GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Overexpression of protein kinase B/AKT induces phosphorylation of p70S6K and 4E-BP1 in goat fetal fibroblasts

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Genet. Mol. Res. 13 (4): 9931-9938 (2014)
Received January 9, 2014
Accepted June 3, 2014
Published November 27, 2014
DOI http://dx.doi.org/10.4238/2014.November.27.22

ABSTRACT. Protein kinases regulate many processes, including cell growth, metabolism, molecular interactions, and cell proliferation. Protein kinase B (PKB)/AKT (v-AKT mouse thymoma viral oncogene homolog) is an upstream component of mammalian target of rapamycin (mTOR) signaling and mediates pathophysiological processes in several signaling pathways. This study aimed to construct and overexpress a eukaryotic goat *AKT* expression vector in goat fetal fibroblasts and examine the effects of AKT on the phosphorylation of p70S6K and 4E-BP1. *AKT* was subcloned into the expression vector pIRES2-DsRed2 to generate pIRES2-DsRed2-AKT, which was transfected into goat fetal fibroblasts with Lipofectamine™ 2000. *AKT* was measured by reverse transcription-polymerase chain reaction in the transgenic cells, and the expression of AKT and phosphorylation of p70S6K (Thr389) and 4E-BP1 (Thr37/46) were analyzed by Western blot. Cell clones that stably emitted red fluorescence were obtained after transfection for 48 h, and the exogenous gene was verified. Exogenous *AKT* was transcribed, and AKT was overexpressed, inducing the phosphorylation of p70S6K

(Thr389) and 4E-BP1 (Thr37/46) in goat fetal fibroblasts. Thus, the overexpression of AKT activates mTOR signaling in goat cells.

Key words: Protein kinase B (PKB)/AKT; Fetal fibroblasts; Mammalian target of rapamycin (mTOR); AKT overexpression; Inner Mongolia Cashmere goat

INTRODUCTION

Protein kinase B (PKB)/AKT (v-AKT mouse thymoma viral oncogene homolog) has 3 highly homologous isoforms - PKBα/AKT1, PKBβ/AKT2, and PKBγ/AKT3 - that belong to the AGC kinase family (Nicholson and Anderson, 2002). PKB/AKT comprises 480 amino acids and forms 3 domains, from the N- to C-terminus: a pleckstrin homology domain, which binds phosphoinositide 3-OH kinase (PI3K) to induce the translocation of PKB/AKT to the plasma membrane, a catalytic domain, and a regulatory domain (Downward, 1998).

AKT mediates many signal transduction pathways as a serine/threonine kinase in many species. AKT phosphorylates downstream substrates to regulate cell growth, proliferation, apoptosis, and other processes (Martelli et al., 2010). The integrated activation of AKT requires the phosphorylation of Thr308 in its activation loop by phosphoinositide-dependent kinase 1 (PDK1) (Stephens et al., 1998) and of Ser473 in its C-terminal hydrophobic motif by the rictor-mTOR complex *in vitro* (Sarbassov et al., 2005).

AKT is as an obligate intermediate in certain signaling pathways, particularly insulin signaling; thus, an AKT-independent pathway of insulin activity might be exploited to treat metabolic diseases (Cheng and White, 2012). AKT has critical functions in cell proliferation, apoptosis, and protein synthesis. AKT also governs signaling transduction by nutrients (Jewell and Guan, 2013) and immune modulation (Zhang et al., 2013).

As a central regulator of cell metabolism, PI3K/AKT/mTOR signaling mediates the pathogenesis of many tumors. The mammalian target of rapamycin (mTOR), a PI3K-related protein kinase, controls cell growth in response to nutrients and growth factors and is frequently deregulated in cancer (Yang et al., 2013). In PI3K/AKT/mTOR signaling, AKT is activated by PI3K kinase in the cell membrane and returns to the cytoplasm or nucleus to phosphorylate downstream substrates.

Of the many substrates of AKT, mTOR and its downstream molecules inhibit non-p53-dependent apoptosis (Skeen et al., 2006). As an upstream regulator of mTOR, AKT is negatively regulated by phosphatase and tensin homolog deleted on chromosome 10, which suppresses AKT by dephosphorylating PI(3,4,5)P3 to PI(4,5)P2 (Tong et al., 2009). AKT inhibits tuberous sclerosis complex 1/2 (TSC1/2), a complex that inhibits mTOR by suppressing the activity of the upstream mTOR activator Rheb (Huang et al., 2008). The TSC1/2 complex negatively regulates mTORC1 by converting a small Ras-related GTPase (Rheb) into its inactive GDP-bound state. In its GTP-bound form, Rheb interacts directly with and activates mTORC1 (Alayev and Holz, 2013). The activation of AKT mediates PI3K/AKT/mTOR signaling and governs cell fate.

Progress has been made in identifying AKT and understanding its functions in human, mouse, and rat, but little is known about its activity in small ruminants, specifically goat. In this study, we constructed and overexpressed a eukaryotic goat AKT expression vector in goat fetal fibroblasts (GFbs) to study its function and regulatory effects on the mTOR signaling

pathway. AKT is functional in GFbs and regulates mTOR signaling.

MATERIAL AND METHODS

Cell culture conditions

Inner Mongolia Cashmere GFbs were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT, USA), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Sigma-Aldrich, Inc., St. Louis, MO, USA). Cell cultures were maintained and incubated at 37°C in humidified air with 5% CO_2.

Construction of pIRES2-DsRed2-AKT

pMD19T-AKT and pIRES2-DsRed2 (Clontech Laboratories, Inc., Mountain View, CA, USA) were digested with *Eco*RI and *Bam*HI (TaKaRa, Dalian, China), and an AKT fragment was subcloned into pIRES2-DsRed2 to generate the recombinant vector pIRES2-DsRed2-AKT. The recombinant plasmids were verified by restriction analysis, and photos were taken on an ultraviolet transilluminator (UVItec, UK) after electrophoresis. The maps of the expression vectors are shown in Figure 1A.

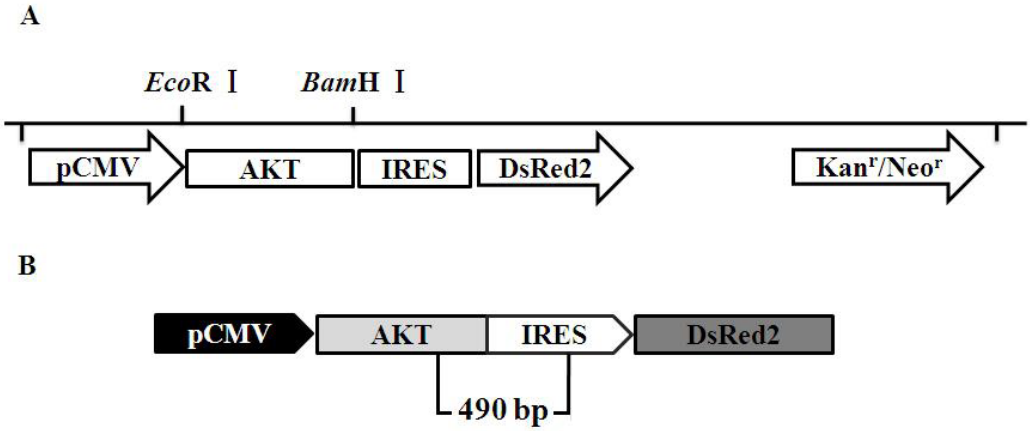

Figure 1. A. Structure of pIRES2-DsRed2-AKT and **B.** schematic of reverse transcription-polymerase chain reaction (RT-PCR) product.

Transfection *in vitro*

pIRES2-DsRed2-AKT (4 μg) was mixed with 8 μL Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) and 5 mL serum-free medium. GFbs, grown from 60 to 70% confluence on 6-well plates, were incubated with this solution for 6 h at 37°C and 5% CO_2; empty vector and untransfected cells were used as controls. The medium was removed after 4 h, and the cells were incubated in DMEM/F12 with 10% FBS. Red fluorescence was observed 48 h after transfection under a fluorescence microscope (Olympus IX71, Japan).

Transcription of exogenous *AKT* in transfected GFbs

Total RNA was isolated from GFb transfectants with RNAzol (RNAiso Plus, TaKaRa Co. Ltd.). RNA was reverse transcribed with oligo$(dT)_{18}$ primer according to manufacturer instructions (TaKaRa Co. Ltd.). An input of 1 µg total RNA was used for each reaction. A 490-bp fragment (Figure 1B) was amplified by polymerase chain reaction (PCR) with cDNA as the annealing template at 55°C using forward (5'-ACCATTACGCCACCTGACC-3') and reverse primers (5'-GAGCCATTTGACTCTTTCCAC-3').

Analysis of AKT overexpression in GFbs

Transfected GFbs were harvested with trypsin, washed with cold phosphate-buffered saline, and dissolved in cell lysis buffer. The cells was then placed on ice for 15 min and centrifuged at 13,000 rpm at 4°C for 20 min. The concentrations of the lysates were measured by Bradford assay (Bio-Rad Laboratories, USA). Equal amounts (30 µg) of protein were electrophoresed on 10% (w/v) sodium dodecyl sulfate polyacrylamide gels, transferred to polyvinylidene fluoride membranes, and incubated with primary antibody overnight at 4°C and peroxidase-conjugated secondary antibody at room temperature for 1 h. Enhanced chemiluminescence reagent Amersham ECL Western Blotting System (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) was used to detect the signals.

RESULTS

Construction and identification of recombinant expression vectors

To express AKT, recombinant pIRES2-DsRed2-AKT was constructed using the pIRES2-DsRed2 backbone. The pIRES2-DsRed2-AKT plasmid was transfected into *Escherichia coli* and identified by restriction analysis with *Eco*RI and *Bam*HI; the *AKT* fragment was 1443 bp (Figure 2). The plasmids were sequenced, and the correct clones were selected.

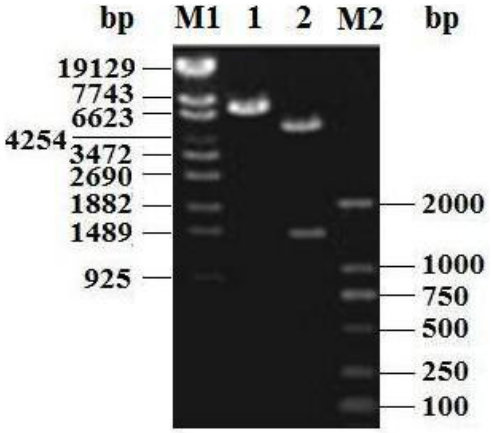

Figure 2. Restriction analysis of pIRES2-DsRed2-AKT. *Lane M1* = λ-*Eco*T14 I-digested DNA marker; *lane 1* = pIRES2-DsRed2-AKT digested with *Eco*RI; *lane 2* = pIRES2-DsRed2-AKT digested with *Eco*RI and *Bam*HI; *lane M2* = DL2000 marker.

Evaluation of transfection efficiency

Lipofectamine™ 2000 was used to transfect GFbs with pIRES2-DsRed2-AKT. pIRES2-DsRed2 and untransfected cells were used as control groups. The expression of DsRed was examined under a fluorescence microscope (Figure 3), and photos were taken with a digital camera. Red fluorescence of the transfectants was observed 48 h after transfection, and the rate was approximately 10%.

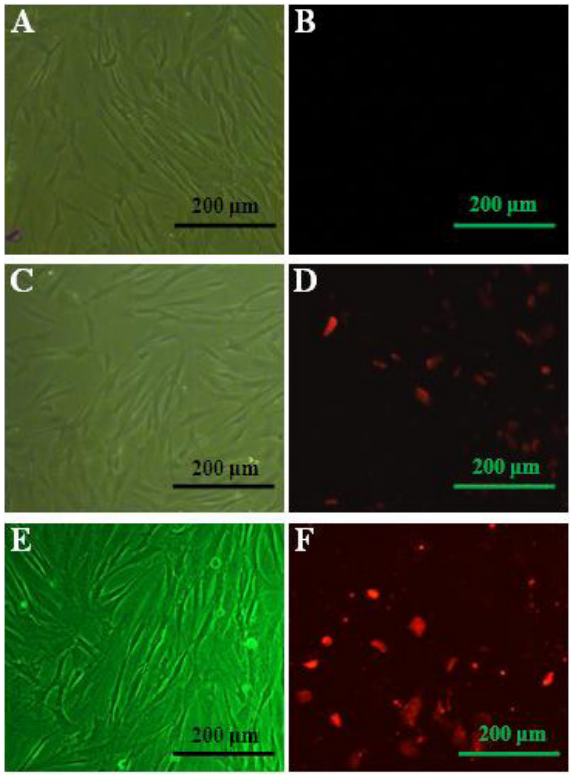

Figure 3. Goat fetal fibroblasts 48 h after transfection. **A.** Bright-field image of control cells. **B.** Red fluorescence of control cells. **C.** Bright-field image and **D.** red fluorescence of cells transfected with pIRES2-DsRed2. **E.** Bright-field image and **F.** red fluorescence of cells transfected with pIRES2-DsRed2-AKT.

Expression analysis of *AKT* in GFbs

Reverse transcription-PCR (RT-PCR) of total RNA from GFbs was performed to amplify a 490-bp fragment that included the 3'-end of *AKT* and the 5'-end of IRES (Figure 4A). The fragment was successfully amplified from the cDNA of the transfectants using forward and reverse primers (P1 and P2, respectively). PCR of the target segment demonstrated that exogenous goat *AKT* was transcribed in GFbs. By Western blot, AKT was overexpressed in transgenic cells, and the molecular mass was estimated to be 55 kDa (Figure 4B). The phosphorylation of p70S6K (Thr389) and 4E-BP1 (Thr37/46) was observed in the transgenic and control cells, and the transgenic cells had a greater level of phosphorylation (Figure 4B).

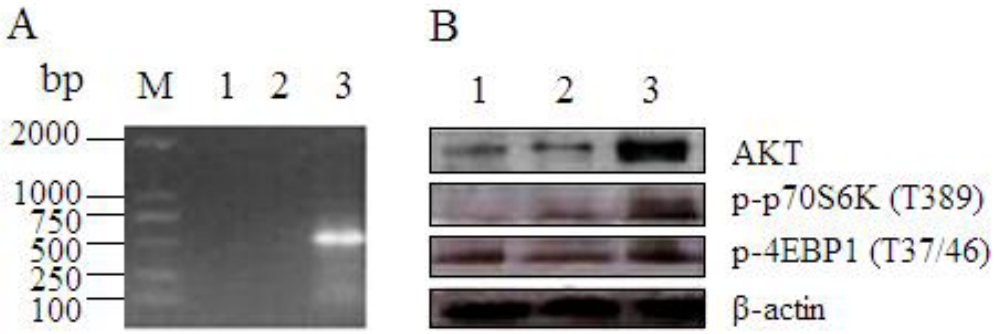

Figure 4. Expression analysis of AKT in transgenic cell clones by RT-PCR and Western blot. **A.** Identification of transgenic cell clones by RT-PCR. *Lane M*, DL2000 marker; *lane 1*, control cells; *lane 2*, cells transfected with pIRES2-DsRed2; *lane 3*, cells transfected with pIRES2-DsRed2-AKT. **B.** Western blot analysis of AKT, phospho-p70S6K (Thr389), and phospho-4E-BP1 (Thr37/46) in goat fetal fibroblasts.

DISCUSSION

As a critical cell-specific serine/threonine protein kinase, AKT is required for cell proliferation and metabolism in mammalian cells, and it is a frequently activated oncoprotein in human cancer cells (Liu et al., 2013; Mao et al., 2013). The PI3K/AKT pathway has significant functions in various cancers. This pathway can be abnormally activated in childhood acute lymphoblastic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, and certain pediatric lymphomas and lymphoproliferative disorders (Barrett et al., 2012). Overactivation of the PI3K/AKT/mTOR pathway mediates cellular growth and survival in the pathogenesis of endometrial cancer (Slomovitz and Coleman, 2012). A positive feedback loop, which is generated by the signaling interactome network of autocrine and paracrine elements from cancer hypoxic microenvironments, induces extreme robustness in metastatic cancer, relapsed leukemia, myeloma, and lymphoma (Radisavljevic, 2013a). Phosphorylated AKT is a cancer multidrug resistance locus. Targeting this locus by modulating the oxidant/antioxidant balance transforms positive feedback loops into negative feedback loops, leading to the disappearance of multidrug resistance (Radisavljevic, 2013b). Targeting the *AKT* locus by locus-directed chemotherapy to induce loop conversion is a new approach that is being used to control integrative angiogenic robustness and multidrug resistance.

Evidence over the past 15 years has demonstrated the presence of active AKT in the nucleus, where it is a fundamental component of important signaling pathways. Nuclear AKT counteracts apoptosis by blocking caspase-activated deoxyribonuclease, inhibiting chromatin condensation, and regulating cell cycle progression, cell differentiation, mRNA export, DNA repair, and tumorigenesis (Martelli et al., 2012). For example, AKT phosphorylates Ser166/186 of MDM2, which induces its nuclear entry to decrease p53 levels and activity (Feng, 2010). The AKT-activated transcription factor nuclear factor kappa B regulates the expression of hundreds of genes through the phosphorylation of IKK (Sempere et al., 2008). The proliferation of endothelial progenitor cells is inhibited by the silencing of endogenous DNA binding 1 (Id1), but the effects of Id1 transfection are mitigated when PI3K/AKT/NFκB signaling is blocked by inhibitors (Li et al., 2012). A β2-adrenergic antagonist significantly induces G1/S phase arrest and apoptosis in pancreatic cancer cells through the Ras/AKT/NFκB pathway (Zhang et

al., 2011). AKT/NFκB signaling is a significant regulator in normal and cancer cells.

Increasing evidence suggests that AKT mediates mTOR signaling. In this pathway, AKT is an upstream regulator of mTOR, and S6K1 and 4E-BP1 are 2 downstream effectors of mTOR. S6K1 is activated by mTOR-dependent phosphorylation, and 4E-BP1 is inactivated by mTOR-dependent phosphorylation. AKT activates mTOR signaling through various mechanisms and affects the phosphorylation of p70S6K and 4E-BP1. In a model, Ser2448 of mTOR is a direct target of phosphorylation by AKT (Navé et al., 1999). In another model, AKT phosphorylates TSC1/2 on multiple sites and inhibits TSC1/2 GTPase activity, which, in turn, activates Rheb and mTORC1 (Inoki et al., 2002; Potter et al., 2002). Phosphorylation and activation of S6K1 by mTORC1 enhance the translation of genes that are involved in ribosomal and mitochondrial biogenesis (Hannan et al., 2003; Holz and Blenis, 2005). In this study, we examined the regulation of 2 downstream targets of mTORC1, S6K1 and 4E-BP1, in Cashmere goat cells with AKT overexpression. Our data confirm that the overexpression of AKT induces the phosphorylation of p70S6K (Thr389) and 4E-BP1 (Thr37/46) and that mTOR signaling is activated in Cashmere GFbs.

CONCLUSIONS

Exogenous goat *AKT* was overexpressed in Cashmere GFbs, increasing the phosphorylation of p70S6K (Thr389) and 4E-BP1 (Thr37/46). The overexpression of AKT activates mTOR signaling in goat cells.

ACKNOWLEDGMENTS

Research supported by grants from the Natural Sciences Foundation of China (#31160469, #31360561) and the Natural Sciences Foundation of Inner Mongolia (#2011MS0521), a Graduate Student Research Project of the Inner Mongolia University, and Major Projects for New Varieties of Genetically Modified Organisms (#2013ZX08008-002).

REFERENCES

Alayev A and Holz MK (2013). mTOR signaling for biological control and cancer. *J. Cell Physiol*. 228: 1658-1664.

Barrett D, Brown VI, Grupp SA and Teachey DT (2012). Targeting the PI3K/AKT/mTOR signaling axis in children with hematologic malignancies. *Paediatr. Drugs* 14: 299-316.

Cheng Z and White MF (2012). The AKTion in non-canonical insulin signaling. *Nat. Med*. 18: 351-353.

Downward J (1998). Mechanisms and consequences of activation of protein kinase B/Akt. *Curr. Opin. Cell Biol*. 10: 262-267.

Feng ZH (2010). p53 regulation of the IGF-1/AKT/mTOR pathways and the endosomal compartment. *Cold Spring Harb. Perspect. Biol*. 2: a001057.

Hannan KM, Brandenburger Y, Jenkins A, Sharkey K, et al. (2003). mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. *Mol. Cell Biol*. 23: 8862-8877.

Holz MK and Blenis J (2005). Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. *J. Biol. Chem*. 280: 26089-26093.

Huang J, Dibble CC, Matsuzaki M and Manning BD (2008). The TSC1-TSC2 complex is required for proper activation of mTOR complex 2. *Mol. Cell Biol*. 28: 4104-4015.

Inoki K, Li Y, Zhu T, Wu J, et al. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol*. 4: 648-657.

Jewell JL and Guan KL (2013). Nutrient signaling to mTOR and cell growth. *Trends Biochem. Sci*. 38: 233-242.

Li W, Wang H, Kuang CY, Zhu JK, et al. (2012). An essential role for the Id1/PI3K/Akt/NFkB/survivin signalling pathway in promoting the proliferation of endothelial progenitor cells *in vitro*. *Mol. Cell Biochem*. 363: 135-145.

Liu YP, Liao WC, Ger LP, Chen JC, et al. (2013). Carboxyl-terminal modulator protein positively regulates Akt phosphorylation and acts as an oncogenic driver in breast cancer. *Cancer Res*. 73: 6194-6205.

Mao Y, Xu J, Song G, Zhang N, et al. (2013). Twist2 promotes ovarian cancer cell survival through activation of Akt. *Oncol. Lett*. 6: 169-174.

Martelli AM, Evangelisti C, Chiarini F and McCubrey JA (2010). The phosphatidylinositol 3-kinase/Akt/mTOR signaling network as a therapeutic target in acute myelogenous leukemia patients. *Oncotarget* 1: 89-103.

Martelli AM, Tabellini G, Bressanin D, Ognibene A, et al. (2012). The emerging multiple roles of nuclear Akt. *Biochim. Biophys. Acta* 1823: 2168-2178.

Navé BT, Ouwens M, Withers DJ, Alessi DR, et al. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J*. 344: 427-431.

Nicholson KM and Anderson NG (2002). The protein kinase B/AKT signalling pathway in human malignancy. *Cell. Signal*. 14: 381-395.

Potter CJ, Pedraza LG and Xu T (2002). Akt regulates growth by directly phosphorylating Tsc2. *Nat. Cell Biol*. 4: 658-665.

Radisavljevic Z (2013a). AKT as locus of cancer positive feedback loops and extreme robustness. *J. Cell. Physiol*. 228: 522-524.

Radisavljevic Z (2013b). AKT as locus of cancer multidrug resistance and fragility. *J. Cell. Physiol*. 228: 671-674.

Sarbassov DD, Guertin DA, Ali SM and Sabatini DM (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307: 1098-1101.

Sempere MC, Fanjul VR, Pérez IS and Perona R (2008). The role of the NFκB signalling pathway in cancer. *Clin. Transl. Oncol.* 10: 143-147.

Skeen JE, Bhaskar PT, Chen CC, Chen WS, et al. (2006). AKT deficiency impairs normal cell proliferation and suppresses oncogenesis in a P53-independent and mTORCI-dependent manner. *Cancer Cell* 10: 269-280.

Slomovitz BM and Coleman RL (2012). The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. *Clin. Cancer Res*. 18: 5856-5864.

Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, et al. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* 279: 710-714.

Tong Z, Fan Y, Zhang W, Xu J, et al. (2009). Pancreas-specific Pten deficiency causes partial resistance to diabetes and elevated hepatic AKT signaling. *Cell Res.* 19: 710-719.

Yang H, Rudge DG, Koos JD, Vaidialingam B, et al. (2013). mTOR kinase structure, mechanism and regulation. *Nature* 497: 217-223.

Zhang D, Ma Q, Wang Z, Zhang M, et al. (2011). β2-adrenoceptor blockage induces G1/S phase arrest and apoptosis in pancreatic cancer cells via Ras/Akt/NFκB pathway. *Mol. Cancer* 10: 146.

Zhang Y, Wang X, Yang H, Liu H, et al. (2013). Kinase AKT controls innate immune cell development and function. *Immunology* 140: 143-152.