
Protein Kinase G-1 α Hyperactivation and VASP Phosphorylation in Promoting Ovarian Cancer Cell Migration and Platinum Resistance

Janica C. Wong and Ronald R. Fiscus

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/53468>

1. Introduction

Platinum-based drugs such as cisplatin (*cis*-diammine-dichloro-platinum, also commonly known as CDDP) have dominated the drug therapy of ovarian cancer during the past three decades [1]. Cisplatin interacts with DNA to form intrastrand crosslink adducts, and its molecular mechanism involves regulation of p53 and the mitogen-activated protein kinase (MAPK) signaling pathway [2]. The phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway is crucial for regulation of survival and for progression and chemoresistance in ovarian cancer, leading to the development of new chemotherapeutic inhibitors targeting the PI3K/Akt pathway and the downstream serine/threonine protein kinase mTOR. [3]. Inhibition of PI3K pathway signaling using PI3K or mTOR inhibitors has been shown to sensitize ovarian cancer cell lines to the apoptosis-inducing effect of platinum compounds [4, 5]. In addition, activation of the PI3K/Akt/mTOR pathway in ovarian cancer cell lines contributes to cisplatin resistance [6]. The anti-apoptotic, pro-angiogenic effects of PI3K/Akt/mTOR may be mediated, at least in part, through a downstream signaling pathway involving endogenous endothelial-form nitric oxide synthase (eNOS, also called NOS3), and subsequently soluble guanylyl cyclase (sGC) and protein kinase G (PKG). Studies have shown that Akt activates eNOS by phosphorylating human eNOS at Ser1177 (equivalent to bovine eNOS at Ser1179), leading to an increase in nitric oxide (NO) production in endothelial cells [7, 8]. In the cases of vascular endothelial growth factor (VEGF) [9, 10], sphingosine 1-phosphate [11, 12], and estrogen [13, 14], there are vast evidences suggesting PI3K-activation of Akt is responsible for regulating the phosphorylation and activation of eNOS. In bovine aortic endothelial cells, eNOS co-immunoprecipitates with Akt, indicating that the two enzymes associate *in vivo*, and Akt directly activates eNOS, increasing eNOS activity by 15-20 fold

[15]. This signaling pathway has been shown to play an essential role in promoting angiogenesis or tumor vascularization [16]. In a very recent study, microgravity stimulated tube formation and migration in human umbilical vein endothelial cells (HUVEC), and the process was mediated through the PI3K-Akt-eNOS signal pathway [17].

Our early studies of the NO/cyclic GMP (cGMP)/PKG signaling pathway have identified PKG as a key mediator of vasodilation and anti-hypertensive effects induced by NO as well as atrial natriuretic peptide (ANP) [18-21]. Recent studies from our laboratory have shown that the PKG-I α splice variant of PKG, at basal or moderately elevated activity, plays an important cytoprotective role in preventing spontaneous apoptosis and promoting cell proliferation in many types of mammalian cells, including neural cells [22-27], human ovarian cancer cells [28-30], primary murine vascular smooth muscle cells [31] and murine bone marrow mesenchymal (stromal) stem cells [32]. Evidence from our laboratory suggested that basal activation of PKG-I α leads to increased attachment of cells to the extracellular matrix and increased cell migration, shown in bone marrow-derived mesenchymal (stromal) stem cells [32]. We have identified certain intracellular proteins that are directly phosphorylated and functionally regulated by PKG-I α , including 1) the apoptosis-regulating protein BAD [26], 2) vasodilator-stimulated phosphoprotein (VASP) [28, 31, 32], 3) the oncogenic tyrosine kinase c-Src [28, 33] and 4) the transcription factor cAMP responsive element binding protein (CREB) [24, 34], which may contribute to the exaggerated proliferation, enhanced chemoresistance and increased cell migration and invasion in ovarian cancer cells (Figure 1). Our recent studies have shown that cisplatin regulates the endogenous expression of nitric oxide synthases (NOSs) in human ovarian cancer cells, upregulating inducible nitric oxide synthase (iNOS, also called NOS2) expression but dramatically downregulating the expression of eNOS and neural-form nitric oxide synthase (nNOS, also called NOS1), which is involved in determining cisplatin resistance in ovarian cancer cells [30]. Our studies show that the chemoresistance/cytoprotective effects of endogenous eNOS involve hyperactivation of PKG-I α in the ovarian cancer cells [28].

Studies from our laboratory suggest that PKG-I α promotes proliferation in ovarian cancer cells, which involves the enhancement of the tyrosine kinase activity of c-Src [28], an oncogenic protein often overexpressed and/or hyperactivated in many types of cancer cells. We showed that PKG-I α plays a key role in activating c-Src and promoting cell proliferation, using the short interfering RNA (siRNA) or RNA interference (RNAi) technique, to knock-down the expression of PKG-I α in ovarian cancer cells [28]. We found that epidermal growth factor (EGF)-induced activation of c-Src tyrosine kinase activity causes tyrosine phosphorylation of PKG-I α , increasing the serine/threonine kinase activity of PKG-I α and its growth-promoting effects in ovarian cancer cells [28]. Later, we have found that PKG-I α directly phosphorylates c-Src at Ser17, which enhances the tyrosine kinase activity of c-Src in both *in vitro* and intact-cell experiments [33]. This novel interaction between PKG-I α and c-Src causes reciprocal phosphorylation, which means PKG-I α and c-Src phosphorylate each other, potentially setting up an "oncogenic reinforcement" resulting in exaggerated DNA synthesis and cell proliferation (Figure 1).

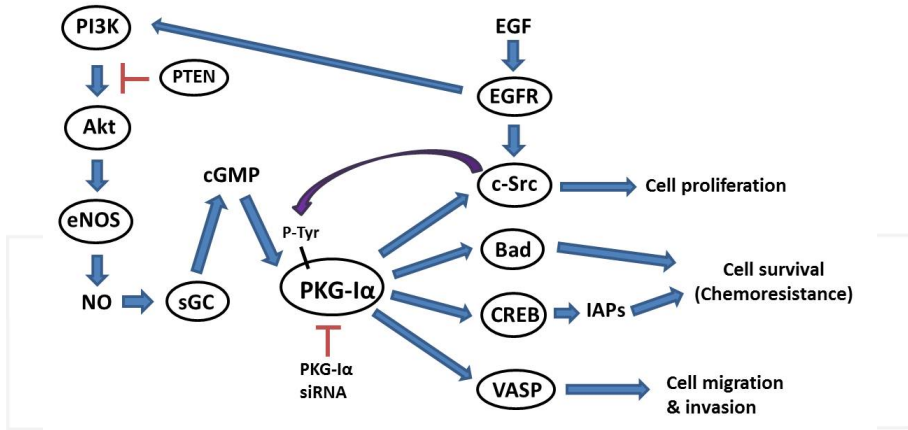


Figure 1. Model of the biological role of PKG-Iα in ovarian cancer cells illustrating the effects of growth factors (e.g. EGF), which stimulates both the PI3K/Akt pathway, enhancing eNOS activity and low-level NO generation and the activation of c-Src. The low, physiological levels of NO activate sGC, elevating cGMP levels that enhance the activation of PKG-Iα. PKG-Iα is further activated (hyperactivated) by the combined effects of cGMP allosteric stimulation and the tyrosine phosphorylation by c-Src. PKG-Iα phosphorylates several downstream proteins, including c-Src, Bad, CREB and VASP, leading to enhanced cell proliferation and cytoprotection, contributing to chemoresistance in ovarian cancer cells and increased cell migration and invasion.

2. Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) at Ser239 as a useful indicator of endogenous PKG kinase activity

Vasodilator-stimulated phosphoprotein (VASP) was first described in 1987 as a protein phosphorylated in platelets in response to vasodilators such as sodium nitroprusside, nitroglycerin and various prostaglandins that elevate cAMP and cGMP [35]. VASP belongs to the Ena/VASP family which includes VASP, Mena (mammalian enabled) and EVL (Ena VASP-like). The Ena/VASP family proteins function as anti-capping proteins [36, 37], regulating the actin cytoskeleton dynamics [38-42] and are therefore important for actin-based adhesion [43, 44], migration [45-47] and cell-cell interaction [48-50]. Many studies from others have suggested the involvement of VASP in invasion, angiogenesis and tumorigenesis. In an *in vitro* model of capillary morphogenesis using human umbilical vein endothelial cells (HUVECs) in three-dimensional collagen gels, the differentiated endothelial cells showed 2 to 3-fold increase in migration with increased VASP mRNA and protein expression [51]. A study on human placenta development showed that VASP may participate in vasculogenesis and endothelial sprouting during placental vasculogenesis, and VASP expression was stimulated by vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) [52]. NIH-3T3 fibroblast deficient in VASP showed loss of contact inhibition, and continued cell division past confluence, while overproduction of VASP by transfection in NIH-3T3 fibro-

blasts resulted in neoplastic transformation, suggesting a role of VASP in tumorigenesis and/or cancer progression [53]. In human osteocarcinoma specimens, higher VASP expression was associated with metastasis and increased migration, and VASP expression was regulated by Rac1 [54]. In lung adenocarcinomas tissues, VASP expression was increased compared to normal lung tissues, and was significantly increased with more advanced tumor stage [55]. Elevated VASP expression was also reported in human breast cancer tissues [56] and was implicated on invasion and migration in breast cancer cells involving the Rac1 pathway [57]. Moreover, it was shown that in mice lacking VASP, melanoma growth was greatly impaired [58]. In gastric cancer cells, VASP was upregulated by epidermal growth factor (EGF) and promoted migration and invasion. Using microRNA (miRNA) expression profiling of the paired normal/tumor gastric tissues, the same group identified miR-610 as a novel miRNA regulated by EGF that targets VASP in gastric cancer cells [59].

VASP has been reported to be phosphorylated by cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) [35, 60]. VASP was found to be primarily present as a 46 kDa membrane-associated protein in its dephosphorylated form in platelets, and VASP is converted to an apparent 50 kDa phosphoprotein upon phosphorylation, as observed on Western blot [61, 62]. VASP contains three phosphorylation sites, Ser157, Ser239 and Thr274, all of which can be phosphorylated by either PKA or PKG [63]. Ser157 is the preferred site of phosphorylation for PKA, Ser239 is the preferred site for PKG, and Ser157 was the site responsible for the phosphorylation-induced mobility shift of VASP on Western blots [63]. Because it was well-characterized that VASP at Ser239 is the preferred phosphorylation site for PKG *in vitro* and in mammalian cells, VASP phosphorylation at Ser239 has been proposed to be a useful indicator of endogenous PKG kinase activity [61, 63, 64]. In fact, VASP at Ser239 was shown to be a functional biomarker of endothelial nitric oxide/cyclic GMP signaling [65], and could be used to indicate defective nitric oxide/cGMP signaling and endothelial dysfunction [66]. In colon cancer cells, VASP Ser239 phosphorylation was used as a biomarker for the action of the anti-cancer drug Exisulind, an inhibitor of type-5 phosphodiesterase (PDE-5) that elevates cGMP and stimulates PKG activation, and that constitutively activated mutants of PKG resulted in direct *in vivo* phosphorylation of VASP Ser239 [67].

We had shown that the endogenous NO/cGMP signaling pathway in ovarian cancer cells causes a constitutive downregulation of p53 protein expression, which likely contributes to the chemoresistance and exaggerated cell proliferation in these cells [29]. Furthermore, we have previously identified that PKG-I α is the predominant isoform of PKG in both OV2008 (cisplatin-sensitive, wild-type p53) and A2780cp (cisplatin-resistant, mutated p53) ovarian cancer cells as determined by Western blot analysis as well as using the new, ultrasensitive Nano-Pro100 capillary electrophoresis-based nano-fluidic protein analysis system [28, 68, 69]. Our more recent data now show that the chemoresistance and exaggerated cell proliferation are likely mediated by the constitutive hyperactivation of PKG-I α (reflected in the high levels of VASP phosphorylation at Ser239) in ovarian cancer cells, and that the PKG-I α is already activated to approximately 90% of maximal activity, described in our previous book chapter [68]. In our recent study, epidermal growth factor (EGF)-induced activation of Src family kinase (SFK) was found to tyrosine-phosphorylate PKG-I α increasing its serine/threonine kinase ac-

tivity in ovarian cancer cells. The EGF-stimulated increase in PKG-1 α kinase activity (indicated by VASP Ser239 phosphorylation) was blocked by both SKI-1 and SU6656 (SFK inhibitors). Using the specific PKG-1 α kinase inhibitor DT-2 and small interfering RNA (siRNA) PKG-1 α gene knockdown, we showed that the inhibition of endogenous PKG-1 α kinase activity reduced VASP Ser239 phosphorylation and DNA synthesis rate in ovarian cancer cells [28]. New data from our laboratory show that the knockdown of PKG-1 α expression inhibits the EGF-stimulated increases in VASP Ser239 phosphorylation and Src/SFK autophosphorylation at the equivalent of Tyr416 (the phosphorylation site for activating the tyrosine kinase activity) in A2780cp (cisplatin-resistant, mutated p53) ovarian cancer cells (see Figure 2 below).

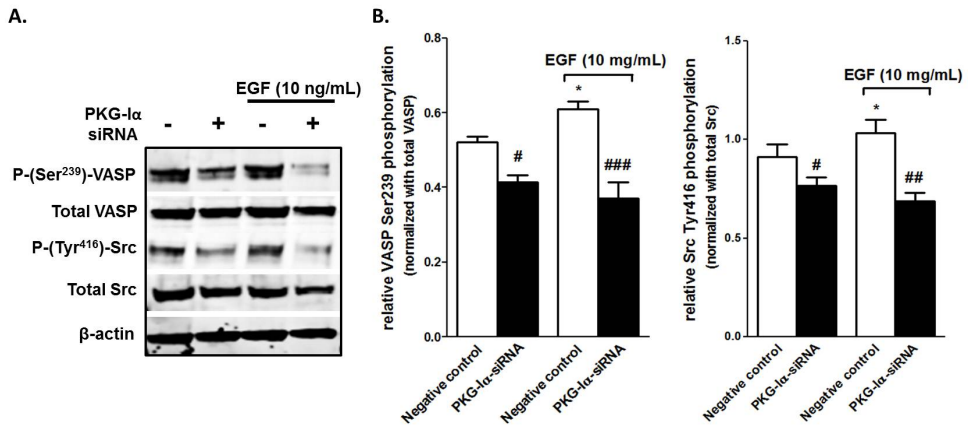


Figure 2. A, EGF (10 ng/mL) elevated VASP Ser239 phosphorylation and Src Tyr416 phosphorylation in A2780cp cells, assessed by Western blot analysis. Gene knockdown of PKG-1 α by PKG-1 α -siRNA partially inhibited the basal VASP phosphorylation and Src/SFK autophosphorylation and completely inhibited the EGF-stimulated increases in VASP phosphorylation and Src/SFK autophosphorylation. The Western blot shown is representative of four experiments. B, Quantification of the relative levels of VASP and Src phosphorylation from Western blot. Bar graphs show mean \pm SEM from four independent experiments. *, $P < 0.05$, compared with no EGF control; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, compared with negative control.

3. Role of PKG in invasion/migration in A2780cp ovarian cancer cells

The role of NO/cGMP/PKG pathway in invasion/migration in cancer cells is largely unknown. However, a significant number of reports have shown that the NO/cGMP/PKG pathway plays a key role in endothelial cell migration and angiogenesis, involving the downstream activation of the mitogen-activated protein kinase (MAPK) family. It has been shown that NO promotes endothelial cell migration and neovascularization by activating the PI3K/Akt signaling pathway in a PKG-dependent manner [70]. Activation of the NO/cGMP/PKG pathway also promoted endothelial cell angiogenesis and increased extracellular signal regulated kinase 1/2 (ERK1/2) and p38 phosphorylation [71, 72], which were blocked by soluble guanylyl cyclase (sGC) inhibitor, 1H-

[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), or PKG inhibitor DT-3 [73-75]. Moreover, the mitogenic effect of vascular endothelial growth factor (VEGF) on endothelial cells appears to be mediated by endogenous NO (from eNOS) and cGMP, which results in PKG activation and PKG-mediated downstream stimulation of MEK and ERK [76, 77]. Although it has not yet been reported which isoform of PKG is involved in the multiple pro-angiogenesis responses of endothelial cells, our recent studies suggest that endothelial cells express predominantly the PKG-I α isoform (unpublished observations by J.C. Wong and R.R. Fiscus), which likely mediates the stimulation of downstream growth-promoting and pro-angiogenesis pathways in endothelial cells.

Interestingly, in colon cancer cells, recent studies showed that activation of PKG inhibited cell migration [78], and cGMP-dependent VASP phosphorylation suppressed the number and length of locomotory (filopodia) and invasive (invadopodia) actin-based organelles [79], suggesting a role of VASP Ser239 in invasion and migration. Our studies suggest that the opposite roles of PKG in regulating apoptosis, proliferation and migration reported by others are likely dependent on cell type, growth conditions (presence of different growth factors), as well as the differential expression of PKG-I α and PKG-I β isoforms. The two splice variants of PKG-I, PKG-I α and PKG-I β , are activated by different concentration ranges of NO and are localized to different subcellular locations within cells. Therefore, the two PKG-I isoforms can phosphorylate different sets of downstream target proteins and can mediate completely different biological responses. The very different biological roles of the two PKG-I isoforms are reviewed in further detail elsewhere in another recent book chapter from our laboratory [68]. For example, PKG-I α ($K_{act} = 0.1 \mu\text{M}$ by cGMP allosteric activation) is activated at low, physiological levels of NO, whereas PKG-I β is activated at higher, pathological levels of NO and requires at least 10-times higher levels of cGMP for activation ($K_{act} = 1 \mu\text{M}$) [68, 72, 80, 81].

In our hypothesis, the PKG-I α and PKG-I β isoforms mediate opposite biological effects on cell proliferation and apoptosis, based on observations in two types of cells that express one isoform of PKG-I or the other. Our studies have shown that human ovarian cancer cells express predominantly the PKG-I α isoform, and that the activation of this kinase by endogenous low-level NO (0.01–1 nM), generated by endogenous eNOS and nNOS, would selectively activate the PKG-I α isoform within ovarian cancer cells from our laboratory, promoting DNA synthesis/cell proliferation and suppressing apoptosis, thus contributing to chemoresistance [28, 30, 68]. Studies in our laboratory, using both normal and malignant cells, including vascular smooth muscle cells, bone marrow-derived mesenchymal (stromal) stem cells and neuroblastoma cells, have suggested that a major role of the low-level-NO/cGMP/PKG-I α signaling pathway is to protect these cells against the toxic/pro-apoptotic effects of high-level NO, as might occur during inflammation and exposure of cells to pro-inflammatory cytokines [22, 24, 25, 31, 32]. In contrast, based in part on published data from the laboratories of Weinstein and Thompson, it appears that when PKG-I β is activated by the higher levels of NO, the growth-inhibitory and pro-apoptotic effects of PKG-I β predominate over the growth-stimulatory and anti-apoptotic effects mediated by PKG-I α . Their laboratories have shown that in colon cancer cells, PKG-I β is the predominant PKG-I isoform expressed. Upon activation, PKG-I β phosphorylates two downstream target proteins, β -catenin and MEKK1, resulting in inhibition of cell proliferation and induction of apoptosis [78, 82, 83].

As stated above, we have previously determined that PKG-1 α is the predominant isoform in A2780cp ovarian cancer cells [30, 68, 69]. To study whether PKG-1 α plays a role in cell migration/invasion in ovarian cancer cells, we performed experiments using small interfering RNA (siRNA) gene knockdown against PKG-1 α in transwell migration studies. Figure 3 shows that siRNA gene knockdown of PKG-1 α dramatically decreases no EGF as well as EGF-stimulated cell migration (as reflected by the quantity of migrated cells at the bottom of the transwell, stained with crystal violet) in A2780cp cisplatin-resistant ovarian cancer cells. These data confirm the role of endogenous PKG-1 α activity, potentially via VASP Ser239 phosphorylation, in promoting cell migration/invasion in ovarian cancer.

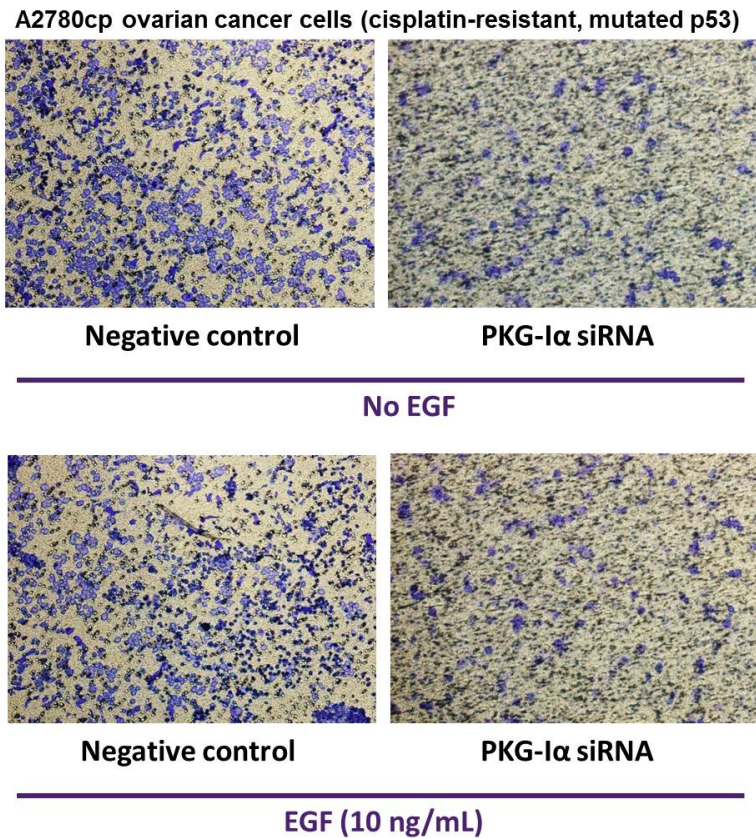


Figure 3. PKG-1 α siRNA gene knockdown in A2780cp cells decreased both basal and EGF-stimulated cell migration as assessed by *in vitro* cell migration (invasion) assay. Migration of cells was assessed using transwells (Corning) with 8 μ m pore polycarbonated inserts, coated with growth factor-reduced matrigel (BD Bioscience). The upper chamber contained 4 x 10⁴ cells and the lower chamber contained 0.6 ml of complete medium with or without EGF. Migration through the membrane was determined after 24 h of incubation at 37°C. Cells remaining on the topside of the transwell membrane were removed using a cotton swab, and cells migrated to bottom were stained with 0.5% crystal violet.

4. Inhibition of the PKG-I α signaling pathway enhances sensitivity of ovarian cancer cells to cisplatin-induced apoptosis – Potential involvement of cAMP-response-element-binding protein (CREB) and inhibitor of apoptosis proteins (IAPs)

Platinum-based drugs such as cisplatin have dominated the drug therapy of ovarian cancer during the past three decades [1]. Cisplatin interacts with DNA to form intrastrand crosslink adducts, and its molecular mechanism involves regulation of p53 and the mitogen-activated protein kinase (MAPK) signaling pathway [2]. It has been shown that inhibition of ERK1/2 activation with the mitogen-activated protein kinase/ERK kinase 1 (MEK1) inhibitor PD98059 resulted in decreased p53 protein half-life and diminished accumulation of p53 protein during exposure to cisplatin [84]. Our data have shown that human ovarian cancer cells express all of the key components of the NO/cGMP/PKG signaling pathway, including all three isoforms of NOSs, thus providing an endogenous source of NO [30]. Furthermore, ovarian cancer cells continuously produce NO at low physiological levels, activating the heme-dependent soluble guanylyl cyclase (sGC) [29], elevating cGMP levels sufficiently enough to cause continuous high-level activation of PKG [28]. Our data suggested that such basal sGC/cGMP activity regulates p53 expression, and promotes cell survival in part through regulation of caspase-3 [29] (now thought to be mediated by downstream hyperactivation of PKG-I α).

Cisplatin is also widely employed in chemotherapy on treating solid tumors such as lung cancer. Recently, we showed that, in NCI-H460 and A549 non-small cell lung cancer (NSCLC) cells, PKG-I α phosphorylates cAMP-response-element-binding protein (CREB) at Ser133 [34]. CREB was first shown to be phosphorylated by PKG *in vitro* by Colbran et al., which showed that PKG effectively phosphorylates CREB at Ser133, although at a slower rate compared to PKA [85]. Interestingly, NO was shown to regulate the c-fos promoter involving soluble guanylyl cyclase (sGC) and PKG [86] in a CREB-dependent manner [87]. They also showed that transfection of PKG in baby hamster kidney (BHK) cells activated the c-fos promoter [88], which required nuclear translocation of PKG and phosphorylation of CREB at Ser133 by PKG [87, 89, 90]. In our recent study, inhibition of the sGC/PKG-I α signaling pathway by ODQ (sGC inhibitor), DT-2 (PKG-I α kinase inhibitor) and PKG-I α -siRNA gene knockdown showed that PKG-I α kinase activity is necessary for maintaining higher levels of CREB phosphorylation at Ser133 and the protein expression of certain inhibitor of apoptosis proteins (IAPs), specifically c-IAP1, livin and survivin, as well as the anti-apoptotic Bcl-2 family member Mcl-1, preventing spontaneous apoptosis and promoting colony formation [34]. In the same study, we discovered that DT-2 and cisplatin have a synergistic effect on the induction of apoptosis, with DT-2 dramatically enhancing the pro-apoptotic effects of cisplatin in A549 cells (a NSCLC cell line that requires higher levels of cisplatin to induce apoptosis). We also showed that prior activation of PKG-I α by 8-bromo-cGMP (8-Br-GMP), a cell-permeable cGMP analog that directly activates PKG [22, 24], has cytoprotective effects against cisplatin. PKG-I α activity stimulated by 8-Br-cGMP was reflected by increased VASP phosphorylation at Ser239. Pretreatment of A549 cells with 8-Br-

cGMP caused significant protection against cisplatin-induced apoptosis, even at higher concentrations of cisplatin. Interestingly, when the same treatments were used on PKG-Iα knockdown cells, the cytoprotective effects of 8-Br-cGMP against cisplatin-induced apoptosis was completely abolished, confirming that the cytoprotection (chemoresistance) was mediated by PKG-Iα [34].

To investigate whether such synergism occurs in ovarian cancer cells, we tested the combined treatment of the specific PKG-Iα kinase inhibitor, DT-2, and cisplatin in the A2780cp cisplatin-resistant ovarian cancer cell line. Our new preliminary data presented in this book chapter (illustrated in Figure 4) verified the synergistic effects of DT-2 and cisplatin. Figure 4 shows the level of apoptosis in A2780cp cells after a 24-hr co-treatment of DT-2 (5 or 10 μM) and cisplatin (2 μM). The Cell Death Detection ELISA^{PLUS} assay (Roche Applied Science), based on quantitative sandwich-enzyme-immunoassay-principle with monoclonal antibodies directed against DNA and histones, were used to quantify apoptotic fragments. DT-2 (5 μM) or cisplatin (2 μM) alone did not cause significant increase in apoptosis. However, combined treatment of DT-2 (5 or 10 μM) and cisplatin (2 μM) significantly (###P<0.001) increased apoptosis, showing a synergistic effect.

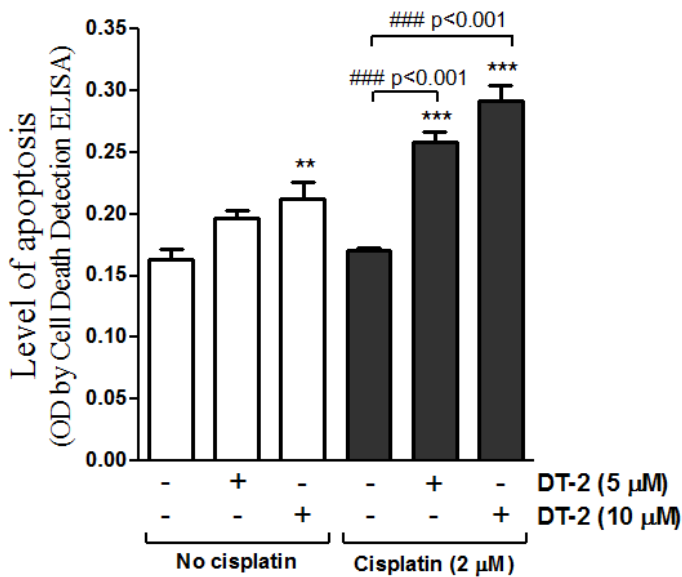


Figure 4. Synergistic effect of DT-2 with cisplatin in A2780cp human ovarian cancer cells. Combined treatment of DT-2 (5 or 10 μM) and cisplatin (2 μM) significantly (###P<0.001) increased apoptosis, compared to cisplatin (2 μM) alone. **P<0.01, ***P<0.001, compared to no DT-2 control. Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls Multiple Comparison Test using GraphPad (PRISM software). Results were expressed as the mean ± SEM of at least six different samples.

Based on our study of the roles of sGC/PKG-I α /CREB/IAPs in cisplatin resistant non-small lung cancer cells, we have proposed the anti-apoptotic role of PKG-I α observed in A2780cp cells is likely mediated through PKG-I α downstream phosphorylation of CREB at Ser133 and activation of certain IAPs. IAPs have been shown to regulate apoptosis and tumorigenesis [91]. Although how CREB regulates apoptosis through IAPs is largely unknown, it was shown that CREB phosphorylation is a key event in the induction of certain IAPs, c-IAP2 and livin, via multiple protein kinases, PKA, ERK1/2 and p38 MAPK, in colon cancer cells [92, 93]. In ovarian cancer cells, X-linked inhibitor of apoptosis protein (XIAP) has been shown to control ovarian tumor growth and regulate Akt activity and caspase-3 in cisplatin-induced apoptosis [94-96], and the ability of cisplatin to down-regulate XIAP may be an important determinant of chemosensitivity [97]. Down-regulation of XIAP sensitized cells to cisplatin in the presence of wild-type p53, and both XIAP and Akt modulated cisplatin sensitivity individually but that XIAP required Akt for its full function [98]. Inhibition of PI3K/Akt/mTOR signaling has been shown to activate apoptosis and inhibit migration and invasion in ovarian cancer cells [3, 4, 99-104]. Furthermore, inhibition of PI3K pathway signaling using PI3K or mTOR inhibitors has been shown to sensitize ovarian cancer cell lines to induction of apoptosis by platinum compounds [4, 5]. Several recent evidences have suggested that such effects involve the matrix-metalloproteinases (MMPs) [105-107], which are zinc-dependent endopeptidases capable of degradation of extracellular matrix proteins.

5. Overall model of NO/cGMP/PKG-I α signaling pathway in ovarian cancer

Figure 5 illustrates our overall model showing the involvement of the NO/cGMP/PKG-I α pathway in promoting cell proliferation and suppressing apoptosis in human ovarian cancer cells, which would contribute to enhanced tumor growth and chemoresistance. Our early studies of the NO/cGMP/PKG pathway have identified PKG as a key mediator of vasodilation and anti-hypertensive effects induced by NO as well as atrial natriuretic peptide (ANP) [18-21]. Recent studies from our laboratory have shown that basal or moderately elevated PKG-I α activity plays a cytoprotective role in preventing spontaneous apoptosis and promoting cell proliferation in many types of mammalian cells, including neural cells [22-27], human ovarian cancer cells [28-30], primary murine vascular smooth muscle cells [31] and murine bone marrow stromal cells [32]. We found that murine bone marrow-derived mesenchymal (stromal) stem cells endogenously produced ANP and that basal NO/cGMP/PKG-I α activity and autocrine ANP/cGMP/PKG-I α activity are necessary for preserving cell survival and promoting cell proliferation and migration in the OP9 bone marrow stromal cell line [32]. Recently, we have identified certain intracellular proteins phosphorylated by PKG-I α , including BAD [26], vasodilator-stimulated phosphoprotein (VASP) [28, 31, 32], c-Src [28] and cAMP responsive element binding protein (CREB) [34]. We have recently shown that PKG-I α directly phosphorylates BAD at Ser155, using *in vitro* experiments, and have further shown that a large part of the Ser155 phosphorylation of BAD within neuroblastoma cells is

dependent on endogenous PKG-Iα kinase activity, contributing to decreased caspase-3 activity and inhibition of apoptosis [26].

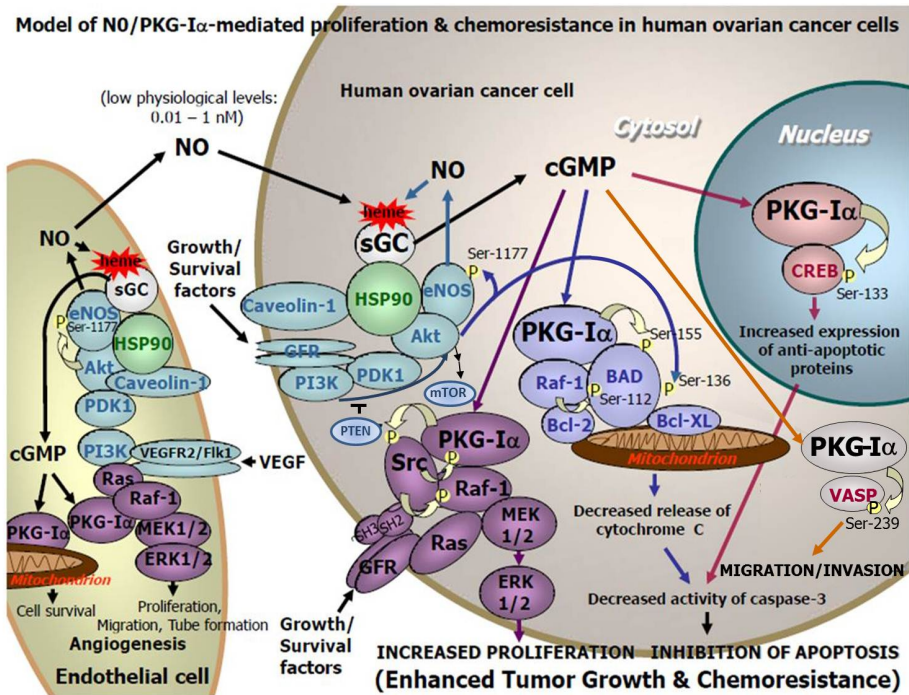


Figure 5. Cellular model of the involvement of the NO/cGMP/PKG-Iα signaling pathway in promoting chemoresistance, tumor growth and angiogenesis in ovarian cancer. A special role of VASP phosphorylation at Ser239 may contribute to enhanced ovarian cancer cell migration and invasion.

As illustrated in the model of Figure 5, we have also identified an important role of c-Src/PKG-Iα interaction in promoting DNA synthesis and cell proliferation in human ovarian cancer cells. Previous studies have shown that PKG-I binds Raf-1 and promotes downstream activation of MEK and ERK1/2 in endothelial cells [76]. In ovarian cancer cells, we proposed that PKG-Iα binds to Raf-1 at the internal surface of the plasma membrane, bringing PKG-Iα in close proximity to one of its downstream target proteins c-Src. This leads to downstream activation of the Raf-1/MEK/ERK signaling pathway, promoting cell proliferation. We found that PKG-Iα directly phosphorylates c-Src at Ser17, which enhances the tyrosine kinase activity of c-Src in both *in vitro* and intact-cell experiments [33]. Our recent studies have shown a clear role of the PKG-Iα-mediated phosphorylation of c-Src at Ser17 in preventing apoptosis and promoting proliferation, attachment and migration in the mesothelioma and NSCLC cells. It is very likely that a similar PKG-Iα catalyzed phosphorylation of c-Src at Ser17 occurs in human ovarian cancer cells, which can explain the dependence of the c-Src activation by EGF on the presence of PKG-Iα [28]. Epidermal growth factor (EGF)-induced activation of c-Src tyrosine

kinase activity was found to cause tyrosine phosphorylation of PKG-I α , increasing the serine/threonine kinase activity of PKG-I α (indicated by phosphorylation of the PKG substrate VASP at Ser239) and its growth-promoting effects in ovarian cancer cells [28]. In human ovarian cancer cells, the c-Src-mediated tyrosine-phosphorylation of the EGF receptor was found to be highly dependent on PKG-I α kinase activity [28].

We hypothesized in ovarian cancer cells, as reported in the lung cancer cells in our recent study [34], that PKG-I α phosphorylated CREB at Ser133, and the cGMP/PKG-I α signaling pathway maintains the expression of certain IAPs such as c-IAP1, livin and survivin as well as the anti-apoptotic Bcl-2 family member Mcl-1, leading to decreased activity of caspase-3 and promoting cell survival. In ovarian cancer cells where PKG-I α is hyperactivated, increased downstream phosphorylation of CREB at Ser133 and increased IAPs expression may explain the development of resistance to cisplatin-induced apoptosis. Moreover, PKG-I α siRNA gene knockdown also decreased both basal and EGF-stimulated cell migration in A2780cp ovarian cancer cells, as shown in Figure 3.

VASP phosphorylation at Ser239 has been shown to be a useful indicator of endogenous PKG kinase activity, both in our recent studies [28, 31, 32] and reports from others [61, 63, 64]. In the current study in this book chapter, we show that siRNA gene knockdown of PKG-I α expression inhibited EGF-stimulated increases in VASP Ser239 phosphorylation and Src/SFK autophosphorylation in A2780cp (cisplatin-resistant, mutated p53) ovarian cancer cells. Therefore, VASP Ser239 phosphorylation may be a useful biomarker in ovarian cancer cells, and hyperactivation of the unique NO/sGC/PKG-I α signaling pathway may be a novel therapeutic target for regulation of cancer cell migration/invasion.

Also shown in the model of Figure 5 is the role of endothelial cells, which would provide an additional source of endogenous NO within the growing tumor, potentially contributing to the “angiogenic switch”, i.e. the increased tumor growth that occurs after the invasion of endothelial cells into the tumor. Endothelial cells also play another important role in tumor growth by providing new blood vessels needed for the vascularization and blood perfusion of the growing tumor. In endothelial cells, heat shock protein 90 (HSP90) and Akt activate eNOS involving the formation of a HSP90-Akt-Calmodulin (CaM)-eNOS complex, leading to an increase in NO production [108-111]. Interestingly, HSP90 activation of eNOS can be Ca²⁺-dependent [112] or Ca²⁺-independent [109, 113].

6. Future experiments

Our future studies will need to determine: 1) whether PKG-I α is the only isoform of PKG expressed in other human ovarian cancer cell lines as well as in tumor samples of ovarian cancer patients, 2) the subcellular localization of PKG-I α (and possibly PKG-I β), for example, membrane, nuclear, and/or cytosolic localization, 3) the roles of PKG-I α , its downstream phosphorylation of CREB at Ser133 (and other transcription factors), expression of the IAPs and anti-apoptotic Bcl-2 family proteins in ovarian cancer cells.

7. Conclusions

The NO/cGMP/PKG-I α pathway and the downstream phosphorylation of the actin-filament/focal-adhesion-regulating protein VASP at Ser239 appear to promote migration/invasion and the downstream phosphorylation of BAD at ser155, CREB at ser133 and c-Src at ser17 appear to promote DNA synthesis, cell proliferation and platinum resistance in ovarian cancer cells. The unique features of this signaling pathway in ovarian cancer cells may provide a novel therapeutic target for disrupting tumor growth and the metastasis and secondary tumor formation during ovarian cancer progression.

Acknowledgements

Financial support for the research and the preparation of this book chapter was provided by Internal Funding from Roseman University of Health Sciences (formerly named University of Southern Nevada), Henderson, Nevada, USA, U.S. Department of Defense (DOD) grant W81XWH-07-1-0543 and Start-up Funding from the Nevada Cancer Institute, Las Vegas, Nevada, USA, awarded to Dr. Fiscus.

Author details

Janica C. Wong^{1,2,3} and Ronald R. Fiscus^{1,3}

1 Center for Diabetes & Obesity Prevention, Treatment, Research & Education, Cancer Research Program, and College of Pharmacy (Pharmaceutical Sciences), Roseman University of Health Sciences, Henderson, Nevada, and South Jordan, USA

2 Department of Chemistry, University of Nevada, Las Vegas, Nevada, USA

3 Cancer Molecular Biology Section, Nevada Cancer Institute, Las Vegas, Nevada, USA

References

- [1] Gershenson. DM. Management of ovarian germ cell tumors. *J Clin Oncol.* 2007 Jul 10;25(20):2938-43.
- [2] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene.* 2003 Oct 20;22(47):7265-79.
- [3] Diaz-Padilla I, Duran I, Clarke BA, Oza AM. Biologic rationale and clinical activity of mTOR inhibitors in gynecological cancer. *Cancer Treat Rev.* 2012 Oct;38(6):767-75.

- [4] Altomare DA, Wang HQ, Skele KL, De Rienzo A, Klein-Szanto AJ, Godwin AK, et al. AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. *Oncogene*. 2004 Jul 29;23(34):5853-7.
- [5] Winograd-Katz SE, Levitzki A. Cisplatin induces PKB/Akt activation and p38(MAPK) phosphorylation of the EGF receptor. *Oncogene*. 2006 Nov 30;25(56):7381-90.
- [6] Lee S, Choi EJ, Jin C, Kim DH. Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. *Gynecol Oncol*. 2005 Apr;97(1):26-34.
- [7] Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 1999 Jun 10;399(6736):601-5.
- [8] Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*. 1999 Jun 10;399(6736):597-601.
- [9] Jesmin S, Mowa CN, Sultana SN, Mia S, Islam R, Zaedi S, et al. Estrogen receptor alpha and beta are both involved in the cerebral VEGF/Akt/NO pathway and cerebral angiogenesis in female mice. *Biomed Res*. 2010 Dec;31(6):337-46.
- [10] Feliers D, Chen X, Akis N, Choudhury GG, Madaio M, Kasinath BS. VEGF regulation of endothelial nitric oxide synthase in glomerular endothelial cells. *Kidney Int*. 2005 Oct;68(4):1648-59.
- [11] Igarashi J, Michel T. Sphingosine 1-phosphate and isoform-specific activation of phosphoinositide 3-kinase beta. Evidence for divergence and convergence of receptor-regulated endothelial nitric-oxide synthase signaling pathways. *J Biol Chem*. 2001 Sep 28;276(39):36281-8.
- [12] Igarashi J, Bernier SG, Michel T. Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase. differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. *J Biol Chem*. 2001 Apr 13;276(15):12420-6.
- [13] Hisamoto K, Ohmichi M, Kurachi H, Hayakawa J, Kanda Y, Nishio Y, et al. Estrogen induces the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem*. 2001 Feb 2;276(5):3459-67.
- [14] Haynes MP, Sinha D, Russell KS, Collinge M, Fulton D, Morales-Ruiz M, et al. Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circ Res*. 2000 Oct 13;87(8):677-82.
- [15] Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, et al. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass

- spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J Biol Chem.* 1999 Oct 15;274(42):30101-8.
- [16] Michell BJ, Griffiths JE, Mitchelhill KI, Rodriguez-Crespo I, Tiganis T, Bozinovski S, et al. The Akt kinase signals directly to endothelial nitric oxide synthase. *Curr Biol.* 1999 Jul 29-Aug 12;9(15):845-8.
- [17] Shi F, Wang YC, Zhao TZ, Zhang S, Du TY, Yang CB, et al. Effects of Simulated Microgravity on Human Umbilical Vein Endothelial Cell Angiogenesis and Role of the PI3K-Akt-eNOS Signal Pathway. *PLoS One.* 2012;7(7):e40365.
- [18] Fiscus RR, Murad F. cGMP-dependent protein kinase activation in intact tissues. *Methods Enzymol.* 1988;159:150-9.
- [19] Fiscus RR, Rapoport RM, Murad F. Endothelium-dependent and nitrovasodilator-induced activation of cyclic GMP-dependent protein kinase in rat aorta. *J Cyclic Nucleotide Protein Phosphor Res.* 1983;9(6):415-25.
- [20] Fiscus RR, Rapoport RM, Waldman SA, Murad F. Atriopeptin II elevates cyclic GMP, activates cyclic GMP-dependent protein kinase and causes relaxation in rat thoracic aorta. *Biochim Biophys Acta.* 1985 Jul 30;846(1):179-84.
- [21] Fung E, Fiscus RR, Yim AP, Angelini GD, Arifi AA. The potential use of type-5 phosphodiesterase inhibitors in coronary artery bypass graft surgery. *Chest.* 2005 Oct;128(4):3065-73.
- [22] Fiscus RR. Involvement of cyclic GMP and protein kinase G in the regulation of apoptosis and survival in neural cells. *Neurosignals.* 2002 Jul-Aug;11(4):175-90.
- [23] Fiscus RR, Tu AW, Chew SB. Natriuretic peptides inhibit apoptosis and prolong the survival of serum-deprived PC12 cells. *Neuroreport.* 2001 Feb 12;12(2):185-9.
- [24] Fiscus RR, Yuen JP, Chan SL, Kwong JH, Chew SB. Nitric oxide and cyclic GMP as pro- and anti-apoptotic agents. *J Card Surg.* 2002 Jul-Aug;17(4):336-9.
- [25] Cheng Chew SB, Leung PY, Fiscus RR. Preincubation with atrial natriuretic peptide protects NG108-15 cells against the toxic/proapoptotic effects of the nitric oxide donor S-nitroso- N-acetylpenicillamine. *Histochem Cell Biol.* 2003 Sep;120(3):163-71.
- [26] Johlfs MG, Fiscus RR. Protein kinase G type-1 α phosphorylates the apoptosis-regulating protein Bad at serine 155 and protects against apoptosis in N1E-115 cells. *Neurochem Int.* 2010 Mar;56(4):546-53.
- [27] Barger SW, Fiscus RR, Ruth P, Hofmann F, Mattson MP. Role of cyclic GMP in the regulation of neuronal calcium and survival by secreted forms of beta-amyloid precursor. *J Neurochem.* 1995 May;64(5):2087-96.
- [28] Leung EL, Wong JC, Johlfs MG, Tsang BK, Fiscus RR. Protein kinase G type 1 α activity in human ovarian cancer cells significantly contributes to enhanced Src activation and DNA synthesis/cell proliferation. *Mol Cancer Res.* 2010 Apr;8(4):578-91.

- [29] Fraser M, Chan SL, Chan SS, Fiscus RR, Tsang BK. Regulation of p53 and suppression of apoptosis by the soluble guanylyl cyclase/cGMP pathway in human ovarian cancer cells. *Oncogene*. 2006 Apr 6;25(15):2203-12.
- [30] Leung EL, Fraser M, Fiscus RR, Tsang BK. Cisplatin alters nitric oxide synthase levels in human ovarian cancer cells: involvement in p53 regulation and cisplatin resistance. *Br J Cancer*. 2008 Jun 3;98(11):1803-9.
- [31] Wong JC, Fiscus RR. Protein kinase G activity prevents pathological-level nitric oxide-induced apoptosis and promotes DNA synthesis/cell proliferation in vascular smooth muscle cells. *Cardiovasc Pathol*. 2010 Nov-Dec;19(6):e221-31.
- [32] Wong JC, Fiscus RR. Essential roles of the nitric oxide (NO)/cGMP/protein kinase G type-Ialpha (PKG-Ialpha) signaling pathway and the atrial natriuretic peptide (ANP)/cGMP/PKG-Ialpha autocrine loop in promoting proliferation and cell survival of OP9 bone marrow stromal cells. *J Cell Biochem*. 2011 Mar;112(3):829-39.
- [33] Fiscus RR, Jholls MG. Protein kinase G type-I phosphorylates c-Src at serine-17 and promotes cell survival, proliferation and attachment in human mesothelioma and non-small cell lung cancer cells. *BMC Pharmacology* 2011;11(Suppl. 1): 031.
- [34] Wong JC, Bathina M, Fiscus RR. Cyclic GMP/protein kinase G type-Ialpha (PKG-Ialpha) signaling pathway promotes CREB phosphorylation and maintains higher c-IAP1, livin, survivin and Mcl-1 expression and the inhibition of PKG-Ialpha kinase activity synergizes with cisplatin in non-small cell lung cancer cells. *J Cell Biochem*. 2012 (In Press).
- [35] Waldmann R, Nieberding M, Walter U. Vasodilator-stimulated protein phosphorylation in platelets is mediated by cAMP- and cGMP-dependent protein kinases. *Eur J Biochem*. 1987 Sep 15;167(3):441-8.
- [36] Bear JE, Svitkina TM, Krause M, Schafer DA, Loureiro JJ, Strasser GA, et al. Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell*. 2002 May 17;109(4):509-21.
- [37] Applewhite DA, Barzik M, Kojima S, Svitkina TM, Gertler FB, Borisy GG. Ena/VASP proteins have an anti-capping independent function in filopodia formation. *Mol Biol Cell*. 2007 Jul;18(7):2579-91.
- [38] Walders-Harbeck B, Khaitlina SY, Hinssen H, Jockusch BM, Illenberger S. The vasodilator-stimulated phosphoprotein promotes actin polymerisation through direct binding to monomeric actin. *FEBS Lett*. 2002 Oct 9;529(2-3):275-80.
- [39] Reinhard M, Rudiger M, Jockusch BM, Walter U. VASP interaction with vinculin: a recurring theme of interactions with proline-rich motifs. *FEBS Lett*. 1996 Dec 9;399(1-2):103-7.
- [40] Jenzora A, Behrendt B, Small JV, Wehland J, Stradal TE. PREL1 provides a link from Ras signalling to the actin cytoskeleton via Ena/VASP proteins. *FEBS Lett*. 2005 Jan 17;579(2):455-63.

- [41] Pasic L, Kotova T, Schafer DA. Ena/VASP proteins capture actin filament barbed ends. *J Biol Chem*. 2008 Apr 11;283(15):9814-9.
- [42] Sechi AS, Wehland J. ENA/VASP proteins: multifunctional regulators of actin cytoskeleton dynamics. *Front Biosci*. 2004 May 1;9:1294-310.
- [43] Brindle NP, Holt MR, Davies JE, Price CJ, Critchley DR. The focal-adhesion vasodilator-stimulated phosphoprotein (VASP) binds to the proline-rich domain in vinculin. *Biochem J*. 1996 Sep 15;318 (Pt 3):753-7.
- [44] Galler AB, Garcia Arguinzonis MI, Baumgartner W, Kuhn M, Smolenski A, Simm A, et al. VASP-dependent regulation of actin cytoskeleton rigidity, cell adhesion, and detachment. *Histochem Cell Biol*. 2006 May;125(5):457-74.
- [45] Zhang Y, Tu Y, Gkretsi V, Wu C. Migfilin interacts with vasodilator-stimulated phosphoprotein (VASP) and regulates VASP localization to cell-matrix adhesions and migration. *J Biol Chem*. 2006 May 5;281(18):12397-407.
- [46] Loureiro JJ, Rubinson DA, Bear JE, Baltus GA, Kwiatkowski AV, Gertler FB. Critical roles of phosphorylation and actin binding motifs, but not the central proline-rich region, for Ena/vasodilator-stimulated phosphoprotein (VASP) function during cell migration. *Mol Biol Cell*. 2002 Jul;13(7):2533-46.
- [47] Samarín S, Romero S, Kocks C, Didry D, Pantaloni D, Carlier MF. How VASP enhances actin-based motility. *J Cell Biol*. 2003 Oct 13;163(1):131-42.
- [48] Krause M, Dent EW, Bear JE, Loureiro JJ, Gertler FB. Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. *Annu Rev Cell Dev Biol*. 2003;19:541-64.
- [49] Kwiatkowski AV, Gertler FB, Loureiro JJ. Function and regulation of Ena/VASP proteins. *Trends Cell Biol*. 2003 Jul;13(7):386-92.
- [50] Scott JA, Shewan AM, den Elzen NR, Loureiro JJ, Gertler FB, Yap AS. Ena/VASP proteins can regulate distinct modes of actin organization at cadherin-adhesive contacts. *Mol Biol Cell*. 2006 Mar;17(3):1085-95.
- [51] Salazar R, Bell SE, Davis GE. Coordinate induction of the actin cytoskeletal regulatory proteins gelsolin, vasodilator-stimulated phosphoprotein, and profilin during capillary morphogenesis in vitro. *Exp Cell Res*. 1999 May 25;249(1):22-32.
- [52] Kayisli UA, Demir R, Erguler G, Arici A. Vasodilator-stimulated phosphoprotein expression and its cytokine-mediated regulation in vasculogenesis during human placental development. *Mol Hum Reprod*. 2002 Nov;8(11):1023-30.
- [53] Liu K, Li L, Nisson PE, Gruber C, Jessee J, Cohen SN. Reversible tumorigenesis induced by deficiency of vasodilator-stimulated phosphoprotein. *Mol Cell Biol*. 1999 May;19(5):3696-703.
- [54] Wu G, Wei L, Yu A, Zhang M, Qi B, Su K, et al. Vasodilator-stimulated phosphoprotein regulates osteosarcoma cell migration. *Oncol Rep*. 2011 Dec;26(6):1609-15.

- [55] Dertsiz L, Ozbilim G, Kayisli Y, Gokhan GA, Demircan A, Kayisli UA. Differential expression of VASP in normal lung tissue and lung adenocarcinomas. *Thorax*. 2005 Jul;60(7):576-81.
- [56] Hu LD, Zou HF, Zhan SX, Cao KM. EVL (Ena/VASP-like) expression is up-regulated in human breast cancer and its relative expression level is correlated with clinical stages. *Oncol Rep*. 2008 Apr;19(4):1015-20.
- [57] Han G, Fan B, Zhang Y, Zhou X, Wang Y, Dong H, et al. Positive regulation of migration and invasion by vasodilator-stimulated phosphoprotein via Rac1 pathway in human breast cancer cells. *Oncol Rep*. 2008 Oct;20(4):929-39.
- [58] Kim YM, Renne C, Seifert S, Schuh K, Renne T. Impaired melanoma growth in VASP deficient mice. *FEBS Lett*. 2011 Aug 4;585(15):2533-6.
- [59] Wang J, Zhang J, Wu J, Luo D, Su K, Shi W, et al. MicroRNA-610 inhibits the migration and invasion of gastric cancer cells by suppressing the expression of vasodilator-stimulated phosphoprotein. *Eur J Cancer*. 2012 Aug;48(12):1904-13.
- [60] Halbrugge M, Walter U. Purification of a vasodilator-regulated phosphoprotein from human platelets. *Eur J Biochem*. 1989 Oct 20;185(1):41-50.
- [61] Eigenthaler M, Nolte C, Halbrugge M, Walter U. Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets. Estimating the rate of cAMP-regulated and cGMP-regulated protein phosphorylation in intact cells. *Eur J Biochem*. 1992 Apr 15;205(2):471-81.
- [62] Halbrugge M, Friedrich C, Eigenthaler M, Schanzenbacher P, Walter U. Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators. *J Biol Chem*. 1990 Feb 25;265(6):3088-93.
- [63] Butt E, Abel K, Krieger M, Palm D, Hoppe V, Hoppe J, et al. cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. *J Biol Chem*. 1994 May 20;269(20):14509-17.
- [64] Smolenski A, Bachmann C, Reinhard K, Honig-Liedl P, Jarchau T, Hoschuetzky H, et al. Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239 phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody. *J Biol Chem*. 1998 Aug 7;273(32):20029-35.
- [65] Ibarra-Alvarado C, Galle J, Melichar VO, Mameghani A, Schmidt HH. Phosphorylation of blood vessel vasodilator-stimulated phosphoprotein at serine 239 as a functional biochemical marker of endothelial nitric oxide/cyclic GMP signaling. *Mol Pharmacol*. 2002 Feb;61(2):312-9.
- [66] Oelze M, Mollnau H, Hoffmann N, Warnholtz A, Bodenschatz M, Smolenski A, et al. Vasodilator-stimulated phosphoprotein serine 239 phosphorylation as a sensitive

- monitor of defective nitric oxide/cGMP signaling and endothelial dysfunction. *Circ Res.* 2000 Nov 24;87(11):999-1005.
- [67] Deguchi A, Soh JW, Li H, Pamukcu R, Thompson WJ, Weinstein IB. Vasodilator-stimulated phosphoprotein (VASP) phosphorylation provides a biomarker for the action of exisulind and related agents that activate protein kinase G. *Mol Cancer Ther.* 2002 Aug;1(10):803-9.
- [68] Fiscus RR, Leung EL, Wong JC, Johlfs MG. Nitric oxide/protein kinase G-1 α promotes c-Src activation, proliferation and chemoresistance in ovarian cancer. In: Farghaly S, editor. *Ovarian Cancer - Basic Science Perspective*: Intech Open Access Publisher; 2012. p. 315-34.
- [69] Fiscus RR, Johlfs MG. Protein kinase G (PKG): Involvement in promoting neural cell survival, proliferation, synaptogenesis and synaptic plasticity and the use of new ultrasensitive capillary-electrophoresis-based methodologies for measuring PKG expression and molecular actions.. In: Mukai H, editor. *Protein Kinase Technologies (in the series NEUROMETHODS)*, Springer; 2012. p. 319-48.
- [70] Kawasaki K, Smith RS, Jr., Hsieh CM, Sun J, Chao J, Liao JK. Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis. *Mol Cell Biol.* 2003 Aug;23(16):5726-37.
- [71] Zaragoza C, Soria E, Lopez E, Browning D, Balbin M, Lopez-Otin C, et al. Activation of the mitogen activated protein kinase extracellular signal-regulated kinase 1 and 2 by the nitric oxide-cGMP-cGMP-dependent protein kinase axis regulates the expression of matrix metalloproteinase 13 in vascular endothelial cells. *Mol Pharmacol.* 2002 Oct;62(4):927-35.
- [72] Pilz RB, Casteel DE. Regulation of gene expression by cyclic GMP. *Circ Res.* 2003 Nov 28;93(11):1034-46.
- [73] Pyriochou A, Zhou Z, Koika V, Petrou C, Cordopatis P, Sessa WC, et al. The phosphodiesterase 5 inhibitor sildenafil stimulates angiogenesis through a protein kinase G/MAPK pathway. *J Cell Physiol.* 2007 Apr;211(1):197-204.
- [74] Pyriochou A, Vassilakopoulos T, Zhou Z, Papapetropoulos A. cGMP-dependent and -independent angiogenesis-related properties of nitric oxide. *Life Sci.* 2007 Nov 10;81(21-22):1549-54.
- [75] Pyriochou A, Beis D, Koika V, Potytarchou C, Papadimitriou E, Zhou Z, et al. Soluble guanylyl cyclase activation promotes angiogenesis. *J Pharmacol Exp Ther.* 2006 Nov; 319(2):663-71.
- [76] Hood J, Granger HJ. Protein kinase G mediates vascular endothelial growth factor-induced Raf-1 activation and proliferation in human endothelial cells. *J Biol Chem.* 1998 Sep 4;273(36):23504-8.
- [77] Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, et al. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular sig-

- nal-regulated kinase1/2 activation in postcapillary endothelium. *J Biol Chem.* 1998 Feb 13;273(7):4220-6.
- [78] Deguchi A, Thompson WJ, Weinstein IB. Activation of protein kinase G is sufficient to induce apoptosis and inhibit cell migration in colon cancer cells. *Cancer Res.* 2004 Jun 1;64(11):3966-73.
- [79] Zuzga DS, Pelta-Heller J, Li P, Bombonati A, Waldman SA, Pitari GM. Phosphorylation of vasodilator-stimulated phosphoprotein Ser239 suppresses filopodia and invadopodia in colon cancer. *Int J Cancer.* 2012 Jun 1;130(11):2539-48.
- [80] Lincoln TM, Dey N, Sellak H. Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J Appl Physiol.* 2001 Sep;91(3):1421-30.
- [81] Francis SH, Busch JL, Corbin JD, Sibley D. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev.* 2010 Sep;62(3):525-63.
- [82] Soh JW, Mao Y, Kim MG, Pamukcu R, Li H, Piazza GA, et al. Cyclic GMP mediates apoptosis induced by sulindac derivatives via activation of c-Jun NH2-terminal kinase 1. *Clin Cancer Res.* 2000 Oct;6(10):4136-41.
- [83] Thompson WJ, Piazza GA, Li H, Liu L, Fetter J, Zhu B, et al. Exisulind induction of apoptosis involves guanosine 3',5'-cyclic monophosphate phosphodiesterase inhibition, protein kinase G activation, and attenuated beta-catenin. *Cancer Res.* 2000 Jul 1;60(13):3338-42.
- [84] Persons DL, Yazlovitskaya EM, Pelling JC. Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin. *J Biol Chem.* 2000 Nov 17;275(46):35778-85.
- [85] Colbran JL, Roach PJ, Fiol CJ, Dixon JE, Andrisani OM, Corbin JD. cAMP-dependent protein kinase, but not the cGMP-dependent enzyme, rapidly phosphorylates delta-CREB, and a synthetic delta-CREB peptide. *Biochem Cell Biol.* 1992 Oct-Nov; 70(10-11):1277-82.
- [86] Idriss SD, Gudi T, Casteel DE, Kharitonov VG, Pilz RB, Boss GR. Nitric oxide regulation of gene transcription via soluble guanylate cyclase and type I cGMP-dependent protein kinase. *J Biol Chem.* 1999 Apr 2;274(14):9489-93.
- [87] Gudi T, Casteel DE, Vinson C, Boss GR, Pilz RB. NO activation of fos promoter elements requires nuclear translocation of G-kinase I and CREB phosphorylation but is independent of MAP kinase activation. *Oncogene.* 2000 Dec 14;19(54):6324-33.
- [88] Gudi T, Huvar I, Meinecke M, Lohmann SM, Boss GR, Pilz RB. Regulation of gene expression by cGMP-dependent protein kinase. Transactivation of the c-fos promoter. *J Biol Chem.* 1996 Mar 1;271(9):4597-600.

- [89] Gudi T, Lohmann SM, Pilz RB. Regulation of gene expression by cyclic GMP-dependent protein kinase requires nuclear translocation of the kinase: identification of a nuclear localization signal. *Mol Cell Biol.* 1997 Sep;17(9):5244-54.
- [90] Chen Y, Zhuang S, Cassenaer S, Casteel DE, Gudi T, Boss GR, et al. Synergism between calcium and cyclic GMP in cyclic AMP response element-dependent transcriptional regulation requires cooperation between CREB and C/EBP-beta. *Mol Cell Biol.* 2003 Jun;23(12):4066-82.
- [91] Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. *Genes Dev.* 1999 Feb 1;13(3):239-52.
- [92] Nishihara H, Hwang M, Kizaka-Kondoh S, Eckmann L, Insel PA. Cyclic AMP promotes cAMP-responsive element-binding protein-dependent induction of cellular inhibitor of apoptosis protein-2 and suppresses apoptosis of colon cancer cells through ERK1/2 and p38 MAPK. *J Biol Chem.* 2004 Jun 18;279(25):26176-83.
- [93] Nishihara H, Kizaka-Kondoh S, Insel PA, Eckmann L. Inhibition of apoptosis in normal and transformed intestinal epithelial cells by cAMP through induction of inhibitor of apoptosis protein (IAP)-2. *Proc Natl Acad Sci U S A.* 2003 Jul 22;100(15):8921-6.
- [94] Sasaki H, Sheng Y, Kotsuji F, Tsang BK. Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. *Cancer Res.* 2000 Oct 15;60(20):5659-66.
- [95] Fraser M, Leung B, Jahani-Asl A, Yan X, Thompson WE, Tsang BK. Chemoresistance in human ovarian cancer: the role of apoptotic regulators. *Reprod Biol Endocrinol.* 2003 Oct 7;1:66.
- [96] Asselin E, Mills GB, Tsang BK. XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. *Cancer Res.* 2001 Mar 1;61(5):1862-8.
- [97] Li J, Feng Q, Kim JM, Schneiderman D, Liston P, Li M, et al. Human ovarian cancer and cisplatin resistance: possible role of inhibitor of apoptosis proteins. *Endocrinology.* 2001 Jan;142(1):370-80.
- [98] Fraser M, Leung BM, Yan X, Dan HC, Cheng JQ, Tsang BK. p53 is a determinant of X-linked inhibitor of apoptosis protein/Akt-mediated chemoresistance in human ovarian cancer cells. *Cancer Res.* 2003 Nov 1;63(21):7081-8.
- [99] Kumar S, Bryant CS, Chamala S, Qazi A, Seward S, Pal J, et al. Ritonavir blocks AKT signaling, activates apoptosis and inhibits migration and invasion in ovarian cancer cells. *Mol Cancer.* 2009;8:26.
- [100] Karam AK, Santiskulvong C, Fekete M, Zabih S, Eng C, Dorigo O. Cisplatin and PI3kinase inhibition decrease invasion and migration of human ovarian carcinoma cells and regulate matrix-metalloproteinase expression. *Cytoskeleton (Hoboken).* 2010 Aug;67(8):535-44.

- [101] Zhou C, Qiu L, Sun Y, Healey S, Wanebo H, Kouttab N, et al. Inhibition of EGFR/PI3K/AKT cell survival pathway promotes TSA's effect on cell death and migration in human ovarian cancer cells. *Int J Oncol*. 2006 Jul;29(1):269-78.
- [102] Peng DJ, Wang J, Zhou JY, Wu GS. Role of the Akt/mTOR survival pathway in cisplatin resistance in ovarian cancer cells. *Biochem Biophys Res Commun*. 2010 Apr 9;394(3):600-5.
- [103] Whitley BR, Beaulieu LM, Carter JC, Church FC. Phosphatidylinositol 3-kinase/Akt regulates the balance between plasminogen activator inhibitor-1 and urokinase to promote migration of SKOV-3 ovarian cancer cells. *Gynecol Oncol*. 2007 Feb;104(2):470-9.
- [104] Park BK, Zeng X, Glazer RI. Akt1 induces extracellular matrix invasion and matrix metalloproteinase-2 activity in mouse mammary epithelial cells. *Cancer Res*. 2001 Oct 15;61(20):7647-53.
- [105] Furuya F, Hanover JA, Cheng SY. Activation of phosphatidylinositol 3-kinase signaling by a mutant thyroid hormone beta receptor. *Proc Natl Acad Sci U S A*. 2006 Feb 7;103(6):1780-5.
- [106] Vasko V, Saji M, Hardy E, Kruhlik M, Larin A, Savchenko V, et al. Akt activation and localisation correlate with tumour invasion and oncogene expression in thyroid cancer. *J Med Genet*. 2004 Mar;41(3):161-70.
- [107] Choi JH, Choi KC, Auersperg N, Leung PC. Gonadotropins activate proteolysis and increase invasion through protein kinase A and phosphatidylinositol 3-kinase pathways in human epithelial ovarian cancer cells. *Cancer Res*. 2006 Apr 1;66(7):3912-20.
- [108] Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, et al. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature*. 1998 Apr 23;392(6678):821-4.
- [109] Takahashi S, Mendelsohn ME. Synergistic activation of endothelial nitric-oxide synthase (eNOS) by HSP90 and Akt: calcium-independent eNOS activation involves formation of an HSP90-Akt-CaM-bound eNOS complex. *J Biol Chem*. 2003 Aug 15;278(33):30821-7.
- [110] Gratton JP, Fontana J, O'Connor DS, Garcia-Cardena G, McCabe TJ, Sessa WC. Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90, and caveolin-1 complex in vitro. Evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. *J Biol Chem*. 2000 Jul 21;275(29):22268-72.
- [111] Fontana J, Fulton D, Chen Y, Fairchild TA, McCabe TJ, Fujita N, et al. Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res*. 2002 May 3;90(8):866-73.

- [112] Brouet A, Sonveaux P, Dessy C, Moniotte S, Balligand JL, Feron O. Hsp90 and caveolin are key targets for the proangiogenic nitric oxide-mediated effects of statins. *Circ Res.* 2001 Nov 9;89(10):866-73.
- [113] Takahashi S, Mendelsohn ME. Calmodulin-dependent and -independent activation of endothelial nitric-oxide synthase by heat shock protein 90. *J Biol Chem.* 2003 Mar 14;278(11):9339-44.

INTECH

INTECH

