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Effects of p21-activated kinase 1 inhibition on 11q13 amplified ovarian cancer cells

Tatiana Y. Prudnikova[1], **Olga Villamar-Cruz**[1], **Sonali J. Rawat**[1], **Kathy Q. Cai**[2], and **Jonathan Chernoff**[1,¶]

[1]Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA 19111

[2]Department of Pathology, Fox Chase Cancer Center, Philadelphia, PA 19111

Abstract

p21-activated kinases (PAKs) are Cdc42/Rac–activated serine-threonine protein kinases that regulate of several key cancer-relevant signaling pathways, such as the Mek/Erk, PI3K/Akt, and Wnt/b-catenin signaling pathways. Pak1 is frequently overexpressed and/or hyperactivated in different human cancers, including human breast, ovary, prostate, and brain cancer, due to amplification of the *PAK1* gene in an 11q13 amplicon. Genetic or pharmacological inactivation of Pak1 has been shown to reduce proliferation of different cancer cells *in vitro* and reduce tumor progression *in vivo*.

In this work, we examined the roles of Pak1 in cellular and animal models of *PAK1*-amplified ovarian cancer. We found that inhibition of Pak1 leads to decreased proliferation and migration in *PAK1* amplified/overexpressed ovarian cancer cells, and has no effect in cell that lack such amplification/overexpression. Further, we observed that loss of Pak1 function causes 11q13 amplified ovarian cancer cells to arrest in the G2/M phase of the cell cycle. This arrest correlates with activation of p53 and $p21^{Cip}$ and decreased expression of cyclin B1. These findings suggest that small molecule inhibitors of Pak1 may play a therapeutic role in the ~25% of ovarian cancers characterized by *PAK1* gene amplification.

Keywords

protein kinases; p21-activated kinase; ovarian cancer; 11q13 amplification

Introduction

The p21-activated kinases (Paks) are cytosolic Cdc42/Rac activated serine-threonine protein kinases that regulate a number of key signal transduction pathways (1). There are 6 Pak isoforms that can be grouped into 2 families - group I (Pak1–3) and group II (Pak4–6) - based on their sequence homology and on presence of a well-defined auto-inhibitory region in group I but not group II Paks (2). The Paks act as downstream effectors of the Rho family

¶To whom correspondence should be addressed: Jonathan Chernoff, Cancer Biology Program, Fox Chase Cancer Center, 333 Cottman Ave, Philadelphia, PA 19111, USA, Tel.: (215) 728 5319; Fax: (215) 728 3616; Jonathan.Chernoff@fccc.edu.

of small GTPases such as Rac and Cdc42 (3), and play important roles in different cellular processes, including cell proliferation, cell survival, adhesion and migration, and anchorage-independent growth (1).

Pak1 regulates a variety of important signaling pathways, including the Ras/Erk (4, 5), PI3K/Akt, and Wnt/β-catenin pathways (6, 7). In Ras/Erk signaling, Pak1 phosphorylates c-Raf at S338 and Mek1 at S298 (4, 5), sites thought to be required for Erk activation. It has been reported that Pak1 is required for activation of Akt, though it is not clear if the catalytic activity of Pak1 is required for this function (8). Recently, it has also been reported that Pak1 can interact with the Wnt/β-catenin pathway (6, 7). In this case, Pak1 has been shown to associate with β-catenin and phosphorylate it at S663 and S675. These modifications lead to stabilization and relocalization of β-catenin to the nucleus and subsequent transcriptional activity, including upregulation of Myc and Cyclin D1.

Pak1 has been shown to inhibit apoptosis, and this function is thought to be mediated by direct phosphorylation of BAD, by indirect phosphorylation of BAD via c-Raf, and also by activation of the NFκB pathway (9, 10). In addition, Pak1 signaling has been identified as a component of the DNA damage response, influencing cellular sensitivity to ionizing radiation (11).

While activating point mutations in the *PAK1* gene have not been reported in human cancer, Pak1 is overexpressed in many malignancies, most often due to chromosomal amplification of genes within the 11q13 region (12–14). Pak1 can also be hyperactivated by mutations in upstream regulators such as Rac or its exchange factors (NR 3–6). Changes to Pak1 mRNA, protein and/or activity in human malignancies, generally positively correlated with advanced tumor grade and decreased survival. In breast and ovarian cancer, amplification of 11q13 is associated with poor prognosis (13, 14). Genetic or pharmacologic inhibition of Pak1 has been reported to decrease proliferation and migration in different human cancer cells *in vitro* and to reduce tumor growth in animal models. Importantly, it has been shown that inhibition or deletion of group I Paks can block transformation by oncogenic forms of Kras, ErbB2, and KSHV in animal models (15–17).

Several studies of 11q13-amplified cells reported that cells with upregulated Pak1 showed marked sensitivity to Pak1 siRNA (12, 18). In this study, we first determined the effect of Pak1 knock-down on the growth, motility and signaling of human ovarian cancer cells with and without amplified 11q13. As Pak1 has important scaffolding functions that are independent of its kinase activity, we also used newly described selective Pak small molecule inhibitors to assess if *PAK1*-amplified ovarian cancer cells show enhanced sensitivity to such agents. We found that ovarian cancer cells with amplified/overexpressed Pak1 were significantly more sensitive to genetic and pharmacologic inhibition of Pak1 *in vitro* and *in vivo*. Thus, the presence of *PAK1* amplification might serve as a useful patient selection criterion for designing clinical trials of anti-Pak1 agents.

Results

Pak1 expression in ovarian cancer

To investigate the roles of Pak1 in growth of ovarian cancer cells, several different human ovarian cancer cell lines were evaluated for PAK1 mRNA and protein expression (Fig. 1A and B). Pak1 was expressed almost in all ovarian cancer cell lines, with the exception of ES-2. The highest levels of Pak1 were observed in the OVCAR-3 and OV-90 cell lines, which are known to have an amplification of the 11q13 region (19).

To examine the effect of Pak1 loss in ovarian cancer cell lines, cells with or without the 11q13 amplification were transiently transfected with scrambled, Pak1, or Pak2 specific siRNA, and the cells were then assessed for proliferation and migration. Knockdown of Pak1 was efficient, in accord with our previous studies with this siRNA pool (6). The proliferation rate was evaluated during 120 h of growth after siRNA transfection and the number of attached cells was measured every hour using an xCELLigence device. Similarly, the migration ability of transfected cells was evaluated hourly for 72 h after transfection.

Pak1 knockdown was accompanied by a decreased rate of proliferation (5– to 8-fold, $p < 0.0001$), Fig. 1B) and migration (Fig. 1) in OV-90 and OVAR-3 cells, which bear an 11q13 amplification, but had no significant effect in SKOV3 cells, which do not bear this amplification (Fig. 1C and 1D). In contrast, knockdown of Pak2 had no significant effect in any of these cell lines (Supplemental Fig. S1A).

To investigate the effect of long-term Pak1 downregulation in ovarian cancer cells, we used a doxycycline inducible short hairpin RNA (shRNA) to reduce Pak1. OVCAR-3, OV-90, and SKOV-3 cells were stably transduced with either empty virus or a virus encoding a Pak1 shRNA construct. Upon addition of doxycycline, shRNA-transduced cells displayed a 75–80% loss of Pak1 protein (Fig.1F). Depletion of Pak1 in OVCAR-3 cells resulted in 2.3- fold inhibition of cell proliferation (cyQuant assay, Supplemental Fig. S1B) and reduced cell migration (wound healing assay, Fig. 1E), compared with corresponding cells without doxycycline induction. Similar results were observed in the OV-90 cell line, in which Pak1 inhibition by shRNA caused 3.2-fold inhibition of cell proliferation (Supplemental Fig. S1B) and reduced cell migration (Fig. 1E), whereas Pak1 depletion in SKOV-3 cells did not affect cell proliferation. These results suggest that Pak1 is required for efficient growth and migration of ovarian cancer cells that express high levels of this protein, as in 11q13 amplified ovarian cancer cells.

Molecular mechanisms and pathways affected by Pak1 in ovarian cancer in vitro

To assess the mechanism by which Pak1 contributes to cellular proliferation, we investigated the effects of Pak1 loss on cell cycle distribution. The cell cycle distribution of OVCAR-3 cells was analyzed by the incorporation of PI using a Guava cell cycle assay. These experiments revealed that Pak1 knockdown induced a significant increase in the number of cells in G2/M phase (Fig. 2A). In contrast, Pak1 knockdown had a milder effect on cell cycle in OV-90 cells, characterized by a slightly larger G1 population.

Next, we evaluated levels and activity of proteins that regulate the cell cycle (Fig. 2B). Knockdown of Pak1 in OVCAR-3 cell line led to upregulation of phospho-ATM, CHK1, and p53. As p53 and its transcriptional target $p21^{Cip}$ can induce cell cycle arrest in the G2/M phase by impeding transcription of cyclin B1 (20), we assessed cyclin B1 levels by western blot and found that they were decreased (Fig. 2A).

Because Pak1 also regulates expression of genes in the DNA damage response pathway (11), we examined whether Pak1 knockdown was associated with DNA double strand breaks by monitoring Ser-139 phosphorylation of H2A.X, which is a common marker of such damage (21, 22). Western blot studies clearly showed upregulation of phospho-H2A.X in Pak1 knockdown OVCAR-3 cells, and, to a lesser extent, in OV90 cells (Fig. 2A). As shown in Fig. 2B, immunofluorescence analysis further confirmed Ser-139 phosphorylation of H2A.X and an increase in the formation of γH2A.X foci in Pak1 knockdown OVCAR-3 cells.

Pak1 loss in OV-90 cells resulted to arrest in G1 phase, and, while we observed slight upregulation in phospho-p53 and p21 protein levels, these changes were not associated with downregulation of cyclin B1. Nevertheless, we found that phosphorylation of BRCA1, which is also involved in DNA damage response, was enhanced. It has been shown that BRCA1 can bind and downregulate pRb1 (23), and we found that Pak1 inhibition in OV-90 cells was associated with downregulation of pRb1 and cyclin D. Thus, in both Pak1-amplified cell lines, Pak1 knockdown had cytostatic effects, but the mechanisms were not identical in OVCAR-3 *vs.* OV-90 cells.

Effects of Pak1 inhibition in vivo

To confirm our *in vitro* results, the role of Pak1 in tumor growth was evaluated using an OV-90 xenograft model (Fig. 3). A doxocyclin regulated shRNA to Pak1 was introduced into OV-90 ovarian cancer cells by retroviral transduction (6). GFP-containing colonies were selected to obtain clones with Pak1 knockdown of >80%.

6-weeks old SCID female mice were injected either with control cells or cells with Pak1 shRNA. After tumor establishment, animals were fed with doxycycline-containing food, and tumor growth was monitored for 21–24 d. Inhibition of Pak1 in OV-90 tumors significantly slowed tumor growth compare to control mice ($p < 0.0001$) (Fig. 3). Furthermore, analysis of xenograft tumors by immunohistochemistry revealed a substantial decrease in K_i-67–positive tumor cells in Dox-treated tumors expressing shPak1 compared with controls (Fig. 3*C*). The proportion of K_i-67–positive nuclei was quantified, and the anti-proliferative effect of Pak1 knockdown *in vivo* was shown to be statistically significant (OV-90 control 59 ± 5%, OV-90 shPak1 46 ± 3%, $p < 0.01$). Interestingly, when tested using OVCAR-3 cell line, transduction of the Pak1 hairpin did not reduce tumor growth. However, we found that all tumors recovered from such xenografted animals expressed high levels of Pak1. Similar results with respect to the difficulty of maintaining stable Pak1 knockdown have been reported by Yi *et al* (24).

Together, these findings show that Pak1 is critically important not only for proliferation of Pak1-overexpressing ovarian cancer cells *in vitro* but also for tumor growth *in vivo*.

Effects of Pak1 small molecule inhibitors

Since genetic silencing of Pak1 reduces or eliminates the entire Pak1 protein, we also asked if pharmacologic inhibition of Pak1 kinase activity would have similar effects on the growth of ovarian cancer cells. We used two types of Pak inhibitors: PF3758309, which inhibits both group I and II Paks (25), and FRAX-597, -716, or -1036, which are specific for group I Paks (26). As shown in Fig. 4, treatment of ovarian cancer cell lines with these Pak small molecules for 72 h dramatically impaired cellular proliferation. Ovarian cancer cell lines with upregulated Pak1 level (OV-90 and OVCAR-3) were more sensitive for Pak1 inhibition compare to SKOV-3 cell line (Fig. 4). We also analyzed an larger series of 11q13 plus and minus ovarian cancer cell lines and found a similar correlation between Pak1 expression levels and sensitivity to FRAX-1036 (Supp. Fig. S2).

As expected, OVCAR-3 and OV-90 cells treated with Pak1 inhibitors showed significant decrease of phospho-Pak1 level and decreased phosphorylation of Mek and Erk. Interestingly, Pak1 or Pak2 RNAi mediated knockdown did not lead to significant changes in Mek and Erk phosphorylation (data not shown). These data suggests that Pak1 and Pak2 may share redundant functions with respect to the Mek/Erk signaling pathway. OVCAR-3 cell treated with FRAX-1036 (the most specific Pak inhibitor) showed upregulation of p53 and p21 and downregulation of cyclin B1, similar to results obtained with Pak1 RNAi mediated knockdown (Fig. 2).

Pharmacologic inhibition of PAK1 in vivo

To determine the effect of FRAX-1036 on tumor growth *in vivo*, we used xenograft model of OV-90, OVCAR-3 and SKOV-3 cell lines. Tumor progression was monitored twice a week for each animal. Three weeks after injection, animals were enrolled randomly into control (vehicle only) or drug-treated groups (50 mg/kg, oral, once daily) for a period of 14 days. Analysis of the tumor growth for the animals in two groups demonstrated a significantly slower tumor growth rate in FRAX1036-treated mice compared with control mice for OV-90 and OVCAR-3 xenografts, and had no effect in SKOV-3 xenografts (Fig. 5*B*). After 14 days of treatment the animals were sacrificed and the tumors excised and weighed. The FRAX-1036-treated cohort showed significantly lower average tumor weight compared with the control cohort (0.32 g *versus* 0.97 g, and 0.45 g versus 0.29 g, $p = 0.0001$ for OV-90 and OVCAR-3, correspondently). Taken together, these data demonstrate that FRAX-1036 has a significant anti-proliferative activity against ovarian cancer cells with elevated Pak1 level *in vitro* and anti-tumor activity *in vivo*.

Discussion

About 25% of ovarian cancers are characterized genetically by amplification of chromosomal region 11q13. A number of candidate oncogenes reside in this region and may contribute to the growth, survival, and spread of ovarian cancer cells. It has been shown that 11q13.5 amplification is associated with shorter overall survival in ovarian cancer patients (27). Recently, one of the genes within this amplicon, *RSF1* (also known as *HBXAP*) was found to be involved in paclitaxel resistance (27). Other nearby genes, including *CCND1, GAB2*, and *EMSY*, have also been suggested to play roles in the genesis and maintenance of

ovarian cancer, and it is likely that several genes in the 11q13 amplicon contribute to tumorigenesis (13).

The gene for p21-activated kinase 1 (*PAK1*), which resides at 11q13.5, has been proposed as a potential oncogenic driver in ovarian cancer and other cancers that contain this amplicon. It has been shown by IHC staining that PAK1 is overexpressed in more than 50% of ovarian carcinomas (28) and correlated with poor overall survival, but rigorous tests for the contribution of *PAK1* to the genesis or maintenance of ovarian cancer are lacking. Also, it is important to note that there is a strong correlation between PAK1 gene copy number and Pak1 mRNA expression levels (12, 29).

In this study we show that ovarian cancer cell lines with 11q13 amplification and elevated Pak1 levels are more sensitive for PAK1 inhibition either by RNAi mediated/shRNA mediated knockdown or by small molecule inhibitors. Loss of Pak1 in these cells led to decreased proliferation *in vivo* and *in vitro*. These observations are in keeping with previous studies showing a crucial role for Pak1 in cancer cell proliferation and survival. Knockdown of Pak1 inhibited the anchorage-dependent (18) and -independent (12) growth of breast cancer cells, the proliferation of NSCLC cancers *in vitro* and *in vivo* (18), colon cancer cell growth *in vitro* (30) and *in vivo* (31), and the growth of gastric cancer cells anchorage-dependently and -independently as well as tumor xenografts (32). In previous studies, gene amplification or increased protein expression Pak1 has been found to correlate with dependence on this pathway for tumor progression(12, 18).

Pak1 has been shown to phosphorylate Mek1 on serine 298 (33). However, in OVCAR-3 cancer cells, inhibition of cell proliferation induced by knockdown of Pak1 did not correlate with consistent reduction in phosphorylation of Mek or Erk. These results suggest that in these ovarian cancer cells, Pak1 promotes tumor cell proliferation independently of the Raf/Mek/Erk pathway. Remarkably, we found that knockdown of Pak1, but not Pak2, led to G_2/M cell cycle arrest, accompanied by activation of p53, strongly increased expression of $p21^{cip}$, and reduced expression of cyclin B1, consistent with the observed cell cycle effects. These findings were unexpected, as OVCAR-3 cells bear a R248Q mutation in p53 that reduces its ability to bind DNA and activate transcription (34, 35). We speculate that loss of Pak1 leads to phosphorylation and reactivation of this mutant p53 protein, partly restoring its function through a conformational change. In that regard, it is interesting to note that p53 activation and G2/M arrest has been reported in OVCAR3 cells in response to treatment with triptolide (36) or TNF-α and IFN-γ (37).

In contrast to OVCAR-3, Pak1 knockdown in OV-90 led to a delay in G1 transit, rather than a G2/M arrest. The p53 S215R mutation in this cell line, like p53 R248Q, is associated with defective transcriptional activity. However, the degree of p53 activation in response to Pak1 knockdown was much less than in OVCAR-3 cells (Fig. 2A and 2C). Interestingly, in OV-90 cells, Pak1 knockdown was associated with activation of the DNA damage response protein, BRCA1 (Fig. 2C). BRCA1 has several Rb binding sites (38, 39) and overexpression of wild-type BRCA1 inhibits expression of pRb and pRb family (p107 and p130) proteins (23). In this work, we confirmed that Pak1 inhibition in OV-90 cells is associated with phospho-

BRCA1 upregulation and pRb and cyclin D downregulation, consistent with the observed G1 delay.

Previously, we reported that treatment of breast cancer mouse xenografts (6) and K-ras-driven skin cancer mouse model with either the pan-Pak inhibitor PF3758309 or the group I-specific Pak inhibitor FRAX-597 decreased tumor growth (40). In the present study, we investigated FRAX-597, -716, and -1036, a series of increasingly specific group I Pak small-molecule inhibitors (26). All three group I Pak inhibitors decreased proliferation and survival of the ovarian cancer cells OV-90 and OVCAR-3 *in vitro*. FRAX-1036, the most specific Pak1 inhibitor (26), was also effective in inhibiting tumor growth *in vivo*. These results support earlier studies with Pak inhibitors (41, 42) and, together with our shRNA data, suggest that inhibition of Pak1 could be an effective approach for suppressing the growth of ovarian carcinomas with increased Pak1 expression, such as those characterized by 11q13 amplification.

Materials and Methods

Animal experiments

All animal experiments were approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee (IACUC) and carried out according to NIH-approved protocols in compliance with the guide for the Care and Use of Laboratory Animals.

Statistical analysis

Statistical analysis was conducted using the unpaired Student *t* test. Values of $P < 0.05$ were considered significant.

Cell lines, cell culture

OVAR-3, OV-90 and SKOV-3 cell lines were acquired from the American Type Culture Collection (ATCC), authenticated, tested for mycoplasma contamination, and maintained in early passages, no more than 6 months after receipt from the ATCC. Cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 95% air/5% CO_2.

Real-time qRT-PCR

The level of *PAK1* gene expression was measured using qRT-PCR as previously described (6). Primers and TaqMan probes for GAPDH and PAK1, and TaqMan master mix were from Applied Biosystems.

Western blot analysis

Following the experimental treatment, Western blot analysis were performed as previously described (6). Immunoblot analyses were carried out on lysates extracted from cells or tumors. Protein concentration was determined, and equal amounts of total proteins were separated on SDS-PAGE. Antibodies used included total (SCT #2602) and phospho-Pak1(SC #2606), Mek (SCT#9121), phospho-Mek pSer298(SCT#9128), Erk (SCT#9102), phospho-Erk1/2 (pThr202/pTyr204) (SCT#9101), phospho-ATM, phospho-ATR, phospho-

Chk1, phospho-Chk2, phospho-p53, $p21^{Cip}$ SCT DNA damage sampler kit, #9947), cyclin B(SCT#4128), cyclin D1(SCT#2978), GAPDH (SCT#4138) were from Cell Signaling Technology.

Transient transfection

Cells were plated at a density of 5×10^5 cells in 6-well dishes and incubated for 4 hours. The cells were transiently transfected with siRNA smartpool oligonucleotides (10 nM) targeting Pak1 or Pak2 (Dharmacon) using RNAiMax (Invitrogen) according to the manufacturer's instructions. Non-target siRNA was used as a control.

Retroviral transductions

An inducible shRNA-bearing retrovirus against Pak1 was previously described (6) and oligonucleotide used in this study are as follows: Pak1 shRNA-1 5'-GAT CCCCGA AGA GAG GTT CAG CTA AAT TCA AGA GAT TTA GCT GAA CCT CTCTTC TTT TTT GGA AA-3'; the ΦNX packaging cell line (Orbigen) was transfected using Lipofectamine 2000 (Invitrogen). Viral supernatants were harvested 48 hr post-transfection and filtered. Ovarian cancer cells were incubated with retroviral supernatant supplemented with 4 μg/ml polybrene for 4 h at 37°C, and then were cultured in growth media for 48 h for viral integration. Green fluorescent protein (GFP)-positive infected cells were selected by flow cytometry (GFP).

Cell viability assay

Cells were plated at 4×10^3 cells per well in 96-well plates overnight and treated with various concentrations of Pak inhibitors for 72 h. Cell viability was measured by Alamar assay and the half maximal inhibitory concentration (IC_{50}) was calculated.

Cell cycle distribution

For cell cycle profiles, cells were transfected with PAK1 siRNA. 72 h after transfection cells were collected, washed, and fixed in 70% ethanol. After fixation, cells were resuspended in 0.5 ml of a Guava Cell cycle solution (1M Tris-HCl pH 8.0, 0.1% Nonidet P-40, 10 mM NaCl, 50 μg/ml propidium iodide and 70 Kunitz units/mlRNase A). DNA content was analyzed using Guava software (Millipore). A minimum of 10,000 events was collected per sample.

Proliferation and Migration assays

Cell proliferation was assessed using E-16-well plates and the xCELLigence technology (Acea Bioscience, San Diego, CA, USA, distributed by Roche Diagnostics) (43). Briefly, cells (4×10^3 OVCAR-3 cells/well) were transfected with non-target or target siRNA (Dharmacon) with RNAiMax (Invitrogen) using reverse transfection protocol in E-16-well. Cell growth was monitored for 72 h. Microelectrodes, placed on the bottom of plates, were used to detect impedance changes proportional to the number of adherent cells and expressed as the cell index. The impedance value of each well was automatically monitored by the xCELLigence system and expressed as a cell index value. The experiments were conducted in triplicate and repeated twice.

Cell migration assays were performed using an xCELLigence system (CIM-plates). Cell were transfected (1×10^4 in OPTI-MEM medium) with non-target or PAK1 siRNA and cultured for 72 hours. 10^5 cells were seeded in the upper chamber of the CIM-plates in serum-free media. The lower chamber was filled with RPMI-1640 medium containing 10% FBS. Cell index as acquired by the software was set to 100% migration after flattening of the slope. Experiments were performed in triplicate. The rate of cell migration was monitored in real-time with the xCELLigence system for duration of 48 h and expressed as a CI value.

Immunofluorescence analysis

cells were fixed in 4% paraformaldehyde at room temperature for 15 min, and processed as described. Confocal analyses were performed with a Nikon TE2000 confocal microscopy system.

Wound-healing assay

Cell monolayers were scratched using a 200-μL pipette tip after the cells had reached 70% confluency in RPMI-1640 medium supplemented with 1% FBS. Photomicrographs were then taken at ×100 magnification with an EVOS fluorescence microscope (EVOS).

Tumor xenografts in SCID mice: FRAX-1036 treatment

Six-week-old female SCID mice were injected with 5×10^6 OVCAR-3, OV-90 or SKOV-3 cells into the flank, and tumors were allowed to develop. Upon identification of a palpable tumor (minimal volume of 150–200 mm^3), mice were randomly divided into 2 groups (10 mice in each group). Vehicle or drug (30 μg/kg body weight) was administered via oral gavage every day for 21 days.

PAK1 stable knockdown

Six-week-old female SCID mice were injected with 5×10^6 OV-90 or shPAK1-OV-90 cells into the flank, and tumors were allowed to develop. 3 weeks after injection mice were randomly divided into 4 groups. Control mice were fed a diet lacking doxycycline, while a doxycycline diet was given to a similar number of mice from each cohort.

Tumor length (L) and width (W) were measured with a caliper and tumor volumes were calculated with the formula $(L \times W^2)/2$. At the end of the treatment period, the animals were euthanized and the tumors were used for biochemical studies.

Tissue preparation, histology, immunohistochemistry

All tumors were fixed overnight in 4% paraformaldehyde, dehydrated and embedded in paraffin. Hematoxylin and eosin (H&E) stained sections were used for diagnostic purposes and unstained sections for immunohistochemical (IHC) studies. IHC was conducted with rat monoclonal antibody for Ki67 (Dako). The evaluation of the IHC was conducted blindly, without knowledge of the origin or genotype. The percentage of Ki67 positive cells was determined by scanning the slides using an Aperio CS Scanscope scanner and nuclear detection software from the same manufacturer (2,000 to 5,000 cells were counted per mouse, 3 to 5 mice/group).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Radu M, Semenova G, Kosoff R, Chernoff J. PAK signalling during the development and progression of cancer. Nature reviews Cancer. 2014; 14(1):13–25. [PubMed: 24505617]
2. Arias-Romero LE, Chernoff J. A tale of two Paks. Biology of the cell / under the auspices of the European Cell Biology Organization. 2008; 100(2):97–108.
3. Prudnikova TY, Rawat SJ, Chernoff J. Molecular Pathways: Targeting the Kinase Effectors of RHO-Family GTPases. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015; 21(1):24–29. [PubMed: 25336694]
4. Wang Z, Fu M, Wang L, Liu J, Li Y, Brakebusch C, et al. p21-activated kinase 1 (PAK1) can promote ERK activation in a kinase-independent manner. The Journal of biological chemistry. 2013; 288(27):20093–20099. [PubMed: 23653349]
5. Wang Z, Pedersen E, Basse A, Lefever T, Peyrollier K, Kapoor S, et al. Rac1 is crucial for Ras-dependent skin tumor formation by controlling Pak1-Mek-Erk hyperactivation and hyperproliferation in vivo. Oncogene. 2010; 29(23):3362–3373. [PubMed: 20383193]
6. Arias-Romero LE, Villamar-Cruz O, Huang M, Hoeflich KP, Chernoff J. Pak1 kinase links ErbB2 to beta-catenin in transformation of breast epithelial cells. Cancer research. 2013; 73(12):3671–3682. [PubMed: 23576562]
7. He H, Shulkes A, Baldwin GS. PAK1 interacts with beta-catenin and is required for the regulation of the beta-catenin signalling pathway by gastrins. Biochimica et biophysica acta. 2008; 1783(10): 1943–1954. [PubMed: 18515095]
8. Higuchi M, Onishi K, Kikuchi C, Gotoh Y. Scaffolding function of PAK in the PDK1-Akt pathway. Nature cell biology. 2008; 10(11):1356–1364. [PubMed: 18931661]
9. Schurmann A, Mooney AF, Sanders LC, Sells MA, Wang HG, Reed JC, et al. p21-activated kinase 1 phosphorylates the death agonist bad and protects cells from apoptosis. Molecular and cellular biology. 2000; 20(2):453–461. [PubMed: 10611223]
10. Tran NH, Frost JA. Phosphorylation of Raf-1 by p21-activated kinase 1 and Src regulates Raf-1 autoinhibition. The Journal of biological chemistry. 2003; 278(13):11221–11226. [PubMed: 12551923]
11. Motwani M, Li D-Q, Horvath A, Kumar R. Identification of Novel Gene Targets and Functions of p21-Activated Kinase 1 during DNA Damage by Gene Expression Profiling. PloS one. 2013; 8(8):e66585. [PubMed: 23950862]
12. Shrestha Y, Schafer EJ, Boehm JS, Thomas SR, He F, Du J, et al. PAK1 is a breast cancer oncogene that coordinately activates MAPK and MET signaling. Oncogene. 2012; 31(29):3397–3408. [PubMed: 22105362]
13. Brown LA, Kalloger SE, Miller MA, Shih Ie M, McKinney SE, Santos JL, et al. Amplification of 11q13 in ovarian carcinoma. Genes, chromosomes & cancer. 2008; 47(6):481–489. [PubMed: 18314909]
14. Lundgren K, Holm K, Nordenskjold B, Borg A, Landberg G. Gene products of chromosome 11q and their association with CCND1 gene amplification and tamoxifen resistance in premenopausal breast cancer. Breast cancer research : BCR. 2008; 10(5):R81. [PubMed: 18823530]

15. Arias-Romero LE, Villamar-Cruz O, Pacheco A, Kosoff R, Huang M, Muthuswamy SK, et al. A Rac-Pak signaling pathway is essential for ErbB2-mediated transformation of human breast epithelial cancer cells. Oncogene. 2010; 29(43):5839–5849. [PubMed: 20711231]
16. Dadke D, Fryer BH, Golemis EA, Field J. Activation of p21-activated kinase 1-nuclear factor kappaB signaling by Kaposi's sarcoma-associated herpes virus G protein-coupled receptor during cellular transformation. Cancer research. 2003; 63(24):8837–8847. [PubMed: 14695200]
17. Tang Y, Chen Z, Ambrose D, Liu J, Gibbs JB, Chernoff J, et al. Kinase-deficient Pak1 mutants inhibit Ras transformation of Rat-1 fibroblasts. Molecular and cellular biology. 1997; 17(8):4454–4464. [PubMed: 9234703]
18. Ong CC, Jubb AM, Haverty PM, Zhou W, Tran V, Truong T, et al. Targeting p21-activated kinase 1 (PAK1) to induce apoptosis of tumor cells. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108(17):7177–7182. [PubMed: 21482786]
19. Lambros MB, Fiegler H, Jones A, Gorman P, Roylance RR, Carter NP, et al. Analysis of ovarian cancer cell lines using array-based comparative genomic hybridization. The Journal of pathology. 2005; 205(1):29–40. [PubMed: 15586366]
20. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. Nature reviews Cancer. 2009; 9(6):400–414. [PubMed: 19440234]
21. de Feraudy S, Revet I, Bezrookove V, Feeney L, Cleaver JE. A minority of foci or pan-nuclear apoptotic staining of gammaH2AX in the S phase after UV damage contain DNA double-strand breaks. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107(15):6870–6875. [PubMed: 20351298]
22. Fernandez-Capetillo O, Lee A, Nussenzweig M, Nussenzweig A. H2AX: the histone guardian of the genome. DNA repair. 2004; 3(8–9):959–967. [PubMed: 15279782]
23. Fan S, Yuan R, Ma YX, Xiong J, Meng Q, Erdos M, et al. Disruption of BRCA1 LXCXE motif alters BRCA1 functional activity and regulation of RB family but not RB protein binding. Oncogene. 2001; 20(35):4827–4841. [PubMed: 11521194]
24. Yi C, Wilker EW, Yaffe MB, Stemmer-Rachamimov A, Kissil JL. Validation of the p21-activated kinases as targets for inhibition in neurofibromatosis type 2. Cancer research. 2008; 68(19):7932–7937. [PubMed: 18829550]
25. Murray BW, Guo C, Piraino J, Westwick JK, Zhang C, Lamerdin J, et al. Small-molecule p21-activated kinase inhibitor PF-3758309 is a potent inhibitor of oncogenic signaling and tumor growth. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107(20):9446–9451. [PubMed: 20439741]
26. Chow HY, Dong B, Duron SG, Campbell DA, Ong CC, Hoeflich KP, et al. Group I Paks as therapeutic targets in NF2-deficient meningioma. Oncotarget. 2014
27. Choi JH, Sheu JJ, Guan B, Jinawath N, Markowski P, Wang TL, et al. Functional analysis of 11q13.5 amplicon identifies Rsf-1 (HBXAP) as a gene involved in paclitaxel resistance in ovarian cancer. Cancer research. 2009; 69(4):1407–1415. [PubMed: 19190325]
28. Davidson B, Shih Ie M, Wang TL. Different clinical roles for p21-activated kinase-1 in primary and recurrent ovarian carcinoma. Human pathology. 2008; 39(11):1630–1636. [PubMed: 18656238]
29. Holm K, Staaf J, Jonsson G, Vallon-Christersson J, Gunnarsson H, Arason A, et al. Characterisation of amplification patterns and target genes at chromosome 11q13 in CCND1-amplified sporadic and familial breast tumours. Breast cancer research and treatment. 2012; 133(2):583–594. [PubMed: 22002566]
30. Huynh N, Liu KH, Baldwin GS, He H. P21-activated kinase 1 stimulates colon cancer cell growth and migration/invasion via ERK- and AKT-dependent pathways. Biochimica et biophysica acta. 2010; 1803(9):1106–1113. [PubMed: 20595063]
31. He H, Huynh N, Liu KH, Malcontenti-Wilson C, Zhu J, Christophi C, et al. P-21 activated kinase 1 knockdown inhibits beta-catenin signalling and blocks colorectal cancer growth. Cancer letters. 2012; 317(1):65–71. [PubMed: 22100495]
32. Liu S, Goldstein RH, Scepansky EM, Rosenblatt M. Inhibition of rho-associated kinase signaling prevents breast cancer metastasis to human bone. Cancer research. 2009; 69(22):8742–8751. [PubMed: 19887617]

33. Park ER, Eblen ST, Catling AD. MEK1 activation by PAK: A novel mechanism. Cellular Signalling. 2007; 19(7):1488–1496. [PubMed: 17314031]
34. Bullock AN, Henckel J, DeDecker BS, Johnson CM, Nikolova PV, Proctor MR, et al. Thermodynamic stability of wild-type and mutant p53 core domain. Proceedings of the National Academy of Sciences of the United States of America. 1997; 94(26):14338–14342. [PubMed: 9405613]
35. Merabet A, Houlleberghs H, Maclagan K, Akanho E, Bui TT, Pagano B, et al. Mutants of the tumour suppressor p53 L1 loop as second-site suppressors for restoring DNA binding to oncogenic p53 mutations: structural and biochemical insights. The Biochemical journal. 2010; 427(2):225–236. [PubMed: 20113312]
36. Wu J, Li QQ, Zhou H, Lu Y, Li JM, Ma Y, et al. Selective tumor cell killing by triptolide in p53 wild-type and p53 mutant ovarian carcinomas. Medical oncology (Northwood, London, England). 2014; 31(7):14.
37. Guan YQ, Li Z, Yang A, Huang Z, Zheng Z, Zhang L, et al. Cell cycle arrest and apoptosis of OVCAR-3 and MCF-7 cells induced by co-immobilized TNF-alpha plus IFN-gamma on polystyrene and the role of p53 activation. Biomaterials. 2012; 33(26):6162–6171. [PubMed: 22682938]
38. Yarden RI, Brody LC. BRCA1 interacts with components of the histone deacetylase complex. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96(9): 4983–4988. [PubMed: 10220405]
39. Aprelikova ON, Fang BS, Meissner EG, Cotter S, Campbell M, Kuthiala A, et al. BRCA1-associated growth arrest is RB-dependent. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96(21):11866–11871. [PubMed: 10518542]
40. Chow HY, Jubb AM, Koch JN, Jaffer ZM, Stepanova D, Campbell DA, et al. p21-Activated kinase 1 is required for efficient tumor formation and progression in a Ras-mediated skin cancer model. Cancer research. 2012; 72(22):5966–5975. [PubMed: 22983922]
41. Hashimoto H, Sudo T, Maruta H, Nishimura R. The direct PAK1 inhibitor, TAT-PAK18, blocks preferentially the growth of human ovarian cancer cell lines in which PAK1 is abnormally activated by autophosphorylation at Thr 423. Drug discoveries & therapeutics. 2010; 4(1):1–4. [PubMed: 22491145]
42. Hashimoto H, Messerli SM, Sudo T, Maruta H. Ivermectin inactivates the kinase PAK1 and blocks the PAK1-dependent growth of human ovarian cancer and NF2 tumor cell lines. Drug discoveries & therapeutics. 2009; 3(6):243–246. [PubMed: 22495656]
43. Limame R, Wouters A, Pauwels B, Fransen E, Peeters M, Lardon F, et al. Comparative analysis of dynamic cell viability, migration and invasion assessments by novel real-time technology and classic endpoint assays. PloS one. 2012; 7(10):e46536. [PubMed: 23094027]

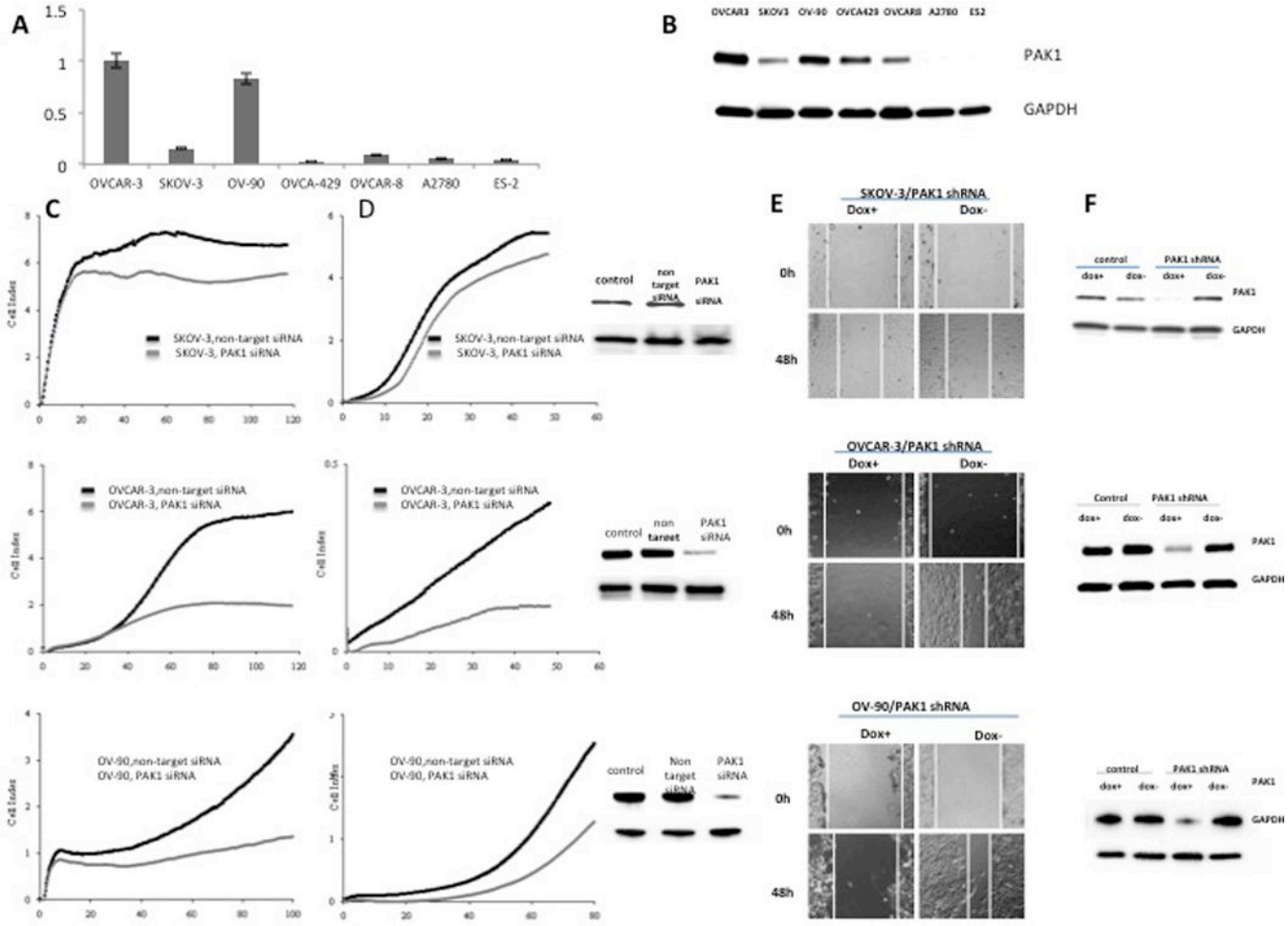

Figure 1.

Pak1 expression in human ovarian cancer cell lines. **A**) The relative expression of Pak1 mRNA was analyzed by Taq-Man Real-Time PCR (values are mean ± SEM). **B)** Pak1 protein levels were determined in different OVCA cell lines by western blot. **C), D)** Proliferation and migration of SKOV-3, OV-90 and OVCAR-3 cell were analyzed using xCELLigence array, Pak1 siRNA mediated knockdown led to decreased proliferation and migration in OV-90 and OVCAR-3 cells and had no effect in SKOV-3 cells. **E)** Wound healing assay for stable Pak1 knockdown. SKOV-3, OV-90 and OVCAR-3 cells bearing bearing shPak1 were grown to 70% confluence and then scratched with 0.2 ml tip. All data are representative of 3 independent experiments.

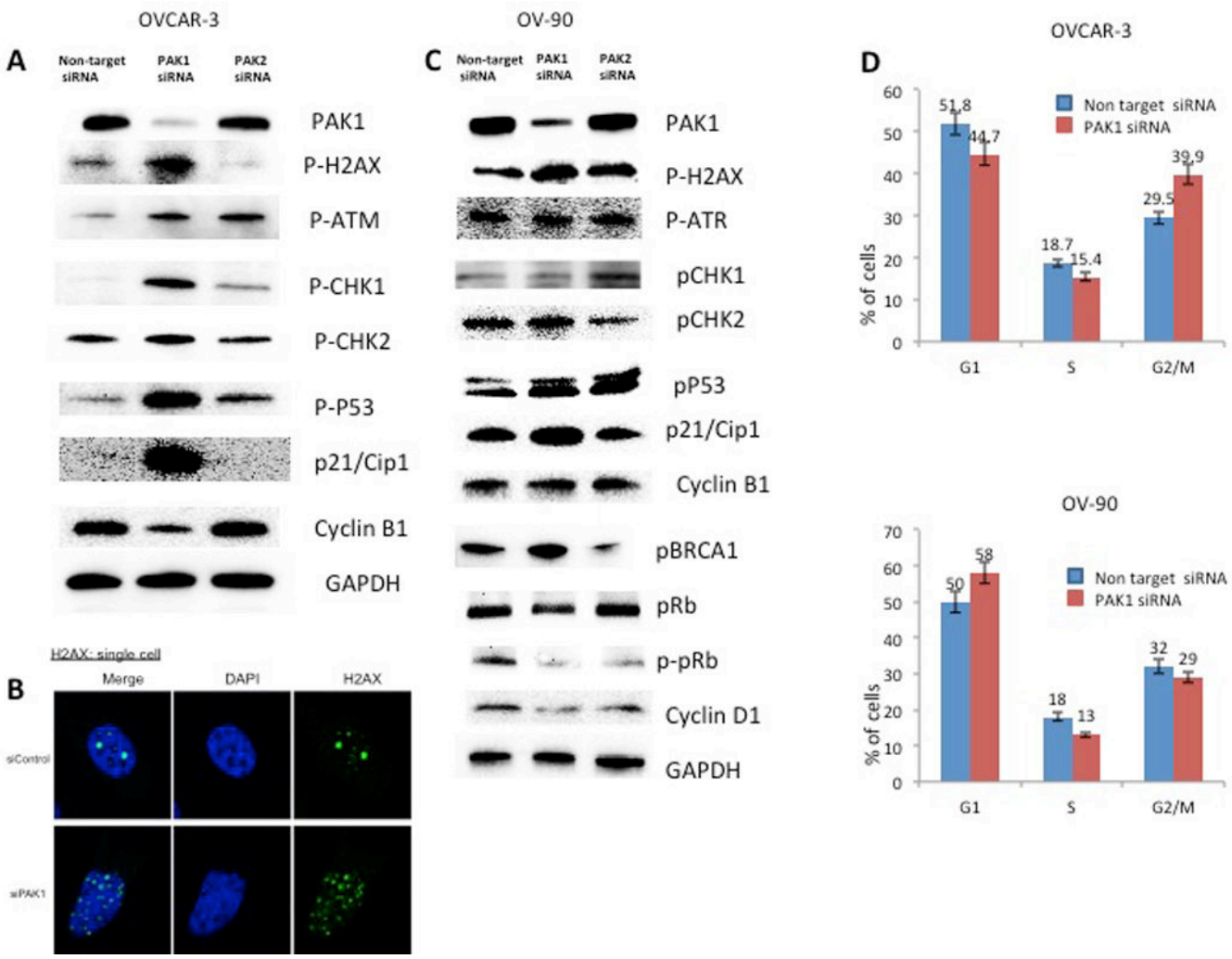

Figure 2.

The effect of Pak1 knockdown on signaling pathways. **A), C)** OVCAR-3 and OV-90 cells were transfected with Pak1 siRNA as described in Materials and Methods. Following SDS/PAGE and transfer to PVDF membranes, expression levels of Pak, pATR, pATM, pCHK1, pCHK2, pp53, $p21^{cip}$, and cyclin B1 were assessed by immunoblot using total and phosphospecific antibodies. GADPH was used as loading control. **B)** Immunofluorescent staining for pH2A.X in OVCAR-3 cells. **D)** Cell cycle distribution of OVCAR-3 and OV-90 cells after RNAi-mediated Pak1 knockdown (values are mean ± SD). All data are representative of 3 independent experiments.

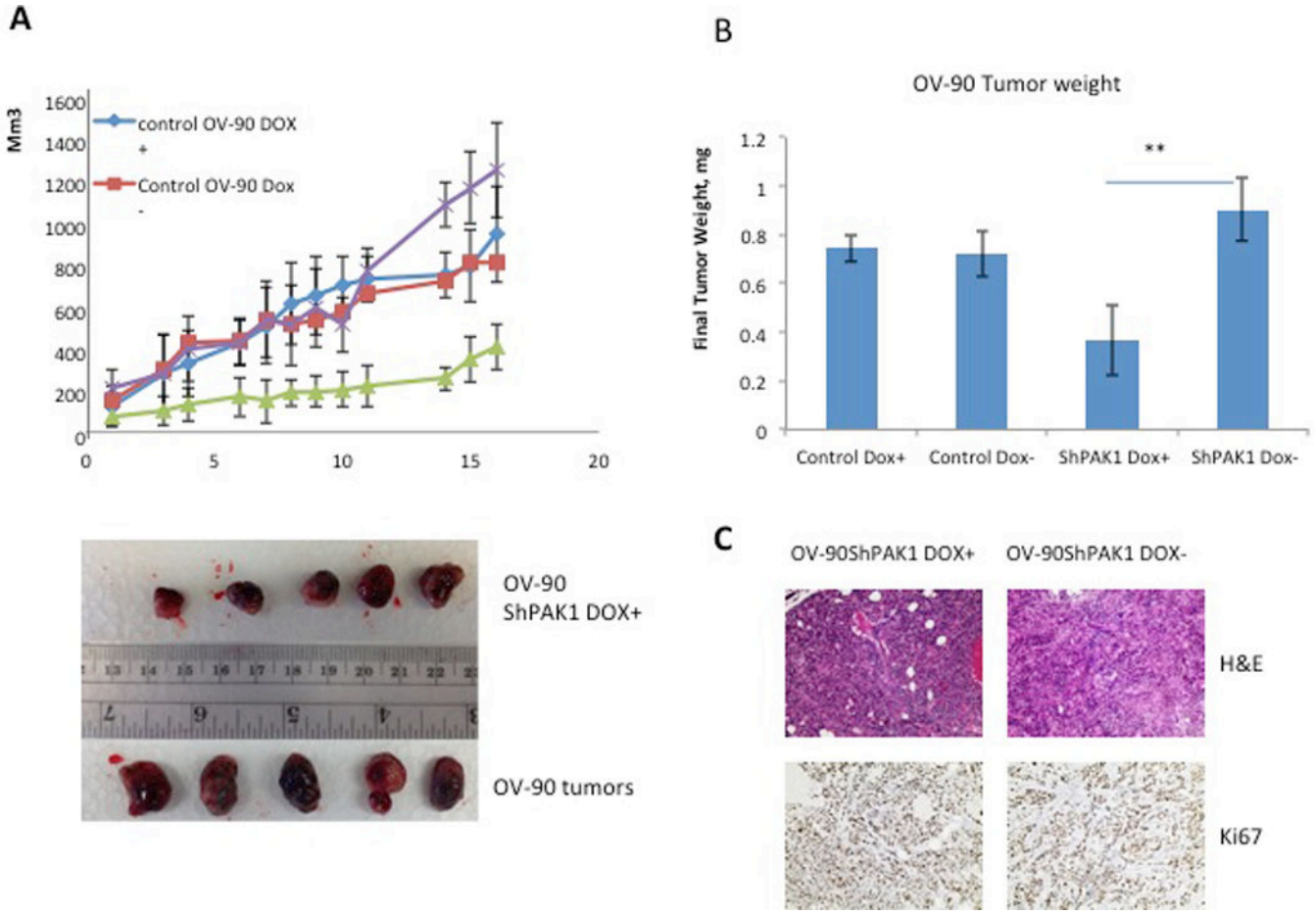

Figure 3.

Genetic Pak1 inhibition *in vivo*. OV-90 control cells or cells harboring shPak1 were subcutaneously injected in mice. After 3 weeks mice were fed with doxycycline diet or normal rodent foods for 2 weeks. **A)** Tumor growth was monitored according to Materials and Methods. **B)** Tumors weight was measured after mice were sacrificed. $^{**}p < 0.005$, t-test **C)** Representative example of tumor sections between Dox+ and Dox-mice, stained with H&E and Ki67.

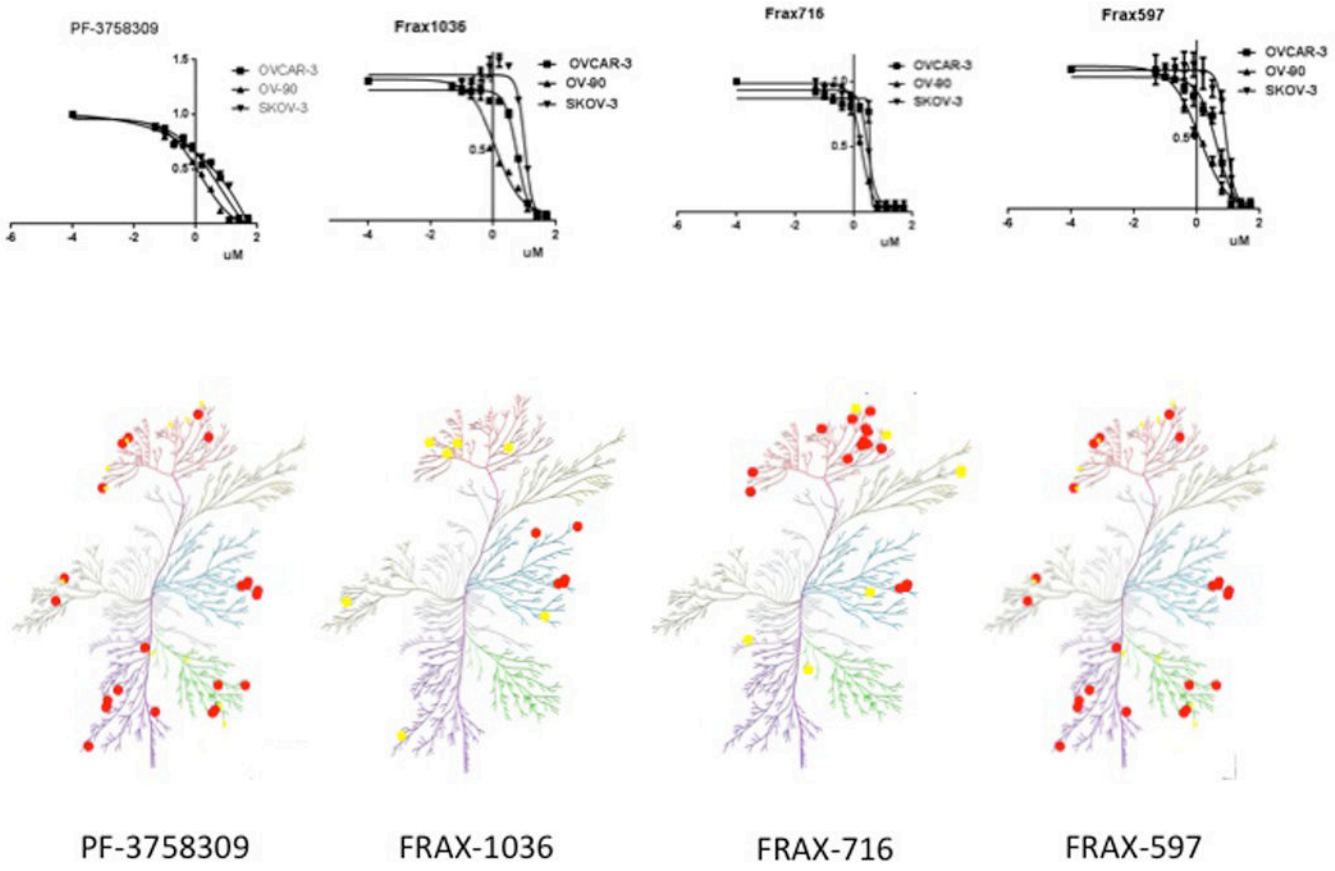

Figure 4.

Effects of Pak small-molecules inhibitors on cell survival. Cell viability analysis of SKOV-3, OV-90, and OVCAR-3 cells after treatment for 72 hours with Pak inhibitors FRAX-597, -716, -1036, or PF3758309 by Alamar assay. All data are representative of 3 independent experiments.

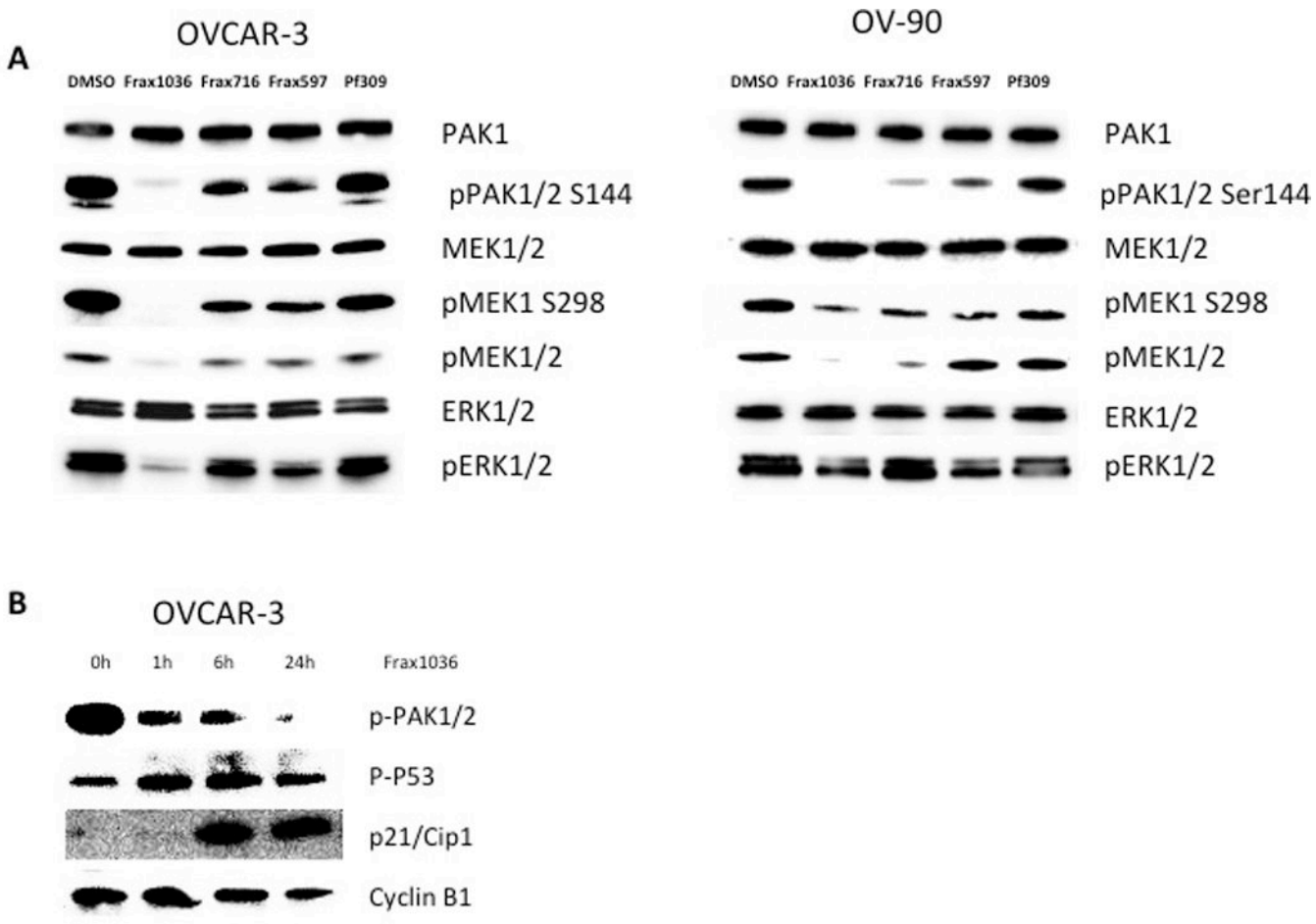

Figure 5.

Effects of Pak inhibitors on signaling pathways. **A)** The effect of Pak inhibitors on Mek/Erk signaling pathways. OVCAR-3 or OV-90 cells were treated with inhibitors for 24 hours. Following SDS/PAGE and transfer to PVDF membranes, expression levels of Pak, Mek, Erk, were assessed by immunoblot using total and phosphospecific antibodies. **B)** Effect of FRAX-1036 on p53, $p21^{cip}$, and cyclin B1 expression in OVCAR-3 cells. OVCAR-3 cells were treated with FRAX-1036 (6 μM) for different timepoints (1 h, 6 h, and 24 h). Following SDS/PAGE and transfer to PVDF membranes, expression levels of Pak, p53, $p21^{cip}$, and cyclin B were assessed by immunoblot using total and phosphospecific antibodies. All data are representative of 3 independent experiments.

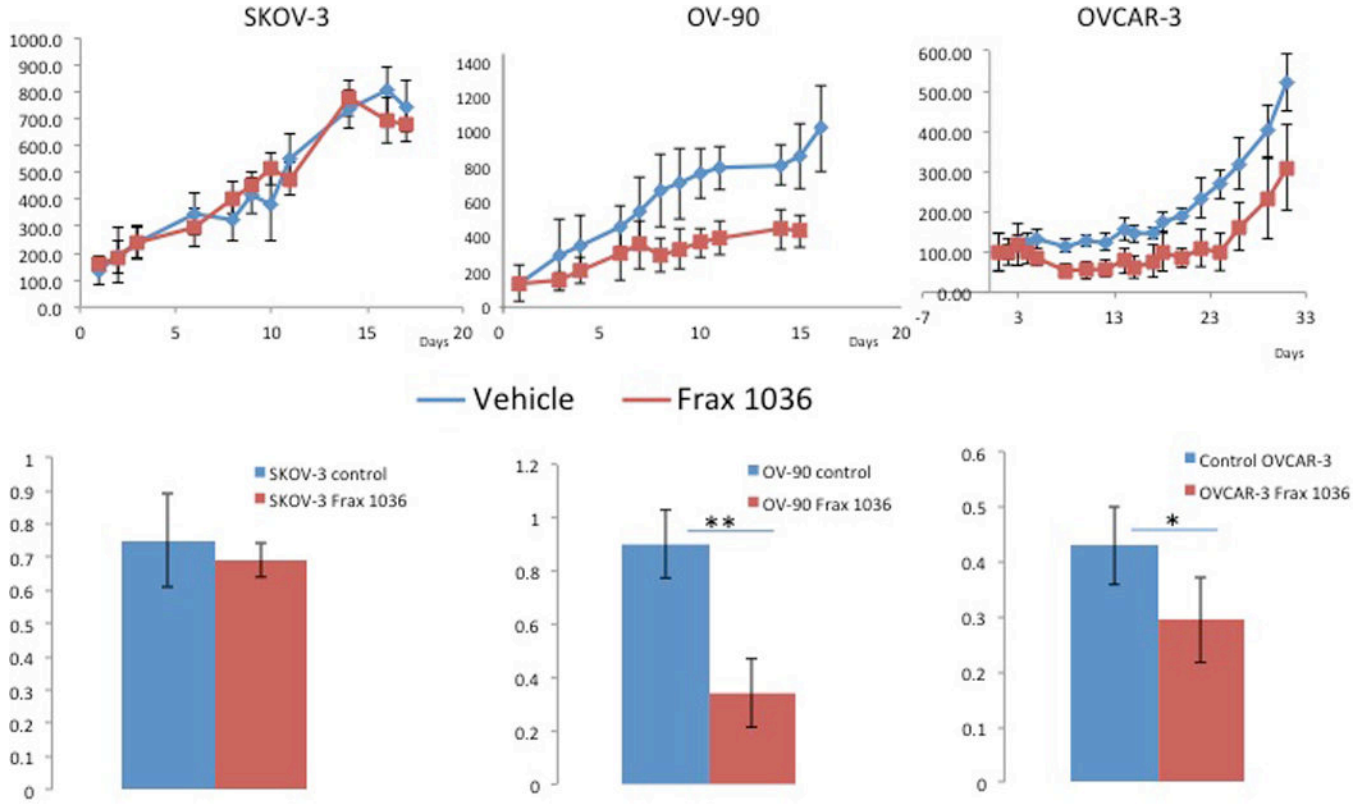

Figure 6.

Effect of Pak inhibition on OV-90, OVCAR-3 and SKOV-3 xenograft growth. OVCAR-3, OV-90 or SKOV-3 cells were injected into the flanks of SCID mice. 3 weeks post-innoculation, the animals were treated with vehicle or inhibitor for 15 days. **A** Volumetric changes in tumor size between untreated mice (vehicle) and mice treated with inhibitor. Tumor growth was monitored according to Materials and Methods. **B)** Tumors weight was measured after mice were sacrificed. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, student *t*-test. NS, not significant.