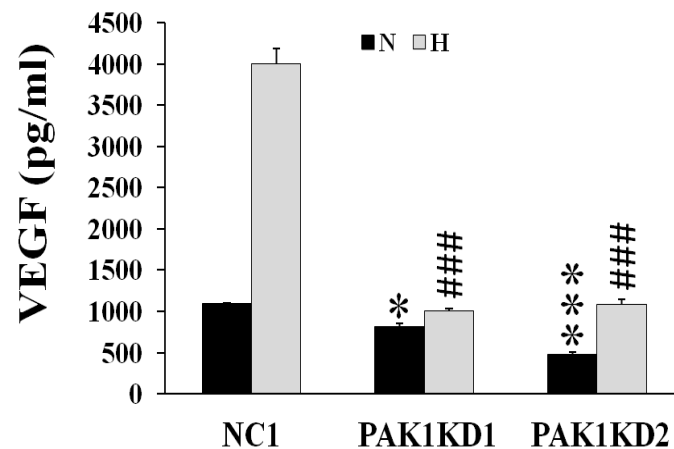
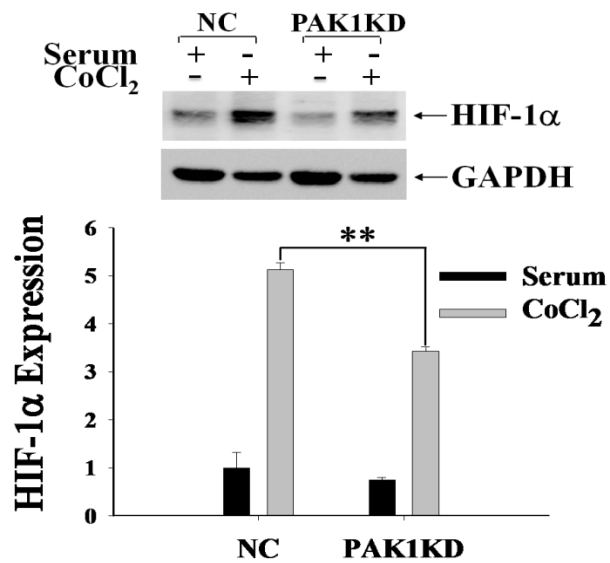
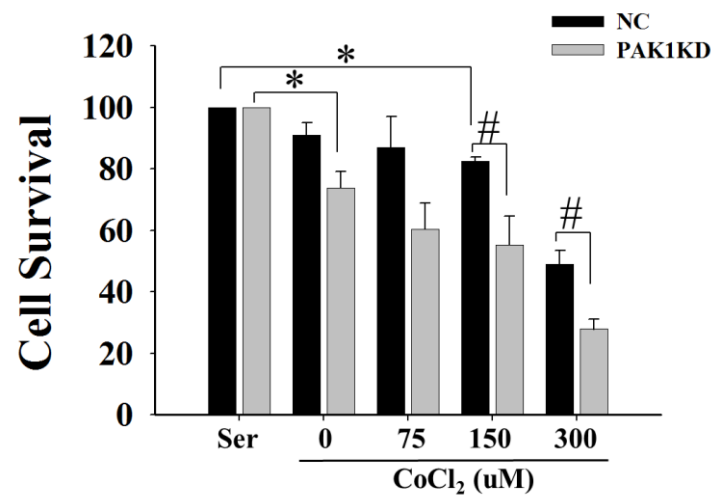
Tumors and adjacent normal tissues were dissected from the colon and rectum of $Apc^{\Delta14/+}$ mice. The protein concentrations of phosphorylated and active PAK1 (pPAK1), HIF-1 and β -catenin were determined by Western blot, normalised to GAPDH, and averaged as described in the legend to Figure 3. Regression lines for the correlation of phosphorylated and active PAK1 (pPAK1) to HIF-1 (A, B) or β -catenin (C, D) in normal tissue (A, C) or tumor tissue (B, D) were plotted using the program Sigma Plot 12. R_N and R_T : regression (R) values for normal and tumour samples respectively.

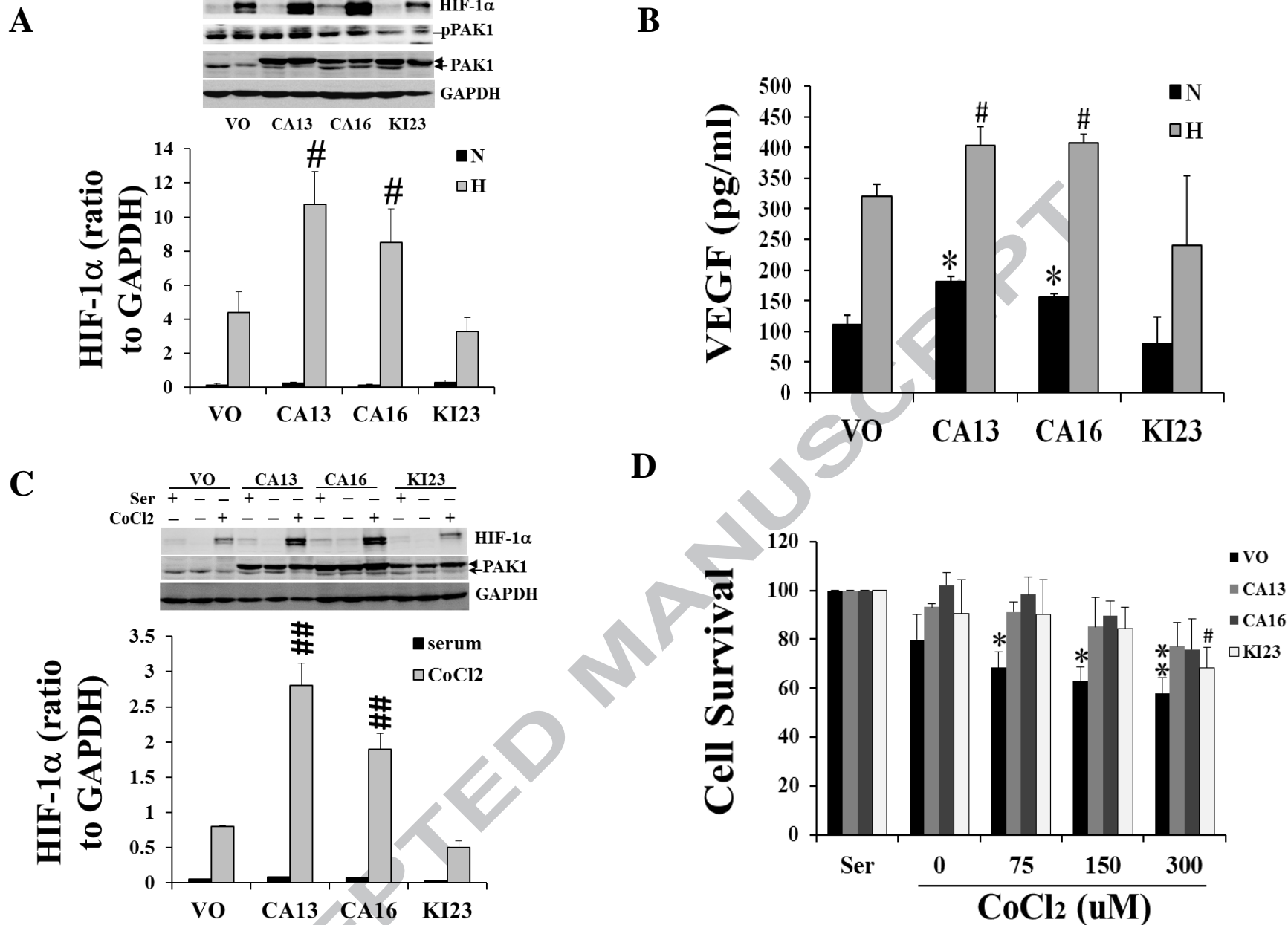
Fig. 5. PAK1 siRNA treatment inhibited tumor growth in $Apc^{\Delta14/+}$ mice by reduction of PAK1 activation and of the expression of β -catenin and HIF-1 .

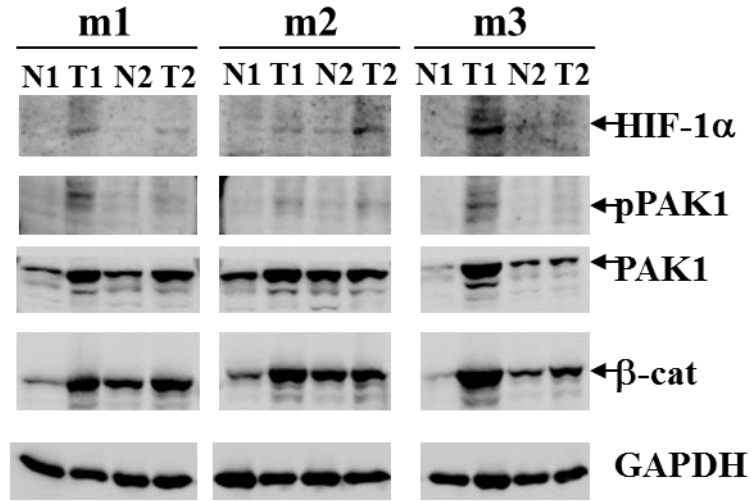
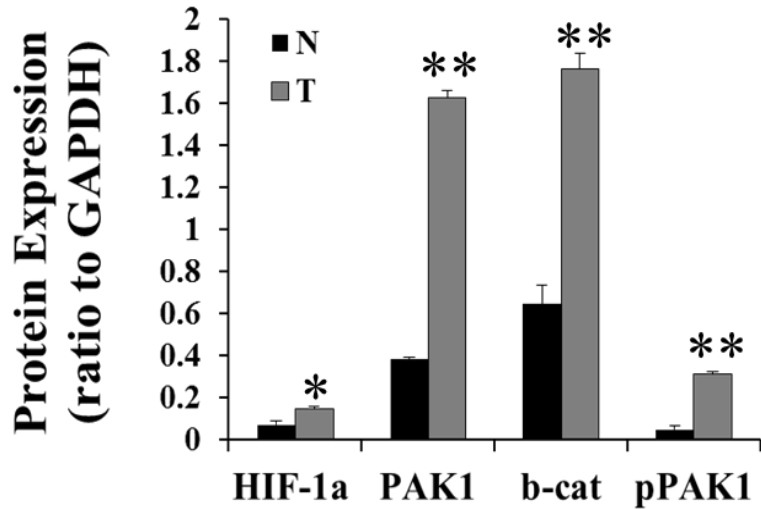
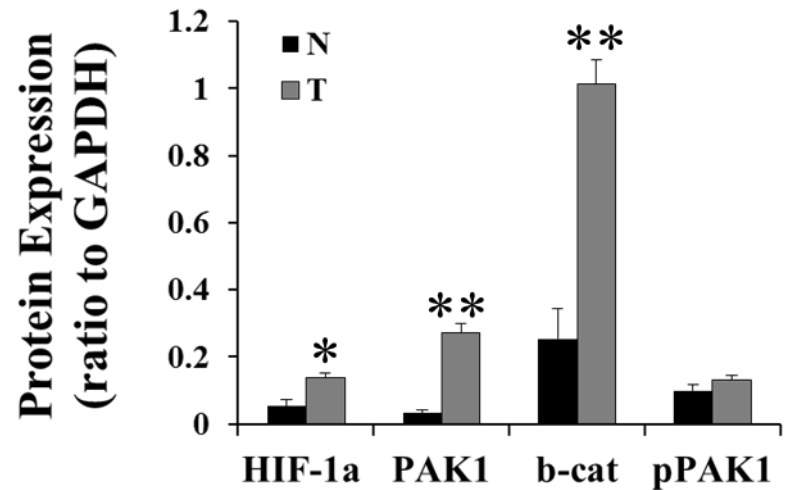
10-week-old $Apc^{\Delta14/+}$ mice were treated with either PAK1 siRNA (11 mice) or luciferase siRNA as control (9 mice) for 13 weeks. Mice were culled at 23 weeks, small intestine, colon and rectum were dissected out, and tumors were counted (B). The expression of total PAK, phosphorylated and active PAK1 (pPAK1), HIF-1 and β -catenin in the tumors from colon and rectum was determined by immunohistochemistry and analysed using the image analysis program Image Pro-Plus 6.0 or Multi-gauge software (A). Cont: control, β -cat: β -catenin, HIF-1a: HIF-1 . *, $p < 0.05$; **, $p < 0.01$, compared to the values obtained from the mice treated with luciferase siRNA (control).

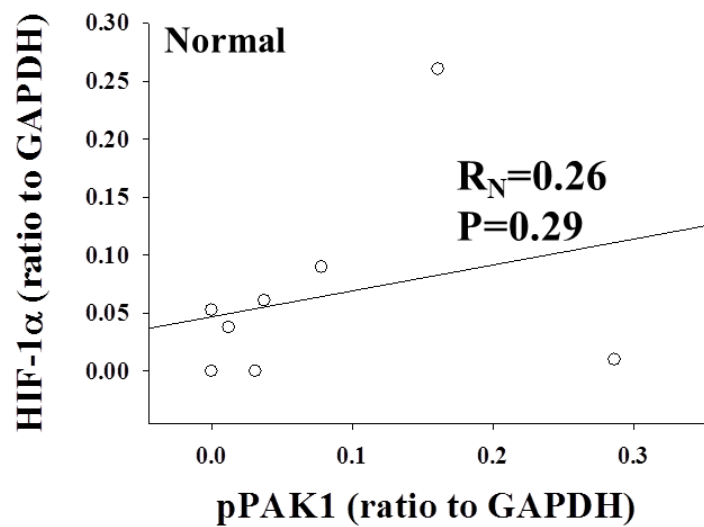
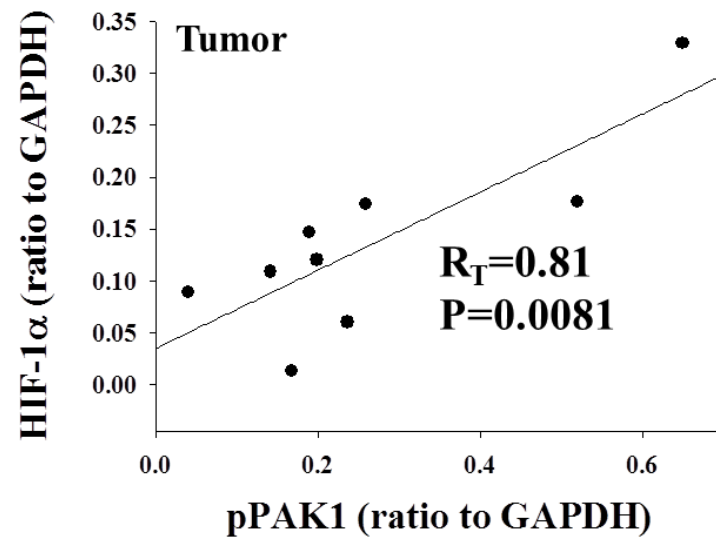
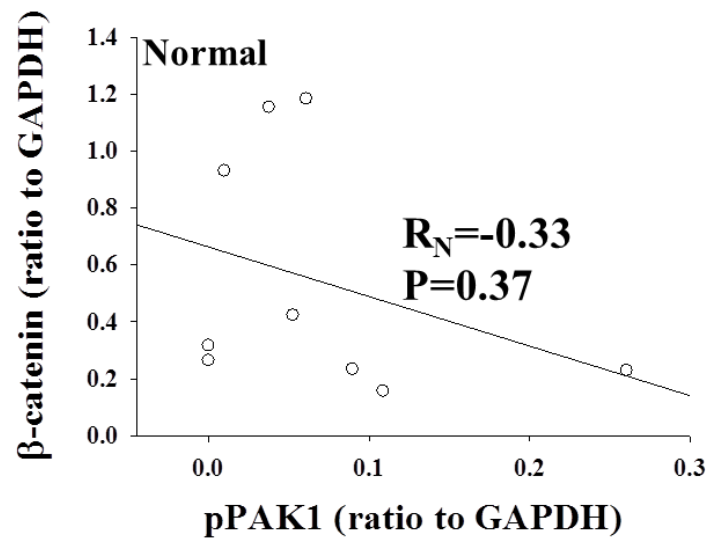
Fig. 6. PAK1 siRNA treatment decreased PAK1 activity, HIF-1 expression and tumour viability in CRC xenografts.

Mice bearing xenografts of the human CRC cell HCT116 were treated with either PAK1 siRNA (8 mice) or lamin A/C siRNA as control (8 mice) for 21 days as described in Material and Methods. The expression of phosphorylated and active PAK1 (A) and of HIF-1 (B) was measured by immunohistochemistry with anti-phospho-PAK1 or anti-HIF-1 antibodies with rabbit IgG as control. Tumor size (C) and viability (D) were determined as described in Material and Methods. The data were analysed with Image J and Multi-gauge software. The ratio of viable area to total tumour area from control siRNA-treated samples was taken as 100% in D. The expression of pPAK1 (A) and HIF-1 (B) in control siRNA-treated samples was taken as 100%. **, $p < 0.01$ compared to the control siRNA-treated samples.

A**B****C****D**



A**B****C**

A**B****C****D**