

# The PI3K/AKT/mTOR pathway in the pathophysiology and treatment of pituitary adenomas

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## Abstract

Pituitary adenomas are common intracranial neoplasms. Patients with these tumors exhibit a wide range of clinically challenging problems, stemming either from results of sellar mass effect in pituitary macroadenoma or the diverse effects of aberrant hormone production by adenoma cells. While some patients are cured/controlled by surgical resection and/or medical therapy, a proportion of patients exhibit tumors that are refractory to current modalities. New therapeutic approaches are needed for these patients. Activation of the AKT/phosphatidylinositol-3-kinase pathway, including mTOR activation, is common in human neoplasia, and a number of therapeutic approaches are being employed to neutralize activation of this pathway in human cancer. This review examines the role of this pathway in pituitary tumors with respect to tumor biology and its potential role as a therapeutic target.

## Key Words

- ▶ pituitary adenoma
- ▶ mTOR
- ▶ mTOR inhibitor
- ▶ rapamycin
- ▶ RAD001

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## Introduction

Pituitary adenomas (PAs) are common intracranial neoplasms accounting for up to 25% of primary brain tumors (Asa & Ezzat 2009). A large proportion of PAs are prolactinomas, although most macroadenomas are clinically nonfunctional variants (NFPA), specifically of gonadotroph differentiation (Mete & Asa 2012). PAs are generally benign but can cause significant morbidity via deregulated hormone production and/or symptoms of mass effect, including visual disturbances.

Current mainstays of therapy include medication, surgery, and radiotherapy (Asa & Ezzat 2009). Currently, medications that have shown efficacy at providing

hormonal control and reducing the size of the PA are dopamine agonists for prolactin-secreting PA (PRL-PA) and somatostatin (SST) analogs for growth hormone-secreting PA (GH-PA) (Asa & Ezzat 2009). While medical therapy is the preferred initial management step for PRL-PA, transphenoidal surgery remains the initial treatment of choice for most GH-, adrenocorticotropic hormone (ACTH)-, thyroid-stimulating hormone-, and NFPA (Oostra *et al.* 2012). Despite these well-studied treatment modalities, there remains a substantial proportion of patients whose tumors are refractory to surgical and medical therapy. New options are needed for these patients, and targeting

aberrantly activated signal transduction pathways that drive the tumor could represent a promising approach for patients with refractory tumors.

Though the understanding of the PA pathobiology has been expanding, precise mechanisms have yet to be elucidated (Asa & Ezzat 2009, Dworakowska et al. 2009). As a result, efficacious medical therapies used in clinical practice are currently limited. Further understanding of cellular pathways involved in PA pathogenesis will help to identify novel treatment targets (Asa & Ezzat 2009). One such pathway that has been implicated in PAs is the phosphatidylinositol-3-kinase (PI3K)/AKT/mTOR pathway. This pathway is involved in numerous crucial cell functions including cell cycle regulation, cell survival, cell growth, protein synthesis, and cellular metabolism. Already implicated in other human cancers, including breast, endometrial, ovarian, prostate, and glioblastoma, recent studies have investigated the activation of this pathway in PAs (Faivre et al. 2006). Furthermore, pre-clinical studies have investigated the therapeutic effects of targeting the PI3K/AKT/mTOR pathway in PAs (Cerovac et al. 2010). The focus of this paper is to review the role of PI3K/AKT/mTOR signaling pathway, with particular attention to novel findings in its activation and potential therapeutic targeting in PAs.

### PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway is a signal transduction cascade involved in cell growth and metabolism (Leslie et al. 2001). The pathway is activated by upstream receptor tyrosine kinases (RTK) which feed the signal through the PI3K complex. PI3K is a lipid kinase consisting of a regulatory (p85) and a catalytic (p110) subunits. It is activated directly via the p85 subunit which interacts with phosphotyrosine residues on the RTK. Alternatively, in the indirect route, PI3K interacts with the RTK via the IRS1 or IRS2 adaptor proteins (White 1998). Both methods of activation lead to the conversion of PIP2 to the second messenger PIP3. PIP3 recruits the kinases PDK1 and Akt to the plasma membrane. Akt is then phosphorylated by PDK1 and mTORC2 on its threonine and serine residues respectively. These phosphorylation events lead to the activation of Akt (Fig. 1).

Akt is the central mediator of the PI3K/AKT/mTOR pathway and phosphorylates several downstream targets which ultimately lead to cell proliferation. These include phosphorylating the apoptosis-inducing factor BAD (Datta et al. 1997) and the FOXO (FOXO1) transcription factors (Medema et al. 2000) which inhibit apoptosis and promote cell survival, or which removes inhibition of

pro-proliferative pathways, phosphorylating glycogen synthase kinase-3 (van Weeren et al. 1998).

mTOR is another phosphorylation target of Akt and is the focus of this paper. mTOR is a kinase that plays an important role in cell growth via modulation of cell cycle regulators or maintenance of nutrient supplies into the cell (Advani 2010). It is affected by Akt through the tuberous sclerosis complex (TSC), which is composed of two subunits: TSC1 (hamartin) and TSC2 (tuberin) (Manning et al. 2002). TSC2 is a negative regulator of mTOR and phosphorylation of TSC2 by Akt relieves TSC2's inhibitory effect on mTOR (Zhang et al. 2003). Once activated, mTOR phosphorylates its downstream effectors, including p70S6K and eIF4E-binding protein 1, which are both involved in protein synthesis (Jefferies et al. 1997, Harrington et al. 2004).

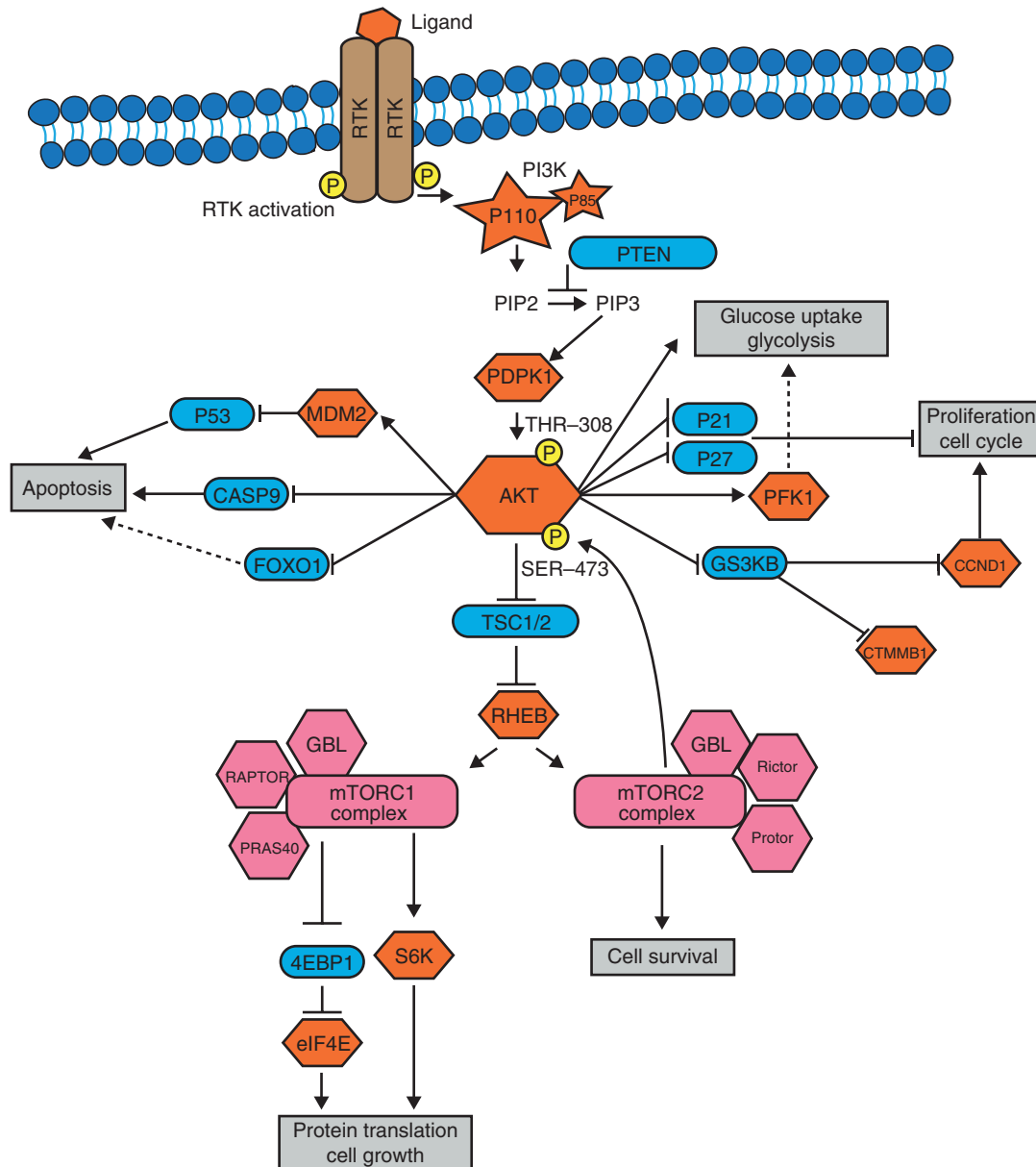
Termination of the PI3K pathway is accomplished via phosphatase-mediated degradation of PIP3, or through the negative feedback induced by the P70S6K-mediated phosphorylation of the upstream IRS1.

### Established links of the PI3K/AKT/mTOR pathway in endocrine and non-endocrine neoplasia

Given the key role of the PI3K/AKT/mTOR pathway in cell growth and metabolism, its role in pathological states such as neoplasia has been investigated extensively in the past decades. As described previously, the PI3K/Akt pathway is activated by upstream ligand-dependent RTKs. One of the most widely studied RTK is the ERBB2 receptor which is frequently overexpressed in breast and other cancers. In breast cancer, for example, ERBB2 is positively associated with worse histological grade, aneuploidy, high rate of cell proliferation, and poor survival (Revillion et al. 1998). Furthermore, transgenic mice overexpressing ERBB2/HER2 develop mammary tumors and lung metastases (Muller et al. 1988, Guy et al. 1992).

The *PIK3CA* gene, which encodes the p110 catalytic subunit of PI3K, appears to be involved in a number of cancers. For example, *PIK3CA* has been reported to have increased amplification in ovarian carcinoma, increased *PIK3CA* expression, and subsequently increased PI3K activity (Shayesteh et al. 1999). Somatic mutations in *PIK3CA* have also been reported in a number of cancers, including tumors of the colon, breast, brain, and lung (Samuels & Velculescu 2004). Furthermore, *PIK3CA* mutations have been shown to upregulate AKT and promote oncogenic transformation *in vitro* (Oda et al. 2008) and *in vivo* (Bader et al. 2006).

## PI3K–AKT–mTOR signaling axis

**Figure 1**

The PI3K/AKT/mTOR pathway.

The tumor suppressor gene *PTEN*, a negative regulator of PI3K signaling, has also been widely studied of its association with cancer. It has been reported that somatic mutations, allelic inactivation, or gene silencing via promoter hypermethylation is present in glioblastoma, melanoma, endometrial, and colon cancers (Nassif *et al.* 2004, Lahtz *et al.* 2010, Chow *et al.* 2011, Mhawech-Fauceglia *et al.* 2014). Furthermore, the inactivation tends

to be associated with poor clinical outcome. In prostate cancer, for example, Yoshimoto *et al.* (2007) demonstrated that homozygous *PTEN* deletion was an indicator of a more advanced disease at surgery and also associated with faster time to biochemical recurrence of disease.

AKT activation is a general feature of malignancy, seen in breast, ovarian, and pancreatic cancers (Kandel & Hay 1999, Altomare *et al.* 2002, Roy *et al.* 2002, Tanno *et al.* 2004).

Of particular note, brain tumors such as meningioma and glioblastoma have increased cell proliferation with concurrent activation of the AKT pathway (Riemenschneider *et al.* 2006, Johnson *et al.* 2010). Furthermore, AKT activation has been linked with poor prognosis in many human cancers (Perez-Tenorio & Stal 2002, Nam *et al.* 2003, Yamamoto *et al.* 2004). In addition, AKT is associated with resistance to chemo- and radiotherapy (Brognard *et al.* 2001, Clark *et al.* 2002, Tanno *et al.* 2004).

It has been shown that a number of cancers have increased mTOR activation. This activation is also associated with clinicopathologic activities. For example, in gastric cancer an increase in phosphorylated cytoplasmic mTOR was associated with depth of tumor invasion, tumor stage, and poorer survival rates (Murayama *et al.* 2009). Nuclear phosphorylated mTOR expression was also associated with poor survival in endometrial cancer (Yoshida *et al.* 2010). Based on the wealth of data available in the literature on the role of PI3K/AKT/mTOR pathway, it is clear that this pathway plays a key role in cancer development and correlates with clinical outcome. Therefore, pharmacologic inhibition of this pathway may be an effective treatment strategy for treating cancer.

### The importance of establishing novel therapeutic targets in PAs

PAs are typically benign nonmetastasizing lesions that may have few characteristics in common with more aggressive cancer types (Asa & Ezzat 2009). However, a large proportion of PAs at diagnosis are macroadenomas that have a tendency to become locally invasive (Mete & Asa 2012). An examination of the molecular determinants of these invasive properties in PA may reveal certain signaling patterns that are also involved in more malignant tumors.

Owing to the aggressive nature of some PAs, complete surgical removal may be impossible in the clinical setting. In such cases, the presence of residual tumor may result in tumor recurrence. Furthermore, some PA may recur several times following multiple survival procedures. Recurrent tumors that are not amenable for reoperation undergo radiation therapy, which may lead to adenoypophyseal damage leading to hypopituitarism, optic nerve damage, or cognitive deficits (Brada *et al.* 1993, Hahn *et al.* 2009).

As mentioned previously, most PAs at first clinical presentation are large macroadenomas, and these macroadenomas consist primarily of NFPA, which are unresponsive to current medications (Mete & Asa 2012). Among functional PA, the available medications, including SST analogs or dopamine agonists, are only effective in

certain subtypes of GH-PA and PRL-PA respectively (Mete & Asa 2012). Thus, there remains a need to identify novel targets for therapy for a large set of patients for whom current options are limited.

The pathobiology of PAs is complex and is yet to be elucidated. It has been established that the classical oncogenes and tumor suppressor genes are not commonly altered in PA (Asa & Ezzat 2009). However, recent interest has focused on exploring whether elements of the PI3K/AKT/mTOR pathway plays a role in the progression of PA, with ultimate hope that targeting this pathway in PAs will provide a novel therapeutic target.

### Analysis of the RTK/PI3K/AKT/mTOR pathways in PA

Several studies have used mouse and rat pituitary cell lines *in vitro* to study PA tumorigenesis (Table 1). The reader is referred to reference (Ooi *et al.* 2004) for a summary of how current mouse and rat PA cell lines were established.

The PI3K/AKT/mTOR pathway is activated by RTK, and RTK activation has been examined in relationship with the pituitary. RTKs are important for normal pituitary function and help to modulate hormone production and cell growth (Ezzat 2001). Aberrant RTK activation possesses the ability to confer proliferative potential and abnormal hormone production to pituitary cells leading to hyperplastic and/or neoplastic growth. Two important RTKs in normal pituitary development that are also reported to be involved in PA are epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR). Currently, studies examining RTK activation in PA and subsequent downstream PI3K/AKT/mTOR signaling remain scant.

EGFR has been reported to be involved in PA. Preclinical studies demonstrate that EGF is able to enhance mRNA levels of PRL in PA cell lines while gefitinib, an EGFR inhibitor, has been shown to block serum-induced cell proliferation and PRL gene expression (Vlotides *et al.* 2008). Furthermore, Lapatinib, a dual EGFR and HER2 inhibitor, has been reported to be more potent than gefitinib at abrogating PRL expression and PA cell proliferation both *in vitro* and *in vivo* (Fukuoka *et al.* 2011). The EGFR-mediated effects on PA cell hormone regulation and proliferation have been shown to be PI3K dependent. In the human situation, EGFR is demonstrable in all types of PA and its expression has been shown to correlate with tumor aggressiveness, especially in GHPA and NFPA (LeRiche *et al.* 1996, Cooper *et al.* 2011).

The FGFR has also been reported to be involved in PA. The FGFR consists of four receptors and mRNA for

**Table 1** Examining elements of the PI3K/AKT/mTOR in models of pituitary adenoma

Study	Model	Species	Treatment	Proteins	Outcome
Vlotides <i>et al.</i> (2008)	GH3 cells	Rat	EGF	EGFR expression	Increase PRL mRNA levels and cell proliferation
Romano <i>et al.</i> (2006)	GH4C1 cells	Rat	IGF1; PI3K/Akt inhibitors	PI3K/AKT	Increased PRL release and PI3K/Akt inhibitors reduce PRL release
Kowarik <i>et al.</i> (2010)	AtT20, MTt/S, and TtT/GF cells	Rat/mouse	PDGF/LY294002	PDGFR expression, PDK1, and AKT	Mixed expression of PDGFR in cells; PDGF-induced cell proliferation and VEGF-A secretion; and VEGF-A secretion blocked by LY294002
Banerjee <i>et al.</i> (2003)	GH3 cells	Rat	17 $\alpha$ -Estradiol/wortmannin	PI3K/AKT	Increased VEGF-A mRNA expression and decreased by wortmannin
Fernandez <i>et al.</i> (2003, 2004)	Lactotroph cells	Rat	IGF1/LY294002	AKT, BAD, and BCL2	Increased cell proliferation and cell survival; reversed by PI3K inhibitor LY294002
Rose <i>et al.</i> (2004)	aT3	mouse	IGF1/LHRH	IRS1, PI3K, and AKT	IGF1 stimulates cell proliferation and survival; co-treatment with LHRH reduces cell survival
Lu <i>et al.</i> (2008)	Genetic mutant developing TSHoma	Mouse	Knock in mutation of the thyroid receptor B gene	AKT, mTOR, p70s6k, and BCL2	Decreased apoptosis; LY294002 reduces pituitary growth
Kenerson <i>et al.</i> (2005)	Renal and pituitary tumor in Eker rats	Rat	Germline mutation of TSC2 gene	mTOR, S6	The mTOR pathway activity is critical for tumor progression
Musat <i>et al.</i> (2005)	ACTH-, GH-, and PRL-NFPAs	Human	NA	p-AKT, PTEN, and p27	Increased p-AKT expression; lower nuclear p27 and PTEN expression as compared with normal pituitary
Sajjad <i>et al.</i> (2013)	GH-, ACTH-, and NFPAs	Human	NA	mTOR (pS6/eIF4e)	Increased mTOR activity in all PA subtypes vs controls
Dworakowska <i>et al.</i> (2009)	ACTH-, GH-, and PRL-NF-adenomas	Human	NA	mTOR, TSC2, and p70s6k	No difference in mTOR, TSC2, p70s6k expression compared with controls; decreased c-MYC and increased cyclin D1 expression only in NF adenomas
Lin <i>et al.</i> (2009)	Pituitary adenomas	Human	Genetic <i>PI3kCA</i> mutations	PI3K/AKT	Some invasive and no noninvasive PA harbored mutation

prototypic isoforms of FGFR1, FGFR2, and FGFR3 are present in the normal pituitary (Abbass *et al.* 1997). A common alteration in the FGFR that promote PA is the presence of an N-terminally truncated variant of FGFR4 called pituitary tumor-derived FGFR4 (ptd-FGFR4; Ezzat *et al.* 2002). N-terminal truncation of FGFR4 results in a constitutively activated protein that is localized to the cytoplasm where it promotes PA oncogenic transformation *in vitro* and *in vivo*. FGFR4 inhibition with a

FGFR-selective inhibitor has been shown to restore membranous FGFR4 and inhibit PA proliferation. Ptd-FGFR4 is present in human PAs, where it has been shown to correlate with tumor aggressiveness (Ezzat *et al.* 2004, 2006). Recently, a SNP in the *FGFR4* gene, resulting in a substitution of glycine to arginine in the transmembrane domain of the FGFR4 protein, has been identified in PAs. The presence of the polymorphic variant has been shown to promote PA cell proliferation and



hormone deregulation *in vitro* and *in vivo*. In humans, the presence of this SNP has been shown to correlate with GH levels and tumor size in GH-PA (Tateno *et al.* 2011). The FGFR alterations with subsequent PI3K/AKT/mTOR signaling have not been examined in PAs; however, the polymorphic variant of FGFR4 and downstream mTOR signaling has been reported in pancreatic neuroendocrine tumors (Serra *et al.* 2012).

Consistent with a role for PI3K activation in PA, in a study by Lin *et al.*, mutations of the *PIK3CA* gene were assessed in 353 pituitary tumors. Nine percent of the invasive, but none of the noninvasive tumors, harbored *PIK3CA* gene mutations. Genomic *PIK3CA* amplifications, defined as more than four copies, were observed in both invasive and noninvasive tumors with a prevalence of 20–40% (Lin *et al.* 2009). Another study examined *PIK3CA* mutations and genomic amplifications in 33 PAs including ACTH-, GH-, PRL-producing, and NFPAs, and found that *PIK3CA* mutations were evident in 12.1% of tumors including one noninvasive ACTH tumor. Genomic amplification (defined as copy number  $\geq 4$ ) was found in 21.2% of cases (Murat *et al.* 2012).

Additional elements of the PI3K/AKT/mTOR pathway have been examined in PAs. In human tissues, AKT, PTEN, and p27 (a target of Akt) mRNA and protein expression were analyzed in ACTH-, GH-, PRL-, and NFPAs, as well as in normal pituitary tissue controls. In PAs, AKT mRNA expression and phosphorylated-AKT protein levels were increased in comparison to normal pituitary tissue. In addition, the levels of PTEN and p27 were lower in PA (Musat *et al.* 2005). The immediate downstream effectors of mTOR, pS6, and eIF4E, have also been studied in human PA tumor samples with more frequent activation of S6/eIF4E evident in all recorded PA subtypes relative to normal pituitary controls (33–71 vs 20%), with GH-PAs exhibiting the highest frequency of overexpression (Sajjad *et al.* 2013). Mouse models harboring genetic mutations resulting in the formation of PA have also been reported to exhibit unregulated p70S6k/S6 expression in PA tissue relative to adjacent CNS tissue (Kenerson *et al.* 2005, Lu *et al.* 2008). In contrast, another study reported that there was no difference in the expression of phosphorylated or total mTOR, TSC2, or p70S6K as compared with controls. However, this group did report an increase in c-MYC levels (a target of AKT) in all PA subtypes, as well as a mild activation of cyclin D1 but only in NFPAs (Dworakowska *et al.* 2009). Furthermore, elevated levels of cyclin D1 staining in NFPAs compared with other tumors have been replicated in a number of other studies. In a study including only NFPAs and GH-PAs, cyclin D1 allelic imbalance, which

may indicate gene amplification, was detected in one quarter of the PAs analyzed (Hibberts *et al.* 1999). Table 1 provides a comprehensive list of the different components of the PI3K/AKT/mTOR pathway examined in PA.

### Current mTOR inhibitors

The proposed role of aberrant PI3K/AKT/mTOR signaling in PA makes this tumor group amenable for the use of mTOR inhibitors in the clinic. Results using mTOR inhibitors in preclinical models of PA have provided further support for mTOR pathway involvement in PA tumorigenesis and as a target for medical therapy.

### Effects of rapamycin (and rapalog)-induced mTOR inhibition on PA cellular proliferation and viability

Rapamycin (sirolimus) is an immunosuppressant and antiproliferative agent that has previously been shown to be effective at abrogating cancer-related properties in a number of tumors (Douros & Suffness 1981, Majumder *et al.* 2004). Specifically, rapamycin binds to the mTORC1 complex, affecting downstream signaling events including cell cycle arrest and protein synthesis inhibition. Tumors that harbor upstream mutations from mTOR, such as *PTEN* deletion or *AKT* overexpression, are ideal targets for treatment with mTOR inhibitors. Everolimus (RAD001) is an orally available analog to rapamycin (rapalog) and has also been shown to be an effective anticancer agent in a number of *in vitro* cell lines and animal models (Beuvink *et al.* 2005).

PAs are typically of low proliferative potential as assessed by the cell cycle antigen ki-67 and of low mitotic index. However, the ability of ki-67 index to predict tumor growth/invasion and recurrence is debatable (Chacko *et al.* 2010). The presence of large macroadenomas that have a propensity for invasion into extrasellar structures could potentially benefit from antiproliferative agents. The ability of rapamycin and its analogs to inhibit cell proliferation is key to its antitumor efficacy. Several studies have examined the antiproliferative properties of rapamycin and rapalog treatment in pituitary and PA cells (Table 2). In normal rat pituitary cells, rapamycin was shown to inhibit basal proliferation and insulin-, cAMP-, and estradiol-induced proliferation of cells (Kawashima *et al.* 2000). Human PRL gene is expressed in the GH3 cell line and its transcription was shown to be inhibited by rapamycin (Wera *et al.* 1995a,b). In an animal model using rats that carry an inactivating germline mutation of the

**Table 2** *In vitro* studies examining effect of mTOR inhibitors on pituitary or pituitary adenoma cells

Cells	Species	Hormone	Drug	Outcome
Lactotrophs (1°)	Rat	PRL	Rapamycin	Inhibit basal and growth factor-induced proliferation of cells (Kawashima <i>et al.</i> 2000)
GH3 (CL)	Rat	GH/PRL	Rapamycin	Transcription inhibition of prolactin gene (Wera <i>et al.</i> 1995a,b); decreased cell viability and proliferation (Gorshtein <i>et al.</i> (2009), Sukumari-Ramesh <i>et al.</i> 2011); co-treatment with RT results in radiosensitization (Sukumari-Ramesh <i>et al.</i> 2011); and decrease cell proliferation/increased apoptosis (Dai <i>et al.</i> 2013)
			RAD001	Decreased cell viability and proliferation (Gorshtein <i>et al.</i> (2009), Sukumari-Ramesh <i>et al.</i> 2011); co-treatment with RT results in radiosensitization (Sukumari-Ramesh <i>et al.</i> 2011)
MtT/S (CL)	Rat	GH	Rapamycin/RAD001	Decreased cell viability and proliferation (Gorshtein <i>et al.</i> 2009)
MMQ (CL)	Rat	PRL	Rapamycin	Decreased cell viability and proliferation (Sukumari-Ramesh <i>et al.</i> 2011) and decreased cell proliferation/increased apoptosis (Dai <i>et al.</i> 2013)
αT3-1 (CL)	Mouse	FSH/LH	Rapamycin	Decreased cell proliferation/increased apoptosis (Dai <i>et al.</i> 2013)
			RAD001	Decreased cell viability (Sukumari-Ramesh <i>et al.</i> 2011)
AtT20 (CL)	Mouse	ACTH	Rapamycin Octreotide/rapamycin	Decreased cell proliferation (Cerovac <i>et al.</i> 2010) Greater decrease in cell proliferation than rapamycin or octreotide alone (Cerovac <i>et al.</i> 2010)
GH-adenoma (1°)	Human	GH	Rapamycin/RAD001	Decreased cell viability and proliferation (Gorshtein <i>et al.</i> 2009)
NFPA (1°)	Human	NA	Rapamycin	Decreased cell proliferation (Cerovac <i>et al.</i> 2010)
			Octreotide/rapamycin	Greater antiproliferative response than either octreotide or rapamycin exposure
			RAD001	Decreased cell viability; blockage of growth factor induced cell proliferation and VEGF secretion (Zatelli <i>et al.</i> 2010)
MENX (1°)	Rat	NA	NVPBEZ235	Greater antiproliferative effect seen when drugs were combined vs individually (Zatelli <i>et al.</i> 2010)
			RAD001	Decreased cell viability (Zatelli <i>et al.</i> 2010)
			NVPBEZ235	Decreased cell proliferation; triggers apoptosis (Lee <i>et al.</i> 2011) Greater antiproliferative effect than RAD001; triggers apoptosis (Lee <i>et al.</i> 2011)

CL, cell line; 1°, primary cells.

TSC2 gene that results in pituitary tumor formation, rapamycin induced regression of the pituitary tumors and a concomitant decrease in the levels of phosphorylated-S6 (the target of p70S6K; Kenerson *et al.* 2005). A recent study using human PA cells in primary culture has also demonstrated that rapamycin induces mTOR inhibition in mTOR-active GH-, ACTH-, and NFPA, although, in this study, cell viability or proliferation was not assessed (Sajjad *et al.* 2013). Nevertheless, these studies provide evidence for rapamycin as a possible anti-PA agent through its mTOR-inhibiting effects.

Gorshtein *et al.*, first demonstrated the antiproliferative effects of rapamycin and its orally bioavailable analog,

RAD001, on GH3 and MtT/S cells as well as on human GH-PA cells in primary culture. They reported that treatment with rapamycin or RAD001 significantly decreased cellular viability and proliferation in a dose- and time-dependent manner, which was reflected by decreased levels of phosphorylated p70S6K (Gorshtein *et al.* 2009). Another study used GH3 and the PRL secreting MMQ cell lines and also noted a time- and concentration-dependent decrease in cellular viability following exposure to rapamycin or RAD001 (Sukumari-Ramesh *et al.* 2011). These findings were attributed to decreased levels of mTOR phosphorylation at the serine-2448 residue, which is a key determinant of mTOR activity. A third study noted a small

but statistically significant antiproliferative effect of 1 nM rapamycin on mouse AtT20 cells and no effect at lower doses (Cerovac *et al.* 2010).

Musat *et al.* (2005) have reported that NFPAs have the highest levels of AKT mRNA expression of all PA subtypes. Furthermore, it has been shown that high levels of phosphorylated AKT may contribute to early recurrence of NFPA (Noh *et al.* 2009). Therefore, it is important to evaluate the antiproliferative efficacy of rapalogs in this histological subtype. Zatelli *et al.* (2010) examined RAD001 sensitivity in 40 NFPAs and reported that 70% (28/40) displayed a dose-dependent reduction in cellular viability. Furthermore, in these 28 samples, RAD001 blocked insulin-like growth factor 1 (IGF1)-induced increases in cell proliferation and VEGF secretion, although RAD001 by itself had no effect on VEGF secretion. Of note, investigation of patient medical records showed that those patients who responded to RAD001 were younger, were more likely to have an invasive macroadenoma, and predominantly female. Lee *et al.* examined a particular strain of rats with a *MENX* mutation that results in the development of multiple endocrine tumors, including NFPA. Administration of RAD001 to *MENX* PA cells in primary culture also resulted in a dose-dependent reduction in cellular viability that reached significance at a dose of 100 nM in 45% of their sample (five out of 11 cultures; Lee *et al.* 2011). Finally, Cerovac *et al.* (2010) exposed 28 human NFPAs in primary culture to rapamycin and reported a small (<20%) reduction in cellular proliferation in 29% (eight of 28 tumors) of their samples.

### Effects of dual PI3K/mTOR inhibition on PA cellular proliferation and viability

One potential pitfall of mTOR inhibition is that mTOR activation normally causes negative feedback on IRS1. O'Reilly *et al.* (2006) reported that inhibition of mTOR by rapamycin does indeed cause a decrease in p70S6K, abolishing its negative feedback to IRS1 and increasing AKT phosphorylation. This mechanism may explain the partial response to the inhibitor treatments mentioned earlier. Theoretically, it seems that co-administration of an agent being able to decrease AKT phosphorylation upstream could potentiate the antiproliferative action of rapamycin treatment downstream.

SST receptor type 2 (SST2) was shown to deactivate the PI3K pathway by inhibiting p85 tyrosine phosphorylation and thereby decreasing AKT phosphorylation (Theodoropoulou *et al.* 2006). SST analogs, such as octreotide, are frequently used for the treatment of neuroendocrine

tumors (Lamberts *et al.* 2002). Cerovac *et al.* (2010) demonstrated that the addition of octreotide to rapamycin increased serine-phosphorylated IRS1 levels, but notably decreased rapamycin-induced pAkt-Ser levels. In AtT20 cells, the addition of octreotide to rapamycin resulted in a 50% decrease in cell viability, an effect that was significantly greater than the actions of octreotide or rapamycin alone (Cerovac *et al.* 2010).

Rapamycin combined with SST analogs are effective at inhibition of human PA cell growth *in vitro*. In one study, among human NFPAs that did not respond to rapamycin treatment alone (20/28), all responded to concurrent rapamycin and octreotide treatment. Moreover, in the co-treatment group a lower dose of rapamycin was required to maintain a significant antiproliferative effect. This study also noted that co-treatment suppressed proliferation to a greater extent in the cells that did respond to rapamycin alone (8/28; Cerovac *et al.* 2010). Similar results were obtained when human NFPAs were exposed to SOM230, a SST receptor multiligand, in combination with RAD001. This study showed higher response rate to RAD001 alone (28/40 cultures responded), but co-treatment with SOM230 significantly potentiated the antiproliferative effect of RAD001 compared with SOM230 or RAD001 alone (Zatelli *et al.* 2010). Similar antiproliferative results were also obtained when ACTH PA cells in primary culture were exposed to SOM230 and RAD001 in combination (Zatelli *et al.* 2010).

The effects of RAD001 on the negative feedback loop which, by inhibiting mTOR, reduces p70S6k phosphorylation and induces IRS1 expression, was examined in pituitary cell lines. In one study, GH3 and AtT20 cells were exposed to rapamycin, which reduced serine-phosphorylated mTOR and phosphorylated p70S6K levels. In AtT20 cells, rapamycin treatment increased phosphorylated AKT levels, but this effect was reversed by octreotide in a serine-phosphorylated IRS1-dependent mechanism (Cerovac *et al.* 2010). In contrast, Gorshtein *et al.* (2009) showed that rapamycin or RAD001 treatment of GH3 and MtT/S cells abrogated phosphorylation of p70S6K without an accompanied rise in phosphorylated AKT levels 24 h after treatment.

Recently, a new class of compounds have been developed that are designed to inhibit both mTOR and upstream PI3K pathway components. Theoretically, these drugs are designed to inhibit mTOR without stimulating an increasing in upstream AKT activity. One of these agents, NVPBEZ235, is a synthetic small molecule that inhibits both PI3K and mTOR kinase activity by binding to the ATP cleft of these enzymes (Maira *et al.* 2008). In a study by



Lee *et al.* (2011), NVPBEZ235 was reported to inhibit the viability of 100% (ten of ten) of MENX rat PAs in primary cell culture. This result was significantly more potent than previous studies of RAD001 effects on this cell line. Furthermore, it was noted that incubation with NVPBEZ235 at concentrations that suppress cellular viability also decreased the phosphorylation of AKT and S6, whereas RAD001 decreased phosphorylated S6, but increased phosphorylated AKT (Lee *et al.* 2011). In another study examining NVPBEZ235, NFPA cells in primary cell culture exposed to the dual inhibitor exhibited a 50% decrease in cell viability in 37 of 40 cases (Zatelli *et al.* 2010).

Dual PI3K/mTOR inhibitors have also been combined with more conventional therapies to yield promising preclinical results in PA cells. One such dual-inhibitor known as XL765 used in combination with temozolomide (TMZ), an orally available alkylating agent previously shown to be effective at reducing PA cell viability (Ma *et al.* 2011, Sheehan *et al.* 2011), synergistically inhibited the proliferation of  $\alpha$ T3-1, MMQ, and GH3 PA cell lines (Dai *et al.* 2013).

### Effects of mTOR inhibition on apoptosis on PA cells *in vitro*

Apoptosis, or programmed cell death, is characterized by a rapid sequence of events leading to the elimination of damaged cells. It is defined by morphological changes such as cell shrinkage and nuclear demarcation. In neoplastic tissue, apoptosis is typically suppressed in favor of cell survival.

PA are clonal lesions that are thought to derive from a single transformed cell and defects within the apoptotic pathway may preserve the survival of the altered cell and promote PA genesis and growth. However, the clinical utility of using indicators of apoptosis as a prognostic tool in PAs is not consistently reported (Guzzo *et al.* 2014). Nevertheless, induction of PA cell apoptosis via mTOR inhibition has been an important characteristic of their anti-PA properties. Caspases are the main protein family effectors of apoptosis and the study by Zatelli *et al.* (2010) demonstrated that the effects of RAD001 on cellular viability of NFPA may be attributed to increased levels of caspase 3/7 activity. Increases in caspase 3/7 activity were also reported by Lee *et al.* (2011) following NVPBEZ235 treatment of MENX rat PAs in primary culture, but did not affect caspase activity in AtT20 cells (Cerovac *et al.* 2010). The use of XL765 and TMZ synergistically increased caspase 3/7 levels and TUNEL-positive  $\alpha$ T3-1, MMQ, and GH3 cells compared with either treatment alone (Dai *et al.* 2013).

### Effects of PI3K and mTOR inhibition on the cell cycle of PAs

The cell cycle is an important regulator of cell proliferation. Many cyclins, cyclin-dependent kinases (CDKs), and cyclin kinase inhibitors have been implicated in PA tumorigenesis (Saeger 2004), and some have been shown to be affected by mTOR inhibition.

Previous *in vitro* studies have reported that induction of cell cycle arrest is an important mechanism by which mTOR inhibitors exert their antiproliferative effects on PA cells (Favre *et al.* 2006). It has been reported that treatment with rapamycin results in the arrest of PA cell lines in the G0/G1 phase.

The early G1 phase is positively regulated by D-type cyclins and their corresponding CDKs (Sherr 2000). Rapamycin and its analogs seem to primarily affect cyclin D3 levels, while cyclin D1 and the CDKs (CDK4 and CDK6) remain unaffected. The dual PI3K/mTOR inhibitor NVP may be a more potent agent at abrogating PA cell cycle progression by attenuating both cyclins D1 and D3.

The KIP1/CIP family of CDK inhibitors (CKDI) negatively regulate cyclins and CDKs in the G1 phase of the cell cycle. Gorshtein *et al.* (2009) have demonstrated that p21/CIP levels were reduced by rapamycin in GH3 cells. A similar downregulation of p21/CIP was also noted when AtT20 cells were exposed to high concentrations of NVPBEZ235 (Cerovac *et al.* 2010). On the other hand, p27/KIP1 was reported to have greater transcriptional and protein expression following combined rapamycin/octreotide treatment in AtT20 cells (Cerovac *et al.* 2010). Increased p27 also seemed to play a role in the antiproliferative action of the dual inhibitor NVPBEZ235. Lee *et al.* (2011) were able to demonstrate that increased levels of p27 positively correlate with the antiproliferative efficacy of NVPBEZ235 in GH3 cells transfected with WT p27 and in MENX rat PA cells in primary culture.

A major determinant of G1/S progression is retinoblastoma (Rb) phosphorylation. CDK4 when associated with D-type cyclins phosphorylates Rb in the G1 phase. Gorshtein *et al.* (2009) showed that rapamycin inhibits Rb phosphorylation in GH3 and MtT/S cells and reduces subsequent E2F transcriptional activity. Octreotide and rapamycin co-treatment of AtT20 cells seems to potentiate this Rb inhibition effect (Cerovac *et al.* 2010).

In response to decreased E2F activity, cell cycle components that contribute to late-G1 phase and S-phase entry, such as the expression of E2F-regulated

genes cyclin E and CDK2, were also reduced by rapamycin in GH3 cells (Gorshtein *et al.* 2009). In AtT20 cells, cyclin E expression is reduced even further by rapamycin and octreotide co-treatment (Cerovac *et al.* 2010).

Thus, it appears that mTOR inhibitors possess the capacity to reduce proliferation of PA cells by inducing apoptosis and restoring some of the inhibitory mechanisms involved in the cell cycle particularly in the G1 phase.

### mTOR inhibition and radiation in PA cells

Radiotherapy is typically reserved for particularly aggressive PAs that are not suitable for surgical resection or are multirecurrent lesions that are resistant to pharmacologic therapies. To date, a number of studies have shown that mTOR inhibition radiosensitizes cancer cells (Altmeyer *et al.* 2012, Mauceri *et al.* 2012, Burris 2013). Existing data suggests that mTOR inhibition may also lead to decreased radioresistance of PA cells *in vitro*. Sukumari-Ramesh *et al.* (2011) report that inhibition of mTOR radiosensitizes GH3 cells such that 2.5 Gy in combination with 0.5 nM rapamycin or RAD001 reduced cellular viability more effectively than 2.5 or 10 Gy alone. Another study utilized nelfinavir, a protease inhibitor, to increase radiation sensitivity of GH3 cells, as well as MMQ and AtT20 cells. At 3 days post-radiation, cellular viability was decreased in all cell lines in a radiation dose-dependent manner (dose range, 0–6 Gy; Zeng *et al.* 2011). In this study, apoptosis was induced *in vitro* at higher rates by co-nelfinavir and radiation treatment compared with either intervention alone. The proposed mechanism of nelfinavir action is decreased phosphorylated S6, a key downstream target of the PI3K/AKT/mTOR pathway. Thus, these data suggest that nelfinavir action may bare similarities to that of rapamycin and its derivatives.

An increase in the radiosensitization of PA cells by mTOR inhibitors is an important therapeutic feature because although radiotherapy is a relatively uncommon form of treatment for PA, it is nevertheless utilized for aiding in the arrest of tumor growth of invasive and aggressive PA. Radiotherapy has been associated with a number of deficits in patients with PA, including optic nerve damage, cranial nerve palsies, and adenohypophyseal damage. Thus, when radiotherapy is employed, coupling this form of therapy with mTOR inhibition in PA patients may reduce the required dose of radiation thus minimizing the subsequent radiation-induced adverse effects.

### mTOR inhibition using xenograft *in vivo* models of PAs

Xenograft models are an important tool for studying the behavior of PA. Unlike transgenic models that may take a prolonged period of time to develop a PA, xenograft models can be generated quickly and reliably and are more reflective of the common sporadic PA which do not usually possess underlying genetic mutations (Asa & Ezzat 1998). Furthermore, some transgenic mouse models first develop pituitary hyperplasia before developing a PA which is not characteristic of the human scenario.

Two studies to date have used xenograft models to examine the effects of mTOR inhibition on PA. Zeng *et al.* demonstrated that GH3 cells implanted into the flanks of nude mice treated with a combination of nelfinavir and radiation experienced delayed tumor size quadrupling time. Co-treatment displayed a synergistic effect compared with nelfinavir or radiation treatment alone. Immunohistochemistry of tumor sections from this study confirmed downregulation of phosphorylated S6 (RPS6KB1) following treatment with nelfinavir (Zeng *et al.* 2011). In another study by Dai *et al.* (2013), GH3 cells were also used and implanted into the flanks of female nude mice. These mice were treated with a combination of XL765 and TMZ which was shown to inhibit tumor growth and induce apoptosis; inhibit GH and PRL secretion; and downregulate AKT, mTOR, and S6 phosphorylation (Dai *et al.* 2013).

Unfortunately, there are currently no intracranial mouse models of PA to study mTOR inhibition. Intracranial models may be more reflective of the human situation by mimicking the intracranial microenvironment of PA. An intracranial xenograft model may provide a more accurate portrait of the nature of sporadic PA growth behavior following mTOR inhibition. One factor hampering the establishment of an intracranial mouse model is the difficulty in accessing the mouse pituitary fossa. Nevertheless, more robust xenograft models in general and intracranial models specifically are needed to better characterize PI3K/AKT/mTOR pathway signaling and how it impacts PA growth dynamics. Utilization of improved preclinical models of PA may provide stronger evidence for the role of this oncogenic pathway in PA and pave the way for clinical trials involving mTOR inhibition in PA patients.

### Future directions: clinical applications for mTOR inhibitors

Despite a promising role for mTOR inhibitors in preclinical models of PA, data regarding mTOR inhibitors as

efficacious therapeutic agents remains limited in clinical management of PA. To date, there is one case report published on the combined use of everolimus and octreotide in a patient with an ACTH pituitary carcinoma resistant to TMZ treatment (Jouanneau *et al.* 2012). Pituitary carcinomas are extremely rare malignant pituitary tumors which are differentiated from PA by their potential for metastatic spread. The combined everolimus–octreotide treatment was unable to control tumor growth in this patient who eventually died shortly after treatment was initiated. It should be noted that the studies attesting to the efficacy of mTOR inhibition in the PA preclinical setting were conducted using human NFPA or GH-secreting PA (Gorshtein *et al.* 2009, Zatelli *et al.* 2010). At this stage, though preclinical results are promising, the enthusiasm for their efficacy in clinical practice has to be moderated by the fact that objective clinical data is lacking to confirm the therapeutic value of mTOR inhibitors in PA patients.

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