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The role of Akt signalling in the mammalian ovary

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ABSTRACT The serine/threonine protein kinase Akt is involved in many cellular processes including cell growth, survival, proliferation and metabolism. Akt activity is modulated downstream of phosphatidylinositol-3-kinase (PI3K) in response to different extracellular stimuli. In the mammalian ovary, Akt collaborates with other kinases in the regulation of coordinate follicle and oocyte development. Akt determines the pool of primordial follicles and the transition from quiescent to growing phase. In addition, the kinase modulates granulosa cell apoptosis throughout folliculogenesis. In oocytes Akt participates in the control of meiosis resumption and, at metaphase II stage, regulates polar body emission and spindle organization. Its inhibition negatively affects preimplantation embryo development. As a consequence of such a central role, Akt dysregulation is associated with several human diseases including infertility and ovarian cancer.

KEY WORDS: *Akt, ovary, cell cycle, ovarian cancer, infertility*

The serine/threonine protein kinase Akt, also known as protein kinase B (PKB), is a critical regulator of human physiology, and controls an impressive array of diverse cellular functions including the modulation of growth, survival, proliferation and metabolism. The Akt kinase family is comprised of three highly homologous isoforms, Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ), which have a highly conserved domain structure formed by an N-terminal pleckstrin homology (PH) domain, a kinase domain and a C-terminal regulatory tail containing a hydrophobic motif (Manning and Cantley, 2007). The functions are not completely overlapping and isoform-specific signalling contributes to the diversity of Akt activities. However, despite these important advances, a thorough understanding about the specific roles of Akt family members and the molecular mechanisms that determine Akt isoform functional specificity will be essential to elucidate the complexity of Akt regulated cellular processes and how Akt isoform-specific deregulation might contribute to disease states (Gonzalez and McGraw, 2009).

Regulation of Akt activity

Akt is one of the most versatile kinase in the human kinome (Hanada *et al.*, 2004; Manning and Cantley, 2007). Its activity is modulated downstream of phosphatidylinositol-3-kinase(PI3K) in response to extracellular stimuli following a multistep process (Fig. 1). Many growth factors and cytokines stimulate an increased activity in the lipid enzyme PI3K, resulting in a subsequent increase in PI(3,4)P2 and PI(3,4,5)P3 on the plasma membrane. Akt, by virtue of the PH domain, binds to the 3-phosphoinositide produced by PI3K, and then is recruited to plasma membrane. Akt is phosphorylated at two sites: the Thr308 residue within the T-loop of the catalytic domain is phosphorylated by the phosphoinositide-dependent kinase 1 (PDK1); the Ser473 residue within the carboxyl terminal hydrophobic domain by the mammalian target of rapamycin complex 2 (mTORC2). Other proteins, including integrin-linked kinase (ILK) and mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK2), have been reported to phosphorylate this residue. Akt can also be activated in a PI3K-independent manner. For example, cAMP elevating agents have been shown to activate Akt through PKA, whilst Ca^{2+}/calmodulin-dependent kinase (CaMK) can directly phosphorylate and activate Akt *in vitro*. Several non-kinase interactors (Hsp90, Hsp27, Tcl1, Geb10, Ft1) have also been described to positively regulate Akt catalytic activity (Xu *et al.*, 2012). It has been suggested that the phosphorylation in Ser473 can facilitate that in Thr308, and may determine Akt specificity rather than activity. Akt is transiently localized to the plasma membrane during activation and, once activated, phosphorylates substrates regulating multiple cellular functions (Manning and Cantley, 2007) (Fig.1A).

Akt1, Akt2 and Akt3 isoforms share approximately 80% amino

Abbreviations used in this paper: mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog delete on chromosome 10.

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acid identity and are thought to have similar primary substrate specificity. They are widely expressed in various tissues: Akt1 is most abundant in brain, heart and lung; Akt2 is predominantly expressed in skeletal muscle and embryonic brown fat; Akt3 is mainly expressed in kidney, brain and embryonic heart. Findings from Akt isoform-specific knockout mice suggest that Akt family kinases are likely to have distinct biological functions *in vivo*. Akt1 knockout mice are smaller than littermate controls and show increased rates of apoptosis in some tissues, reflecting the role of Akt1 in cell survival. By contrast, Akt2 knockout mice develop type 2 diabetes and show impaired glucose utilization, suggesting that Akt2 function is more specific for the insulin receptor signalling pathway. The precise role of Akt3 is less clear, but knockout mice display reduced brain development (Fig.1B).

Some of the Akt substrates carry out more than one function, and one process is often mediated by several downstream targets. Identification of substrates has been greatly aided by the definition of a minimal recognition sequence for Akt, RXRXX(pS/pT)ψ, where R denotes Arginine, X any aminoacid, pS/pT phosphoserine and phosphothreonine and ψ represents a bulky hydrophobic residue (Hers *et al.*, 2011). This consensus phosphorylation site is similar to that of two other AGC kinases as p90 ribosomal S6 kinase (p90Rsk) and p79S6K. Phosphorylation by Akt has various effects on protein substrates, including inhibition/stimulation of their activity, alteration of subcellular localization, protection against degradation or regulation of binding to protein partners (Vasudevan and Garraway, 2010).

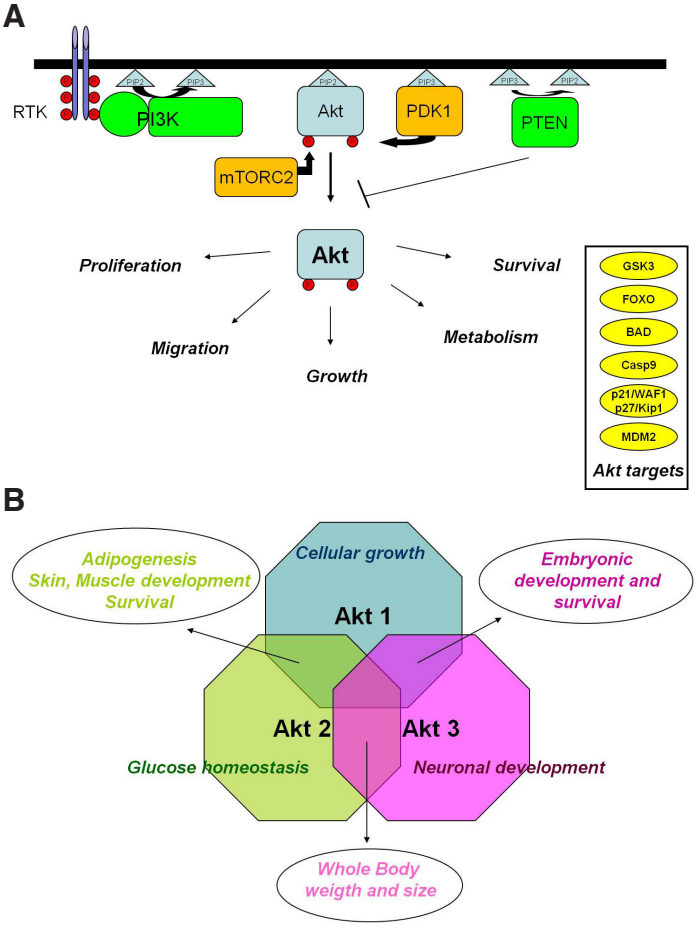

Fig. 1. Schematic regulation of the Akt pathway. (A) *In response to numerous growth factors and cytokines Akt is activated downstream of PI3-kinase following a multistep mechanism. Activated PI3-kinase converts PIP2 into PIP3 providing sites of recruitment at the plasma membrane of proteins containing PH domains, including Akt and PDK1 kinases. Then, Akt is phosphorylated/activated by PDK1 and mTORC2. Activated Akt, through the phosphorylation of numerous downstream targets, regulates a wide array of cellular functions.* **(B)** *Overlapping and specific functions of the Akt family members elucidated from the phenotypic analysis of single and double Akt isoform knockout mice.*

AKT in physiological metabolism

Although consensus motif analysis indicates that there are potentially thousands of cellular substrates for Akt, about 50 have been characterized so far (Bononi *et al.*, 2011). As a proto-oncoprotein and primary target of PI3K, Akt was firstly characterized for its capacity to regulate cell survival and proliferation. Indeed, the constitutive activation of Akt leads to uncontrolled cell proliferation, inhibition of apoptotic pathways and cell cycle dysregulation, typical hallmarks of many human cancers. Akt is able to directly or indirectly modulate apoptosis. The direct effects are linked to phosphorylation events or interactions with cell death actors, whereas the indirect regulation is mediated through transcriptional responses to apoptotic stimuli. In this context, Akt regulates the activity of transcriptional factors necessary for the expression of pro- or anti-apoptotic molecules (e.g. YAP, CREB, FOXO proteins, NF-κB, E3 ubiquitin ligase MDM2). In particular, Akt phosphorylates members of the Forkhead family of transcription factors (FOXOs), leading to binding of 14-3-3 proteins and cytoplasmic localisation: FOXO1 is phosphorylated on Thr24, Ser256 and Ser319; FOXO3a and FOXO4 on equivalent sites. These phosphorylations prevent the expression of target genes, such as those expressing the BH3-only family proteins and Fas-ligand. Moreover, FOXOs modulate proliferative effects by increasing the expression of p27/Kip1, which inhibits the cyclin/CDK complexes. Finally, FOXOs are also involved in mediating the effects of Akt on gluconeogenesis and fatty acid oxidation.

Akt positively regulates transcription factors controlling the expression of genes implicated in cell survival. The kinase has an important role in the regulation of NF-κB-dependent pro-survival genes such as c-IAP1 and c-IAP2, and positively regulates the CREB-dependent binding of accessory proteins that are necessary for the transcription of pro-survival genes such as Bcl-2 and Mcl-1 (Hers *et al.*, 2011). Bad, a proapoptotic member of the Bcl-2 family, is one of the first discovered targets of Akt phosphorylation. Its phosphorylation on Ser136 disrupts interaction with Bcl- 2/Bcl-XL, while Akt-dependent phosphorylation of proapoptotic Bax protein on Ser184 suppresses its translocation to mitochondria.

One of the best-conserved functions of Akt is the capacity to promote cell growth. Indeed, Akt activation increases the transcription of c-Myc, a strong promoter of cell cycle progression by inducing the expression of cyclins D and suppressing the expression of multiple negative regulators such as p21/Cip1, p27/Kip1 and p15/INK4b. However, the stability of c-Myc and cyclin D1 are indirectly controlled via the glycogen synthase kinase (GSK3). It is noteworthy that Wnt signalling activates β-catenin through the blocking of GSK3 (Metcalfe and Bienz, 2011).

In somatic cells, GSK3 is present in two isoforms encoded by

two distinct genes, designated GSK3α and GSK3β. Akt regulates cell cycle by preventing GSK3β-mediated phosphorylation and degradation of cyclin D1, and by negatively regulating the cyclin-dependent kinase (Cdk) inhibitors p27/Kip1 (Viglietto *et al.*, 2002). Akt also stimulates G1/S progression by inactivating Cdk inhibitor p21/Cip1 and p27/Kip1 with consequent activation of cyclin E-Cdk2 (Viglietto *et al.*, 2002). Remarkably, cytoplasmic mislocalization of p27/Kip1 has been observed in various human tumors, and in about 40% of primary human breast cancers, cytoplasmic p27/Kip1 correlates with Akt activation (Viglietto *et al.*, 2002).

It has been reported previously that Akt activity is high in the G2/M phase of the cell cycle through direct phosphorylation of both Cdk1 activators and inhibitors. In fact, during the G2/M transition, Cdc25B phosphatase shuttles between the nucleus and the cytoplasm. This event is caused by the opposing actions of the nuclear localization (NLS) and nuclear export signals (NES) in association with 14-3-3 family members. After Akt-mediated Ser353 phosphorylation, Cdc25B is exported in the cytoplasm and contributes to mitotic entry. In oocytes collected from the starfish *Asterina pectinifera*, Akt phosphorylates Cdk1 inhibitor Myt1 at Ser75, thus inducing cyclin B-Cdk1 activity. A recent study suggests that Akt can also regulate the progression from S to G2 phase via phosphorylation and cytoplasmic translocation of Cdk2 (Xu *et al.*, 2012). The predominant mechanism for promoting cell growth appears to be mediated by activation of the mammalian target of rapamycin complex 1 (mTORC1), which is regulated by both nutrients and growth factor signalling. Akt has been suggested to directly phosphorylate mTOR on Ser2448, but the role of this phosphorylation remains still unclear (Xu *et al.*, 2012). mTORC1 activation seems due to the inhibitory phosphorylation of the tumour suppressor tuberous sclerosis complex 2 (TSC2, also known as tuberin). This complex functions as a GTPase-activating protein (GAP) for the small G protein Ras homologue enriched in brain (RHEB). The decreased GAP activity of the complex leads to accumulation of RHEB-GTP and activation of mTORC1. However, it remains poorly understood how TSC2 phosphorylation by Akt leads to decreased GAP activity. Moreover, a second Akt substrate has been found to be involved in mTORC1 regulation, i.e. the proline-rich Akt substrate of 40 kDa (PRAS40). Indeed, Akt directly phosphorylates PRAS40 on Thr246, and this phosphorylation is fundamental for 14-3-3 binding, thereby enhancing mTORC1 activity.

Akt has a key role in glucose transport downstream of the insulin receptor, and can exert a positive feedback on this pathway via the phosphorylation of the protein tyrosine phosphatase PTP1B on Ser50. Furthermore, Akt regulates the storage of glucose in the form of glycogen by inhibiting GSK3 after specific serine phosphorylations (Hers *et al.*, 2011). Interestingly, GSK3β is a target of Akt phosphorylation/inactivation also inside mitochondria, even if the role of this interaction remains to be clarified (Bononi *et al.*, 2011). Akt has a key role in regulating lipid metabolism within the cell, and activates cyclic nucleotide phosphodiesterases (PDE) via phosphorylation on Ser273, that reduces cAMP levels and inhibits lipolysis (Hers *et al.*, 2011).

Akt in pathological disease

As a result of the central importance in such a wide range of cellular processes, Akt dysregulation is associated with several human diseases including diabetes, cardiovascular, neurological and cancer diseases.

Sensitivity of cells to insulin is critical for glucose uptake into responsive tissues. Clinical insulin resistance of peripheral target tissues (a feature of Type II diabetes) can be defined as a failure of such tissues to increase whole body glucose disposal in response to insulin. Akt regulates glucose uptake into muscle and fat cells by stimulating the translocation of GLUT4 glucose transporter to the plasma membrane. Furthermore, this kinase stimulates survival and proliferation of insulin-secreting β-cells in the pancreas, and represses insulin-dependent gluconeogenesis in the liver. Akt de-regulation has been implicated in diabetes, as Akt2 knockout mice are diabetic. Defective glucose transport causes elevated circulation of free fatty acids (FFA) and inflammatory cytokines that contribute to insulin resistance by inducing serine phosphorylation of IRS-1. This modification determines the uncoupling between IRS-1 and its receptor or downstream effectors, followed by reduction of PI3K/Akt activity (Hers *et al.*, 2011).

Akt has a clearly defined role also in the functional behaviour of cells in the cardiovasculature, and regulates cardiac growth, contractile function and coronary angiogenesis. In addition, Akt suppresses neuronal cell death via multifarious mechanisms including alterations in gene expression, inhibition of caspase-9 and suppression of cytochrome-C release by mitochondria. In dopamine neurons of the substantia nigra, overexpression of a constitutively active Akt (Myr-Akt) prevents neurotoxin-induced cell death and maintenance of axonal sprouting. Accordingly, Akt activation represents a potential therapeutic approach for the treatment of several neurodegenerative diseases including Parkinson's disease (Hers *et al.*, 2011).

Akt and cancer

Akt signalling is frequently activated in human cancers (Hers *et al.*, 2011). Although Akt gene mutations are not widely reported, amplification, overexpression and activation of this gene occur at high frequency in a number of cancers (Bononi *et al.*, 2011). Interestingly, there is evidence for hyperactivation of specific Akt isoforms in certain tumors, suggesting that in some cases there is Akt isoform-specificity to cell transformation. Indeed, Akt2 gene amplification has been reported in ovarian and pancreatic cancers; Akt1 amplification has been reported in gastric cancers, and Akt3 amplification in melanoma. Overexpression of specific Akt isoforms independent of gene amplification has also been found to differ among different cancers. For example, Akt2 has been found to be upregulated in hepatocellular carcinomas and colorectal cancers, Akt1 in breast cancers and Akt3 mRNA has been shown to be upregulated in estrogen receptor-negative breast tumors and melanoma. Recently, a somatic activating mutation in Akt1 PH domain has been identified in human ovarian, breast and colorectal cancers, but no changes in Akt2 or Akt3 has been found (Gonzalez and McGraw, 2009).

Alterations of Akt signalling in human cancer also result from mutations of the upstream PI3K and PTEN (Phosphatase and tensin homolog delete on chromosoma 10). Many activating mutations have also been described in PI3KCA gene, which encodes one of the p110 catalytic subunits of PI3K. PI3KCA mutations (hotspots: E542K, E545K, H1047R) are able to constitutively activate Akt and enhance its oncogenic activity (Xu *et al.*, 2012). PTEN is one of the most frequently mutated genes, second only to p53 in

human cancers, and its activity can be lost by mutation, deletion, or promoter methylation silencing (Xu *et al.*, 2012). PTEN acts as a tumor suppressor that negatively regulates Akt pathway by hydrolysing PI(3,4,5)P3 to PI(4,5) P2 (Hers *et al.*, 2011).

PTEN pathway

PTEN influences multiple cellular functions in a context-dependent manner. The loss of PTEN leads to constitutively high levels of PIP3, which promotes the recruitment of a subset of proteins that contain a pleckstrin homology domain to cellular membranes, including PDK1 and Akt (Chow and Baker, 2006). Ser380, Thr382 and Thr383 phosphorylations in the C-terminal tail of PTEN appear to increase its stability, but may also decrease activity by inhibiting PTEN recruitment to the membrane through disruption of electrostatic interactions or decreased interaction with other proteins.

PTEN can be inactivated either by mutation with loss of heterozygosity or by other mechanisms (e.g. promoter methylation, micro-RNA interference with or without pseudogene loss, phosphorylation and delocalization from the plasma membrane). PTEN transcriptional regulation is also a possibility in tumors. In fact c-Jun, NF-κB and HES-1 have been shown to repress PTEN transcription downstream of Ras, MKK4 and Notch activation, respectively. PTEN also induces a marked decrease of proliferation and inhibits the migration of cells, likely by involvement of Rac and cdc42, but not of RhoA. Recently, a structural role has been proposed for PTEN in the maintenance of apical-basal polarity in epithelial cells and of a segregated apical pool of PIP2, which should compartmentalize PIP2-binding proteins to the apical membrane. It is possible that loss of this function contributes to the epithelial-mesenchymal transition (EMT) observed in the progression of epithelial cancers (Georgescu, 2010). The activity of PTEN is modulated by posttranslational modifications (oxidation, acetylation, phosphorylation, ubiquitination, proteolytic cleavage) and by protein-protein interactions. It is therefore straightforward to examine in tumors the deregulation of the enzymes acting on PTEN or of its interacting proteins that may be modified (Georgescu, 2010). Mutation, homozygous deletion, promoter methylation and translational modification can all account for PTEN silencing. In conclusion, the integration of all these functions and connections makes of PTEN a central inhibitory node with a major impact in cancer (Georgescu, 2010).

The mTOR Pathway

mTOR is an atypical serine/threonine protein kinase that belongs to the PI3K-related kinase family and interacts with several proteins to form two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2). Both mTOR complexes have shared and unique components, but have different sensitivities to rapamycin as well as upstream inputs and downstream outputs.

mTORC1 is the better characterized of the two mTOR complexes. The mTORC1 pathway integrates inputs from at least five major intracellular and extracellular cues—growth factors, stress, energy status, oxygen and amino acids—to control many processes, including protein and lipid synthesis and autophagy. The heterodimer consisting of tuberous sclerosis 1 (TSC1; also known as hamartin) and TSC2 (also known as tuberin) is a key upstream regulator of mTORC1, and functions as a GTPase-activating protein (GAP) for the Ras homolog enriched in brain (Rheb) GTPase. The GTP-bound form of Rheb directly interacts with mTORC1 and strongly stimulates its kinase activity. TSC1/2 negatively regulates mTORC1 by converting Rheb into its inactive GDP-bound state. TSC1/2 transmits many of the upstream signals that impinge on mTORC1 as insulin and insulin-like growth factor 1 (IGF1), that stimulate the PI3K and Ras pathways. The effector kinases of these pathways phosphorylate both TSC1/TSC2 complex, which is inactivated, and mTORC1, which is activated. Akt also signals to mTORC1 in a TSC1/2-independent fashion by causing the dissociation from raptor of phosphorylated PRAS40, an mTORC1 inhibitor. Many stresses (low energy and oxygen levels, DNA damage) also act, at least in part, through TSC1/2 in response to hypoxia or a low energy state. Adenosine monophosphate-activated protein kinase (AMPK) phosphorylates TSC2 and increases its GAP activity toward Rheb. DNA damage also signals to mTORC1 through multiple mechanisms, all of which require p53-dependent transcription. DNA damage induces either the expression of TSC2 and PTEN, causing down-regulation of the entire PI3K-mTORC1 axis, or activates AMPK through a mechanism that depends on the induction of Sestrin1/2. mTORC1 amino acid-dependent activation requires Rag GTPases, and these interactions result in the translocation of mTORC1 from a poorly characterized cytoplasmic location to the lysosomal surface, where Rag GTPases dock it on a multisubunit complex called Ragulator. Protein synthesis is by far the best-characterized process controlled by mTORC1, that directly phosphorylates eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1(S6K1). mTORC1 also controls the synthesis of lipids required for proliferating cells to generate membranes (Laplante and Sabatini, 2012) by acting through the sterol regulatory element-binding protein 1/2 (SREBP1/2) transcription factors. In addition, mTORC1, positively regulates cellular metabolism and ATP production.

Compared to mTORC1, much less is known about the mTORC2 pathway. mTORC2 signalling is insensitive to nutrients but does respond to growth factors such as insulin through poorly defined mechanism(s) that require PI3K. One potential mechanism involves mTORC2 binding to ribosomes in a PI3K-dependent fashion. mTORC2 controls several members of the AGC subfamily of kinases including Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1), and protein kinase C-α (PKCα). mTORC2 directly activates Akt by phosphorylating its hydrophobic motif (Ser473), a site required for its maximal activation. Defective Akt-Ser473 phosphorylation associated with mTORC2 depletion impairs the phosphorylation of some Akt targets, including FOXO1/3a, while other targets, such as TSC2 and GSK3β remain unaffected. The fact that Akt activity is not completely abolished in cells lacking mTORC2 probably explains these results. Moreover, mTORC2 directly activates SGK1, a kinase controlling ion transport and growth (Laplante and Sabatini, 2012) that, in contrast to Akt, is completely blocked by the loss of mTORC2. Because SGK1 controls FOXO1/3a phosphorylation on residues also phosphorylated by Akt, loss of SGK1 activity is probably responsible for the reduction in FOXO activity in mTORC2-depleted cells. PKCα is the third AGC kinase activated by mTORC2. Along with other effectors, such as paxilin and Rho GTPases, the activation of PKCα by mTORC2 regulates cell shape in cell type-specific fashion by affecting the actin cytoskeleton (Laplante and Sabatini, 2012).

Several observations support the importance of mTOR pathway in cancer pathogenesis. Many components of the PI3K signalling pathway, which is upstream of both mTORC1 and mTORC2, are

mutated in human cancers. In addition, several familial cancer syndromes arise from mutations in genes encoding proteins that lie upstream of the mTOR complexes, including TSC1/2, serine/threonine kinase 11 (Lkb1), PTEN and neurofibromatosis type 1 (Nf1). A growing body of evidence points to the deregulation of protein synthesis downstream of mTORC1 at the level of 4E-BP1/eIF4E as playing a central role in tumor formation. 4E-BP1/eIF4E also mediates the effects of oncogenic Akt signalling on mRNA translation, cell growth, and tumor progression (Hsieh *et al.*, 2010). However, despite the evidence in the literature data, how the mTORC1/4E-BP1/eIf4E axis contributes to cancer is still unclear. In addition, it has been reported that the constitutive activation of PI3K-mTORC1 signalling in cancer cells strongly inhibits autophagy. There is also emerging evidence for a role of mTORC2 in cancer. Many gliomas overexpress the mTORC2 subunit RICTOR, and its forced overexpression promotes mTORC2 assembly and activity with increased proliferative and invasion potential of cancer cells (Laplante and Sabatini, 2012). In mice, the development of prostate cancer induced by the loss of the tumor suppressor PTEN requires mTORC2 function. Although these results point to new anticancer therapies, at present there is no pharmacological way to inhibit mTORC2 without also affecting mTORC1 (Laplante and Sabatini, 2012).

The PI3K/Akt gene family in the mammalian ovary

Among the several regulative pathways involved in the regulation of oogenesis (Edson *et al.*, 2009; Cecconi *et al.*, 1996; Cecconi *et al.*, 2004; Canipari *et al.*, 2012), the PI3K signalling pathway is a critical regulator of follicle growth, differentiation and survival. The observation that deletions of these genes cause infertility and premature ovarian failure (POF) sustains their essential role in preserving the normal female reproductive lifespan (Edson *et al.*, 2009).

In mouse ovary the tumor suppressor protein PTEN is specifically expressed by oocytes and contributes to the maintenance of the pool of primordial follicles in a quiescent but surviving condition (Reddy *et al.*, 2009). Its deletion causes Akt hyperactivation, thereby determining rapid depletion of primordial follicle pool and the onset of POF (Reddy *et al.*, 2008).

Also PDK1 is expressed in the oocyte, and appears to be indispensable for maintaining the survival of primordial follicles. For this reason, female mice deficient for PDK1 are infertile and show accelerated ovarian aging and POF(Reddy *et al.*, 2009). At molecular level, the absence of this kinase determines in oocytes the suppression of Akt/S6K1 (p70 ribosomal protein S6 kinase 1)/rpS6 (ribosomal protein S6) signalling. A low activity of rpS6 leads to accelerated loss of primordial follicles; in contrast, its over-activity enhances follicular activation and survival (Reddy *et al.*, 2008). It is noteworthy that while PTEN loss in oocytes leads to POF due to excessive follicular activation and degeneration, PDK1 deficiency causes POF as a result of abnormal recruitment of primordial follicles from quiescence (Reddy *et al.*, 2008; Reddy *et al.*, 2009). Both mutations have been involved in the onset of POF in humans.

In bovine granulosa cells, bone morphogenetic proteins 4 and 7 (BMP-4, BMP-7) inhibit granulosa cell apoptosis via PI3K/PDK1/Akt and PI3K/PDK1/PKC, respectively. In these cells, BMP-4 or BMP-7 may stimulate the different site of phosphorylation of PDK1 (Shimizu *et al.*, 2012). Also in humans, PDK1 can be phosphorylated at various residues of serine (Ser25, Ser241, Ser393, Ser396 and Ser410), that differentially regulate its enzymatic activity.

The role of Akt in the mammalian ovary has been mainly assessed by analyzing null mice. Akt1-deficient males are fertile, but are more sensitive to both genotoxic and nongenotoxic insults. Akt1-deficient female mice have a reduced fertility, a delay of about 5 days in the onset of estrus, an increase in maternal age at first litter, and a reduction in average litter size. The oocytes of primary follicles of Akt1-deficient females are larger than wild type animals, and sometimes follicles contain multiple oocytes, suggesting alterations in the process of cyst breakdown. Akt3-deficient females are reported to have normal fertility, while the consequences of Akt2 deletion on fertility are still unclear. In the porcine ovary, Akt1 has been localized in granulosa cells of primordial follicles and in the basal layers of the granulosa cells of preantral and antral follicles, but not in atretic follicles or corpora lutea. In the human ovary, Akt1 is expressed in oocytes, in granulosa cells and in the thecal cells of primordial follicles, in follicles at each growing stage, and in the luteal cells (Goto *et al.*, 2007). In rodents, Akt1 has been found in both granulosa cells and in oocytes (Reddy *et al.*, 2009).

PI3K/Akt interactions with signalling pathways controlling follicular and oocyte development

Several lines of evidence have indicated that a strict correlation exists between Kit ligand (KL)-Kit receptor (kit) pathway and PI3K/Akt pathway, both necessary for follicle development especially at FSH-independent stages (Liu, 2006) (Fig. 2). KL is fundamental in the control of primordial germ cells (PGCs) migration into the genital ridges, because of its chemoattractant action. In purified 11.5-12.5 dpc PGCs, KL stimulates the phosphorylation of Akt by either PI3K or SRC kinases. The crucial role of Akt in promoting PGC survival and proliferation has been confirmed by Kimura *et al.,* (Kimura *et al.*, 2008), who found that PGC apoptosis significantly decreased and proliferation significantly increased upon Akt induction.

The formation of primordial follicles occurs usually during the latter half of pregnancy in humans and immediately following birth in mice. The postnatal mouse ovary is mainly populated by primordial follicles, each composed of a meiotically arrested (germinal vesicle, GV) primary oocyte enclosed within several flattened pregranulosa cells. The primordial to primary follicle transition is controlled by KL-kit signalling through the induction of PI3K/AKT pathway. The interaction between KL, which is expressed primarily by granulosa cells, and kit receptor, which is present in oocytes (Cecconi *et al.*, 2004), stimulates PI3K recruitment from the cytoplasm to cell membrane through the binding of the SRC homology 2 (SH2) domain of the p85 subunit of PI3K to the phosphorylated tyrosine on Kit (Liu, 2006). This binding promotes Akt activation and subsequent phosphorylation of downstream target proteins.

Activated Akt controls the phosphorylation of tumor suppressor protein tuberin (TSC2) that, in association with hamartin (TSC1), controls mTOR. This protein is active in oocytes and granulosa cells, and collaborates in regulation of primordial follicle pool (Tanaka *et al.*, 2012). In germ cells, its expression level increases from the GV stage to the MII stage (Lee *et al.*, 2012).

Phosphorylated Akt controls the activity of the serine-threonine glycogen synthase kinase 3 (GSK3) and of the FOXO family members (FOXO1, FOXO3a and FOXO4). AKT1-mediated phosphorylation of GSK3α at Ser21 or GSK3β at Ser9 results in GSK3 inactivation. In mouse oocytes, granulosa cell-derived KL regulates

phosphorylation of GSK3α and GSK3β through the PI3K/Akt pathway, because the specific PI3K-inhibitor LY294002 inhibits this process. However, even if GSK3 is one of the kinases with most substrates in the cell, in oocytes the classical substrates β-catenin and cyclin D1 are not implicated, and the downstream effectors are still unknown. In the bovine ovary, GSK3β is localised to cytoplasm in granulosa cells and in oocytes throughout folliculogenesis. In metaphase-II (MII)-arrested oocytes, the kinase is present in a region between the oocyte and the first polar body. The use of specific inhibitors evidenced a role for GSK3β in the regulation of MI-MII transition, probably participating in the activation of Aurora A kinase (Uzbekova *et al.*, 2009). Also mouse oocytes contain GSK3 mRNA and protein, and its pharmacologic inhibition causes abnormal meiotic spindle configuration and function, chromatin organization, and bivalent chromatin segregation. On the other hand, also phosphorylated PKC zeta inactivates GSK3β by phosphorylation on Ser9, with the aim of maintaining spindle stability during meiotic metaphase arrest (Baluch and Capco, 2008).

As in other cell systems, also in germ cells the members of the FOXO family are phosphorylated in response to activation of PI3K/Akt signalling, and their transcriptional functions are turned off following nuclear exclusion.

Concerning female fertility, FOXO1, FOXO3 and FOXO4, members of a small subfamily of the FOXO family, are involved in the control of gonadal functions as well as in crucial steps of embryogenesis and folliculogenesis (Uhlenhaut and Treier, 2011). In the mammalian ovary, FOXO3a controls follicular activation and early development. In fact, the transcription factor suppresses the initiation of follicular growth and controls the rate of utilization of primordial follicle activation during the reproductive lifespan. FOXO3a has been detected in the nucleus of oocytes enclosed in primordial and early primary follicles, while its expression is dramatically downregulated in oocytes of primary and growing follicles (Edson *et al.*, 2009; Liu, 2006). FOXO3a pathway is activated by the signalling cascade involving KL-Kit/PI3K/Akt. In fact, when oocytes are treated with KL, FOXO3 is phosphorylated and inactivated. Intra-oocyte FOXO3a inhibits the production of BMP-15 and may also suppress the activation of Smad pathways that control the proliferation and differentiation of surrounding granulosa cells. Other specific target genes regulated by FOXO3 are the cell cycle inhibitor p27 and the enzyme galactose-1-phosphate uridyltransferase (Galt), both involved in the control of follicular activation. In particular, FOXO3a enhances p27 expression and prevents its transfer from nucleus to ooplasm, thereby maintaining the growth-inhibitory function of this protein (Richards and Pangas, 2010).

The functional consequences of FOXO3a inactivation are evident in null mice, that undergo rapid decline in fertility, evidenced by increase of atretic follicle and by depletion of primordial follicle pools. In these knockout mice, POF and infertility are definitively established after 15 weeks of age (John *et al.*, 2007). The absence of oocyte-specific FOXO3 produce the same phenotype of global primordial follicle activation as PTEN deletion (Reddy *et al.*, 2008). However, in the absence of PTEN, PI3K is capable of directly stimulating Akt-dependent phosphorylation and nuclear export of FOXO3a, thereby triggering uncontrolled primordial follicle activation and POF. John *et al.,* (John *et al.*, 2008) proposed that the only role of oocyte PI3K is to regulate FOXO3a activity. In contrast, overexpression of FOXO3a leads to female infertility due to an impaired follicle development. In fact, in neonatal rat ovaries the overexpression of FOXO3a and of its downstream factors Bim (B-cell lymphoma-2), FasL and p27 stimulate apoptosis of naked oocytes. In addition, constitutively active FOXO3a dramatically affects BMP-15, connexin 37 and connexin 43, thereby inhibiting the growth of oocytes and follicle production.

In granulosa cells FSH regulates ovarian atresia via the PI3K/Akt/FOXO3a pathway. Indeed, in the porcine ovary FSH inactivates FOXO3a and prevents its binding to pro-apoptotic protein Bim promoter and the consequent follicular apoptosis (Wang *et al.*, 2012).

Also FOXO1 mRNA and protein are expressed in mouse oocytes (Edson *et al.*, 2009), even though this transcription factor regulates different functions or is not expressed in sufficient quantities to substitute for FOXO3a. FOXO1 expression level and activity are modulated by FSH and LH, that act via PI3K/Akt pathway (Liu *et al.*, 2009). FOXO1 expression level is elevated in granulosa cells of growing follicles, because it regulates the expression of genes controlling granulosa cell development and metabolism. Expres-

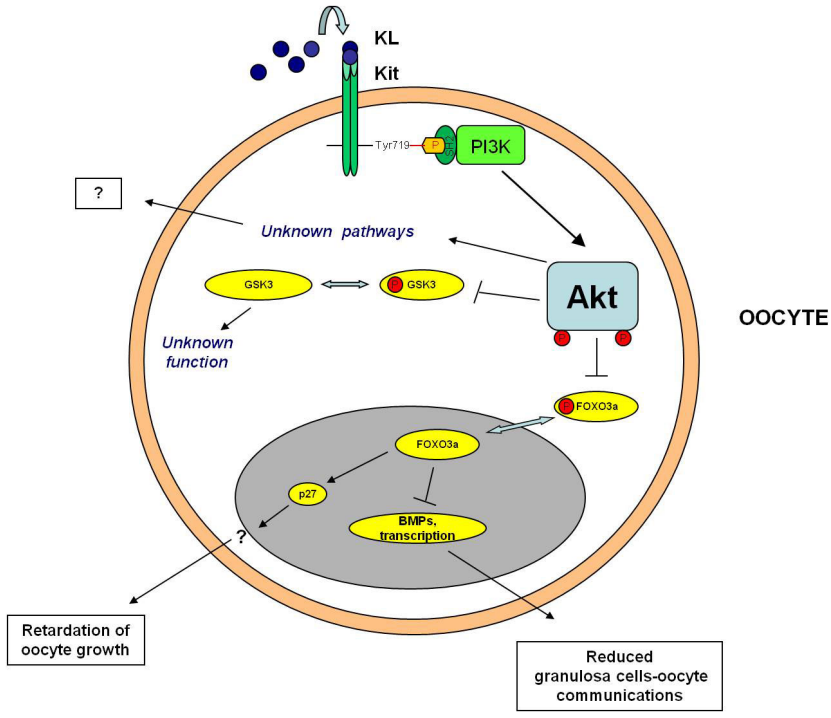

Fig. 2. The PI3K/Akt cascade and regulation of oocyte growth. *KL is produced by granulosa cells. Dimers of KL bind to the Kit receptor and cause its dimerization and activation.The phosphorylated Tyr719 provides a docking site for the SH2 domains of PI3K that, following recruitment to oolemma, is activated. This leads to Akt activation. Among the many targets of Akt, GSK3 and FOXO3a are the most important in mammalian oocytes. When phosphorylated GSK3 is inactivated, but the physiological significance needs to be definitively clarified. The oocyte FOXO3a is a negative regulator of oocyte growth because it downregulates BMP-15 activity and induces the expression of p27, the cycline/CDK inhibitor. The phosphorylation of FOXO3a results in cytoplasmic localization and inhibition of apoptosis.*

sion of a constitutively active nuclear form of FOXO1 in granulosa cells causes the absence of cyclin D2, Cyp19a1, FSH receptor (FSHR), LH/choriogonadotropin receptor (LHCGR) and of enzymes of the cholesterol biosynthetic pathway (Richards and Pangas, 2010). FOXO1 protein is concentrated also in the cytoplasm of eCG-stimulated granulosa and cumulus cells of healthy antral follicles. Following ovulation and luteinization, protein expression is down-regulated, and the most intensive FOXO1 staining has been observed predominantly in the nucleus of granulosa cells of atretic follicles. This finding suggests a causal relationship between follicular atresia and increased nuclear FOXO1 protein levels. In addition, FOXO1 is negatively regulated by Wnt signalling (Fan *et al.*, 2010). Unlike to that reported for the other FOXO members, FOXO4 null mice are viable and do not show developmental defects or histological abnormalities. The existence of a functional redundancy between members of the same subfamily has been suggested to occur in these animals.

The PI3K/Akt pathway is subjected to TGF-β/BMP regulation. In the mammalian ovary, the TGF-β superfamily includes factors derived from granulosa cells (activin, BMP-2, -5 and -6) and theca cells (BMP-2, -4 and -7) that are involved in the regulation of granulosa cell proliferation and follicle survival/apoptosis (Edson *et al.*, 2009). Recent data support a link between BMP signalling, PI3K/Akt and RAS/ERK pathways in the regulation of cell survival. In particular, it has been found that BMP-4 suppresses the apoptosis of granulosa cells by maintaining CAD protein in the cytosol instead of the nuclei, where the protein triggers DNA digestion.

AKT roles in oocyte maturation and in preimplantation embryos

In rodents and humans, elevated levels of cyclic adenosine monophosphate (cAMP) are necessary to maintain oocytes under meiotic arrest. cAMP is transfer from the cumulus cells to the oocyte through intercellular junctional communications, and endogenous production of cAMP occurs by activation of G-protein coupled receptors 3 and 12 (GPR3, 12) (Vaccari *et al.*, 2008) that activates adenylyl cyclase type 3 (AC3). Also cyclic guanosine monophosphate (cGMP) is fluxed from the cumulus cells into the oocyte to inhibit the activation of oocyte cAMP-phosphodiesterase (PDE3A), that specifically degradates cAMP (Zhang *et al.*, 2011). Elevated PKA and Akt kinase activity are necessary to phosphorylate PDE3A to enhance its enzymatic activity at the onset of oocyte maturation. Indeed, mRNAs for the Akt isoforms and the phosphorylated form of the kinase have been detected in GV oocytes (Cecconi *et al.*, 2010). Even though PKA phosphorylates PDE3A in Ser291 and Akt phosphorylates PD3A in Ser291 as well as in other residues, no synergic effect between PKA and Akt on PDE activity has been hypothesized (Han *et al.*, 2006).

In granulosa cells, FSH-dependent activation of Akt regulates apoptotic/survival signalling (Fan *et al.*, 2010) and cell differentiation (Wayne *et al.*, 2007). In cumulus cells, active kinase participates in the maintenance of cell survival and the synthesis/retention of hyaluronic acid (Nemcova *et al.*, 2007).

The processes of ovulation, luteinization and oocyte meiotic maturation are triggered by LH spikes at estrus, when this gonadotropin induces some elaborate signalling cascades that activate specific intracellular substrates. Oocyte meiotic resumption requires a drastic drop in cAMP levels and in cGMP concentration. At the same time, LH-dependent activation of EGFR signalling pathway induces ERK/MAPK and the progressive closure of gap junctions. LH surge stimulates also PI3K/AKT (Han *et al.*, 2006; Hoshino and Sato, 2008; Tomek and Smiljakovic, 2005). Akt activity increases during early steps of mouse oocyte maturation to activate either PDE3A (Han *et al.*, 2006) or downstream mTOR pathway, which phosphorylates factors promoting protein synthesis (Kang and Han, 2011; Kalous *et al.*, 2009). Suppression of Akt activity determines a significant decrease of CDK1 activity and delayed resumption of meiosis in mouse oocytes (Kalous *et al.*, 2009). Recently, it has been demonstrated that PIK3/Akt pathway can be stimulated also by oocyte-derived interleukin 7 (IL7), that promotes the nuclear maturation of preovulatory oocytes. IL 7 stimulates in antral granulosa cells the phosphorylation of Akt, GSK3 and STAT5 proteins, thereby exerting an antiapoptotic effect (Cheng *et al.*, 2011).

Following germinal vesicle breakdown, the active kinase is associated with microtubules of the first meiotic spindle (Cecconi *et al.*, 2010; Hoshino *et al.*, 2004a,b; Kalous *et al.*, 2009). *In vitro* experiments clearly indicated that Akt inhibition induces failure of MI itself, and that at MII stage Akt control polar body 2 emission and normal chromosomal alignment on microtubules (Hoshino and Sato, 2008). This role is confirmed by treatment with LY294002 that induces a significant decrease of phosphorylated Akt-Thr308, alters localization of phosphorylated Akt-Ser473 during oocyte maturation and impairs polar body extrusion (Hoshino *et al.*, 2004b; Hoshino and Sato, 2008). Also in cattle oocytes, Akt is involved in the regulation of meiotic MI/MII transition (Tomek and Smiljakovic, 2005). Spindle migration and asymmetric division during meiotic maturation are controlled also by mTOR (Lee *et al.*, 2012). By comparing mRNA levels during *in vivo* maturation, a significant decrease of Akt1 mRNA level from GV to MII stage has been detected. In vitro, Akt expression level can be differentially modulated by the different hormonal supplements (Cecconi *et al.*, 2010). In fact phosphorylated Akt content is higher in FSH- than in EGF-stimulated oocytes. This could be due to anomalous regulation of PTEN and/or of the phosphatases of the PHLPP family. At MII stage, phosphorylated Akt is still expressed and its role may be to support oocyte survival, at least during the fertilization window. The findings that Akt 1, 2 and 3 mRNA levels are very low in oocytes retrieved from ampullae 29h post hCG, and that protein content dramatically decreases within 33h post hCG confirms this hypothesis (Cecconi *et al.,* personal communication). Following fertilization, Akt is present in one-cell stage fertilized eggs, where the active kinase plays a key role in the first round of mitosis. The elevated Akt activity detected in the fertilized mouse egg clearly highlights the important role of this kinase in G2/M transition. Indeed, Akt changes the subcellular localization of p21(Cip1/WAF1) from nucleus to cytoplasm, most likely by phosphorylating this protein, and indirectly induces suppression of cdc2/cyclin B activity (Wu *et al.*, 2011).

In preimplantation embryos, PI3K and Akt are expressed from 1-cell stage to morula on cell surface, while at blastocyst stage both are present in the trophectoderm cells. Akt induces the phosphorylation and activation of MDM2 that degradates p53 and coordinates processes necessary maintaining the survival of blastomeres while its inhibition determines a significant delay in blastocyst hatching. Maternal PI3K signalling is now considered as a new maternal effector that regulates embryonic genome activation at 2 stage (Rai *et al.*, 2010).

Pathological aspects of altered Akt stimulation

Polycystic ovary syndrome

In women, polycystic ovarian syndrome (PCOS) is a common metabolic disorder associated with a number of metabolic disorders, as insulin resistance and anovulation. Several genes have been identified that may be implicated in the pathogenesis of PCOS, including those involved in the biosynthesis and action of androgens, or correlated with insulin resistance, or encoding for inflammatory cytokines. In the ovary, PCOS is characterized by increased preantral follicle numbers, arrested follicular maturation and disturbances in dominant follicle selection due to reduced sensitivity to FSH or enhanced LH action. Insulin resistance is reported to affect 50% to 80% of women with PCOS. PCOS patients show abnormal gonadotropin-releasing hormone (GnRH) secretion and excessive LH release, that stimulates theca cells to produce more androgens. Peripheral conversion of circulating androgens in the adipose stroma and skin results in increased estrogen production in PCOS women.

Cell growth and antiapoptotic actions of androgens are mediated by activation of PI3K/Akt and MAPK pathways. Mammalian oocytes express Insulin-like growth factor I (IGF-I) receptor mRNA. IGF-I binding to its receptor stimulates mammalian granulosa cell proliferation and survival, increases steroid secretion and responsiveness to gonadotropins of ovarian follicles (Purcell *et al.*, 2012). PI3K/Akt pathway enhances the protective and proliferative effect of IGF-I against GnRH- dependent and inhibits FasL-dependent apoptosis (Goto *et al.,* 2007).

Also following insulin binding, the activated insulin receptor triggers a signalling pathway that regulates several metabolic functions, such as the transport of glucose and synthesis of glycogen via activation of PI3K. As PI3K is expressed in mice and human cumulus cells, insulin-stimulated glucose uptake occurs in both compact and expanded cumuli (Purcell *et al.*, 2012). Insulin signalling is present in mammalian oocytes, as both growing and fully grown oocytes express insulin receptor (Acevedo *et al.*, 2007). However, oocytes do not display insulin-stimulated glucose uptake (Purcell *et al.*, 2012). A prolonged exposure of follicles to insulin during the growth phase causes increased phosphorylation of oocyte GSK3, leading to its inactivation and chromatin condensation errors, which may cause misalignment of chromosomes on the meiotic spindle. As a consequence of congression failure, subsequent embryonic developmental competence is compromised (Acevedo *et al.*, 2007). Insulin may affect granulosa cell proliferation via PTEN. In luteinizing human granulosa cells, insulin-dependent expression of PTEN impairs the phosphorylation of Akt and counterbalances IGF-I-induced phosphorylation of Akt, thereby exerting an antiproliferative effect (Iwase *et al.*, 2009). This means that not only the balance between PI3K and PTEN affects Akt and folliculogenesis, but also that LH and insulin affect PTEN expression. Activation of insulin signalling pathways in mammals negatively regulates members of the FOXO transcription factors that, following Akt-dependent phosphorylation, undergo active nuclear export and inhibition of their transcriptional activities. Thus, in PCOS patients, the increased level of insulin in follicular fluid increases the expression levels of PTEN in granulosa cells (Iwase *et al.*, 2009) and since this protein antagonizes PI3K activity, an inverse relationship with phospho-Akt expression is evident.

Ovarian cancer and PI3K/Akt pathway

Deregulation of the PI3K/Akt pathway is responsible for oncogenesis. In ovarian cancer, activation of the PI3K/AKT/mTOR pathway has a stronger and more direct role in the serous subtypes. Constitutive activation of the PI3K pathway occurs by mutations in PI3KCA gene, a 34-kb gene located on chromosome 3q26.3, that is present in approximately 8% of human ovarian cancers. Also PI3KCA amplification is a recurrent genetic alteration in ovarian cancer and leads to mTOR phosphorylation and to enhanced tumour cell survival. Other alterations in PI3K pathway are characterized by high expression of PTEN, CASP3, BAD, PAK1, XIAP, GSK3B, and NFkB1 (Weberpals *et al.*, 2011).

Several drugs are being investigated in vitro, in cell culture and *in vivo* in mouse models to develop targeted therapies for inhibition of the PI3K/Akt cascade. Akt overexpression confers cisplatin resistance in ovarian cancer lines, indicating that the combination of specific inhibitors and chemotherapy might be a rational and effective treatment strategy.

The first-generation compounds acting on PI3K are wortmannin, quercetin and LY294002, all inhibitors at the ATP binding sites. For example, combination treatment of various ovarian cancer cell lines with the inhibitor LY294002 and cisplatin results into significant reduction in invasion, migration and expression of a number of matrix metalloproteins (Karam *et al.*, 2010). However, the selectivity of action of these drugs on PI3K has been recently criticized, being shown that they bind other targets than PI3K-related kinases (McNamara and Degterev, 2011). More selective compounds acting on p110 catalytic isoform or inhibiting the three isoforms of Akt have been recently synthesized as lead compounds for development of effective anticancer therapies (McNamara and Degterev, 2011).

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