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Targeting the Akt/mTOR pathway in Brca1-deficient cancers

T Xiang[1], **Y Jia**[1], **D Sherris**[2], **S Li**[3], **H Wang**[4], **D Lu**[5], and **Q Yang**[1]

[1] Department of Radiation Oncology, Washington University School of Medicine, St Louis, MO, USA

[2] Paloma Pharmaceuticals, Jamaica Plain, MA, USA

[3] Department of Medicine and Siteman Cancer Center, Washington University School of Medicine, St Louis, MO, USA

[4] Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, St Louis, MO, USA

[5] Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO, USA

Abstract

The breast cancer susceptibility gene 1 (*Brca1*) has a key role in both hereditary and sporadic mammary tumorigenesis. However, the reasons why *Brca1*-deficiency leads to the development of cancer are not clearly understood. Activation of Akt kinase is one of the most common molecular alterations associated with human malignancy. Increased Akt kinase activity has been reported in most breast cancers. We previously found that downregulation of Brca1 expression or mutations of the *Brca1* gene activate the Akt oncogenic pathway. To further investigate the role of Brca1/Akt in tumorigenesis, we analyzed Brca1/Akt expression in human breast cancer samples and found that reduced expression of Brca1 was highly correlated with increased phosphorylation of Akt. Consistent with the clinical data, knockdown of Akt1 by short-hairpin RNA inhibited cellular proliferation of *Brca1* mutant cells. Importantly, depletion of Akt1 significantly reduced tumor formation induced by *Brca1*-deficiency in mice. The third generation inhibitor of mammalian target of rapamycin (mTOR), Palomid 529, significantly suppressed *Brca1*-deficient tumor growth in mice through inhibition of both Akt and mTOR signaling. Our results indicate that activation of Akt is involved in *Brca1*-deficiency mediated tumorigenesis and that the mTOR pathway can be used as a novel target for treatment of Brca1-deficient cancers.

Keywords

Brca1; PKB/Akt; mTOR

Introduction

Germline mutations of the *Brca1* gene account for 50% of hereditary breast cancers and 85% of the families whose members have a high incidence of breast cancers (Miki *et al.*, 1994; Narod and Foulkes, 2004). Although *Brca1* gene mutations are rare in sporadic breast

Correspondence: Professor Q Yang, Division of Radiation and Cancer Biology, Department of Radiation Oncology, Washington University School of Medicine, 4511 Forest Park, St Louis, MO 63108, USA. qyang@wustl.edu.

Conflict of interest
The authors declare no conflict of interest.

cancers, Brca1 protein expression is frequently reduced or absent in sporadic cases (Thompson *et al.*, 1995), suggesting a much wider role of Brca1 in both hereditary and sporadic mammary tumorigenesis. The Brca1 protein contains an N-terminal ring domain and C-terminal tandem BRCT motifs that are phosphoprotein binding motifs. The BRCT domains are important for the tumor-suppressor function of Brca1. Most *Brca1* mutations result in truncated *Brca1* gene products that lack one or both C-terminal BRCT domains. Clinically relevant missense mutations identified at the C-terminus of Brca1 abolish the structure of BRCT. Loss of the Brca1 BRCT domains leads to tumor formation in mice (Ludwig *et al.*, 2001; Xu *et al.*, 2001; Brodie and Deng, 2001; Deng, 2006). We previously showed that Brca1-deficiency activates the Akt oncogenic pathway (Xiang *et al.*, 2008). Mutation of *Brca1* gene increases the phosphorylation and the kinase activity of Akt. The Brca1-BRCT domains directly bind to phosphorylated Akt (pAkt), leading to its ubiquitination towards protein degradation. *Brca1* mutant cells lacking the BRCT repeats accumulate nuclear pAkt and consequently inactivate the transcriptional activity of FOX-O3a, a main nuclear target of pAkt (Brunet *et al.*, 1999; Tran *et al.*, 2003). Here, we demonstrate that the Brca1/Akt1 pathway contributes to tumorigenesis and mammalian target of rapamycin (mTOR) is a therapeutic target for *Brca1*-deficient cancers.

Results

Up to 40–80% of human breast cancers exhibit decreased expression of Brca1 or increased activation of Akt1 (Thompson *et al.*, 1995; Sun *et al.*, 2001; Narod and Foulkes, 2004; Wickenden and Watson, 2010). These alterations can be detected by immunohistochemical (IHC) analysis in breast cancers with Brca1 antibodies or antibodies against pAkt (S473), respectively. To test whether defects in Brca1 expression correlate with activation of Akt1 in breast cancers, we performed IHC analysis on a panel of human breast cancer samples with antibodies of Brca1 (Ab-1, Oncogene, Cambridge, MA, USA) and pAkt (S473, Cell Signaling Technology (Danvers, MA, USA)) as described (Wilson *et al.*, 1999; Kirkegaard *et al.*, 2005; Puc *et al.*, 2005; Dinesh *et al.*, 2006). Cancer tissue samples were obtained from 101 breast cancer patients who underwent mastectomy or breast conserving surgery (Supplementary Table 1). Competitive peptides of pAkt and Brca1 blocked the immunostaining signals of pAkt and Brca1 antibodies, respectively, confirming the specificity of these antibodies (data not shown). The positive reaction of Brca1 and pAkt were scored into four grades according to the intensity of the staining (0, none; 1+, weakly positive; 2+, moderately positive; and 3+, strongly positive). In all, 0 and 1+ were recorded as negative, and 2+ and 3+ were recorded as positive (Supplementary Figure 1). Sections were independently scored by two investigators with double-blind evaluations. The results showed that about 69% (n=70) of tumor tissue samples had decreased Brca1 staining, whereas about 61% of the 101 specimens displayed positive staining for pAkt (Figure 1a). In total, 71% (50/70) of the samples with reduced expression of Brca1 also displayed increased pAkt, indicating that Brca1 expression inversely correlates with Akt1 activation in human breast cancers and supporting our hypothesis that activation of Akt1 is involved in Brca1-deficient cancer development. Figure 1b showed positive and negative IHC staining samples of Brca1 and pAkt in the same cases. There is no statistical difference of clinical-pathological features between Brca1 and pAkt groups (Supplementary Table 1).

We examined Ki67 expression, a proliferation marker, by IHC and found that 63% (61/97) of the samples showed Ki67 positive staining (Supplementary Table 2). The frequency of Ki67-positive tumors was significantly higher in pAkt-positive (71%, 43/61) and Brca1-negative tumors (77%, 47/61), compared with pAkt-negative (47%, 17/36) and Brca1-positive tumors (56%, 20/36), respectively. These results are consistent with previous reports (Ding *et al.*, 2004; Pallares *et al.*, 2005; Aaltonen *et al.*, 2009; van der *et al.*, 2009; Aleskandarany *et al.*, 2010), suggesting that pAkt-positive and Brca1-negative tumors are

associated with tumor proliferation. It has been suggested that *PIK3CA* mutations activate Akt function through its phosphorylation, thus, we investigated the relationship between *PIK3CA* mutations and the expression of pAkt and Brca1. In all, 44 mutations in *PIK3CA* (exon 9 and 20) were identified out of a total of 99 samples (Supplementary Table 2). The frequency of *PIK3CA* mutations was significantly higher in pAkt-positive tumors (77%, 34/44) than pAkt-negative tumors (23%, 10/44). However, Brca1 negative/pAkt positive tumors were not significantly associated with *PIK3CA* mutations (47%, 23/49) compared with *PIK3CA* wild type (54%, 26/49) (Supplementary Table 2), suggesting that there are different mechanisms for pAkt activation.

Brca1 directly binds to pAkt kinase and down-regulates its activation through the ubiquitination pathway leading to its degradation (Xiang *et al.*, 2008). To investigate the effects of interaction of Brca1/Akt on tumorigenesis, we determined the Akt1 binding domains to Brca1. Akt1 contains three functional domains, the N-terminal pleckstrin homology (PH) domain, the kinase domain (KD) and the hydrophobic motif (HM) domain. To map the Brca1 binding sites of Akt1, we generated HA-tagged Akt1 deletion mutants, including HA-ΔPH (deletion amino acids 6–107), HA-ΔKD (deletion amino acids 149–408) and HA-ΔHM (deletion amino acids 409–476). HA-tagged full-length or deletion mutants of AKT1 were transfected into immortalized mouse embryonic fibroblasts (MEFs). The cell lysates were incubated with the GST-BRCT domain of Brca1. The GST complex was eluted and analyzed by western blotting with an anti-GST or anti-HA antibody. The HA-ΔHM mutant was not present in GST-Brca1 BRCT complex, indicating that the HM domain of Akt1 binds to BRCA1 (Supplementary Figure 2).

Next, we addressed whether inhibition of Akt1 affects cell proliferation. Loss of the Brca1-BRCT domains that bind to pAkt leads to mammary cancer formation in mice (Ludwig *et al.*, 2001). We previously found that MEFs from the mouse expressing a truncated *Brca1* allele lacking the BRCT repeats ($Brca1^{tr/tr}$) showed higher levels of pAkt, compared with $Brca1^{+/+}$ MEFs (Xiang *et al.*, 2008). Using the MEFs, we performed the MTT assays. Depletion of Akt1 by short-hairpin RNAs (shRNAs) (shAkt1-A&B) significantly reduced cell viabilities in $Brca1^{tr/tr}$ MEFs compared with GFP-shRNA control cells (shGFP) or $Brca1^{+/+}$ MEFs (Figure 2a). The pAkt level was markedly decreased in cells stably expressing shAkt1. To further examine the relationship among Akt, Brca1 and proliferation, the colony formation assays in soft agar were performed. Only $Brca1^{tr/tr}$ MEFs, but not $Brca1^{+/+}$ MEFs formed colonies (Figure 2b and data not shown). Depletion of Akt1 by shRNAs (shAkt1-A&B) significantly reduced colony formation in $Brca1^{tr/tr}$ MEFs compared with that of shGFP. Overexpression of Akt1-S473D, a constitutively active form, increased colony formation in $Brca1^{tr/tr}$ MEFs with depletion of Akt1 (Figure 2c). Expression of Akt1-ΔHM mutant that does not have the binding domain to Brca1 did not rescue colony formation by depletion of Akt1. These results indicate that the interaction of Brca1 with Akt1 is responsible for Brca1/Akt1-mediated proliferation.

To test whether depletion of Akt1 inhibits the tumorigenicity associated with Brca1-deficiency, $Brca1^{tr/tr}$ MEFs stably expressing either the shGFP or shAkt1 were implanted into SCID mice, and tumor formation was monitored for 9 weeks. Implantation of control shGFP $Brca1^{tr/tr}$ MEFs resulted in tumor formation in 18 of 20 mice (90%, Figure 3a). Expressions of shAkt1-A and shAKT-B were sufficient to dramatically suppress tumor development and only 5–6 of 20 mice generated tumors, respectively. In addition, depletion of Akt1 also resulted in a striking reduction in tumor size compared with those from shGFP control $Brca1^{tr/tr}$ MEFs (Figure 4a). Western blot and IHC analysis of dissected tumors revealed a marked reduction in pAkt levels in tumors from shAKT1-expressing cells compared with those from shGFP control $Brca1^{tr/tr}$ MEFs (Figures 3b and c). Thus,

depletion of Akt1 in *Brca1* deficient cells results in decrease of pAkt level, arrest of cell proliferation, and, most importantly, suppression of tumor growth.

The mTOR is a critical downstream effector of Akt, which contributes to tumorigenesis (Hay, 2005; Sabatini, 2006; Guertin and Sabatini, 2007; Efeyan and Sabatini, 2010). The third generation mTOR inhibitor, Palomid 529 (P529), is a novel small molecular drug inhibiting the TORC1 and TORC2 complexes as well as the Akt activity (Xue *et al.*, 2008; Diaz *et al.*, 2009). We tested the effects of P529 on *Brca1*-deficient tumors in mice and found that P529 significantly inhibited *Brca1*-deficient tumor growth (Figures 4a and b). Tumor growth in mice (n=18, $Brca1^{tr/tr}$ MEFs with shGFP group) was arrested by treatment with P529 along with actual regression of the tumor size. Treatment of shAkt tumor-bearing mice with P529 also inhibited tumor growth, but with less inhibitory effect compared with those of $Brca1^{tr/tr}$ MEFs induced tumors (shGFP), suggesting that P529 may have better therapeutic effects on tumors with high expressing level of pAkt, such as *Brca1*-deficient tumors. Consistent with this hypothesis, P529 treatment for MCF7 (Brca1 wild type)-induced tumors with knockdown of Akt1 also showed less inhibitory effect compared with those of control (shGFP) or $Brca1^{tr/tr}$ MEFs-induced tumors (Figure 4b). Analyses of tumor lysates by western blotting and IHC assays indicated that P529 reduced the levels of pAkt and pS6 (Figures 4c and d and Supplementary Figure 3), suggesting that P529 inhibits both the pAkt and mTOR signaling.

Discussion

The tumor suppressor Brca1 is frequently inactivated in human breast cancers. However, the extent to which Brca1-activated molecular pathways contribute to its tumor suppressor activity remains unclear. Our preliminary results indicate that downregulation of Brca1 expression or mutations of the *Brca1* gene activate the Akt1 oncogenic pathway (Xiang *et al.*, 2008). Brca1 directly binds to Akt1 kinase and downregulates its activation through the ubiquitination pathway leading to its degradation. Here, we further provide evidence that the Brca1-Akt1 pathway contributes to tumorigenesis and Akt1/mTOR is a novel therapeutic target for Brca1-deficient cancers. In human breast cancers, reduced expression of Brca1 is correlated with increased phosphorylation of Akt. These results support our hypothesis that activation of Akt1 is involved in Brca1-deficiency mediated tumorigenesis.

The Akt/PKB kinase is a well-characterized effector of phosphoinositide 3-kinase, and its hyperactivation has an important role in the pathogenesis of human cancers. Cellular processes that are regulated by Akt1 include cell proliferation and survival, intermediary metabolism, angiogenesis and tissue invasion. We found that knockdown of Akt1 reduces Brca1-deficient cell proliferation. Moreover, the Akt1 mutant lacking the Brca1-binding domain could not rescue this phenotype, suggesting that the interaction of Akt1 with Brca1 regulates cell proliferation mediated by Brca1 deficiency.

Mammalian cells contain three Akt isoforms (Akt1–3). Akt1 is the predominantly expressed Akt isoform, and its deficiency reduced total Akt activity by ~50% (Chen *et al.*, 2006). By targeted disruption of the *Akt1* gene, Chen *et al.* (2001) created an *Akt1*-null mouse model with aberrant signaling in the Akt1 pathway. Recent reports show that partial inhibition of Akt activity or inhibition of individual Akt isoform (Akt1) is sufficient to suppress cancer development (Bellacosa *et al.*, 2005; Chen *et al.*, 2006; Faivre *et al.*, 2006). In $Akt1^{-/-}Pten^{+/-}$ mouse model, a deficiency of *Akt1* is most effective as an inhibitor of neoplasia induced by *Pten* inactivation (Chen *et al.*, 2006). Even haplodeficiency of *Akt1* can significantly attenuate the cancer development induced by *Pten* deficiency. Mice expressing truncated $Brca1^{tr/tr}$, which eliminates the C-terminal half of the protein product (missing the BRCT domain), develop a variety of tumors in about ~85% of the mice

(Ludwig *et al.*, 2001). Loss of the Brca1-BRCT domains that bind to Akt1 leads to mammary cancer formation in mice implicates the potential role of Akt1 in Brca1-related tumorigenesis. We implanted *Brca1*$^{tr/tr}$ MEFs in mice and generated tumors. The depletion of Akt1 in *Brca1*$^{tr/tr}$ MEFs significantly reduces tumor formation, which provides direct evidence that Akt1 is involved in Brca1-deficient cancer formation.

The mTOR is a critical downstream effector of Akt (Hay, 2005; Sabatini, 2006; Guertin and Sabatini, 2007; Efeyan and Sabatini, 2010). The mTOR is associated with increased cell proliferation and is activated by Akt (Hay, 2005; Inoki and Guan, 2006; Lee *et al.*, 2007). Akt activates mTOR through direct phosphorylation of the tuberous sclerosis complex 2, which otherwise inhibits mTOR activity (Inoki *et al.*, 2002, 2005; Hay, 2005). Tuberous sclerosis complex 1 and tuberous sclerosis complex 2 form a heterodimer with GTPase activity that inhibits the activity of Rheb, a small GTPase required for mTOR activation. Upon activation, mTOR, which forms a rapamycin-sensitive complex with Raptor (regulatory-associated protein of mTOR), activates S6-kinase, a downstream substrate of mTOR-Raptor (Hay, 2005; Inoki and Guan, 2006). First and second generation mTOR inhibitors, such as rapamycin, have shown promise to inhibit tumor development. However, because of the complex nature of mTOR signaling, these inhibitors can result in counterproductive feedback signaling to potentiate upstream Akt signaling, leading to minimal tumor inhibition in breast cancer clinical trials (Sarbassov *et al.*, 2005; Guertin and Sabatini, 2009; O'Reilly and McSheehy, 2010). P529 is the third generation mTOR inhibitor and inhibits both the TORC1 and TORC2 complexes as well as the Akt activity (Xue *et al.*, 2008; Diaz *et al.*, 2009). Our results showed that P529 not only significantly suppresses tumor growth, but also reduces the size of tumors with high expressing level of pAkt, such as *Brca1*-deficient tumors, suggesting a better therapeutic effect on Brca1-deficient tumors.

Our results demonstrate that Akt1 has a key role in Brca1-deficient caner development. Reduced expression of Brca1 is correlated with increased phosphorylation of Akt1 in human breast cancers. Interactions of Brca1 and Akt1 mediate cell proliferation. Depletion of Akt1 prevents cancer formation induced by *Brca1*-deficiency in mice. Targeting mTOR by P529 sufficiently suppresses *Brca1*-deficient cancer development. Thus, these results delineate the contributions of Brca1/Akt1 pathway to tumorigenesis and establish the mTOR pathway as novel therapeutic target for Brca1-deficient cancers.

Materials and methods

Cell culture, lentivirus infection and western blot analysis

Brca1$^{+/+}$ and *Brca1*$^{tr/tr}$ MEFs and MCF7 breast cancer cells were cultured as described previously (Xiang *et al.*, 2008). To generate lentiviral particles, 293 T cells were cotransfected with the lentiviral vectors and compatible packaging plasmids mixture using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and the lentivirus supernatant was collected 40 h after transfection. For virus infection, cells were exposed to lentivirus supernatant for 24 h in the presence of polybrene (Sigma, St Louis, MO, USA). Protein extracts from MEFs and xenograft tumors were extracted to conduct western blot analysis as described previously (Xiang *et al.*, 2008).

Antibodies and reagents

Anti-pGSK-3β (no. 9336), anti-Akt antibodies (Akt1 (no. 2938), pAkt^{S473} (no. 4058 and no. 3787)) and anti-S6 ribosomal protein antibodies (S6 (no. 2217) and pS6$^{Ser235/236}$ (no. 4857)) were from Cell Signaling Technology. Anti-Ki67 antibody was from Vector Laboratories (Burlingame, CA, USA), anti-β-actin (no. A5441) antibody was from Sigma and anti-Brca1 antibody (Ab-1, N-terminus) was from Calbiochem (Darmstadt, Germany). The goat anti-

mouse IgG-HRP, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-biotin and goat anti rabbit-IgG-biotin second antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Vectastain ABC kit and DAB substrate kit were from Vector Laboratories. P529 was provided from Paloma Pharmaceuticals Inc. GFP and Akt1 shRNAs were obtained from Sigma.

IHC assay

Human breast cancer tissue samples were obtained from 101 primary breast cancer patients who underwent mastectomy or breast conserving surgery during the period from 1989 to 2008 at Barnes-Jewish Hospital in St. Louis. IHC was performed by using Brca1, $pAkt1^{S473}$ and Ki67 antibodies. Paraffin slides were deparaffinized and rehydrated by sequential incubations in xylene, 100% ethanol and 95% ethanol. Endogenous peroxidases were quenched for 20 min with 3% H_2O_2 at room temperature. An antigen retrieval step was performed by placing slides in preheated sodium citrate buffer (10mM, pH 6.0) and heated for 10 min in a pressure cooker. The slides were allowed to cool to room temperature. Slides were blocked with 5%of goat serum in Tris buffered saline (TBS) buffer for 60 min at room temperature. The diluted primary antibody was then added and incubated in a moist chamber at 4 °C overnight. Biotinylated secondary antibody was added for 30 min at room temperature. To detect primary antibody binding, ABC and DAB kits were applied according to the manufacturer instruction (Vector Laboratories). After mounting, the slides were observed under microscope and pictures were taken.

Mutation analysis of *PIK3CA*

Genomic DNA was prepared from the tumor samples as previously described (Yang *et al.*, 2000). PCR amplification was performed with primers previously described for exons 9 and 20 of *PIK3CA* (Samuels *et al.*, 2004). Sequencing of the PCR products was conducted using ABI 3300 automated capillary sequencer. The sequence data of *PIK3CA* gene from GenBank (accession no. NM_006218). Genomic DNA from corresponding normal tissues was subjected to sequence analysis to confirm the detected mutations.

MTT and soft agar assays

For the MTT assay, cells were plated and cultured 4 days to measure cell viability. All data were normalized relative to the control. For the Soft agar assay, cells (10 000/dish) were suspended in 2ml of 0.3% agar and poured over a 3 ml 0.6% solidified layer of agar in 60mm dishes. Cells were incubated for about 14–16 days until colonies were large enough to visualize. Colonies were observed under phase contrast microscope. Experiments were performed in triplicate.

Tumor growth experiments

Animal experiments were performed according to institutional guidelines for animal welfare. Female NOD.SCID/NCR mice of 6–8 weeks of age were purchased from NCI-Frederick Animal Production Program (Frederick, MA, USA). In all, 2×10^6 *Brca1*$^{tr/tr}$ MEFs or MCF7 cells expressing GFP or Akt1 shRNA in 0.1ml PBS were mixed with equal volume of matrigel. The cell mixture was implanted into mouse breast fat pad. After 9 weeks, the tumors became rigid and the volume of tumor ($(L\times W^2)/2$) is measured in range of 180–250mm^3. The mice were assigned to vehicle and P529 treatment groups and mean tumor volumes at the start point for treatment were indistinguishable between the vehicle and P529 treatment groups. P529-treated mice received 40mg/kg of P529 diluted in 100μl of vehicle. Control mice received 100 μl of vehicle only. Drug or vehicle was delivered orally every day for 18 days. At the end of treatment, the mice were killed and the tumors were excised and processed to paraffin section and protein extraction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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a

N=101		pAkt S473		P
	n	Positive 62	Negative 39	
Brca1				
Positive	31	12	19	0.004
Negative	70	50	20	

b

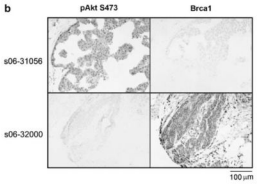

Figure 1.

Brca1 expression inversely correlates with Akt1 activation in human breast cancers. (**a**) Immunohistochemical (IHC) analysis of pAkt (S473) and Brca1 expression. Chi-square test was used for statistical analysis. (**b**) Immunohistochemical staining shows the expression of pAkt (S473) and Brca1 in the same cases of breast cancer tissues. Original magnification ×200.

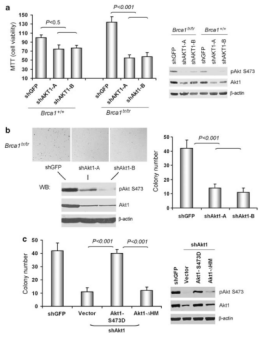

Figure 2.

Deletion of mouse Akt1 reduces proliferation of *Brca1*$^{tr/tr}$ MEFs. (**a**) The MTT assays were performed to measure the cell viability of *Brca1*$^{+/+}$ *and Brca1*$^{tr/tr}$ MEFs with or without knockdown of Akt1. The quantitative data were summarized from three experiments. All data were normalized relative to the control. The right panel showed western blot analysis of pAkt (S473) and Akt1 levels in these MEFs. (**b**) Proliferation of *Brca1*$^{tr/tr}$ MEFs expressing GFP and Akt1 shRNAs was detected by the soft agar assay. The lower panel showed western blot analysis of pAkt (S473) and Akt1 levels. The quantitative data were summarized from three colony formation assay experiments (right panel). (**c**) Active form of Akt1-S473D, but not deletion of hydrophobic motif (HM) mutant, rescues colony formation in *Brca1*$^{tr/tr}$ MEFs with Akt1 knockdown (shAkt-B, target sequence in 3′UTR). The right panel showed western blot analysis of pAkt (S473) and Akt1 levels.

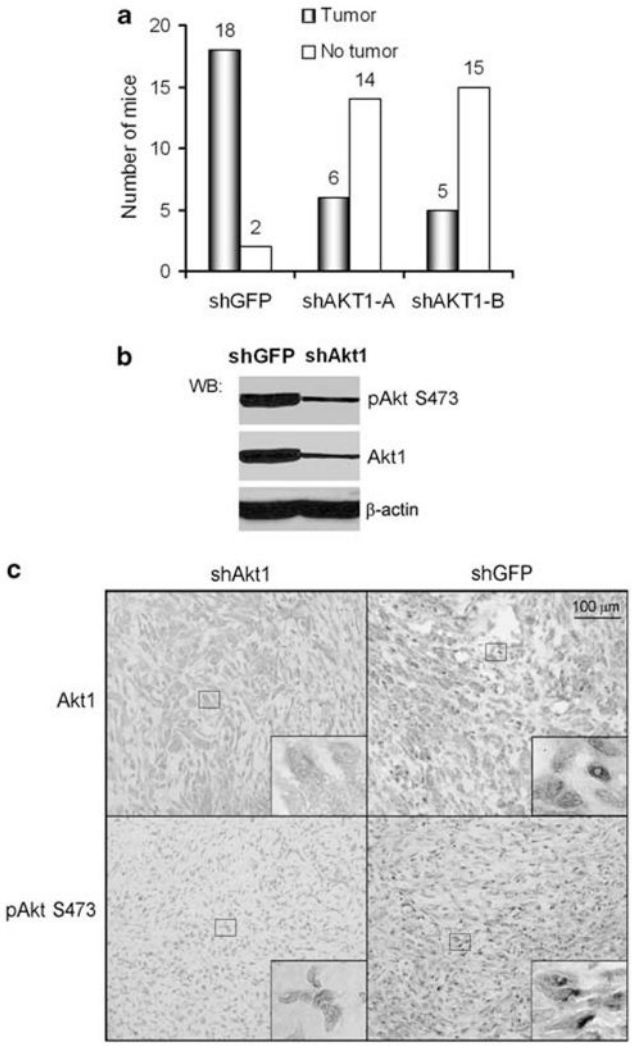

Figure 3.

Depletion of Akt1 suppresses tumor development induced by *Brca1*$^{tr/tr}$ MEFs in mice. (**a**) After 9 weeks, implanting *Brca1*$^{tr/tr}$ MEFs with or without knockdown of Akt1, the tumors became rigid and the volume of tumor (($L \times W^2$)/2) is measured. (**b**, **c**) Western blot and IHC analysis of Akt1 and pAkt (S473) in xenograft tumors. Original magnification ×200. Insides are high magnification (×1000).

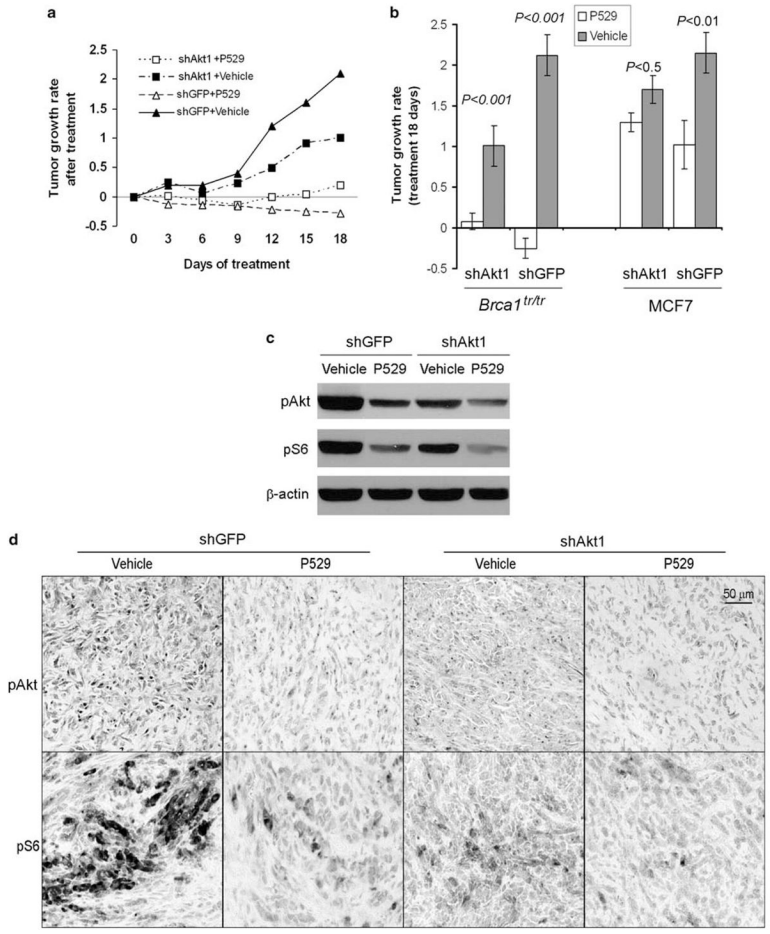

Figure 4.

P529 significantly suppresses tumor growth in *Brca1*$^{tr/tr}$ xenograft tumors. (**a**) Tumor growth rate after P529 treatment. The mice bearing xenograft tumors were assigned to vehicle and P529 treatment groups and mean tumor volumes at the start point (after 9 weeks implanting cells) for treatment were indistinguishable between the vehicle and P529 treatment groups. (**b**) Statistical analysis of effects of P529 on tumor growth for mice bearing *Brca1*$^{tr/tr}$ MEFs or MCF7 xenograft tumors (after 18 days treatment). (**c**) P529 treatment inhibits both pS6$^{S235/236}$ and pAkt^{S473} expression levels in *Brca1*$^{tr/tr}$ xenograft tumors. (**d**) IHC analysis of pS6$^{S235/236}$ and pAkt^{S473} in *Brca1*$^{tr/tr}$ xenograft tumors with or without P529 treatment. Original magnification ×200.