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AKT down-regulates insulin-like growth factor-1 receptor as a negative feedback

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As a member of receptor tyrosine kinase (RTK) family, insulin-like growth factor-1 (IGF1) receptor (IGF1R) activates several downstream pathways to transmit proliferative signals from extracellular stimulation. AKT as a major effector plays a pivotal role in integrating various survival signalling cascades. Our data here show that hyperactive AKT leads to the decrease of IGF1R at the transcriptional level, which could be partly restored by phosphatidylinositol-3 kinase (PI3K) inhibitors including wortmannin and LY294002. Moreover, the decrease of IGF1R impairs the sensitivity of IRS1 to the stimulation by IGF1. mTOR as a main downstream target of AKT is not involved in the AKT-mediated down-regulation of IGF1R.

Keywords: AKT/IGF1/IGF1R/IRS1/mTOR.

Abbreviations: AKT, protein kinase B; Foxo, forkhead box O; IGF1, insulin-like growth factor-1; IGF1R, insulin-like growth factor-1 receptor; IRS, insulin receptor substrate; mTOR, mammalian target of rapamycin; MEF, mouse embryonic fibroblast; PDK1, phosphoinositide-dependent kinase-1; PIP2, phosphatidylinositol (4, 5)-bisphosphate; PIP3, phosphatidylinositol (3, 4, 5)-trisphosphate; PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homolog; RTK, receptor tyrosine kinase; TSC1/TSC2, tuberous sclerosis complex 1/2.

Insulin-like growth factor-1 (IGF1) receptor (IGF1R) belongs to the receptor tyrosine kinase (RTK) family. Binding with IGF1 induces its dimerization and the consequent autophosphorylation of several key tyrosine residues in the catalytic kinase domains (*1*). The autophosphorylation triggers the transmission of survival and proliferative signals via three major downstream pathways: RAS/RAF/MEK1/2/ERK, JAK/STAT and IRS/PI3K/AKT. A number of oncogenes are located in these cascades, and their aberrant activations are always found in human malignancies. Several key molecules including both docking proteins and kinases perform as the central nodes to integrate various upstream anti-apoptotic signals, such as insulin receptor substrate (IRS) and AKT. IRS is activated by binding to the phosphorylated IGF1R via the interaction between the conserved PH and PTB domains. IRS performs mainly as a scaffold helping the activation of phosphatidylinositol-3 kinase (PI3K). Phosphorylated IRS recruits PI3K into a multicomponent complex through the interaction between its phosphotyrosine-containing motifs and the SH2 domain of PI3K (*2*). Besides multiple tyrosine phosphorylation motifs, the over 30 potential serine/threonine phosphorylation sites on IRS make it subjected to a variety of regulation (*3*).

AKT, also named protein kinase B (PKB), plays a central role in promoting cell survival and proliferation. Dysregulation of AKT has been implicated in many types of human cancer (*4*). Via directly phosphorylating over 100 substrates, AKT could regulate various cellular processes, such as cell survival, growth, proliferation, glucose uptake, metabolism and angiogenesis (*5*). Activated PI3K catalyzes the conversion of phosphatidylinositol (4, 5)-bisphosphate (PIP2) to phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3), which can be reversed by phosphatase and tensin homolog (PTEN). PIP3 binds to AKT and phosphoinositide-dependent kinase-1 (PDK1) at the plasma membrane where PDK1 activates AKT through the phosphorylation at T308. One major downstream target of AKT is the tuberous sclerosis complex 1/2 (TSC1/TSC2) heterodimer that suppresses the mammalian target of rapamycin (mTOR) (*6*). While AKT inhibits TSC1/TSC2 thus activating mTOR, hyperactivated mTOR was found to down-regulate AKT as a negative feedback (*7*). mTOR induces the degradation and subcellular redistribution of IRS, thus diminishing IRS/PI3K/AKT cascade (*8–10*).

Another major target of AKT is forkhead box O (Foxo), a family of transcription factors. Phosphorylation by AKT facilitates its interaction with 14-3-3 proteins that repel it from the nucleus to the cytosol (*11*). Foxo1 could bind to the promoter of insulin-like growth factor-binding protein-1 (*Igfbp1*), thus modulating IGF1/IGF1R pathway (*12*). Moreover, constitutively activated Foxo1 up-regulates IGF1R and increases the phosphorylation of both MEK and AKT (*13*). Such evidence stimulated us to postulate that AKT may regulate IGF1R as a feedback in this pathway.

Here, we report that hyperactive AKT down-regulates IGF1R at the transcriptional level, and this regulation is mTOR independent. Considering the well-established IGF1R/IRS/PI3K/AKT pathway, our finding shows a novel mechanism accounting for the negative feedback from AKT to IGF1R.

Materials and Methods

Reagents

Reagents were obtained from commercial sources: wortmannin (W1628), rapamycin (R0395) and IGF1 (I8779) were from Sigma-Aldrich; LY294002 (#9901) was from Cell Signaling Technology; Dulbecco's Modified Eagle's Medium (DMEM) (07-02I) was from Neuronbc; foetal bovine serum (FBS) (SV30087.02) was from Thermo Scientific; TRIzol (15596-026), 4–12% Bis–Tris Nu-PAGE gels (NP0323BOX) and Lipofectamine 2000 (11668-019) were from Invitrogen.

Antibodies

phospho-S6 (Ser235/236) and S6 were kindly provided by Dr Hongbing Zhang as gifts; β-actin (SC-47778) was from Santa Cruz Biotechnology Inc.; IRS1 (#06-248) was from Upstate USA Inc.; PTEN (#9559), AKT (#9272), phospho-AKT (Ser473, #9271), IGF1Rα (#3022), IGF1Rβ (#3027), phospho-IRS1 (Tyr895, #3070), Foxo1 (#9462) and phospho-Foxo1 (Ser256, #9461) were from Cell Signaling Technology.

Cell culture

All MEF cells used here have been described previously (*6*, *14*). Cells were cultured in DMEM with 10% FBS, penicillin/streptomycin in 5% CO_2 at 37°C.

Small interfering RNA knockdown

Small interfering RNA (siRNA) were designed and synthesized by GuangZhou RiboBio. Cells were seeded in six-well plates and transfected with 100 nM siRNA by Lipofectamine 2000 following the manufacturer's instructions. The cells were used for immunoblotting and real-time RT–PCR after 48 h.

Western blot

Whole cell extracts were prepared by boiling for 10 min after the harvest using lysis buffer (2% SDS, 10% glycerol, 10 mM Tris, pH 6.8, 100 mM DTT), and then subjected to immunoblotting. Band density was determined by AlphaEaseFC software.

Plasmids and transfection

The plasmids expressing PTEN and myristoylated AKT were kindly provided by Dr Hongbing Zhang as gifts. The plasmid expressing constitutively activated AKT through glutamic acid-to-lysine substitution at amino acid 17 (E17K) was kindly provided by Dr Haiyong Peng. All plasmids were transfected into the retroviral packaging cell line PT67 (Clontech) using Lipofectamine 2000. Conditioned culture medium containing viruses was used to transduce MEF cells, and the transduced cells were selected with 100 μg/ml hygromycin B for stably expressing cells.

Real-time RT–PCR

Total RNA was extracted from cells using TRIzol according to the instruction. Genomic DNA was eliminated by DNase treatment. For all analyses, retrotranscription of 0.5 μg total RNA was performed with PrimeScript™ RT reagent kit (Takara). Real-time PCRs were performed using SYBR® Premix Ex Taq (Takara) and run in triplicate on 96-wells reaction plates with iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). The relative expression levels of IGF1Rα and IGF1Rβ were acquired by calculating the cycle threshold (Ct) values which were defined as the ratio to β-actin, the internal control amplified simultaneously. All products were subsequently run on 2% agarose gels to check for size and specificity. The primer sequences were as follows: IGF1Rα forward, 5′-GTGGGG GCTCGTGTTTCTC-3′ and reverse, 5′-GATCACCGTGCAGTTT TCCA-3′; IGF1Rβ forward, 5′-GCATCTGATCATTGCTCTGC-3′ and reverse, 5′-GCCCAACCTGCTGTTATTTC-3′; β-actin forward, 5′-ACGTTGACATCCGTAAAGAC-3′ and reverse, 5′-GAA GGTGGACAGTGAGGC-3′.

Statistical analysis

Results of experimental data were reported as mean ± SD. Mann–Whitney test with SPSS 13.0 was used to determine significance levels. Differences were considered significant when $P<0.05$.

Results

Hyperactive AKT leads to the decrease of IGF1R at the transcriptional level

To detect whether AKT could regulate IGF1R, we introduced two models with the hyperactivation of AKT. First, since PTEN deactivates AKT through reversing PI3K-promoted conversion of PIP2 to PIP3, we introduced PTEN-deficient MEFs and their wild-type (WT) control cells. Indicated by the robust p-AKT, PTEN-deficient MEFs exhibited enhanced AKT activity (Fig. 1A). As a canonical substrate of

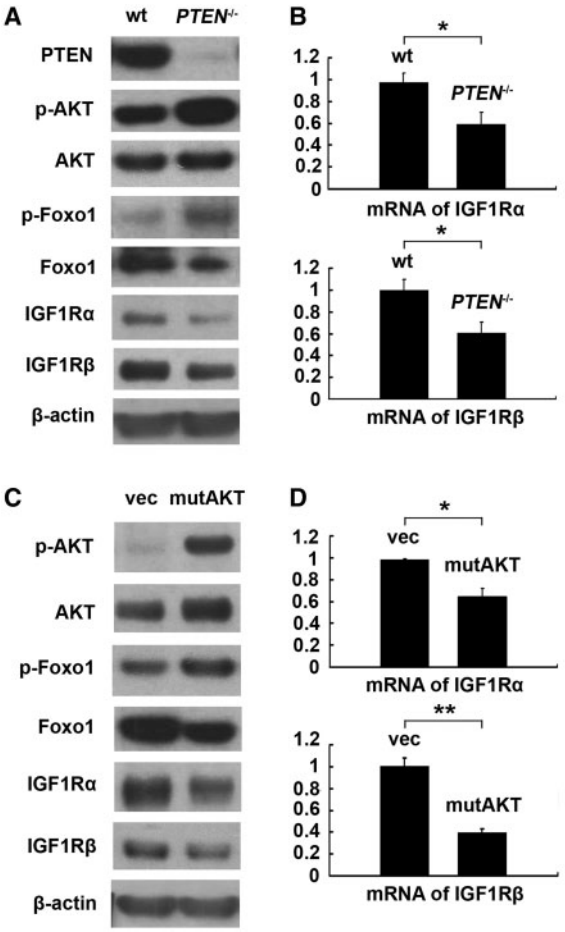

Fig. 1 AKT hyperactivation negatively regulates IGF1R at the transcriptional level. (A) and (B) WT and PTEN-deficient ($PTEN^{-/-}$) MEFs (C) and (D) WT MEFs transfected with the control vector (vec) and the vector expressing E17K mutant AKT (mutAKT). (A) and (C) Cell lysates were analysed for the levels of PTEN, p-AKT, AKT, p-Foxo1, Foxo1, IGF1Rα, IGF1Rβ and β-actin via western blot. The experiments were repeated three times independently. (B) and (D) The mRNA of IGF1Rα and IGF1Rβ were analysed by real-time quantitative RT–PCR. Data represent mean ± SD of triplicate samples. $^{*}P<0.05$ and $^{**}P<0.01$.

AKT, Foxo1 was detected as the reporter of AKT activity. The elevated p-Foxo1 revealed the increased AKT activity in PTEN-deficient MEFs (Fig. 1A). The levels of both IGF1Rα and IGF1Rβ decreased in PTEN-deficient MEFs compared with their WT counterparts (Fig. 1A). Secondly we constructed the vector expressing constitutively activated AKT through glutamic acid-to-lysine substitution at amino acid 17 (E17K), and transfected the WT MEFs. Compared with their counterparts, AKT-overexpressing MEFs exhibited higher AKT activity indicated by the increased p-AKT and p-Foxo1, and the levels of both IGF1Rα and IGF1Rβ dropped dramatically (Fig. 1C). To examine the mechanism of IGF1R reduction, we used real-time quantitative RT–PCR analysis. A substantial decrease in IGF1R mRNA was shown in both PTEN-deficient and AKT-overexpressing cells (Fig. 1B and D). Such observation suggested that IGF1R is negatively regulated by AKT at the transcriptional level.

PI3K inhibitors and AKT knockdown partly restore AKT-induced down-regulation of IGF1R

To further confirm the AKT-induced IGF1R down-regulation, we treated *PTEN*$^{-/-}$ cells with two widely used PI3K inhibitors: wortmannin and LY294002. Both the protein and mRNA expression of IGF1R were analysed via western blot and real-time quantitative RT–PCR. The decrease of p-AKT and p-Foxo1 reflected the drop of AKT activity under 24 h treatments of both wortmannin and LY294002 (Fig. 2A). Moreover, the treatments increased IGF1R at both protein and mRNA level (Fig. 2A and B). Another supporting evidence was derived from AKT knockdown via siRNA in *PTEN*$^{-/-}$ cells. IGF1R were greatly restored after 48 h treatment of

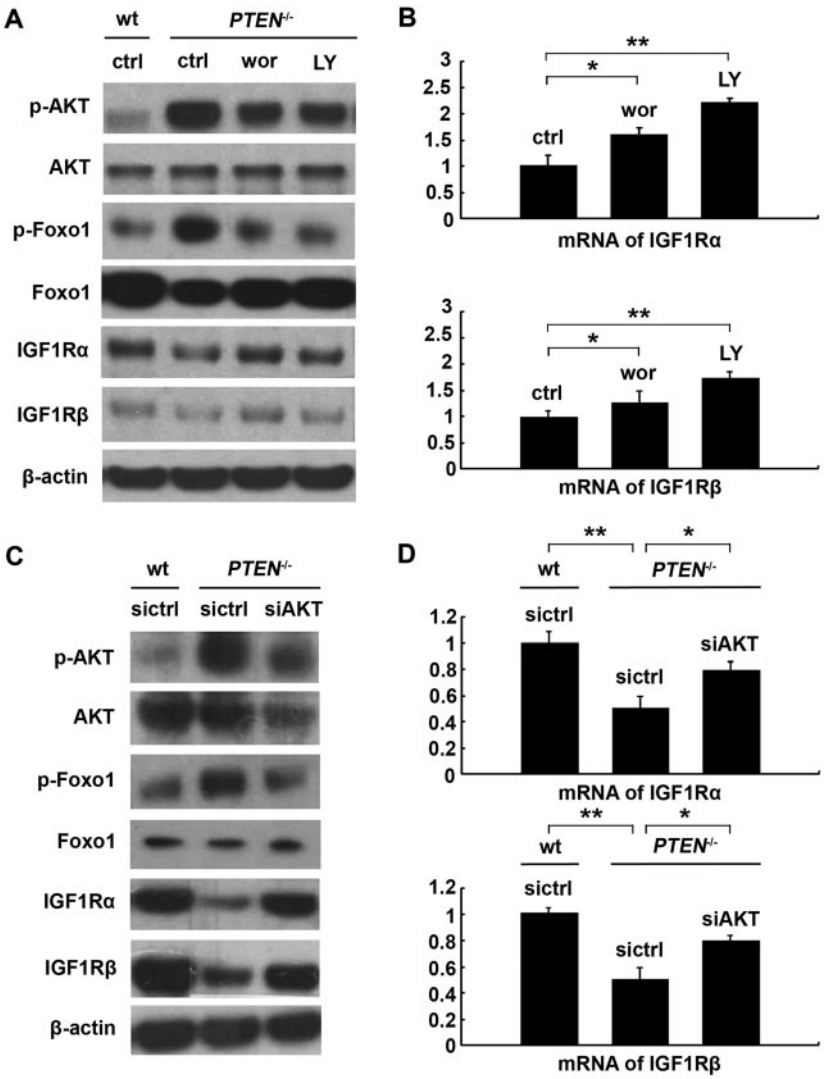

Fig. 2 PI3K inhibitors and AKT-knockdown via siRNA partly restore AKT-mediated down-regulation of IGF1R. (A) and (B) WT MEFs were treated with DMSO as control (ctrl) for 24 h. PTEN-deficient (*PTEN*$^{-/-}$) MEFs were treated with DMSO (ctrl), wortmannin (wor, 5 μM) and LY294002 (LY, 10 μM) for 24 h. (C) and (D) WT MEFs were treated with control siRNA (sictrl) for 48 h. PTEN-deficient (*PTEN*$^{-/-}$) MEFs were treated with control siRNA (sictrl) and siRNA against *AKT* (siAKT) for 48 h. (A) and (C) Cell lysates were analysed for the levels of p-AKT, AKT, p-Foxo1, Foxo1, IGF1Rα, IGF1Rβ and β-actin via western blot. The experiments were repeated three times independently. The mRNA of IGF1Rα and IGF1Rβ in (B) PTEN-deficient MEFs (D) both WT and PTEN-deficient MEFs were analysed by real-time quantitative RT–PCR. Data represent mean ± SD of triplicate samples. $*P<0.05$ and $**P<0.01$.

siRNA against *AKT* (Fig. 2C and D). These data further confirmed that AKT negatively regulates IGF1R at the transcriptional level.

Hyperactive AKT impairs the sensitivity of IRS1 to the stimulation by IGF1

The observation that AKT down-regulates IGF1R promoted us to detect its effect on the signal transmission from IGF1 to IRS. Cells were stimulated with IGF1 after 24 h depletion of serum. Indicated by the decreased tyrosine phosphorylation of IRS1, both PTEN-deficient and AKT-overexpressing cells exhibited reduced sensitivity to the stimulation by IGF1 (Fig. 3A–D). Interestingly, not only p-IRS1, but also the total IRS1 dropped in AKT-hyperactive cells. Since mTOR as a major downstream effector of AKT could result in the degradation of IRS1, the decrease of total IRS1 in AKT-hyperactive cells may be attributed to the activated mTOR. The robust p-S6 as a reporter of mTOR activity indicated the activation of AKT/TSC/mTOR pathway (Fig. 3A and C).

AKT-mediated down-regulation of IGF1R is mTOR independent

As a main downstream target of AKT, mTOR was found to increase the serine phosphorylation thus degradation of IRS1. Here we detected whether AKT-mediated down-regulation of IGF1R is via mTOR activation. Both PTEN-deficient and AKT-overexpressing cells were treated with rapamycin, a specific mTOR inhibitor. While mTOR activity dropped sharply, the levels of IGF1R did not change in the two cell lines after 24 h treatment (Fig. 4A and B). Such results excluded mTOR out of the AKT-mediated IGF1R reduction. To further confirm its absence in this regulation, we introduced TSC2-deficient MEFs. Since AKT activates mTOR via the inhibition of TSC1/TSC2 heterodimer, mTOR could not be activated by AKT with TSC2 deletion. $TSC2^{-/-}$ cells were transfected with the vector expressing myristoylated thus activated AKT. As a result, no difference of mTOR activity was found between AKT-overexpressing and the control $TSC2^{-/-}$ MEFs

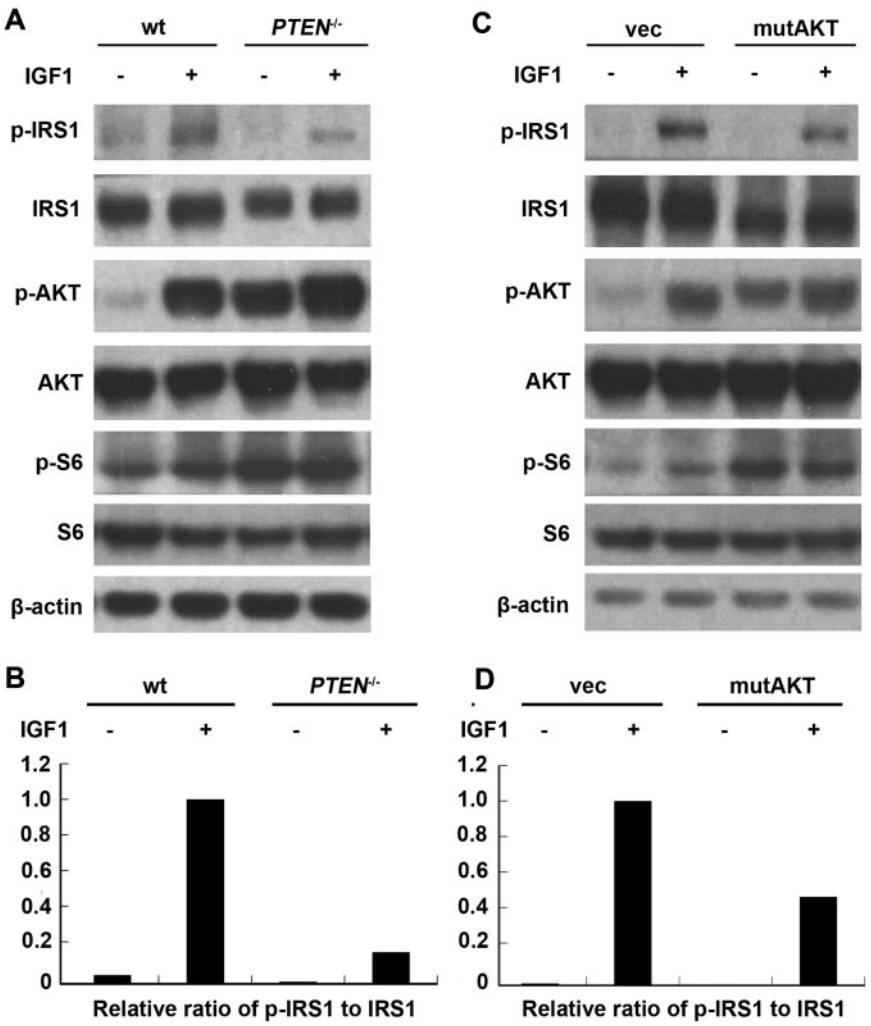

Fig. 3 AKT hyperactivation reduces the sensitivity of IRS1 in response to the stimulation by IGF1. (A) and (B) WT and PTEN-deficient ($PTEN^{-/-}$) MEFs (C) and (D) WT MEFs transfected with the control vector (vec) and the vector expressing E17K mutant AKT (mutAKT). Cells were starved for 24 h and then stimulated by IGF1 (20 nM) for 10 min. Cell lysates were analysed for the levels of p-IRS1, IRS1, p-AKT, AKT, p-S6, S6 and β-actin via western blot. The experiments were repeated three times independently. (B) and (D) Relative ratio of p-IRS1 band density to that of IRS1 was determined by AlphaEaseFC software.

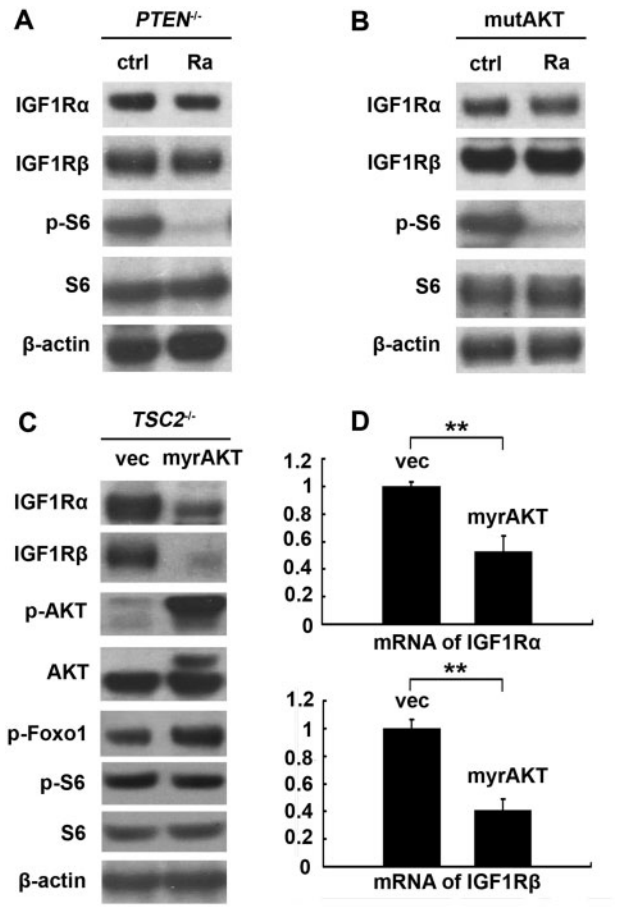

Fig. 4 mTOR is not involved in AKT-mediated down-regulation of IGF1R. (A) PTEN-deficient ($PTEN^{-/-}$) MEFs and (B) WT MEFs transfected with the vector expressing E17K mutant AKT (mutAKT) were treated with DMSO as controls (ctrl) and rapamycin (Ra, 10 nM) for 24 h. (C) and (D) $TSC2^{-/-}$ MEFs transfected with the control vector (vec) or the vector expressing myristoylated thus activated AKT (myrAKT). (A), (B) and (C) Cell lysates were analysed for the levels of p-AKT, AKT, p-Foxo1, IGF1Rα, IGF1Rβ, p-S6, S6 and β-actin via western blot. The experiments were repeated three times independently. (D) The mRNA of IGF1Rα and IGF1Rβ were analysed by real-time quantitative RT–PCR. Data represent mean ± SD of triplicate samples. $^{**}P < 0.01$.

(Fig. 4C). However, AKT-overexpressing $TSC2^{-/-}$ MEFs exhibited dramatically decreased IGF1R at both protein and mRNA levels compared with the control $TSC2^{-/-}$ MEFs (Fig. 4C and D). These data confirmed that mTOR is not involved in AKT-induced down-regulation of IGF1R.

Discussion

In summary, we have shown that AKT negatively regulates IGF1R at the transcriptional level, and it could be partly restored by PI3K inhibitors. mTOR is not involved in this regulation. Moreover, the AKT-induced IGF1R down-regulation diminishes the sensitivity of IRS1 to the stimulation by IGF1.

As a major signalling pathway promoting cell survival and proliferation, IGF1R/IRS1/PI3K/AKT has been implicated in various human diseases, such as atherosclerosis, diabetes and many kinds of malignancies (*15*). Increasing evidence implicate the negative feedback in this pathway. As a scaffold protein mainly responsible for the recruitment and activation of PI3K, IRS1 is considered as the key target for the feedback. mTOR could enhance its serine phosphorylation which accelerates its proteasome-mediated degradation (*8–10*). mTOR inhibitors were found to augment RTK/PI3K/AKT pathway by elevating IRS1 (*16*, *17*). Moreover, mTOR was also reported to down-regulate platelet-derived growth factor receptor (PDGFR), another member of RTK family (*6*, *14*).

Although insulin receptor (IR) has been found to undergo extensive regulations which are involved in insulin resistance, little is known about the regulation of IGF1R. Protein kinase Cα (PKCα) was showed to associate with the IGF1Rβ only after acute IGF1 exposure. Both its inhibitor and siRNA could completely block activation of the IGF1R internal tyrosine kinase (*18*). Kermit 2/XGIPC was identified as an IGF1 receptor interacting protein which is required for IGF signalling in Xenopus eye development (*19*). Growing evidence implicates AKT-mediated negative regulation of IGF1R. Somatostatin-14 (SS-14) was found to inhibit mRNA and functional expression of IGFR1 via direct stimulation of both extracellular signal-regulated kinase (ERK) and AKT in gill filaments of rainbow trout (*20*, *21*). Moreover, a number of evidence also revealed the controversial role PTEN plays in regulating IGF1R. One report suggested that loss of PTEN results in a block in IGF1/insulin signalling, including the reduced activation of PI3K and ERK, as well as the decreased expression of IRS1 and IGF1R (*22*). However, some contradictory reports showed that PTEN overexpression induces the reduction of not only the expression of IGF1R, but also IGF1-induced tyrosine phosphorylation of IRS1 in PC3, SNU-484 and SNU-663 cells (*23–25*). A possible explanation for these contradictory data is that PTEN could mediate IGF1R via other mechanism rather than AKT pathway. The supporting observation is that most evidence showing the negative effect of PTEN on IGF1R came from the study of PTEN overexpression (*23–25*), while the seemingly opposite data were always derived from the model with loss of PTEN (*22*). Here, our data also showed that the loss of PTEN results in the activation of AKT, which down-regulates IGF1R at the transcriptional level. Therefore, we postulate that under the situation of PTEN deletion, the robust AKT activation plays a dominant role in IGF1R down-regulation, thus achieving the same result with that in AKT hyperactivation model. On the other hand, PTEN overexpression itself could negatively regulate IGF1R via other unknown AKT-independent mechanisms. Another possible mechanism may be that PTEN and AKT could regulate IGF1R at different levels.

The supporting evidence about the negative role AKT plays in IGF1R regulation also came from the report that overexpression of Foxo1 activates both AKT and MEK pathways via elevating IGF1R (*13*). Considering that AKT reduces IGF1R at the transcriptional level, Foxo1 as a transcription factor may be responsible for AKT-mediated down-regulation of

IGF1R. However, there is no evidence that Foxo1 directly promotes the transcription of *IGF1R*. The future work should also be focused on the detection *in vivo* to confirm the change of IGF1R in AKT-hyperactivated tissues.

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Conflict of interest

None declared.

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