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Interactions of the Protein Kinase A Signaling Pathway: Implications for the Treatment of Endocrine and Other Tumors

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1. Introduction

Cell signaling is known to be involved in the development and activation of many cancers, and has been the subject of abundant research in the field of cancer (Levitzki & Klein, 2010; Robinson-White & Stratakis, 2002). In response to extracellular and intracellular stimuli, protein kinase mediated pathways orchestrate the activation of cell signaling cascades to control cell growth, proliferation, differentiation and survival. However, there is tremendous complexity in tumor cell signaling (Iqbal et al., 2010). Signaling experiments have demonstrated that many different stimuli may activate the same pathways and many pathways interact ("cross talk") to modify and influence the outcome of a specific extracellular signal. In particular, the protein kinase A (PKA) pathway has been viewed as the central hub in the mediation of signals in endocrine tumorigenesis (Robinson-White & Stratakis, 2002). Depending on the species, tissue, cell type and ligand involved, the PKA hub interacts with high versatility with the mitogen activated protein kinase (MAPK), protein kinase B (akt/PKB), protein kinase C (PKC) and Wnt pathways to promote endocrine and other tumor cell development and progression (Almeida et al., 2010; Robinson-White & Stratakis, 2002). The purpose of this review is to highlight recent findings on the involvement of the PKA cell signaling pathway in endocrine tumorigenesis, and to define current clinical strategies and future perspectives for the treatment of endocrine and other tumors.

2. The cell signaling pathways

2.1 Description of the PKA pathway

PKA is a major factor in eukaryotic cell signaling. The PKA holoenzyme, in its inactive state, consists of two PKA isoforms (type I and type II), each in a tetramer of two homo or heterodimer regulatory subunits (RIα, RIβ, RIIα and RII β). Each R-subunit is bound to one of four catalytic subunits (Cα, Cβ, Cγ, and Prkx) (Azevedo & Stratakis, 2011). The regulatory subunits contain a dimerization/docking domain at their amino terminus and two tandem binding domains for cyclic AMP (cAMP) at their carboxyl terminus, and a linker region that contains a main docking site for the catalytic subunit (Zawadzki & Taylor, 2004). PKA may be activated (Figure 1A) indirectly by ligand binding to a heterotrimeric G-protein coupled

receptor (GPCR), and/or by activation of adenyl cyclase (AC) and ATP, which stimulate the production of cAMP via the GSα subunit of the G-protein (*e.g.* GSα, Bγ); or directly by endogenous cAMP. CAMP binds to the PKA regulatory subunit leading to its subsequent activation. This, in turn, promotes dissolution of the holoenzyme and release of the catalytic subunits. The free catalytic subunits then go on to phosphorylate downstream targets that regulate effector enzymes, ion channels and transcription factors for specific genes that regulate cell growth and differentiation (Pearce et al., 2010); for example, CREB, CREM, NF-kB and other nuclear receptors (Robinson-White & Stratakis, 2002).
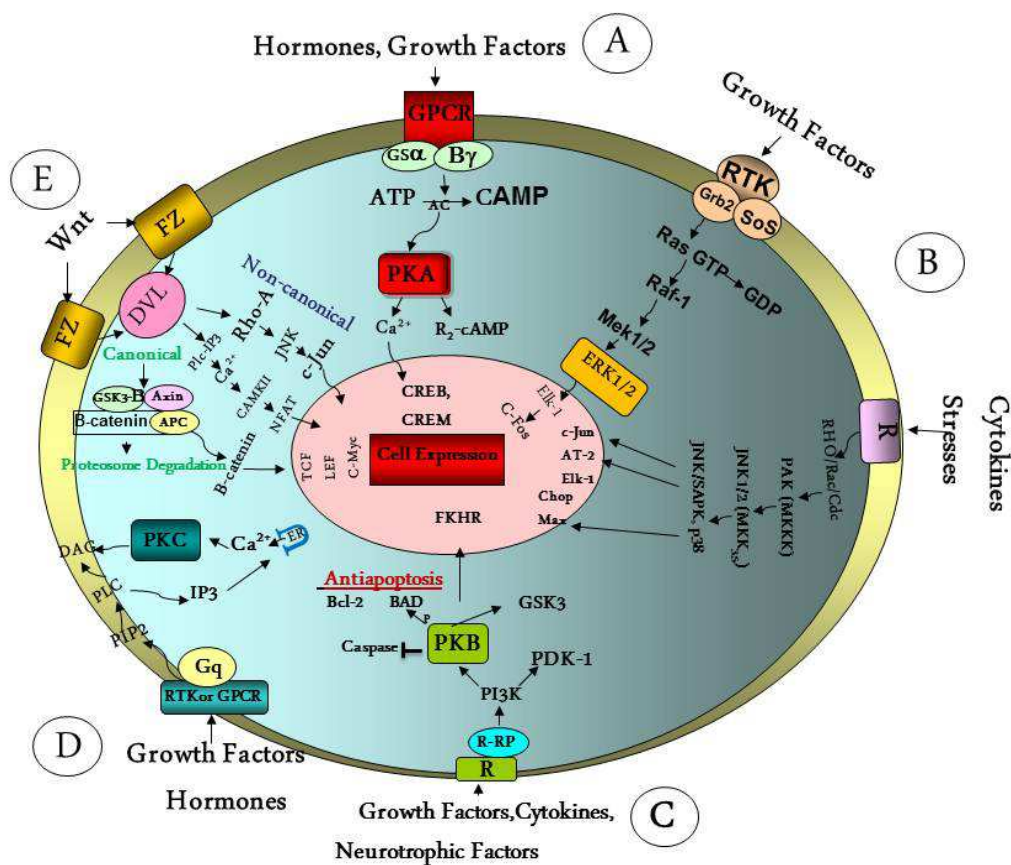

Fig. 1. General overview of the major signal transduction pathways in mammalian cells. Signal transduction in mammalian cells is controlled by five major signaling pathways (A-E), which upon activation, control gene expression and a cells' response (*e.g.* proliferation, differentiation, apoptosis, survival). Each pathway is independent, yet interacts in a complex manner with other pathways. An explanation of each pathway is given in the text.(A) Protein kinase A (PKA) pathway. (B) Mitogen-activated protein kinase (MAPK) pathway. (C) Protein kinase B (PKB) pathway. (D) Protein kinase C (PKC) pathway. (E) Wnt Pathway.

2.2 Description of other major cell signaling pathways in endocrine tissue

2.2.1 MAP-kinase signaling pathway

The MAP kinase proteins are signal transduction enzymes that mediate diverse cellular processes (*i.e.* cell proliferation, differentiation, apoptosis, survival and cytokine production)

(Robinson-White & Stratakis, 2002). The family includes more than a dozen members, arranged in separate but interacting cascades, based on sequence homology and function. These include ERK (extracellular signal-regulated kinase), JNK/SAPK (c-Jun N terminal kinase/stress activated protein kinase), and p38 MAPK. Each cascade consists of a three-core module, MKKK (MAPKK kinase), MKK (MAPK kinase) and MAPK. Upon cell activation (Figure 1B), the first core member activates the succeeding member in a serial manner (Garrington & Johnson, 1999). The final activated core member then regulates gene expression through the phosphorylation and activation of nuclear transcription factors (Cargnello & Roux, 2011) The best studied MAPK is ERK1/ERK2 (ERK1/2), 44- and 42-kDa proteins that are stimulated by a wide range of ligands (*e.g.* serum, growth factors, cytokine stresses, other mitogens). Upon ligand-induced stimulation of receptor tyrosine kinase (RTK) activity, adapter molecules (Grb2, SoS) bind to the receptors to enhance the activation of the Ras GTP-binding protein. Activated Ras binds to the first core member of the cascade, ubiquitous Raf-1 (MKKK, one of three Raf isoforms: A-Raf, B-Raf and c-Raf-1), or rap, which activates B-Raf, and transports the isoform to the plasma membrane for activation by other protein kinases (*e.g.* Src, PKC, PAK). Active Raf-1 or B-Raf dissociates from the Ras complex and phosphorylates its substrate and the second core member, MEK1/2 (MKK). Activated MEK1/2 then phosphorylates and activates the final core member, ERK1/2, which phosphorylates and activates its downstream substrates and various transcription factors (elk-1, c-fos, c/n-Myc) for expression of early response genes leading to a cell response (*e.g.* cell proliferation, differentiation, survival and apoptosis)). Other MAPK subfamilies, not as well characterized as the ERK1/2 family are the JNK/SAPK (46- and 54 kDa proteins, isoforms JNK1, JNK2 and JKN3) and p38 kinases (isoforms p38α, -β, δ, γ) . These kinases are often activated by inflammatory cytokines and cellular stresses (*e.g.* ionizing radiation, heat shock). They are associated with promotion of apoptosis and cytokine production (Robinson-White Stratakis, 2002).

2.2.2 PKB signaling pathway

PKB, a serine/threonine kinase, is named so because of its structural similarity to protein kinases A and C (Coffer & Woodgett, 1991). Its activation (Figure 1C) can occur at the plasma membrane by growth factors, cytokines and neurotrophic factors. The activated receptor phosphorylates a key regulatory protein (through monomeric Ras or Ras-related protein, R-RP), phoshatidylinositol-3 kinase (PI3K). PI3K, a heterodimer of both regulatory and catalytic subunits, has molecular masses of 85 kDa (p85) and 110 kDa (p110), respectively. Each subunit has at least five isoforms. PI3K is activated when p85 and p110 form a heterodimer that interacts with an activator protein. Binding of the activator protein causes a conformational change in the heterodimer, leading to its activation. PI3K has both lipid and protein kinase activity. It has the ability to activate several proteins such as PKB, PKC phosphoinositide-dependent kinase-1 (PDK-1), Ras and Rac cd42. It is then activated in two major steps: first, its pleckstrin homology domain binds with the main products of a PI3K, Ptdlns P and Ptdlns P2 catalyzed lipid reaction. Second, it is phosphorylated by PDK-1 kinase. Activated PKB acts to preferentially control anti-apoptotic mechanisms. It acts through several independent mechanisms: 1), it phosphorylates BAD to prevent its binding to antiapoptotic Bcl-2; 2) it inhibits caspase protease activity; and 3) it directly phosphorylates and activates cAMP-induced ribosomal protein S6 kinase (p70S6k), a key player in the PI3K/mTor/p70S6k pathway. PKB also directly phosphorylates glycogen

synthetase kinase (GSK-3) to decrease its activity and prevent the induction of apoptosis. Lastly, PKB phosphorylates the winged-helix family of transcription factors, *forkhead* (FKHR) (Robinson-White & Stratakis, 2002).

2.2.3 PKC signaling pathway

PKC presents a large super family of protein kinases that comprise at least ten members or isozymes. Each isozyme has a distinct tissue distribution and functional pattern. They are grouped according to their activation requirements. Conventional PKCs (PKC-α, -B_I,-B_{II} and γ) are activated by phosphatidyl serine, Ca^{2+} and diacylglycerol (DAG). Novel PKCs (PKC-δ, -ε, -η, and -φ) do not require Ca^{2+}, while atypical PKCs (PKC-ζ and -λ) require only phosphatidyl serine. Other isozymes, the protein kinase C-related kinases (PRKs) are insensitive to Ca^{2+}, DAG and phorbol esters (Robinson-White & Stratakis, 2002). Stimulation of the PKC pathway (Figure 1D), by various agents (*e.g.* mitogenic growth factors, hormones), leads to the breakdown of plasma membrane phospholipid (PIP_2) by phospholipase C (PLC). The resultant hydrolysis generates inositol triphosphate (IP_3) and membrane located DAG. IP_3 causes the release of Ca^{2+} from the endoplasmic reticulum (ER). DAG production is transient and is frequently followed by a more sustained release following hydrolysis of phosphatidylcholine (PC) by phospholipase D. Ca^{2+} mobilization then causes PKC to bind to the plasma membrane cytosolic leaf where it is activated by DAG. PKC activation results in the activation of cell-specific transcription factors (e.g. c-Fos, c-Jun, NF-kB) for an array of cell responses (Lodish et al., 2000).

2.2.4 Wnt signaling pathway

Wnt (wingless and integration site growth factor) signal transduction is induced by lipid-modified and secreted Wnt glycoproteins. Nineteen Wnt family members and ten 7-transmembrane Frizzled (FZ) receptors exist in humans. Wnt proteins bind to receptors of the FZ family to activate both the canonical (canonical-Wnt/B-Catenin) and non-canonical pathways (Figure 1E), of which the disheveled scaffolding protein (DVL) acts as the central hub (Hu &Li, 2010). In the cytoplasm, in the absence of Wnt protein activation, B-Catenin forms a multi-protein destruction complex with adenomatous polyposis coli (APC), axin and glycogen synthase kinase -3β (GSK3-β), which leads to proteasomal degradation through β-Trep-mediated ubiquitination. Upon Wnt protein binding to activate the canonical pathway, DVL is activated. The destruction complex is disassembled, leaving free and stable B-Catenin in the cytosol. B-Catenin travels to the nucleus and forms a heterodimer with the TCF/LEF transcription factor for activation of target genes, *e.g. c-myc,* to regulate cell proliferation, stem cell maintenance or cell differentiation. Wnt proteins also stimulate B-Catenin -independent non-canonical pathways. These pathways are called the Wnt/jun N-terminal kinase (JNK) and Wnt/calcium pathways. The Wnt/JNK pathway involves the activation of small GTPases of the rho family (*e.g.* rac, cdc42) and downstream protein kinases such as JNK or rho kinase. JNK activates c-Jun, which goes into the nucleus to regulate polarized cell movement. Activation of the Wnt/calcium pathway occurs when Wnt proteins bind to FZ receptors to activate DVL and heterotrimic G-proteins that activate PLCγ and IP_3 and cause the release of Ca^{2+} from the endoplasmic reticulum. Released Ca^{2+} then activates calcium-dependent enzymes, calcium/calmodulin-dependent kinase, CaMKII, PKC or calcineurin. Calcineurin activates NFAT transcription factor and gene

expression for cardiac development and hypertrophy. CaMKII activation can lead to activation of a nemo-like kinase (NLK), which prevents β-Catenin intranuclear signaling (Hu & Li, 2010; Roa & Khul, 2010).

2.3 Interaction of PKA with other signaling pathways in endocrine tissue

Signaling pathways "talk" to each other forming a complicated network of interactions that amplify and/ or dampen the activation of extracellular and intracellular signals. PKA plays the role of the central hub in endocrine signal transduction. Depending on the species, tissue, cell type and cell type involved, PKA can mediate and communicate cAMP effects to MAPK, PKC, PKB, and other pathways to modify and influence a cellular response (Robinson-White & Stratakis, 2002). We present here examples of cAMP/PKA signaling pathway interactions in endocrine tissue.

2.3.1 The hypothalamic-pituitary axis signal transduction

The hypothalamic-pituitary axis (Figure 2A) is regulated via the stimulation of gonadotropin releasing hormone (GnRH) receptors (GnRHR) and the activation of multiple G-proteins by GnRH, from hypothalamic neurosecretory cells. GnRHRs couple to heterotrimeric G-proteins and can activate PKC, PKA and MAPK simultaneously. GnRHRs activate PKC through extracellular Ca^{2+} via the G-protein $Gq/11\alpha$, and activates PKA via $GS\alpha$ and cAMP. GnRHRs also activate $GB\gamma$-mediated PI3K and Src. While both PKC and PKA can independently activate c-Raf-1 of MAPK, PI3K can activate Ras to affect MAPK. The βγ subunit released by activation of Giα also stimulates adenyl cyclase (AC) to produce cAMP. Thus, GnRHR activation of MAPK is mediated by Gsα, Gq/11α, Giα and Gβγ (Cuny et al., 2011; Robinson-White & Stratakis, 2002). Therefore, in pituitary somatolactotroph cells, both the PKA and PKC pathways converge to activate MAPK for the regulation of gonadotropin hormone secretion and for cell proliferation. Recent studies in mouse AtT-20 corticotroph pituitary cells (not shown), suggest that PKA and the canonical Wnt signaling pathway interact to induce tumorigenesis. PKA alters the GSK-3B phosphorylation status at phosphor-GSK-3B (Ser9) thereby altering B-Catenin degradation and controlling cell proliferation (Khattak et al., 2010).

2.3.2 Thyroid Signal Transduction

In normal thyroid follicular cells, the PKA, PKC signaling pathways and protein tyrosine kinase (PTK) predominate (Figure 2B1). Thyrotrophic stimulating hormone (TSH) stimulates the thyroid via a GPCR mediated increase in cAMP levels, and activates PKA for cell differentiation. The phorbol ester, 12-O tetradecanoylphorbol 13-acetate (TPA), activates protein kinase C, and inhibits PKA to stimulate cell proliferation. Epidermal growth factor-1 (EGF-1), acting on a receptor tyrosine kinase (RTK) activates the PKC signaling pathway to induce cell proliferation. The PKA and PKC pathways interact with a high degree of antagonism and coordination. PKA inhibits thyroid cell proliferation as induced by EGF, and PKC inhibits PKA induced differentiation. Upon TSH stimulation, cAMP simultaneously stimulates PKA and influences the selection of the Ras effector (*i.e.* PI3K vs. c-Raf-1), by which PI3K is selected over c-Raf-1 as the effector. Thus, in normal thyroid, MAPK appears to be minimally (or not) involved in cell signaling. Signal transduction in the control of human thyroid carcinoma, presents a different picture (Figure 2B2). Human
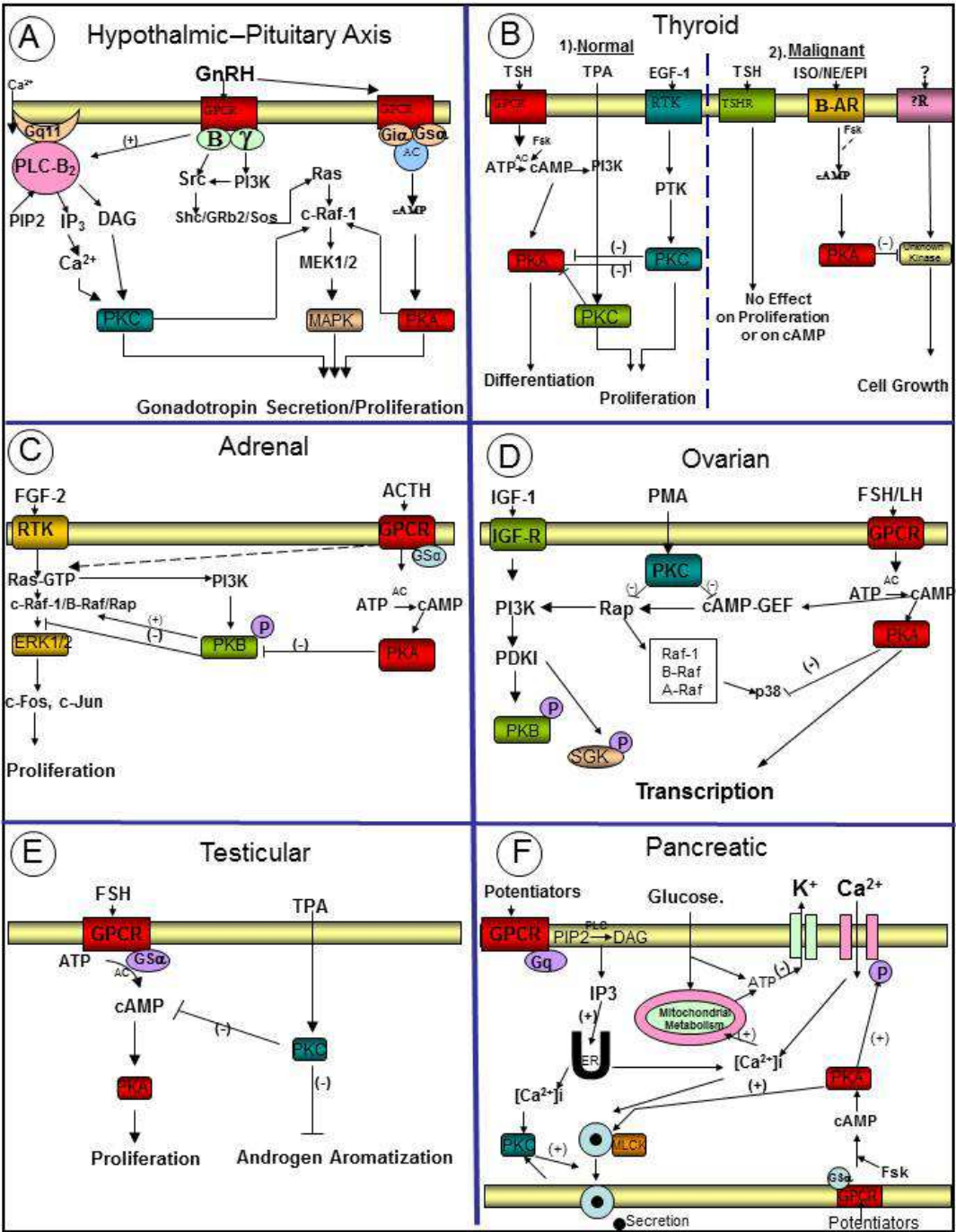
Fig. 2. Cell Signaling in Endocrine Organs. The PKA signaling pathway interacts in a complex manner with other signaling pathways in endocrine tissue (A-F), and acts as a central hub in endocrine signal transduction. An explanation of these interactions is given in the text. (A). Hypothalamic/Pituitary Axis Signal Transduction. (B) Thyroid Signal Transduction; Normal and Mutant tissue. (C) Adrenal Signal Transduction. (D) Ovarian Signal Transduction. (E) Testicular Signal Transduction (F) Pancreas Signal Transduction

thyroid papillary carcinoma cells were treated with TSH, forskolin (Fsk), 8-Br-cAMP (cAMP analogue and PKA stimulant) and the B-adrenergic receptor agonists, norepinephrine (NE), epinephrine (EPI) and isoproterenol (ISO). There was no effect on proliferation by TSH, or

on the accumulation of cAMP. Fsk decreased cell growth, but had no effect on cAMP accumulation. The B-adrenergic agonists induced cAMP accumulation, and reduced cell growth via an unknown kinase. Other studies with the specific B-adrenergic receptor (B-AR) agonist, CGP-12177, showed that thyroid carcinoma cells have B-ARs that have high sensitivity to the growth inhibitory effects of cAMP (Robinson-White & Stratakis, 2002). The mechanism of this inhibition by PKA is yet to be determined.

2.3.3 Adrenocortical signal transduction

In adrenocortical signaling (Figure 2C), MAPK (ERK1/2), plays a large part in signal transduction and interacts with the PKA and PKB pathways, as seen in mouse Y1 (G0/G1 cell cycle–arrested) adrenocortical tumor cells, in normal human cortical tissue and in primary pigmented nodular adrenocortical (PPNAD) tumors. A full discussion on PPNAD will occur in later sections. In mouse Y1 cells, PKA is stimulated by GPCR activation through adrenocorticotropin (ACTH) and adenyl cyclase. Fibroblast growth factor-2 (FGF-2) acting through a receptor tyrosine kinase (RTK) activates ERK1/2 of MAPK for cell proliferation, as well as activates PI3K. PI3K causes the activation of PKB. ACTH also has a small and transitory effect on ERK1/2 early in G1, but has a large negative mitogenic effect on PKB. PKA inhibits PKB activity and its effect on ERK1/2. A similar effect on ERK1/2, to that in mouse, has been seen in normal and mutant human adrenocortical tissue (PPNAD, see Figure 3B). Therefore, PKA and PKB interact in adrenocortical tissue to either inhibit or enhance cell proliferation via ERK1/2, depending on the tissue tumor status (Robinson-White et al., 2006a; Robinson-White & Stratakis, 2002).

2.3.4 Ovarian signal transduction

Rat ovarian follicle growth and the differentiation of ovarian granulosa cells depend on sequential stimulation by FSH and LH. FSH regulates granulosa cell proliferation in small follicles. As follicles mature, FSH induces the expression of genes that encode P450 aromatase and the LH receptor. During granulosa cell development (Figure 2D), FSH and LH bind to GPCRs and stimulate the production of cAMP. CAMP then acts as a molecular switch to control several signaling pathways: In addition to the stimulation of PKA activity for the induction of transcription, it stimulates PI3K via cAMP-GEF/Ras/Rap. This action can be abolished by myristate acetate (PMA) stimulation of PKC. Insulin-like growth factor-1 (IGF-1) can also act in ovarian cells to mediate the activation of PKB, via PI3K and PDKI PKA interacts with MAPK in ovarian cells and tissues. PMA abolishes FSH, but not IGF-1 mediated PKB phosphorylation. Rap can induce the three Raf isoforms to activate the MAPK, p38, which is then inhibited by FSH/LH induced PKA activity (Robinson-White & Stratakis, 2002).

2.3.5 Testicular signal transduction

In the testis (Figure 2E), FSH stimulates PKA via the production of cAMP, to induce cell proliferation. In rat Sertoli cells, most cAMP-dependent activity is mediated by the RIIβ PKA subunit, although other subunits (RIα, RIIα, RIIβ, Cα) have been found. It has been shown that TPA induces PKC to inhibit both FSH-stimulated cAMP formation and androgen aromatization. TPA can transiently stimulate RIα and RIIβ mRNA with no effects

on other PKA subunits. Rat testicular cells treated with both 8-CPTcAMP and TPA showed additive effects on stimulation of RIα mRNA and on inhibition of RIIβ mRNA levels. However, treatment with cycloheximide (a protein synthesis inhibitor) completely blocked the effect of TPA on RIIβ mRNA, but not on RIα. Therefore the inhibitory effect of PKC on RIIβ was dependent on ongoing protein synthesis. Although the precise site(s) of PKC-PKA interaction has not been determined, these data indicate that multiple and distant mechanisms are involved in the stimulation and inhibition of RIα, RIIβPKA subunit activity, respectively (Robinson-White & Stratakis, 2002).

2.3.6 Pancreatic signal transduction

Insulin secretion from the pancreas (Figure 2F) is potentiated by hormones and neurotransmitters that activate PKA and PKC through cAMP and phospholipase C (PLC), respectively, as well as via myosin light chain kinase (MLCK). High glucose concentrations generate an elevated intracellular ATP/ADP ratio that leads to closure of K^+ channels, and the subsequent opening of Ca^{2+} channels via phosphorylation by PKA, which leads to an increased intracellular Ca^{2+} load. Other potentiators of insulin secretion cause IP_3 to release Ca^{2+} from intracellular stores for the activation of PKC. Increased PKC activity induces MLCK and insulin granules to travel to the cells periphery. At the periphery, PKA plays a role in insulin secretion. Therefore release of insulin from secretory granules is orchestrated by PKA, PKC, and MLCK (Robinson-White & Stratakis, 2002).

3. PKA and apoptosis

3.1 Programmed cell death

Due to advancements in our knowledge of cell signaling pathways, programmed cell death (PCD), has recently been expanded from the inclusion of only a single cell death module (apoptosis) to include both necrosis and autophagic cell death as additional modules. Each cell death type is characterized by distinct morphological features and is regulated by different signaling pathways. Of these, necrosis is the least studied and least understood module. Necrosis was originally defined as accidental cell death that does not require gene activity. It is now recognized as a genetically controlled event (Taylor et al., 2008). It involves cell swelling, organelle dysfunction and cell lysis, caused by stress that is incompatible with cell survival. Necrotic cells trigger the immune system due to the release of danger-associated molecular patterns (DAMOs or alarmins) that stimulate recognition receptors in immune cells (Bialik et al., 2010; Degterev & Yuan, 2008). The immune system interprets the presence of necrotic cells as danger and a signal for activation of its response. In contrast to necrotic cell death, our understanding of autophagy cell death is more advanced, but not yet complete. It has been described as a pro-survival pathway that is essential for cell homeostasis and stress responses. It is mediated by ATG genes in which damaged or dysfunctional intracellular contents are engulfed by autophagosomes and degraded by lysosomal enzymes. For the purposes of this review, however, we will concentrate on the third and best studied module of PCD, apoptosis. Apoptosis is characterized by chromatin condensation and fragmentation, membrane blebbing and disintegration of the cell into smaller apoptotic bodies. It is a multistep process and involves two separate pathways, the intrinsic mitochondrial-induced pathway and the extrinsic

death receptor dependent pathway. The intrinsic pathway is activated by a variety of stimuli within the cell (*e.g.* glucocorticords and staurosporin) that provoke cell stress or damage. The B cell CLL/lymphoma-2 (BCL-2) protein family is involved in this pathway. It consists of three types of proteins, anti-apoptotic proteins (*e.g.* BCL-2, BCL-XL, and MC-1), pro-apoptotic proteins (*e.g.* BAK, BAD and BAX) and BH3–only proteins (*e.g.* BID, BIM and PUMA). The pro-apoptotic members act on the mitochondria to cause the release of intramembrane space proteins, such as cytochrome C, for the formation of an apoptosome complex (*i.e.* 7 molecules of APAF-1 and 7 caspase-9 homodimers). Active caspase-9 then causes a proteolytic cascade of further effector caspase activation events which carry out cell "demolition" events, *i.e.* cell blebbing, nuclear condensation and cell fragmentation (Bialik et al., 2010). The extrinsic pathway is activated by a family of transmembrane death receptors of the tumor necrosis factor family (*e.g.* Fas/CD95, Fas/APO-1 and 2) (Walazak & Krammer, 2000). The Fas ligand binds to the receptor and Fas-associated death domain (FADD; an adaptor molecule) is recruited, followed by the recruitment of Caspase -8 and the formation of a death inducing complex (DISC). Caspase -8 then autoactivates and initiates apoptosis by the cleavage of downstream pro-caspase effector molecules (caspase-3, caspase-6 and caspase-7) as mentioned above (Bialik et al., 2010; Zimmerman et al., 2001).

3.2 Effect of PKA and its interaction with other signaling pathways on apoptosis in endocrine tissue

In endocrine tissue, PKA plays a role in regulating PCD through its effects on apoptosis. Although other modules of PCD exist in endocrine tissue, because of the high complexity of cell signaling networks and variation in pathways present in different endocrine tissue, we present examples of the interaction of PKA with other signaling pathways and the action of PKA alone only on apoptosis. PKA appears to act on apoptosis at both its upstage induction stage and downstage effector (demolition) stage. However, due to a lack of experimentation, the data does not reveal the precise molecular points of interaction of PKA within the apoptotic pathways of endocrine tissue.

3.2.1 Apoptosis in adrenal tissue

In the normal human adrenal cortex, evidence suggests that endogenous ligands (neuropeptides, NPB and NPW) of two G-protein coupling receptors, GPR7 and GPR8, stimulate glucocorticoid secretion by activating PKA and PKC signaling pathways. To study human adrenal functional regulation, human adrenocortical carcinoma-derived NCI-H29 (H295R) cells, the main model cell system for human adrenocortical tumors (Groussin et al., 2000), were employed. In H295R cells, mRNA for GPR7 and GPR8 was expressed. However, NPB and NPW did not affect secretion. Yet, both peptides enhanced cell growth by increasing proliferation and inhibiting apoptosis via stimulation of MAPK and not through PKA or PKC. The authors conclude that although GPR7 and GPR8 are expressed in H295R cells, they are a variation of those found in normal adrenal cells and may be uncoupled to PKA and PKC (Andreis et al., 2005). ACTH, the major regulator of adrenal cortex, function acts mainly through PKA (Kirschner, 2002). However, because of increasing evidence that the pro-apoptotic tumor necrosis factor-a (TNF-α) is involved in the regulation of adrenal control and function (Gonzalez-Hernandez et al. ,1996), the interaction of PKA with TNF-α on adrenocortical cell proliferation and apoptosis was

studied. Activation of PKA by dibutyl cAMP [(Bu)$_2$ cAMP] inhibited proliferation and increased TNF-α-induced apoptosis. (Bu)$_2$ cAMP also increased the expression of the transcription factor c-Myc. Thus, PKA may augment TNF-α-induced apoptosis in H295R cells through increased c-Myc expression (Liu et al., 2004). In several other studies, PKA was shown to affect apoptosis in adrenal tissue. In mutant tissue, a dysregulation of the balance of the regulatory PKA subunits, RIα and RIIβ may be involved in adrenal tumorigenesis (Robinson-White et al, 2003). The impact of this imbalance on cell proliferation and on apoptosis was investigated using the specific cAMP site selective analogues, 8-Chloro cyclic-adenosine -monophosphate (8-Cl-cAMP) and 8-(4-chlorophenylthio) cAMP (8-CPT-cAMP). 8-Cl-cAMP activates both RIα and RIIβ, while 8-CPT-cAMP activates only RIIβ. Studies showed that by targeting the PKA RIIβ regulatory subunit, the balance between RIα and RIIβ is disturbed. This disturbance influences cyclin B accumulation, inactivation of CDC2 kinase, cell cycle progression at G$_2$ phase cell cycle arrest, and induces apoptosis (Bouizar et al, 2010). In another study, the effect of inactivation of the RIα PKA subunit on the interaction of the PKA and TGFB (stimulates SMAD proteins through type I and type II serine/threonine kinase receptors) pathways, and on apoptosis was investigated. *PRKAR1A* (specifics of which will, be given in later sections), the gene encoding the RIα subunit of PKA was silenced by siRNA. Silencing *PRKAR1A* stimulated PKA activity and increased transcriptional activity of a PKA reporter construct and expression of the endogenous PKA target, NR22. *PRKAR1A* inactivation also decreased SMAD3 mRNA and protein levels, thus altering the cells response to TGFB. Since TGFB normally stimulates apoptosis in H295R cells, this effect was counteracted by the inactivation of *PRKAR1A*. Thus, PKA and TGFB signaling pathways interact in endocrine tumorigenesis (Ragazzon et al, 2009). In another species, in mouse Y1 cells, which possess an over expressed *c-Ki-Ras* proto-oncogene and exhibits high constitutive levels of Ras-GTP, FGF2 can up-regulate Ras-GTP, activate PI3K and promote PKB phosphorylation and deactivation. This induces a mitogenic, anti-apoptotic effect in Y1 cells. ACTH activates PKA to cause a rapid dephosphorylation of PKB and a downstream de-regulation of the c-Myc protein. This data presents one mechanism of the blocking of G1-S transition in Y1 cells and induction of apoptosis (Robinson-White & Stratakis, 2002).

3.2.2 Apoptosis in thyroid tissue

Thyroid cells are highly resistant to apoptosis, which is actively suppressed by cell signaling pathways. CAMP is known to rescue cells from apoptosis as stimulated by diverse stimuli, yet the survival pathways activated by cAMP are poorly understood. The role of cAMP in thyroid cell survival was investigated in Wistar rat thyroid (WRT) follicular cells. In WRT, cAMP activates multiple signaling pathways (*e.g.* PKA, PI3K, p70S6k and Rap1), and multiple pathways modulate thyroid cell survival. Inhibition of cAMP -stimulated p70S6k, but not PI3K, abolished survival. Treatment with PKA inhibitors (H89 and RI209K) stimulated apoptosis and enhanced cell death after treatment with sodium nitroprusside (SNP). Cells expressing an activated Rap1A mutant also showed enhanced sensitivity to SNP-induced apoptosis. However, cells expressing dominant negative Rap1A were resistant to apoptosis. Although the exact mechanism has not been ascertained, these results show that PKA and Rap1 and PI3K/mTor/p70S6k pathways are involved in anti-apoptotic effects in WRT cells (Saavedra et al, 2002).

3.2.3 Apoptosis in ovarian tissue

Human ovarian surface epithelial (OSE) and Caov-3 (derived from an ovarian adenoma) cells were used to determine the involvement of PKA in the protection of ovarian cancer cells from apoptosis. Fas binds to the CD95 receptor, to stimulate the extrinsic apoptotic pathway in the ovary to subsequently activate caspase-3. Co-treatment of cells with luteinizing hormone (LH) (stimulates PKA activity) and Fas, reduced the number of apoptotic cells in a transient manner. However, LH alone had no effect on apoptosis or on proliferation. The cell permeable cAMP analogue 8-CPT-cAMP mimicked the effect of LH. This finding suggests that the PKA signaling pathway is involved in protecting OSE cells from Fas-induced apoptosis (Slot et al., 2006). Thrombopoietin (TPO), a hormone/growth factor/cytokine plays an essential role in stimulating homatopoietic stem cell function, differentiation, megakariocytosis and platelet production. It also plays a role in controlling ovarian function through various signaling pathways (JAK/STAT, MAPK and PKA). Studies with cultured porcine ovarian follicles determined that TPO regulates proliferation, apoptosis and secretion in ovarian cells and identified intracellular mediators (possibly PKA). Results showed a TPO-induced increase in PCNA (associated with proliferation), BAX (pro-apoptosis), Tyrosine kinase, cdc2/p34, PKA activity and CREB-1. The PKA blocker, KT5720 given alone, reduced BAX and TGF-2B expression and augmented PKA, CREB and oxytocin expression. TPO and K5720 together prevented/reversed the action of TPO on PKA and CREB, but not on BAX. The data suggest that PKA has a role in mediating the effect of TPO on proliferation and apoptosis in the ovary. However, the exact mechanism involved is yet to be determined (Sirotkin et al., 2004). It is well established that leptin, a product of adipose tissue, has direct effects on the control of ovarian steroidogenesis, prostaglandin secretion and IGFBP-3 and IGG-1 release. Leptin also controls the effects of LH-RH on reproduction. The role of leptin on proliferation, apoptosis and PKA was uncertain. Studies in human ovarian granulosa cells showed that leptin stimulates ovarian cell cycle proteins (PCNA and cyclin B), Bax and PKA activity. This data represents the first evidence of leptins' involvement in the control of the ovarian cell cycle, and that PKA is a potential target of leptin and mediator of ovarian cell function (*i.e.* proliferation and apoptosis) (Sirotkin et al, 2008).

3.2.4 Apoptosis in testicular tissue

The putative Leydig cell receptor, peripheral-type benzodiazepine (PBR), modulates steriodogenesis in the testis. Since benzodiazepines are extensively used in regional anesthetics, a determination was made of their peripheral effects on Leydig cell steriodogenesis and underlying transduction pathways. The effect of the benzodiazepine, Midazolam (MDZ), on primary mouse Leydig cells and MA-10 Leydig tumor cells was assessed. MDZ stimulated steriodogenesis in both cell types and induced the expression of PBR and StAR (steriodogenesis acute regulatory protein) proteins. Inhibition of PKA (by H89) and PKC (by GF109203X) decreased MDZ-induced steroid production, indicating that MDZ stimulates steroidogenesis in mouse Leydig cells and the expression of PBR and StAR proteins via PKA and PKC. Also, at high MDZ concentrations, cell round-up, membrane blebbing and later, cell death occurred in MA-10 cells. The authors conclude that MDZ can induce steriodogenesis in both cell types, and at high doses can induce apoptosis in Leydig tumor cells. Thus PKA and PKC may play a role in the induction of apoptosis in Leydig

tumor cells (So et al., 2010). A growing interest exits in the regulation of mammalian fertility, by the endogenous ligand of the cannabinoid CB1 and CB2 receptors, endoicannobinoid anandamide N-arachidonoylethanolamine (AEA). Evidence has shown that endogenous cannabinoids can interfere with critical functions of mammalian and nonmammalian reproduction (*e.g.* regulation of embryo development, oviduct transport and implantation) in females, through apoptosis. However, since the impact of AEA on fertility in the male remained to be elucidated, a study using mouse primary Sertoli cells was performed to determine the effect of AEA on proliferation/apoptosis. It was shown that FSH stimulates PKA to enhance the activity of the AEA hydrolase, fatty acid amide hydrolase (FAAH), resulting in the hydrolysis of AEA. Thus, Sertoli cells are protected from the pro-apoptotic action of AEA. FSH also triggers PI3K signaling, which is required for the stimulation of cytochrome P_{450}-aromatase expression (ARO). ARO can irreversibly transform androgens into estrogens and plays a large role in male reproduction (*e.g.* spermatogenesis). FSH appears to be responsible for total number of sperm produced and not the quality of the sperm. Stimulation of FAAH activity was abrogated by inhibition of PKA by the PKA inhibitor, myristoylated amide 14-22 and the cytochrome P450 inhibitor 4-androsten-4-ol-3, 17-dione, and by the interaction of the PKC pathway with FSH-induced cAMP activity (see Figure 2E). The effects of FSH were reproduced with the PKA stimulant, $(Bu)_2$ cAMP and by estrogen. The data showed that PKA and estrogen mediate the protective effect of FSH on Sertoli cells, and may prevent apoptosis. The action of FSH via PKA and estrogen may be a means for modifying male infertility and Sertoli cell number, respectively, and points to AEA as a critical regulator of male fertility (Rossi et al., 2007).

3.2.5 Apoptosis in pancreatic tissue

Postprandial insulin secretion is regulated by hormonal factors (*i.e.* incretin hormones) released from the gut in response to nutrient ingestion. One hormone, glucose-dependent insulinotropic polypeptide (GIP) causes the release of insulin from pancreatic Beta cells in the presence of glucose via its G-protein coupled receptor. The activated GIP receptor induces stimulation of membrane bound adenyl cyclase which results in insulin secretion, influx of Ca^{2+} and cAMP production (Volz et al, 1995). Other studies showed that GIP and glucose act synergistically as anti-apoptotic factors in B-cells. Using a well differentiated B-cell line, INS-1, it was shown that mitogenic and anti-apoptotic signaling in INS-1 cells occurs in response to the synergism of GIP and glucose via multiple interacting signaling pathways (PKA, PI3K, MAPK, and PKB). The release of intracellular Ca^{2+} appeared to be at the hub of signaling. Using a panel of various pathway inhibitors, many interacting signals were seen: 1) stimulation of PKA/CREB, MAPK, and PI3K/PKB; 2) inhibition of PKA by MEK1/2 of MAPK; 3) activation of ERK1/2 of MAPK by PI3K and PKA and; 4) activation of PKB by MAPK and PKA. This study showed that GIP functions as a mitogenic and anti-apoptotic factor for B-cells by activating a tightly woven network of excitatory and inhibitory "cross-talk" of signaling pathways (Trumper et al., 2002).

4. Bilateral adrenal hyperplasia: Involvement of PKA

Bilateral adrenal hyperplasia (BAH), an adrenocorticotropin-independent form of adrenocortical disease that leads to Cushing Syndrome has been divided into two groups of disorders, micronodular and macronodular hyperplasia, based on the size of the associated

nodules. The size criteria have biological significance since a continuum in the same patient is rarely found. Most patients have either micronodular or macronodular hyperplasia, and rarely both. The PKA signaling pathway and its interaction with other signaling pathways is involved in the pathogenesis of both types. Both types are, therefore, presented, however, with an emphasis on the PKA associated micronodular syndromes.

4.1 Bilateral micronodular hyperplasia

Micronodular hyperplasias consists of diseases of the adrenal cortex with multiple nodules of less than 1 cm in diameter and can be divided into 3 groups, i.e. primary pigmented nodular adrenocortical disease (c-PPNAD), usually found in the context of Carney Complex (CNC), isolated primary pigmented nodular adrenocortical disease (i-PPNAD, and isolated micronodular adrenocortical disease (i-MAD) (Stratakis, 2007; Lodish & Stratakis, 2010a).

4.1.1 Carney complex

Endocrine tumors can present as sporadic events, or as part of an inherited tumor syndrome. One such syndrome, CNC, will be highlighted in this review. CNC is a unique and relatively rare autosomal dominant multiple endocrine neoplasia syndrome first described by Dr. J. Aidan Carney and co-workers at the Mayo Clinic, is characterized by spotty skin tumors and pigmented lesions (lentigenosis), myxomas, endocrine over activity and schwannomas (Carney et al, 1986). Endocrine tumors associated with CNC include primary pigmented adrenocortical disease (PPNAD), growth hormone secreting pituitary tumors, large cell–calcifying Sertoli cell tumors, Leydig cell tumors and thyroid follicular adenomas. The clinical manifestations and molecular genetics of CNC have been well described elsewhere (Rothenbuhler & Stratakis, 2010). Genetic linkage analysis has identified two independent loci for CNC, CNC1 located on chromosome 17p22-24 and CNC2, located on chromosome 2p16 (Stratakis et al., 1996). Most cases (> 60%) of CNC are caused by inactivating mutations in the gene encoding the PKA regulatory subunit, RIα (PRKAR1A), located on chromosome 17p22-24 (Kirschner et al., 2000b) The gene responsible for CNC at locus 2p16 is as yet unknown. However, this locus is thought to contain genes responsible for at least some CNC patients, or the progression of the complex (Stratakis et al., 1996).

4.1.2 PPNAD

PPNAD is a rare bilateral adrenal disease and is the most frequent endocrine manifestation in CNC patients. From 80 to 90 % of PPNAD cases are associated with CNC. However, it may also occur independently of CNC. PPNAD received its name (Primary Pigmented Adrenocortical Disease) from the macroscopic appearance of the adrenal glands, characterized by small cortisol-producing, pigmented micronodules (black or brown, ~6mm-1 cm in diameter) set in a usually atropic adrenal cortex. The micronodules secrete cortisol independently of pituitary signaling. Adrenal atrophy is pathognomonic and reflects the autonomic function of the nodules and suppressed levels of ACTH. The combination of atrophy and nodularity gives the gland an abnormal appearance (though commonly normal in size) that is highly diagnostic. PPNAD occurs mostly in children and young adults, and peaks in the second decade. It is rare under the age of 4 years and after the age of 40. In rare cases, one or both glands may be large and contain adenomas with a

calcified center. Macronodules, larger than 10 mm may also occur in older patients (Rothenbuhler et al., 2010; Stratakis, 2007). PPNAD patients present with adrenocorticotropic hormone (ACTH)-independent Cushing Syndrome (CS), which may be subclinical or cyclic, but often present with a variant CS called "Atypical" Cushing Syndrome (ACS) (Sarlis et al., 1997). Up to 90 % of CNC cases present with CS, due to PPNAD. Hypercortisolism develops progressively over years in PPNAD, or there may be a rapid outburst of excess cortisol that spontaneously regresses (Groussin et al., 2005). The 6-day Liddle test is used to establish a diagnosis in PPNAD, where patients show a "paradoxical" increase in 24-hour urinary free cortisol (UFC) and/or 17-hydroxysteroids. The increase is progressive and peaks on day 2 of high–dose dexamethasone administration in patients with CS. Patients with ACS tend to have normal or near normal 24-hour UFC production with an occasional interruption of days or weeks of hypercortisolism (Lodish & Stratakis, 2010a; Rothenbuhler & Stratakis, 2010; Stratakis, 2007). Most Patients with PPNAD have germline mutations in PRKAR1A and in some cases mutations in genes encoding the cAMP-binding phosphodiesterases (PDEs), PDE11A and PDE8B. It should be noted that PRKAR1A and the PDE genes PDE11A and PDE8B control the action and levels of cAMP in the cell with a final impact on cell proliferation (Horvath et al., 2006, 2008a).

4.1.3 Isolated PPNAD (i-PPNAD)

PPNAD, typically observed in CNC, can also present as a sporadic and isolated disease (i-PPNAD). It occurs in children and young and middle aged adults. It is characterized by micro-adrenomatous hyperplasia with mostly internodular atrophy and nodular pigment (lipofusin). It is autosomal dominant but is not associated with CNC nor does it present any other CNC manifestations. It also has no obvious family history, which would be suggestive of CNC. In a minority of patients, however, other familial cases of PPNAD are known, suggesting a genetic cause (Bourdeau et al., 2003). It is caused by inactivating mutations in PRKAR1A of chromosome 17p22-24, and possibly mutations of gene(s) found in the 2p12-16 locus. Inactivating mutations of phosphodiesterase 11A4 (PDE11A) are also involved. In most cases (~ 65%), these are de novo mutations, which explains the lack of family history (Rothenbuhler et al., 2010; Stratakis, 2007).

4.1.4 Isolated Micronodular Adrenal Disease (i-MAD)

I-MAD occurs mostly in children and young adults. It occurs with hyperplasia of the surrounding zona fasciculate with absent or limited nodular pigment. It is autosomal dominant and isolated and does not occur in the context of CNC. I-MAD is caused by mutations in PDE11A and PDE8B and possibly genes of the 2p12-p16 locus (Stratakis, 2007). It was initially thought that i-MAD patients were CNC patients, but it is now clear that it is not the same as PPNAD (Gunther et al., 2004).

4.2 Bilateral macronodular hyperplasia

Bilateral macronodular hyperplasia, another form of BAH, also causes Cushing Syndrome, but has no other clinical findings. Macronodular hyperplasia is characterized by multiple adrenocortical nodules of greater than 1Cm each, and can be divided into three groups. The

first two groups include bilateral macro-adenomatous hyperplasia (BMAH) and BMAH of childhood (c-BMAH). BMAH occurs in middle age and c-BMAH occurs in infants, and very young children. In both groups, adenomas (~2-3) with intranodular atrophy and an occasional microadenoma can be seen in histological preparations. BMAH is caused by mutations in menin (the ubiquitous nuclear protein coded for by the MEN1 gene), APC, (Adematous Polyposis Coli tumor suppressor gene), GNAS (codes for GSα Gs protein subunit), and FH (codes for the tricarboxylic acid cycle enzyme fumarate hydralase). C-BMAD is caused by mutation in GNAS (Stratakis, 2007). The third group, ACTH-independent macronodular adrenocortical hyperplasia (AIMAH) is also known as massive macronodular disease (MMAD; AIMAH/MMAD) occurs always in middle age. It is a cause of CS due to large bilateral cortisol secreting macronodules. The nodules cause a large increase in the size (up to 100 times the normal size) and weight of the adrenal glands (Stratakis et al., 2001). In this group, adenomatous hyperplasia with internodule hyperplasia of the zona fasciculate has been observed. MMAD is autosomal dominant and isolated. It is associated with ectopic GPCRs, WISP-2 and Wnt–signaling, and genes of the chromosome 17q22-24 locus (Stratakis et al., 2007).

5. Pituitary tumors in CNC

Pituitary adenoma is a benign neoplasia with excess proliferation of each subtype of pituitary cell. The tumors can give rise to severe illness due to hormonal excess or to visual disturbance due to mass effect. This tumor can present as an isolated event or as part of an inherited syndrome (Tichomirowa et al., 2009; Zhang & Nose, 2011). Currently, at least 5% of all patients with pituitary adenoma have been found to have a family history, mainly due to multiple endocrine neoplasia type 1 (MEM1) and CNC (Tichomirowa et al., 2009). These patients have growth Hormone (GH) as well as prolactin (PRL) secreting adenomas, however, PRL-secreting adenomas predominate in MEM1. In CNC, GH-producing tumors are seen in ~10% of patients. Acromegaly with elevated Insulin-like growth factor (IGF1) and GH levels, and subtle hyperprolactinemia, can be seen in up to 75 % of patients. However, clinical acromegaly is rare (Rothenbuhler et al., 2010; Zhang & Nose, 2011). Lesions in the pituitary gland range from pituitary cell hyperplasia to multiple microadenomas to invasive macroadenomas. CNC-related acromegaly is distinguished microscopically by multifocal hyperplasia of somatomammotropic cells (Zhang & Nose 2011, Pack et al., 2000; Stergiopoulos et al., 2004). Studies with a tissue-specific knockout (KO) mouse, pointed to the Prkar1a gene as the causative agent. These studies showed that complete loss of Prkar1a allows the formation of pituitary tumors and abnormalities of the GH-axis, with close analogy to tumors in human patients with CNC (Zhirong et al., 2007).

6. PKA mutations

6.1 PRKAR1A

The gene that codes for the RIα subunit of PKA is a key component of the cAMP/PKA signaling pathway, located in the q22-24 region of chromosome 17. The finding of tumor specific loss of heterozygosity (LOH) within the q22-24 locus (Krischner et. al, 2000a) and the complete or partial loss of RIα in CNC tumors suggested that PRKAR1A is a tumor suppressor gene (Kirschner et al., 2000a). PRKAR1A is mutated in almost half of CNC

patients. Mutant PRKAR1A codes for the only PKA subunit that has been shown to lead to human disease. Its genomic region is approximately 21 kb-long, and the open reading frame contains 11 exons that code for a protein that has 384 amino acids. Over one hundred disease causing pathogenic sequence variants have been identified (Rothenbuhler & Stratakis, 2010). These have been extensively recorded elsewhere (Horvath et al., 2010). Most PRKAR1A mutations are small deletions and insertions, rearrangements, or base substitutions (Rothenbuhler & Stratakis, 2010). Large deletions can also occur, however, rarely (Horvath et al., 2008b). However, the most frequently found PRKAR1A mutation is a 2 bp deletion at position 578 in exon 4B of PRKAR1A (c-578delTG). Over 70 % of CNC patients have a classical phenotype with a PRKAR1A mutation that leads to a premature stop codon and subsequently non-sense mediated mRNA decay (NMD), and thus PRKAR1A haploinsufficiency (Bertherat et al., 2009; Kirschner et al., 2000a; Stratakis et al., 2001). Less frequently, mutations do escape NMD, causing the expression of abnormal RIα proteins (Groussin et al., 2006; Meoli et al., 2008). PRKAR1A haploinsufficiency leads to excess cAMP/PKA signaling in affected tissues, and increased cell proliferation and survival (Robinson-White et al., 2006b), through an increase in total cAMP-stimulated PKA activity. Two mechanisms have been proposed to explain the increase in signaling: 1) RIα-haploinsufficiency leads to a higher intracellular C to R subunit ratio, and therefore, increased availability of the free catalytic subunits that phosphorylate downstream targets. 2). RIα- haploinsufficiency leads to an upregulation of other PKA subunits including PRKAR1B and PRKAR2A or PRKAR2B, depending on the tissue type. It is also possible that the other regulatory subunits do not act as effectively as RIα in controlling cAMP signaling (Rothenbuhler, 2010).

6.2 M1V PRKAR1A

Another mutation has recently been found in the PRKAR1A gene, M1V PRKAR1A, caused by a mutation in the initiation codon of PRKAR1A. This mutation results in a phenotype characterized by PPNAD alone, and sometimes mild ACS. There is significant variation in age of onset and clinical severity, but no other classical manifestations of CNC. Mutant mRNA is expressed equally with wild type levels. However, the mutant protein is not expressed in cells. In a cell free system, the RIα regulatory subunit was shorter than the wild type protein. This observation has highly significant implications for RIα's role in adrenal function and tumorigenesis (Pereira et al., 2010).

7. Mechanism of action of mutant PRKAR1A in endocrine tumorigenesis

The Mechanism of action of PRKAR1A inactivation was investigated using a model cell system, endocrine tissue and mouse models, to determine the manner in which mutant PRKAR1A affects cell proliferation, cell survival and apoptosis, leading to tumorigenesis.

7.1 A Cellular Model System

In varied cell types, including B and T-lymphocytes (Robinson-White et al., 2003), RIα type PKA interacts with the ERK1/2 cascade of MAPK at c-Raf-1, causing a cell-type specific inhibition of MAPK and of cell proliferation. Since RIα is present in B-lymphocytes of CNC patients (Kirschner et al., 2000b), and these cells carry the c.578delTG inactivating PRKAR1A

mutation, B-lymphocytes from CNC patients were used as a model cell system (Figure 3A) to determine if a MAPK/PKA interaction could be responsible for CNC tumorigenesis. In PRKAR1A-mutant cells (Mutant), PKA activity, both at baseline and after stimulation with cAMP was increased. Quantitative mRNA analysis indicated that type I PKA subunits (RIα and RIβ) were the main subunits expressed in both normal and mutant cells. However, RIα was decreased in mutant cells. The cell and pathway specific stimulant, lysophosphatidic acid (LPA), stimulated ERK1/2 levels in both cell types with greater stimulation in mutant cells. Forskolin (Fsk; activates adenyl cyclase for the production of cAMP) and Isoproternol (ISO) stimulated PKA activity, inhibited LPA-induced ERK1/2 activity and cell proliferation in normal cells, but stimulated these parameters in mutant cells. These data were replicated in a pituitary tumor cell line carrying the c.578delTG mutation and in COS-7 cells transfected with an in vitro construct bearing the RIα 184-236 mutation that leads to increased PKA-mediated physphorylation (Robinson-White et al., 2003). The differences in the effect of PKA stimulants on normal and mutant B-lymphocytes, was postulated to occur due to the sensitivity of the Raf isoforms to PKA. In cells having both B and c-Raf-1, PKA inhibits c-Raf-1 and stimulates B-Raf to inhibit and stimulate cell proliferation, respectively, through ERK1/2 (Erhardt et al., 1995). The action of PKA on B-Raf, in some cells, is through activation of the G-protein, Rap1 (Vossler et al., 1997). In normal cells, a balance exists between the stimulation and inhibition of ERK1/2 activity by PKA, with inhibition being more dominant. This dominance is due to the presence of a greater number (70-80%) of the type I PKA isoform than the type II isoform (20-25 %) in lymphocytes (Schmitd & Stork, 2000), and a higher affinity of cAMP for the RIα subunit (Amieux et al., 1997). In RIα-haploinsufficient (PRKAR1A-mutant) lymphocytes, as in CNC-affected cells, the balance is thought to shift towards activation of another PKA subunit (perhaps the RIβ subunit) (Robinson-White et al., 2003). Thus, the underlying mechanism was suggested to be through biochemical compensation or substitution of one subunit (e.g. RIα) for another (e.g. RIβ), as seen in other cell systems (Amieux et al., 1997), which may lead to cell proliferation instead of inhibition, as seen in normal cells (Robinson-White et al., 2003). The data suggested that PKA acts to inhibit ERK1/2 activity (through inhibition of c-Raf-1) and cell proliferation in normal cells, but stimulate ERK1/2 activity (through B-Raf) and cell proliferation in mutant cells. The authors hypothesized that the reversal of PKA-mediated inhibition of MAPK by mutant-PRKAR1A may contribute to CNC tumorigenesis (Figure 3A). These studies were extended to confirm the previous data and to determine the depth of the interaction of the PKA and MAPK signaling pathways in CNC cells. An analysis of the effect of PKA stimulation on components of the ERK1/2 cascade (B and C-Raf-1, and MEK1); on the ERK1/2 activating transcription factor c-Myc, on cell cycle progression and proliferation and on apoptosis was performed. Data in mutant cells was compared to that in normal cells from matched controls. Both Fsk and ISO inhibited cell proliferation in normal cells, but stimulated proliferation in mutant cells. Analysis of the phosphorylation patterns of B and c-Raf-1 in mutant cells upon stimulation by both Fsk and ISO showed that phosphorylation of B-Raf is increased, whereas phosphorylation of c-Raf-1 is inhibited. In normal cells, both B and c-Raf-1 phosphorylation is inhibited. This implied a switch from inhibition to stimulation of ERK1/2 in mutant cells. Likewise, in mutant cells, phosphorylation of MEK1/2 was also increased (Robinson-White et al., 2006b), possibly due to high levels of phosphorylated B-Raf in mutant cells. Fsk also increased LPA-induced stimulation of c-Myc in mutant cells, but c-Myc phosphorylation was inhibited in normal cells. The increased phosphorylation of B-Raf, MEK1/2, ERK1/2 and c-Myc in mutant cells suggested that PRKAR1A inactivation leads to c-Myc activation. C-Myc can respond to mitogenic signals
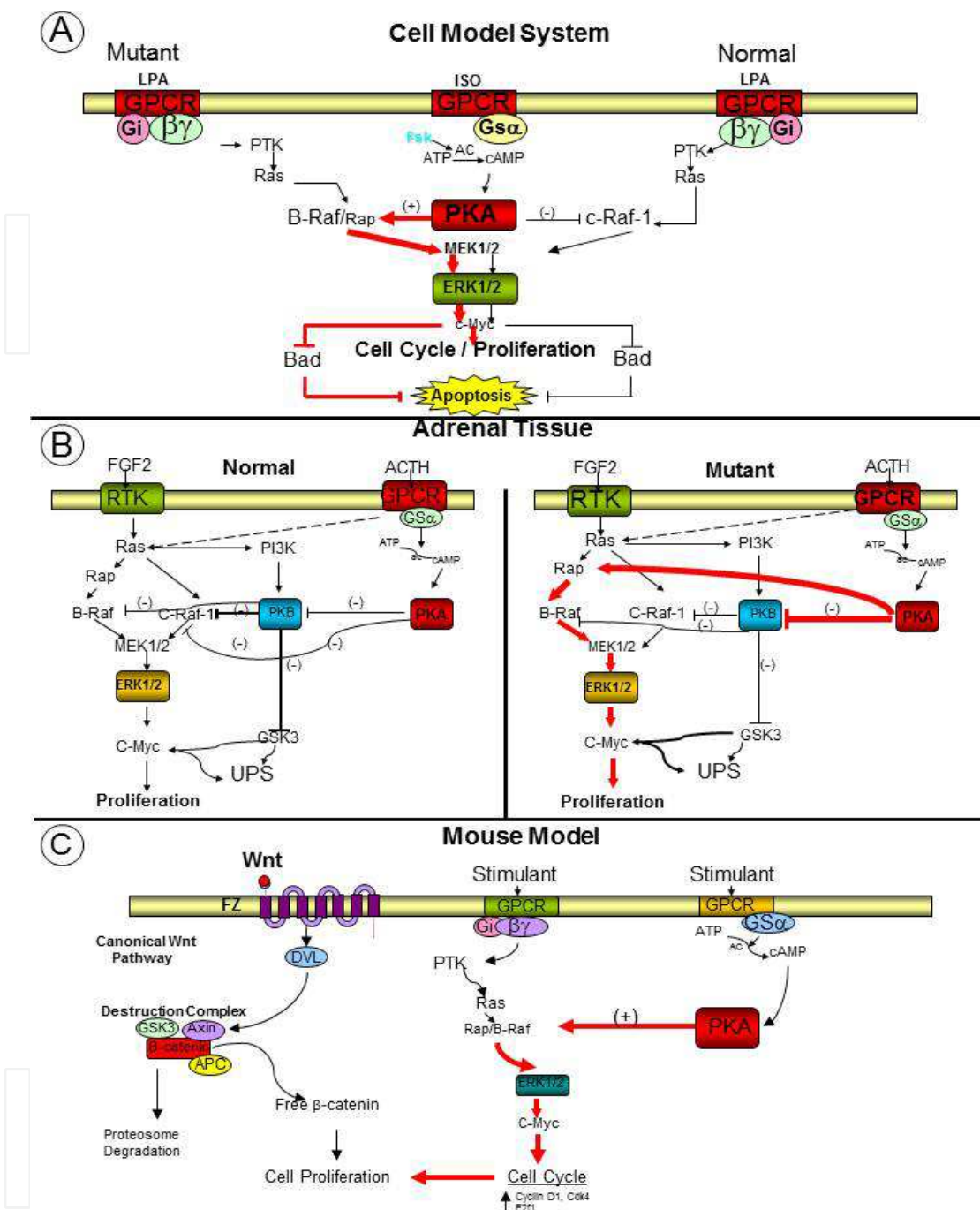
Fig. 3. Mechanism of action of Mutant-*PRKAR1A* in Endocrine Tumorigenesis. **(A). Signaling in normal and *PRKAR1A*-mutant B Lymphocytes.** Lysophosphatidic acid (LPA), and isoproterenol (ISO) stimulate G-protein coupled receptors (GPCRs) in B- lymphocytes. LPA activates cytosolic protein tyrosine kinase (PTK) via βγ G-proteins, to stimulate Ras, followed by the activation of B-Raf and c-Raf-1. ISO, via GSα and adenyl cyclase (AC) activates cAMP/PKA, or AC is activated directly by forskolin (Fsk). In normal lymphocytes, PKA inhibits the ERK1/2 signaling pathway at c-Raf-1, but stimulates Rap/B-raf in *PRKAR1A*- mutant cells, to inhibit and stimulate the cell cycle and proliferation, respectively. ERK1/2, in both cell types, phosphorylates and inhibits BAD of the intrinsic apoptotic pathway. ERK1/2 due to the enhanced ERK1/2 activity in mutant cells, inhibition

of apoptosis is greater in these cells. Line thickness indicates degree of action. **(B) Hypothetical Signaling Mechanism in PPNAD Adrenocortical Tissue.** 1) In both normal and *PRKAR1A*-mutant adrenocortical tissue, fibroblast growth factor2 (FGF2) activates receptor tyrosine kinase (RTK) to stimulate Ras, followed by the sequential activation of B-Raf and c-Raf-1, MEK1/2 and ERK1/2 of the MAPK signaling pathway. Activated ERK1/2 then activates c-Myc, leading to cell proliferation. ERK1/2 is also transiently activated by ACTH, at a point upstream of MEK1/2. 2) Ras stimulates PI3K, which phosphorylates/ activates the PKB pathway. PKB, to a small extent, then inhibits B-Raf and c-Raf-1 in mutant tissue, but inhibits these isoforms to a larger extent (mostly c-Raf-1) in normal tissue. 3) ACTH, via a GSα type G-protein and adenyl cyclase (AC), stimulates cAMP production and PKA activity. In normal tissue, PKA inhibits PKB, c-Raf-1 and B-Raf. A different effect occurs in mutant tissue. PKA greatly inhibits PKB, but has a large stimulatory effect on Rap, which overrides the inhibitory effect that PKB has on the ERK1/2 cascade. PKA, thus, greatly enhances the activity of the ERK1/2 cascade and cell proliferation in mutant tissue. 4) The phosphorylation/deactivation of GSK3 by PKB is reduced in mutant tissue due to a less active PKB, allowing more c-Myc to be targeted for degradation by the ubiquitin proteosome system (UPS). Line thickness indicates degree of activity. **(C) Signaling in a *Prkar1a* mouse model** 1) Wnt proteins bind to a 7-transmembrane Fizzled (FZ) receptor to activate the canonical Wnt pathway. Upon binding of Wnt, the disheveled scaffolding protein (DVL) is activated, causing the disassembly of the destruction complex (GSK3, Axin, B-catenin and APC). Free β-catenin travels to the nucleus to activate target genes for cell proliferation. 2) PKA stimulates the ERK1/2 cascade of MAP kinase. PKA, in *PRKAR1A*-haploinsufficient cells, stimulates ERK1/2 (at rap/B-raf) to increase c-Fos and c-Myc activity and to promote activation of cell cycle cyclin D1, cdk4 and E2f (Go/G1 of the cell cycle) and facilitates cell cycle progression. β-catenin and ERK1/2 may act synergistically to promote cell proliferation.

and move cells from Go to G1 and/or from G1 to S phases of the cell cycle. Cell cycle analysis by Flow Cytometry indicated an increased rate of cell cycle transition by synchronized mutant cells from Go/G1 to S phase, even in the absence of PKA stimulants. The data suggested that this fast transition rate leads to increased cell proliferation in mutant cells. Next, the possibility that apoptosis could account for the decrease in proliferation seen in normal cells was investigated. Staurosporin and CH11 were used as control stimulants for the intrinsic and extrinsic apoptotic pathways, respectively. Both normal and mutant cells were relatively resistant to staurosporin-induced apoptosis with significantly less apoptosis in mutant cells. On stimulation by ISO, apoptosis in mutant cells was again significantly less. The mediator of this decreased apoptosis was found to be the pro-apoptotic protein, BAD. Since ERK1/2 is known to phosphorylate/inactivate BAD, the data suggested that the higher levels of ERK1/2 in mutant cells induced by PRKAR1A deficiency resulted in a small but significant inhibition of the intrinsic apoptotic pathway, contributing to cell survival. The extrinsic apoptotic pathway was induced by the Fas/CD95 receptor agonist CH11. Like staurosporin and ISO, apoptosis was less pronounced in mutant cells. This data was confirmed by increased levels of cleaved caspase-3 in normal versus mutant cells, indicating the increased ability of normal cells to activate the effector caspases leading to apoptosis. In general, this data indicated that the large decrease in cell proliferation in normal cells when stimulated by Fsk and ISO was not due to apoptosis or necrosis, but suggested that PRKAR1A inactivation leads to significant changes in both

apoptotic pathways that control enhancement of mutant cell survival. This study suggested that the balance between cell proliferation and death that occurs in tissue homeostasis (Fulda & Debatin, 2004) may be disturbed in PRKAR1A-defficent cells, and presents a model (Figure 2A) by which partial inactivation of PRKAR1A may increase cell cycle progression, proliferation and survival (Robinson-White et al., 2006b).

7.2 Adrenal tissue

An investigation was made to determine how PKA and its subunits act in the presence of PRKAR1A mutations in adrenocortical tissue. As a result of this investigation, a hypothetical cell proliferation pathway was designed (Figure 3B). This pathway was devised mainly from studies on the activity of PKA and its subunits, ERK1/2, other immunoassays and immunohistochemistry in adrenocortical samples from normal subjects and from patients with germline PRKAR1A mutations (Robinson-White et al., 2006a). Patient samples included multiple cortical–producing adenomas, single cortical-producing adenomas, micronodular hyperplasia, and i-PPNAD. However, the majority of samples were from patients with PPNAD or CNC. Data obtained from other studies on Y1 adrenocortical tumor cells was also included in the formulation of the hypothetical pathway. Since, in many cell types (Jun et al., 1999), the PKB signaling pathway inhibits both the B and c-Raf-1 kinase activity of MAPK, data from PKB was included. Data in adrenocortical tissue showed increased total PKA activity that was associated with mutant PRKAR1A. Quantitative mRNA analysis and immunoblotting showed a 2-fold and 1.8-fold decrease in RIα mRNA and protein expression, respectively; and an increase in levels of other PKA subunits. ERK1/2 immunoassays showed a 2-fold and 6-fold decrease in baseline ERK1/2 (unphosphorylated/non-activated ERK1/2) and corresponding increases in phosphorylated/activated ERK1/2 (p-ERK1/2) in mutant-PRKAR1A samples. Other components of the ERK1/2 cascade, i.e. basal B-raf kinase activity, phosphorylated-MEK1/2 (p-MEK1) were increased in mutant tissue, as well as levels of the phosphorylated transcription factor c-Myc (p-c-Myc). There was no difference in levels of phosphorylated c-Raf-1 (p-c-raf-1) in both tissue types. Likewise, levels of phosphorylated PKB (p-PKB) were not increased. These data were all supported by immunohistochemistry studies (Robinson-White et al., 2006a). Data showed that Y1 cells possess an amplified and constitutively expressed c-Ki-Ras that elicits a mitogenic response to fibroblast growth factor2 (FGF-2) through a tyrosine kinase receptor (RTK). ERK1/2 is then rapidly activated for the induction of transcription factors (c-Fos and c-Jun), the phosphorylation of c-Myc, expression of cyclins D and E, and phosphorylation of the retinoblastoma protein; all leading to the activation of the cell cycle, DNA synthesis and cell proliferation. Phosphorylated PKB (p-PKB) can enhance the mitogenic response to FGF2 by phosphorylating and inactivating glycogen synthetase kinase (GSK) and preventing the targeting of c-Myc for degradation by the ubiquitin proteasome system (UPS) (Lepique et al., 2004). PKB has also been shown to inhibit B-Raf and c-Raf-1 kinase activity in many mammalian cell types (Guan et al., 2000; Jun et al., 1999). However, basal B-Raf is normally 4-fold greater than basal c-Raf-1 (Mercer & Pritchard, 2003). Y1 cells can also exert a strong anti-mitogenic effect, mediated by PKA acting to dephosphorylate p-PKB. This releases PKBs' inhibitory effect on GSK3 (Lepique et al., 2004), leading to the degradation of c-Myc by UPS. Thus, FGF-2 S-phase entry can be blocked by a PKA-dependent process. S-phase entry may also be blocked by PKA in normal adrenocortical tissue through an inhibition by PKA of c-Raf-1 kinase, and a subsequent

decrease in phosphorylated MEK1/2, ERK1/2 and c-Myc, which may lead to a decrease in cell proliferation (Robinson-White et al., 2006a). Therefore, the collection of evidence presented suggested that cell proliferation in normal and mutant-PRKAR1A adrenocortical tissue results from the convergence of at least three signaling pathways, PKA, PKB and MAPK, and are consistent with data found in other human tissue with RIα deficiency (Robinson-White et al., 2003; Robinson-White et al., 2006b; Vossler et al. 1997). In general, the following cell proliferation pathway was presented (Figure 3B 1 and 2): 1). In Normal and PRKAR1A-mutant tissues, FGF-2/RTK induced stimulation of ERK1/2 leads to cell proliferation through activation of the transcription factor c-Myc. In normal tissue (Figure 3B 1), PKA (as activated by ACTH at a GPCR) inhibits PKB to a lesser degree than in mutant-PRKAR1A tissue, allowing activated PKB to inhibit ERK1/2 at B-Raf and c-Raf-1 kinase levels, as well as to directly inhibit c-Raf-1. In mutant-PRKAR1A containing tissue (Figure 3B 2), PKA has a greater inhibitor effect on PKB than in normal tissue and a large stimulatory effect on Rap, which then directly activates B-Raf kinase, for the stimulation of ERK1/2. The net effect of these interactions is the stimulation of cell proliferation in mutant-PRKAR1A tissues.

7.3 Mouse models

For a better understanding of the mechanism by which mutant PRKAR1A causes disease in CNC, several investigative approaches have been made using mouse model systems. Studies with RIα knockout mice confirmed the role of RIα as the key compensatory regulatory subunit of PKA activity in tissue where the three other regulatory subunits are expressed. RIα knockout caused deficits in the morphogenesis of the embryonic germ layer, and early embryonic lethality due to failed cardiac morphogenesis. Embryonic germ layer failure could be rescued by crossing RIα with Cα knockout mice, suggesting that inappropriately regulated PKA C-subunit activity is responsible for the phenotype (Amieux & McKnight, 2002). In other studies, transgenic mice (tTA/X2AS), carrying an antisense transgene for the mouse Prkar1a exon 2(X2AS) developed thyroid follicular hyperplasia, adenomas, adrenocortical hyperplasia and other PPNAD-like features. Allelic loss of the mouse chromosome 11 Prkar1a locus, increase in type II PKA activity and greater RIIB protein levels were associated with these lesions. The authors conclude that the tTA/X2AS mouse, having a down-regulated Prkar1a gene, replicates findings in CNC patients. This data supports the role of RIα as a tumor suppressor gene (Griffin et al., 2004). In another study, conventional and conditional null alleles for Prkar1a were developed in mice. Prkar1a[+/-]mice developed a spectrum of tumors that overlapped those observed in CNC patients, e.g., those arising in cAMP-responsive tissues (bone, Schwann and thyroid follicular cells). This suggested that a complete loss of Prkar1a plays a role in tumorigenesis. Likewise, tissue specific ablation of Prkar1a from facial neural crest cells caused the formation of schwannomas, although with aberrant differentiation than those seen in CNC. Since data with the Prkar1a^{+/-} mouse suggested the importance of increased cAMP/PKA signaling for tumor formation, in vitro studies were performed in Prkar1a [-/-] mouse embryonic fibroblasts and in transformed adrenal PRKAR1A-haploinsufficent cells. These studies showed that dysregulation of cyclins and E2F1 (cell cycle factors and mediators of proliferation via defective RIα, respectively), were key changes in the process of immortalization of the two cell lines. These observations confirmed the identity of PRKAR1A as a tumor suppressor gene (Kirschner et al, 2005; Nadulla & Kirschner, 2005;

Nesterova et al., 2008). Prkar1a$^{+/-}$ mice, however, did not develop skin and other CNC tumors (e.g., heart myxomas and pituitary adenomas); therefore, an investigation was made to determine if the Prkar1a defect is a generic, but a weak tumorigenic signal that depends on tissue-specific or other factors. Since p53 (product of the Trp53 gene) and Rb1 (retinoblastoma; product of the Rb1 gene) are essential for cell cycle control, mouse models were bred to determine if the tumorigenic properties of Prkar1a-haploinsufficiency emulate more accurately the CNC phenotype in the background Trp53 or Rb1 (Almeida et al., 2010). Prkar1a mice were bred within the Trp53$^{+/-}$ or Rb1$^{+/-}$ background, or treated with a skin carcinogenic protocol, including 7, 12-dimethylbenz (a) anthracene (DMBA; causes irreversible activation of the HRas oncogene) and 12-O-tetradecanoylphorbol-13-acertate (TPA; promotes the formation of papillomas). The data showed that Prkaria$^{+/-}$ Trp53$^{+/-}$ and Prkar1a$^{+/-}$ Rb1$^{+/-}$ mice developed more sarcomas and endocrine tumors, respectively than Trp53$^{+/-}$ and Rb1$^{+/-}$ mice. The authors suggest that Prkaria-haploinsufficiency has a synergistic and not an additive effect when combined with Tr53$^{+/-}$ and Rb1$^{+/-}$ defects in mice. A genome wide transcriptome profiling of tumors, produced along with qRT-PCR arrays, immunohistochemistry and cell cycle analysis of siRNA down-regulation of cell cycle genes, indicated that Wnt-signaling is the main signaling pathway activated by abnormal cAMP/PKA signaling. They conclude that in the mouse, Prkar1a-haploinsufficiency is a relatively weak tumorigenic signal that can act synergistically with other tumor suppressor gene defects or chemicals to induce tumors, mostly through Wnt-signaling and cell cycle dysregulation (Figure 3C) (Almeida et al., 2010). From these studies in mice, Wnt signaling appears to be the major signaling pathway for PRKAR1A-induced tumorigenesis. However, since Wnt–induced cell proliferation has been shown to be mediated via the ERK pathway in mouse NIH3T3 cells (Kim & Choi, 2007), the possibility exist that inter-pathway crosstalk (above the level of the cell cycle) between PKA/MAPK/Wnt may also occur in mice and in PRKAR1A-mutant cells and tissues. Differences in pathway interactions may also occur in humans vs. mice, as well as between different cell and tissue types, bringing about a much more complex picture.

8. Targeting PKA activity in endocrine and other cell types

8.1 Effect of 8-Cl-cAMP and 8-Cl- Adenosine (8-Cl-ADO) on cell proliferation and apoptosis

Recent investigations have provided a plethora of information on the pathophysiology and genetics of CNC tumors. However, effective treatment strategies for many tumors of CNC are not available. PKA affects cell proliferation in many CNC cell types and is thus a potential target for treatment of CNC tumors. PKA activity is stimulated by cAMP and its cAMP analogues. One such analogue, the 8-Cl- derivative of cAMP (8-Cl-CAMP), the most potent of the cAMP analogues initially tested (Katsaros et al., 1987), and its dephosphorylated metabolite, 8-Cl-ADO are known inhibitors of cancer cell proliferation. However, their mechanisms of action were debated. Questions asked were: 1) does 8-CL-ADO have inhibitory effects on cell growth in human thyroid, HeLa and other tumor cell types; 2) does 8-Cl-ADO act on adenosine receptors to stimulate adenyl cyclase and PKA activity; 3) are the inhibitory effects of 8-Cl-cAMP due to its by-product 8-Cl-ADO; and 4) does 8-Cl-ADO exert its inhibitory effects by changing the RI to RII subunit ratio as has been suggested for 8-Cl-cAMP (Katsaros et al., 1987; Robinson-White et al., 2008). In [^{3}H]

thymidine uptake and Cell Titer 96 AQ (MST) cell proliferation assays using human thyroid tumor cells (WRO), HeLa cells and other cancer cell types (colon carcinoma/ARO and melanoma/ NPA) both 8-CL-cAMP and 8-Cl-ADO inhibit cell growth and proliferation (IC$_{50}$ values ranged from 0.5 μM to 1.7 μM for 8-CL-ADO and 0.55 μM to 4.4 μM with 8-Cl cAMP, depending on the cell type). The inhibition by 8-Cl-ADO was decreased with time (beginning on the 5th day of incubation). To determine if this decrease was due to intracellular or extracellular 8-CL-ADO metabolism, preincubation experiments were performed. When 8-Cl-ADO was preincubated without cells for 7 days (37oC), and cells were then incubated using the same previously preincubated solution for 5 days; or the culture was replenished with fresh drug on day 4, the decrease in inhibition was eliminated, with no difference seen in inhibition with the preincubated drug and the replenished drug. This suggested that 8-Cl-ADO is not metabolized in the media at 37oC (Robinson-White et al., 2008), but, as others have reported (Halgren et al., 1998; Langeveld et al., 1992) may be degraded within the cell with time, by intracellular adenosine deaminase to an inactive product, 8-Cl-inosine. Studies with adenosine receptor agonists (adenosine and NECA) showed no effect on cell proliferation and the adenosine receptor antagonists, XAC and MRS 1523, did not affect 8-Cl-ADO-induced inhibition, excluding the possibility that 8-Cl-ADO's effect is by an effect on adenosine receptors. The question as to whether 8-Cl-cAMP acts as a pro-drug and is metabolized to 8-Cl-ADO by extracellular phosphodiesterase and 5' nucleotidase, was addressed when cells were incubated with 8-Cl-ADO, 8-Cl-cAMP and 8-Cl-cAMP plus the phosphodiesterase inhibitor 3-isobutytl-1-methylxanthaine (IBMX) in media containing serum or with 8-CL-cAMP in serum–free media. The inhibition by 8-Cl-cAMP was greatly reduced by IBMX (to 62%) and further reduced in serum free media (to 75%). This data suggested that 8-Cl-cAMP is metabolized to 8-Cl-ADO, and the inhibitory effect of 8-Cl-cAMP is due to its by-product, 8-Cl-ADO. The involvement of 8-Cl-ADO in PKA cell signaling was examined. 8-Cl-ADO altered PKA activity in cell types in a differential manner, with a small increase in activity in thyroid cells. No reduction occurred in the ratio of RI to RII subunits, in all, suggesting that PKA and the R subunits have no direct effect on the inhibition by 8-Cl-cAMP and 8-Cl-ADO. To examine the mechanism of the inhibition by the two drugs, the effect of 8-Cl-ADO on apoptosis and on the cell cycle was measured by Flow Cytometry using the Annexin V/7AAD assay and BrdU incorporation, respectively. Apoptosis was induced in all cell lines, in the rank order in cells matching the IC$_{50}$ values in MTS assays. Both drugs also caused rapid and late apoptosis/necrosis. Cell cycle analysis showed that 8-Cl-ADO caused an arrest of the cell cycle with an accumulation of cells in G2/M (NPA) and G1/S (WRO and ARO) phases. The data suggested that 8-Cl-ADO (or 8-Cl-cAMP) may be a potent chemotherapeutic agent for the treatment of thyroid and other cancers and its' effect will not depend on PKA signaling (Robinson-White et al., 2008).

8.2 PKA-dependent and Independent effects of 8-Cl-ADO on proliferation and apoptosis

Because PRKAR1A mutations are present in B-lymphocytes of CNC patients (Kirschner et al, 2000b), B-lymphocytes from CNC patients and their normal matched controls were used in a multi-parametric approach (i.e. growth and proliferation assays, PKA and PKA subunit assays, cAMP and cAMP-binding assays and apoptosis assays) to study the effects of 8-Cl-ADO on growth, cell proliferation and apoptosis; and on the relationship between these

parameters and PKA signaling. Since adenosine (a structural analogue of 8-CL-ADO) is transported across the lymphocyte plasma membrane by specialized membrane nucleoside carrier proteins (equilibrate nucleoside transporters; ENTs) by facilitated diffusion for subsequent metabolism and to induce apoptosis, the possibility that 8-Cl-ADO is also transported across the plasma membrane to induce apoptosis was explored. 8-Cl-ADO inhibited cell proliferation in a time and concentration-dependent manner, with greater inhibition in mutant cells. IC_{50} values were higher (1μM and 2μM in normal and mutant cells, respectively) than those previously reported for other cell types. The manner in which 8-Cl-ADO affects PKA signaling was explored. Cyclic AMP levels were increased, while [^{3}H]-cAMP binding was decreased in both cell types. However, binding experiments suggested a time-required down-regulation of cAMP binding. PKA subunit levels were altered with an overall reduction in the RI/RII subunit ratio, whereas levels of the catalytic subunit Cα were 2-fold that of RII. Basal and cAMP-stimulated PKA activity was increased by 8-Cl-ADO (Robinson-White et al., 2009). These data suggested that 8-Cl-ADO can change the site selectivity of cAMP toward R-subunits, as shown for 8-Cl-cAMP (Ally et al., 1988; Rohlff et al., 1993). Experiments with the ENT protein inhibitor dipyridamole (DIP) and 8-Cl-ADO indicated a competition between 8-Cl-ADO and DIP for the ENT protein and suggested that 8-Cl-ADO is transported into lymphocytes by facilitated diffusion. Also, an analysis of components of the intracellular adenosine metabolic pathway, as affected by 8-Cl-ADO, showed that adenosyl-homocysteine (ADOHcy, the main product of adenosine metabolism) alone inhibited cell proliferation, while the inhibition by 8-Cl-ADO in both cell types was increased. This inhibition was greater in mutant cells. Likewise, 8-Cl-ADO also increases levels of p53, another component of adenosine metabolism. Since adenosine metabolism results in apoptosis, whether or not 8-CL-ADO induces apoptosis in B-lymphocytes and if this apoptosis is reversed by DIP was explored. Apoptosis was induced by 8-Cl-ADO, was reversed by DIP and was greater in mutant cells. As in proliferation studies, the effect indicated a competition by DIP and 8-Cl-ADO for the ENT protein. Together, the data suggested that 8-Cl-ADO is transported in and metabolized to ADOHcy to induce apoptosis in B-lymphocytes. Data also indicated that other cancer cell types (e.g. MCF-7 breast carcinoma and SW-13 adrenal cortex carcinoma cells, HeLa, NPA, WRO, and ARO) transport 8-Cl-ADO via ENTs and may indicate a general mechanism of action of 8-CL-ADO on cell proliferation. 8-Cl-ADO also inhibits ISO-induced GPCR activation of cell proliferation, LPA-stimulation of RTK-induced proliferation, as well as pERK1/2 levels in lymphocytes, but had only a small and nonspecific inhibitory effect on A1, A2a, and A2b adenosine receptors, and no effect on A3 receptors, suggesting that 8-Cl-ADO can also inhibit proliferation induced by different GPCRs and proliferation induced by other receptor types (e.g. RTK). The authors present a hypothesis (Figure 4) for the mechanism of action of 8-Cl-ADO on cell proliferation. The inhibition may be due to: 1) stimulation of an ENT protein to transport 8-Cl-ADO intracellularly for metabolism to adenosyl-homocysteine (ADOHcy) to induce apoptosis; 2), intracellular 8-Cl-ADO may stimulate PKA activity. However, the ability of transported 8-Cl-ADO to inhibit proliferation would depend on the cell's ability to use the PKA signaling pathway to induce proliferation (Robinson-White et al, 2008); 3) PKA activity may be increased by the selective binding of 8-Cl-ADO to R-subunits; 4) 8-Cl-ADO may inhibit GPCR-and RTK induced proliferation; and 5) ENT proteins may transport 8-Cl-ADO in other cell types to inhibit proliferation. Additionally, the effect of 8-Cl-ADO on PKA may be overlooked because of the large and

direct effects of transported 8-Cl-ADO on proliferation and on the induction of apoptosis. The conclusions from this work may have applications in solid tumors of CNC and even in non-PRKAR1A-mutant systems (Robinson-White et al., 2009).
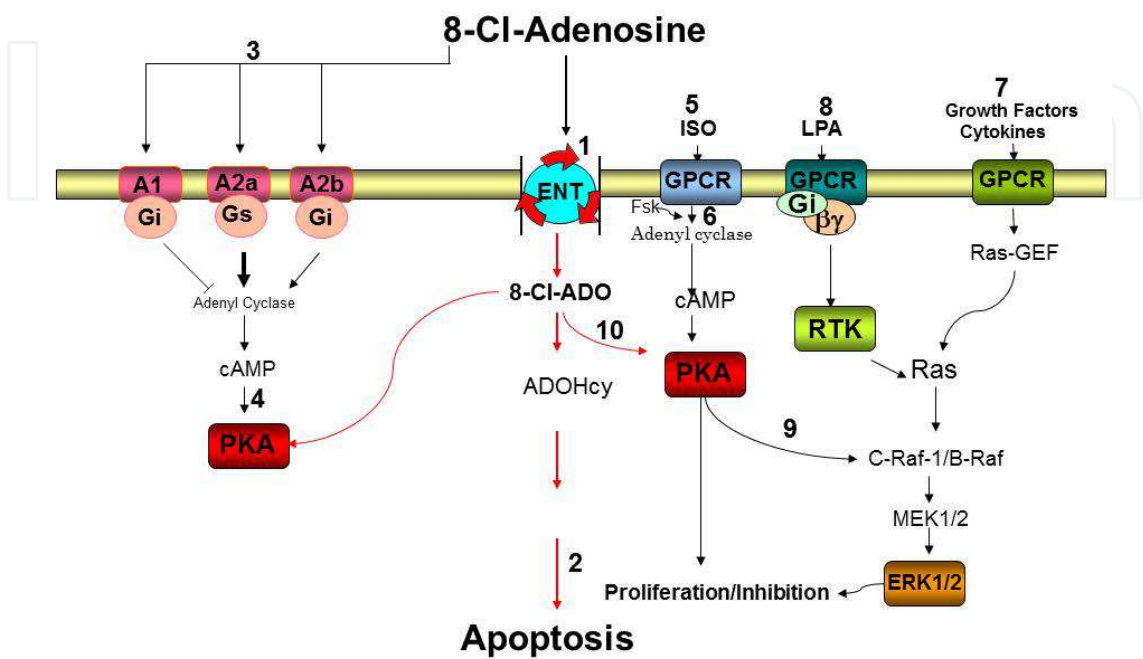

Fig. 4. Mechanism of the Inhibition by 8-Cl-Adenosine (8-Cl-ADO) on proliferation in B Lymphocytes 1), 8-C-ADO is transported into the cell by an *es*-type equilibrate nucleoside transporter protein (ENT) and metabolized to adenosyl-homocysteine (ADOHcy) to (2) induce apoptosis. 3), Extracellular 8-CL-ADO alters the action of adenosine receptors (A1, A2a and A2b) that (4) activate or inhibit PKA. 5), PKA is activated extracellularly at G-protein coupled receptors (GPCR) by isoproterenol (ISO) or intracellularly at (6) adenyl cyclase by forskolin (Fsk). 7), Growth factors or cytokines stimulate GPCRs to activate the ERK1/2 cascade of the MAPK signaling pathway. Lysophosphatidic acid (LPA) stimulates GPCRs that interact with receptor tyrosine kinase (RTK) that activates ERK1/2. 9), PKA alters c-Raf-1 or B-Raf activity to inhibit or stimulate, respectively, cell proliferation. 10), 8-CL-ADO alters the activity of PKA.

9. Future perspectives

9.1 Targeted therapy and drug design for endocrine tumors

Targeted therapy refers to the use of anti-cancer drugs that are designed to interfere with a specific molecular target in a cell signaling pathway that plays a crucial role in tumorigenesis and in its progression. Appropriate targets within the pathways must be identified based on a detailed understanding of the molecular mechanisms underlying the cancer progression. Targeted therapy has improved the therapeutic landscape among solid tumors and offers new treatment strategies beyond surgery, conventional chemotherapy

and radiation. Use of small molecular compounds that regulate oncogenic transduction pathways and monoclonal antibodies have emerged as promising treatment options in solid tumors.

9.1.1 Tyrosine kinase inhibitors

Of the greater than 500 different protein kinases in humans, only 91 are tyrosine protein kinases and are serine/threonine/kinases. A subset of serine/threonine kinases (e.g., such as PKB, ERK1/2, PKC), are involved in signal transduction as key regulators of cell proliferation, differentiation and apoptosis (Levitzki & Klein, 2010). Of the small molecular inhibitors that affect signaling pathways, tyrosine kinase inhibitors (TKIs) are designed to affect tyrosine kinase-dependent oncogenic pathways. TKIs selectively inhibit tyrosine kinase activity and provide a relatively high therapeutic window and low toxicity, in comparison with conventional chemotherapy. Some TKIs currently in use for the treatment of endocrine cancers include erlotinib, axitinib and vatalanib for pancreatic cancer, axitinib and vandetanib, imatinib and motesanib for thyroid cancer, while others are in ongoing clinical trials. There is conservation in the structure of the ATP binding site in all protein kinases. This allows TKIs to act as small molecules with structural similarity to ATP, and to bind to and disrupt tyrosine kinases catalytic activity. However, the homology of structure may also afford TKIs the ability to act against a broad range of protein kinases, to affect multiple pathways and bring about important side effects. Recently endocrine-related side effects have been seen, some of which include alterations in thyroid function, bone metabolism, growth, gonadal function, fetal development, glucose metabolism and adrenal function (Lodish & Stratakis, 2010b). Although TKIs offer promising treatment options, further investigation is needed to determine the molecular mechanisms underlying endocrine dysfunction, as well to bypass TKI related side effects to obtain wider use of TKIs in the treatment of cancer.

9.1.2 Monoclonal antibodies

A number of monoclonal antibodies (mAbs) have been developed for cancer therapy in recent years and many more are in clinical trials. Monoclonal antibodies interfere with cancer progression by three basic mechanisms: inhibiting signal transduction, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). The hallmark of an effective therapeutic mAb is its ability to target antigens that are crucial for tumor maintenance, inhibit signaling by that antigen and induce cytotoxicity. Antibodies inhibit signal transduction by binding to, e.g. cell surface receptors, or by preventing ligand binding to specific receptors, to prevent the activation of signaling pathways. ADCC occurs when mAbs bind to tumor cells and to Fc receptors on immune cells (e.g., neutrophils, mononuclear phagocytes, but mainly to natural killer cells). This activates the cells to engulf and destroy the bound tumor cell. The same mAbs can also activate the complement system to kill cells directly, by the formation of a "membrane attack complex" (MAC). MAC produces a pore within the tumor cell membrane, which leads to tumor cell destruction (Levitzki & Klein, 2010). Over expression or activation of epidermal growth factor receptors (EGFRs) has been reported in many cancers, including ovarian cancer. Cetuximab (Erbitux®), panitumumab (Vestibix®) and trastuzumab (Herceptin®) are three EGFR targeting mAbs that are licensed for clinical use. Cetuximab blocks EGFR ligand binding

and induces receptor internalization and degradation. It inhibits tumor growth and proliferation via Go/G1 cell cycle arrest, induction of apoptosis and inhibition of metastasis and enhances radiosensitivity (Stoffel, 2010). Trastuzumab is so far, the most successful anti-EGFR antibody, having been used for HER2 positive breast cancer. It binds to the juxtamembrane portion of the HER2 receptors' extracellular domain, to prevent the activation of its intracellular tyrosine kinase domain. The endothelial growth factor (VEGF) can increase tumor vasculature to enhance tumor growth, and is also implicated in various other aspects of tumorigenesis (e.g. angiogenesis, mitogenesis and permeability enhancing effects). Bevacizumab (Avastin®) is the most advanced VEGF targeted agent for treatment of solid tumors (Levitzki & Klein, 2010, Stoffel, 2010), and the first VEGF inhibitor approved by the FDA (2004) as a first-line treatment of metastatic colorectal cancer. It binds to and neutralizes all VEGFA type isoforms, preventing binding to VEGF tyrosine kinase receptors. Combination therapy with mAbs and other anti-tumor agents in clinical trials showed, in most cases, an increase in response rate over monotherapy. However, median survival showed no significant improvement. Clinical use of mAbs has also shown that antibodies can have deleterious side effects, mainly because the same antigens present on cancer cells are also present on normal cells (Stoffel, 2010). Investigations are ongoing for the improvement of (Vossler et al., 1997) mAb therapy for efficacy and the relief of side effects.

9.2 Viral therapy

Advances in cancer biology and virology has allowed the compilation of a growing arsenal of naturally occurring and genetically engineered oncolytic viruses (OV) to treat human cancer. These viruses give a promise of killing cancer cells with low toxicity to normal tissue. They act by infecting tumor cells and selectively replicating inside cells, achieved by modification of the viral genome for targeting of specific tumor cell molecules or signal transduction pathways. Infected cells then become "factories" for the replication, subsequent release of new viruses, and a cycle of infection-replication-release ensues. This cycle is repeated indefinitely to effectively kill the tumor mass. Adenoviruses (Ad), Herpes simplex virus (HSV), and vaccinia (VV; the prototype poxvirus) are the most studied viruses and have shown promising results in clinical trials. Ad infects cells via receptor mediated endocytosis using two separate receptors. The virus first binds to the primary receptor, the host cell receptor (Coxsakie-adenovirus receptor), and is then internalized by binding of its viral capsid with host avB integrins. It enters the cell via endosomes where it is dissembled and lysed. Ads have also been engineered to selectively infect and lyse tumor cells for the purpose of targeting specific cancer cell mutations. Like Ad, HSV are genetically engineered for tumor-selective replication (i.e., it only targets tumor cells), accomplished by deletion of viral genes to restrict replication to tumor cells. VV relies on its own encoded proteins as well as host proteins and functions for its life cycle. This allows for rapid and efficient translation, transcription and replication in poxvirus "factories', in the host cell cytoplasm. VV can enter and replicate in many cell types from various species, although it has a propensity toward human cells. It enters the cell via an endosome or through the plasma membrane. Since VV is a large (250 x 350 mm) virus it may require a leaky vasculature for extravasations from the vasculature into tumor tissues. Tumors are known for their leaky vasculature and production of vascular endothelial cell growth factor (VEGF). With this in mind, a number of strategies are being investigated to enhance replication in tumors cells, and concurrently reduce replication in normal cells. Another new and exciting approach to viral therapy is the use of carrier cells to deliver tumor-specific OV to

tumors. These are autogenous cells, e.g. mesenchymal progenitor stem cells and immune cells that have a great potential to by-pass pre-exiting immunity and to reduce "off target toxicity" as well. In all, viral therapy has greatly progressed in recent years, and much success has indicated possible widespread future use. However, there are still unmet requirements to overcome, 1) overcoming pre-existing host immunity; 2) since only small fractions of carrier cells reach the tumor target, strategies for enhanced trafficking should be investigated and, 3) there is a need for increased OV distribution throughout the tumor tissue for more effective therapy (Guo et al., 2008).

9.3 Modulation of apoptosis

Apoptotic pathways have recently garnered considerable attention in the development of cancer treatment strategies, and studies have focused on key regulatory points in these pathways. In preclinical and clinical investigations, pro-apoptotic and anti-apoptotic Bcl-2 proteins, that control the intrinsic pathway, have been used as drug targets (Elkholi et al., 2011). In particular, studies on the pro-apoptotic BH3-only proteins have shown alterations in protein expression in several cancers, (e.g., colon, lung and other cancers). For example, use of Oblimersen, designed to target Bcl-2 mRNA (Klasa et al., 2002), is one of the first attempts to regulate Bcl-2 proteins. However, treatment has not been successful, possibly due to Oblimersen's targeting of only BCL-2 protein, with no effect on other anti-apoptotic proteins, and in some cases an enhanced effect on other proteins that increase BCL-2. To remedy this problem, several small molecules (BH3 mimetics) have been developed to functionally mimic the BH3 domain of Bcl-2. These proteins bind within the hydrophobic groove of Bcl-2 and inhibit its activity. Obatoclax®, the first pan anti-apoptotic Bcl-2 protein inhibitor, not only inhibits Bcl-2, Bcl-xL and MC-1, but presumably other anti-apoptotic proteins. However, Obatoclax® may also have Bcl-2–independent targets, decreasing its specificity. The development of synthetic protein mimetics and the manipulation of these peptides (e.g. "stapling to maintain α-helicity") are valuable tools for designing cancer therapeutics and studying the exact nature of protein-protein interactions, as well as for the development of models to target individual proteins (Elkholi et al, 2011). The MAP kinase, ERK1/2, has been shown to phosphorylate and deactivate the pro-apoptotic protein BAD for inhibition of apoptosis in PRKAR1A-mutant B-lymphocytes (Robinson-White et al., 2006b). Studies on the pro-apoptotic ability of cAMP/PKA showed that PKA induces G1 phase cell cycle arrest and apoptosis in wild type S49 cells by up-regulation of pro-apoptotic BIM, but not in deathless (D) S49 lymphoma cells which lack cAMP promoted apoptosis. Up-regulation of BIM may, thus, be an important element of cAMP/PKA mediated apoptosis (Zambon et al., 2011). Therefore, the manipulation of various proteins, small molecular inhibitors and signaling pathways can be used in therapeutic strategies to regulate apoptosis. However, the specific protein, signaling pathway, stimulant and cell and tissue type must be considered. Further investigation into the mechanisms involved in the interactions of these proteins and signaling pathways with apoptosis in cancer cells will certainly bring to light other therapeutic targets to benefit cancer patients.

9.4 Challenges of anticancer efforts

Tremendous advances in certain areas of cancer diagnosis and treatment have occurred within the last 20 years. However, we still face many challenges in the treatment of most

cancer types. More in-depth research and improved treatment strategies are needed to overcome these obstacles.

9.4.1 Lack of biomarkers

There is a need for reliable biomarkers for endocrine and other cancers. Diagnosis and treatment of certain cancers are still impeded by the lack of specific and sensitive biomarkers that correlate with disease burden and respond to surgical and systemic treatment. For example, pancreatic adenocarcinoma, the fourth highest cause of cancer-related deaths in the United States, has the most aggressive presentation, resulting in a very short median survival time, and one of the lowest survival rates for solid tumors. At the present time there is still no reliable method for early detection, and less than 10% of cases are diagnosed at an early stage. Because surgical resection remains the only option for a potential cure and most patients present with inoperable disease, early and accurate diagnosis is essential. Carbohydrate antigen 19-9, i.e. sialylated Lewis antigen, is the only widely used biomarker for pancreatic cancer (sensitivity 80.8%, specificity 89%). It, however, is expressed in other cancers and in other benign diseases, and is thus an ineffective screening tool in the general population (Castellanos et al., 2011). Recently, a novel urine steroid metabolomics approach was designed to detect adrenocortical carcinoma (ACC) and to differentiate ACC from adrenocortical adenoma (ACA). The method is based on gas chromatography/mass spectrometry steroid profiling, followed by machine learning analysis. Recent data showed that 9 steroids differentiate ACC from ACA (specificity= sensitivity = 90%). Thus, urine steroid metabolism is both sensitive and specific and has great promise for differentiating benign from malignant disease (Arlt et al., 2011). Epithelial ovarian cancer (EOC) is the most common gynecological cancer and the ninth most common cancer overall. Diagnostic and treatment options lag due to poor characterization of disease progression, disease heterogeneity and lack of early detection biomarkers. Early detection and treatment of EOC would significantly benefit from specific and sensitive routine screening tests on biofluids. Over 200 potential biomarkers for EOC have been identified in the past twenty years. Of the many biomarker candidates, only a small number are validated with clinical samples, due to the lack of biomaterials that are linked with accurate clinical data. Research is now geared toward identifying more stratified biomarkers to accurately follow disease progression as well as efficacy of treatment (Elschenbroich et al., 2011).

9.4.2 Resistance to drug therapy

Resistance to drug therapy is a major cause of treatment failure. For example, relapse after successful treatment of patients with chronic myeloid leukemia with the TKI, imatinib, occurred due to the emergence of cells having a second-site mutation in the BCR/ABL oncoprotein, which prevented imatinib binding. Likewise, second-site mutations were identified in epidermal growth factor receptors (EGFRs) in non-small cell lung carcinoma. These cells acquired resistance to the TKI, erlotinib. Many second-site mutations exist prior to therapy; therefore there is a need for mutational analysis of the cancerous tissue and combination drug therapy in the form of a "drug cocktail" as the first line of therapy to prevent the emergence of resistant clones. Studies have also shown that drug efficacy can be compromised by mutations in other un-related genes. Mutations in genes such as PTEN and p53 can control the outcome of targeted therapy. The mechanism of this reaction is

unknown. Another cause of relapse after successful treatment is the ability of cancer cells to adapt to treatment. Cancer cells can re-wire signal transduction networks to become less dependent on the molecular target (McCormick, 2011). The mechanism that drives this is part of a process called "oncogene addition" (Weinstein & Joe, 2008). Cancer cells are addicted to and depend on a particular oncogene for survival. For example, certain cancer cells that depend on, e.g. mutated B-Raf are addicted to MEK. These cells are exquisitely sensitive to MEK1/2 inhibitors. But other tumor cells or normal cells are not. The confounding aspect of this observation is that "oncogene addicted" cancer cells, after treatment can lose their addition and fail to respond to the same therapy (McCormick, 2011). Some cancer cells can switch to alternate signaling pathways. Successful treatment of estrogen positive breast cancer with aromatase inhibitors, was eventually decreased due to adaptive changes in these cells; leading to a decrease in the expression of estrogen receptor α and aromatase, and a switch to use of the MAPK signaling pathway (Brodie et al., 2011). Other outcomes of targeted therapy have also left investigators perplexed, and go beyond the present level of understanding of signaling pathways. In particular, patients who have K-Ras mutations and were treated with EGFR inhibitors plus chemotherapy, showed increased tumor progression in spite of the combined therapy (McCormick, 2011). In all, these examples of events occurring in cancer cells, allow us to realize the extent of the adaptive ability of cancer cells to resist therapeutic strategies. Therefore a comprehensive knowledge of all mutations and signaling pathways in a tumor should be sought to predict a drug response.

9.4.3 Complexity of signaling

Most anticancer strategies focus on attacking specific targets in a given biological pathway to achieve target specificity and to minimize toxicity. However, due to resistance to therapy, this approach has not produced the desired effect, i.e. eradication of all tumor cells. The major problem has been our incomplete understanding of the multiplicity and complexity of genetic and epigenetic changes, and the redundancies and "cross-talk" found in key signaling pathways occurring in the majority of cancer cells, as well as the ability of cancer cells to use alternative signaling pathways to resist therapy (Azmi et al., 2010). We are now aware that much more knowledge should be gathered on tumor genetics and the complexities of tumor signaling pathways and pathway interactions versus that in normal cells for the development of more refined therapeutic strategies. Instead of a single-pathway targeted approach or the use a single therapeutic moiety, innovative approaches (e.g. combination therapy with chemotherapy, mAb and TKI moieties) are needed to overcome these obstacles to treatment efficacy.

9.4.4 Lack of individualized therapy

The one size fits all approach to cancer therapeutics is based on standards of care, obtained by epidemiological studies of large cohorts. It is a reactive approach that focuses on treatment of signs and symptoms. It does not take into account the vast underlying biological heterogeneity of tumors, host genetics, and inter-individual variations in drug response. The need for a more tailored approach to treatment is apparent. Likewise, biomarkers do not predict which patients will respond positively, which are non-responders and which patients will experience adverse reactions for the same medications and dose. At present, dosage must be optimized by a trial and error method. Recent advances in medical

genetics and molecular profiling, e.g. genetic and proteomic profiling, genetic testing and improvement in treatment response assessment, has allowed the advancement of the field of personalized medicine. Personalized medicine, considers each persons unique clinical, genetic, genomic and environmental information (Shastry, 2006). The implementation of personalized medicine would allow the selection of the right drugs and manner of treatment for a given patient, and to define lifelong health maintenance strategies.

10. Conclusions

In the last 25 years, an understanding of endocrine tumorigenesis has greatly advanced, due to the increase in our knowledge of underlying molecular signaling pathways and cellular and molecular genetics. In endocrine and other cells, various signaling pathways communicate ("talk") to each other forming a highly complex network of interactions that amplify or dampen extracellular and intracellular signals. The PKA signaling pathway appears to be at the hub of signaling in endocrine tissue. It interacts with versatility with the MAPK, PKC, PKB, Wnt and other signaling pathways, as well as with apoptotic pathways, in a manner that depends on the species, tissue, cell type and ligand involved. From pathophysiological and other studies, endocrine tumors have been well characterized and classified. Of these tumors, we have focused here, in particular, on CNC and its main clinical manifestation, PPNAD. Mutations in the gene encoding the RIα subunit of PKA, *PRKAR1A*, have been shown to be central to the development of tumors of CNC. Through studies in *PRKAR1A* mutant cells and tissues and in mouse models, we are beginning to understand *PRKAR1A*s role in the intricate molecular mechanisms involved in CNC tumor formation   Despite the fact that effective treatment strategies for many tumors of CNC are currently lacking, studies with the cAMP analogue, 8-Cl-adenosine, have shown that this drug may be a useful therapeutic agent for the treatment of CNC tumors. Although the future looks bright for the use of other highly innovative approaches in the treatment of endocrine and other tumors, it is apparent that there are still many challenges to overcome before these strategies use be widely implemented. More comprehensive research into the complexity of extracellular/intracellular signaling, inherent biological heterogeneity of tumors, host genetics and inter-individual variations in host response should be undertaken to overcome these challenges.

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12. References

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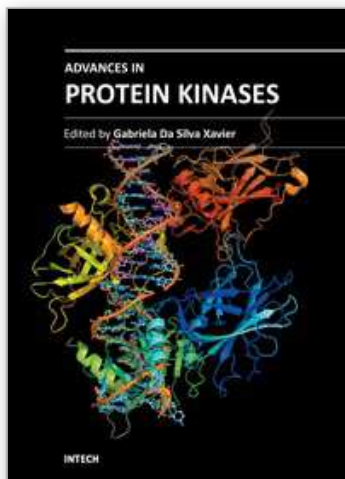
Advances in Protein Kinases

Edited by Dr. Gabriela Da Silva Xavier

Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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