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cAMP/PKA signaling defects in tumors: genetics and tissue-specific pluripotential cell-derived lesions in human and mouse

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Abstract

In the last few years, bench and clinical studies led to significant new insight into how cyclic adenosine monophosphate (cAMP) signaling, the molecular pathway that had been identified in the early 2000s as the one involved in most benign cortisol-producing adrenal hyperplasias, affects adrenocortical growth and development, as well as tumor formation. A major discovery was the identification of tissue-specific pluripotential cells (TSPCs) as the culprit behind tumor formation not only in the adrenal, but also in bone. Discoveries in animal studies complemented a number of clinical observations in patients. Gene identification continued in parallel with mouse and other studies on the cAMP signaling and other pathways.

Keywords

adrenal glands; cAMP signaling pathway; adrenocortical hyperplasia; *PRKARIA* gene; tumor

1. Introduction

Several years ago, we proposed the hypothesis that studying hyperplasias of the adrenal cortex was likely to “*identify molecular pathways involved in the first steps of tumor formation*” (Stratakis; 2009, 2007). This approach has led to fruitful research over the last one-and-a-half decades: Our first studies led to the identification of the main regulator of the cAMP-signaling pathway, regulatory subunit-type 1A (R1 α) of PKA (coded by the *PRKARIA* gene on chromosome 17q22-24), as responsible for primary pigmented nodular adrenocortical disease (PPNAD) and Carney complex (Kirschner et al., 2000a; Kirschner et al., 2000b), a multiple endocrine neoplasia (MEN) whose main endocrine manifestation is PPNAD (Almeida and Stratakis, 2010; Rothenbuhler and Stratakis, 2010). More recently, we focused on trying to delineate clinically the various types of primary bilateral adrenal hyperplasias (BAH) (Stratakis and Boikos, 2007; Stratakis, 2008). We described isolated micronodular adrenocortical disease (iMAD), a disorder likely to be inherited in an autosomal dominant manner that is unrelated to Carney complex (Gunther et al., 2004) or other MENs: the identification of *PRKARIA* mutations in PPNAD led to the recognition that non-pigmented forms of BAHs existed, and a new nomenclature was proposed that has

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since been in use worldwide (Stratakis and Boikos 2007; Stratakis, 2008; Bourdeau et al., 2007). In 2006, a genome-wide association (GWA) study (Horvath et al., 2006a) led to the identification of mutations in phosphodiesterase (PDE) genes (Levy et al., 2011; Libé et al., 2011), *PDE11A* -a dual specificity PDE (Boikos et al., 2008; D'Andrea et al., 2005; Horvath et al., 2006b), and *PDE8B* (Horvath et al., 2008a, 2008b), a cAMP-specific PDE (Conti and Beavo, 2007; Gamanuma et al., 2003), (coded by the *PDE11A* and *PDE8B* genes, respectively) in iMAD. Following the identification of cAMP/PKA involvement in PPNAD and iMAD, we and others found that increased cAMP levels and/or PKA activity and abnormal PDE activity may be found in most benign adrenocortical tumors (ACTs), including the common adrenocortical adenoma (ACA) (Bertherat et al., 2003; Mantovani et al., 2008; Bimpaki et al., 2009). We then found *PDE11A* and *PDE8B* mutations or functional variants in adrenocortical cancer (ACC), and other forms of adrenal hyperplasia like massive macronodular adrenocortical disease (MMAD), also known as ACTH-independent adrenocortical hyperplasia (MMAD/AIMAH) (Horvath et al., 2006b; Libe et al., 2008; Rothenbuhler et al., 2012). Germline *PDE11A* sequence variants may also predispose to testicular cancer (testicular germ cell tumors or TGCTs) and prostate cancer (Horvath et al., 2009; Faucz et al., 2011), indicating a wider role of this pathway in tumor formation on cAMP-responsive, steroidogenic, or related tissues (Libé et al., 2011). There is significant pleiotropy of *PDE11A* and *-8B* defects. Histo-morphological studies on human adrenocortical tissues from patients with these mutations showed that iMAD is highly heterogeneous (Carney et al., 2010) and, thus, likely to be caused by several genes of the cAMP/PKA-signaling pathway or its regulators and/or downstream effectors. Likewise, the G-protein coupled receptor (GPCR)-linked MMAD/AIMAH is a disease that includes a range of adrenal phenotypes (Hsiao et al., 2009) from very similar to iMAD to the *GNAS*-caused primary bimorphic adrenocortical disease (PBAD) and McCune-Albright syndrome (Carney et al., 2011), caused by somatic mutations of the *GNAS* gene (coding for the G-protein stimulatory subunit alpha or Gs α) (Boston et al., 1994; Brown et al., 2010). Although a few of the patients with MMAD/AIMAH have germline *PDE11A*, *PDE8B*, and somatic *GNAS* mutations (Horvath et al., 2006b; Libe et al., 2008; Rothenbuhler et al., 2012; Hsiao et al., 2009; Fragoso et al., 2003) others have germline fumarate hydratase (*FH*) (Hsiao et al., 2009; Matyakhina et al., 2005; Lehtonen et al., 2006), menin (*MEN1*) (Hsiao et al., 2009; Gatta-Cherifi et al., 2012; Simonds et al., 2012), and adenomatous polyposis coli (*APC*) (Hsiao et al., 2009; Gaujoux et al., 2010; Beuschlein et al., 2000) mutations pointing to the range of possibilities and the pathways that may be involved. From these, particularly interesting is the connection with *FH* mutations associated with mitochondrial oxidation defects that have been linked to adrenomedullary tumors (Timmers et al., 2008; Lodish et al., 2010). This led us to investigate a disorder known as Carney Triad, the only known disease that has among its clinical manifestations both adreno-cortical, MMAD/AIMAH) and medullary tumors (pheochromocytomas [PHEOs] and paragangliomas [PGLs]), in addition to hamartomatous lesions in various organs (pulmonary chondromas, pigmented and other skin lesions) and a predisposition to gastrointestinal stromal tumors or sarcomas (GISTs) (Carney et al., 1977; Zhang et al., 2010). A subgroup of patients with PHEOs, PGLs and GISTs (Carney and Stratakis, 2002), were identified to harbor mutations in succinate dehydrogenase (SDH) subunits B, C and D (coded by the *SDHB*, *SDHC* and *SDHD* genes, respectively) (McWhinney et al., 2007; Perry et al., 2006), they also rarely have adrenocortical lesions, ACAs and/or hyperplasia, and their disease is known as the dyad or syndrome of PGLs and GISTs (Carney and Stratakis 2002; McWhinney et al., 2007; Perry et al., 2006) or, as named by a group of pathologists (Daum et al., 2006) and now in wide use, Carney-Stratakis syndrome (CSS) (<http://www.ncbi.nlm.nih.gov/omim;entry#606864>, Stratakis and Carney 2009; Vaughan et al., 2011; Wang et al., 2011). PPNAD appears to be less heterogeneous and is mostly caused by *PRKAR1A* mutations (Kirschner et al., 2000a; Kirschner et al., 2000b; Groussin et al., 2002a; Groussin et al., 2002b), but up to 1/3 of patients with the classic features of PPNAD do not have *PRKAR1A* mutations,

deletions or 17q22-24 copy-number variant (CNV) abnormalities (Bertherat et al., 2009; Horvath et al., 2010a). A subset of these patients may have defects in other molecules of the PKA holoenzyme and their study is important for understanding how PKA works and the tissue-specificity of each defect. Still, about 1/4 of all patients with PPNAD do not have PKA-related defects (unpublished data).

The *Wingless/int* (*Wnt*)-signaling was suggested as one of the downstream effectors of tumor formation in the context of increased cAMP/PKA activity (Almeida and Stratakis, 2011). Somatic β -catenin (*CTNNB1*) mutations were found in large ACAs that formed in the background of PPNAD caused by germline *PRKAR1A* mutations (Tissier et al., 2005; Gaujoux et al., 2008; Tadjine et al., 2008). Our transcriptomic studies had previously identified *WNT1*-inducible signaling pathway protein 2 (*WISP2*) as the main molecule overexpressed in GIP-dependent Cushing syndrome caused by MMAD/AIMAH (Bourdeau et al., 2004), and our recent micro-RNA studies showed that genes that regulate *Wnt*-signaling were major targets of micro-RNAs that were found dysregulated in both PPNAD and MMAD/AIMAH (Iliopoulos et al., 2009; Bimpaki et al., 2010). Cells from tumors or other lesions from animals with R1 α deficiency showed increased β -catenin expression and/or aberrant *Wnt*-signaling (Almeida et al., 2010; Tsang et al., 2010), and similarities to adult stem cells or cancer stem cells in other models of dysregulated *Wnt*-signaling. However, it appears that β -catenin activation in R1 α -deficient cells is an event preceded by yet unknown molecular abnormalities that take place within the still benign and R1 α -haploinsufficient tissues in the early stages of tumor formation (Almeida et al., 2011b; Almeida et al., 2012). The discovery that neural crest (Jones et al., 2010b), heart (Yin et al., 2008a), adrenal (Sahut-Barnola et al., 2010)-specific knockouts (KO) of R1 α or other mice with R1 α defects (Almeida et al., 2010; Tsang et al., 2010; Kirschner et al., 2005; Griffin et al., 2004; Griffin et al., 2004; Pavel et al., 2008; Molyneux et al., 2010; Jones et al., 2010a), develop lesions caused by proliferation of stem cell-like TSPCs in adult tissues, such as the adult skeleton (Tsang et al., 2010; Pavel et al., 2008) was an important one that further supported the possible role of *Wnt*-signaling in R1 α defects, since *Wnt*-signaling is critical for the maintenance and/or proliferation of pluripotential cells.

2. Human studies: genetics of adrenal hyperplasias and related disorders

2.1. Genetic defects in BAHs

BAHs are mostly benign adrenocortical lesions that are characterized on the basis of their hormone secretion, size nodularity, and lipofuscin content, as we first suggested in the previous review cycle (Stratakis and Boikos, 2007; Stratakis, 2008). The presence of lipofuscin determines pigmentation (Stratakis, 2008; Stratakis et al., 1999a): heavily pigmented BAHs contain mostly lipofuscin and only occasionally (and always in addition to lipofuscin) melanin (Stratakis and Boikos, 2007; Stratakis, 2008). BAHs are mostly cortisol- or aldosterone-producing (Stratakis and Boikos, 2007; Stratakis, 2008; Gunther et al., 2004; Bourdeau et al., 2007; Geller et al., 2008), lately, an adrenal androgen-producing form of BAH was also described (Ghayee et al., 2011). Combined gluco- and mineralo-corticoid hormone-producing adrenal lesions have also been described (Onoda et al., 2009; Murakoshi et al., 1995), and we have seen them in our patients; almost certainly BAHs that produce sex steroids in combination with gluco- or mineralo-corticoid hormones also exist (unpublished data). On the basis of size, cortisol-producing BAHs are divided into two groups: macronodular (when the size of their nodules mostly exceed 1 cm) and miconodular (when nodules are smaller than 1 cm) (Stratakis and Boikos, 2007; Stratakis, 2008; Bourdeau et al., 2007). There is no such subclassification on aldosterone-producing or other forms of BAHs which appear to be mostly macronodular; however, miconodular non-cortisol producing BAHs certainly exist (unpublished data) but have not been studied.

Up to recently, the only phenotype-genotype correlation for aldosterone-producing lesions was their biochemical response to dexamethasone (Torpy et al., 1998): glucocorticoid-remediable aldosteronism (GRA) or familial hyperaldosteronism type-I (FH-I) is caused by the chimeric gene *CYP11B1/CYP11B2* defect on chromosome 8q21 (Taymans et al., 1998); all other forms of aldosterone-producing BAHs have not been characterized with the exception of a few rare phenotypes (Geller et al., 2008). Familial hyperaldosteronism type-II (FH-II) is the term that we and others have used to describe familial non-GRA aldosterone-producing BAH that is inherited in an autosomal dominant manner (Torpy et al., 1998; Carss et al., 2011). We have mapped a subset of families with this disorder to chromosome 7p22, a locus that has also been seen in GWA studies of hypertension (Lafferty et al., 2000; Elphinstone et al., 2004). However, the responsible gene(s) remain(s) elusive. The recent description of *KCNJ5* mutations in a subset of aldosterone-producing BAHs known as familial hyperaldosteronism type-III (FH-III) led to the screening of our patients (Choi et al., 2011) and allowed us to focus on kindreds with BAH or FH-II that do not have *KCNJ5* mutations (Xekouki et al., 2012b); others have found various frequencies of *KCNJ5* mutations (Taguchi et al., 2012; Boulkroun et al., 2012; Scholl et al., 2012). Genetic studies are ongoing in our laboratory for the identification of the responsible gene defect(s) in these patients in collaboration with other groups (Mathur et al., 2010). The rest of this text focuses on BAHs that are not responsible for hyperaldosteronism, those that produce cortisol and/or other steroids and maybe associated with other manifestations.

Macronodular forms of (mostly) cortisol-producing BAHs are collectively known as MMAD or AIMAH (Bourdeau et al., 2007), as mentioned in the introduction. MMAD/AIMAH is a clinically and genetically heterogeneous condition that is mostly sporadic, occurs in middle age, and is most often associated with a mild form of Cushing syndrome that develops over a number of years (Bourdeau et al., 2007; Hsiao et al., 2009). When familial, the disease is inherited in an autosomal dominant manner (Hsiao et al., 2009; Gagliardi et al., 2009; Vezzosi et al., 2007; Nies et al., 2002). Although MMAD/AIMAH was thought to be infrequently familial our clinical studies indicated that this disorder may be inherited in at least a tenth to a fifth of the cases (unpublished observations). In our most recently published cohort, mutations or sequence variants in *FH*, *MEN1*, *APC*, *PDE11A* (in the germline) and *GNAS* (somatic) were found in up to a quarter of the patients (Hsiao et al., 2009); in a separate investigation of a larger cohort of MMAD/AIMAH patients, we also found germline *PDE8B* sequence variants, albeit infrequently (Rothenbuhler et al., 2012); these variants did affect PDE activity of the PDE8B enzyme *in vitro*. An ongoing investigation also examines the role of *FH* sequence variants in a larger, unselected group of patients with MMAD/AIMAH; however, *FH* mutations appear to be rare among sporadic MMAD/AIMAH, despite the relatively frequent presence of ACTs among patients with hereditary leiomyomatosis and renal cancer (HLRC), the disease that is caused by germline *FH* mutations (Matyakhina et al., 2005; Lehtonen et al., 2006; Smit et al., 2011). Our laboratory was the first to document MMAD/AIMAH in HLRC (Matyakhina et al., 2005), but several patients have been described since, in international HLRC cohorts (Lehtonen et al., 2006; Smit et al., 2011). Currently our efforts are focused on continuing characterization of patients with MMAD/AIMAH, the identification of familial cases, and the application of GWA, transcriptomic, and deep sequencing (DSeq)-based strategies for the identification of the responsible genes.

Phenotyping and identification of homogeneous subgroups of patients (for comparison studies and grouping for segregation and linkage studies) are assisted by careful biochemical studies and histopathological analysis. Following the identification of excess 17-hydroxysteroids (17OHS) in the urine of our patients with MMAD/AIMAH (Hsiao et al., 2009) despite their normal or near normal urinary free cortisol (UFC) and the realization that many of the tumors of these patients are combined gluco-, mineralo-corticoid and, rarely,

androgen hormone-secreting, we study the steroid content of serum and/or urine by liquid chromatography tandem mass spectrometry (LC/MS/MS). LC/MS/MS is on its way to replace standard assays for steroid hormones in the clinical care of patients with steroid overproduction and is ideal for the search of novel metabolites that may indicate steroidogenic pathways that participate in the pathogenesis of these tumors (Koal et al., 2011; Arlt et al., 2011).

Micronodular forms of cortisol-producing BAHs include PPNAD (Carney et al., 1985; Carney, 1995), the first hyperplasia that we started working on almost two decades ago (Stratakis et al., 1996; Sarlis et al., 1997; Stratakis et al., 1999b; Stratakis et al., 2001; Bourdeau et al., 2003; Louisset et al., 2009). Genetic studies of families with PPNAD led us to identify *PRKAR1A* as the gene responsible for it and the syndrome known as Carney complex (Kirschner et al., 2000a; Kirschner et al., 2000b). We recently completed a phenotype-genotype study of patients with PPNAD and Carney complex (Bertherat et al., 2009; Horvath et al., 2010a; Horvath et al., 2008c; Greene et al., 2008; Meoli et al., 2008; Patronas et al., 2012; Anselmo et al., 2012; Gaujoux et al., 2011; Gennari et al., 2008; Pereira et al., 2010); the *PRKAR1A* mutations are included in on-line, publicly available, and continuously updated database at <http://prkar1a.nichd.nih.gov/hmdb/intro.html>.

To date more than 121 *PRKAR1A* mutations have been identified. The molecular changes involved single base substitutions and small deletions, insertions, or rearrangements that were spread along the whole open reading frame (ORF) of the gene; in addition, a few relatively large deletions were reported (Horvath et al., 2008c; Blyth et al., 2008). Most of these mutations are unique; however, 5 mutations have been found in several unrelated pedigrees: c.491_492del, c.1A>G (start-site Met1Val mutation) (Pereira et al., 2010), c.82C>T, c.709 (Pavel et al., 2008) del (Groussin et al., 2006), and c.439A>G (Anselmo et al., 2012). The last 4 of these mutations led to isolated PPNAD (Bertherat et al., 2009; Horvath et al., 2010a); indicating a specific genotype-phenotype correlation; the last mutation is the first one to be linked to ACC, since the patient who presented with PPNAD died of metastatic disease (Anselmo et al., 2012); one more patient with ACC and PPNAD was just reported (Morin et al., 2012). Even though most *PRKAR1A* mutations led to nonsense mRNA decay (NMD), several were identified that were expressed at the protein level, and were typically (although not all) associated with a more aggressive phenotype (Groussin et al., 2002b; Horvath et al., 2010a; Greene et al., 2008; Moili et al., 2008). *PRKAR1A* mutations that extended the ORF and did not undergo NMD were also not expressed at the protein level; we showed that this was due to proteosomal degradation (Patronas et al., 2012), adding proteosome-mediated surveillance of R1α to NMD-control of *PRKAR1A* mRNA. These patients had an overall milder phenotype. In addition to the molecular data, and the clear evidence for some genotype-phenotype correlation, the database allowed us to identify at least three new associations for Carney complex: (1) ACC, as mentioned above (Anselmo et al., 2012; Morin et al., 2012), (2) hepatocellular carcinoma (Gennari et al., 2008), and (3) various types of pancreatic malignancies such as acinar cell carcinoma, adenocarcinoma and intraductal mucinous tumors (Gaujoux et al., 2011). Loss-of-heterozygosity (LOH) studies confirmed *PRKAR1A*'s role in these lesions.

The database identified a total of 95 patients who met the diagnostic criteria for Carney complex or PPNAD but had no *PRKAR1A* gene or 17q22-24 (the *CNC1* locus) defects. Most of these patients had sporadic disease, but there were also families that mapped to chromosome 2 (the *CNC2* locus on 2p16) (Stratakis et al., 1996). Analysis of their phenotype revealed for the first time some phenotypic differences for *CNC2* patients: they presented later in life, were unlikely to have family history of the disease, and were less likely to develop myxomas, schwannomas, thyroid and testicular tumors. Only few *CNC2* patients with three or more manifestations had clinically proven PPNAD, whereas

acromegaly was equally present. CNC2 patients appear to suffer from a hamartomatosis syndrome with fewer and/or later presenting endocrine and other tumors. Since tumors with *PRKAR1A* defects had 2p16 dosage changes (Matyakhina et al., 2003), it is tempting to speculate that the responsible genetic defect(s) in CNC2 patients are related to PKA function. CGH, CNV, WES and DSeq are ongoing in our laboratory for the identification of the responsible gene defect(s) in these patients; our recent study excluded mutations in the 2p16-located protein kinase C epsilon (PKCε) (Toledo et al., 2012).

A distinct group of patients in this database had PPNAD only. Patients with *PRKAR1A* defects presented with PPNAD later; in addition, after the teenage years, female patients with PPNAD exceeded males, and by the age of 40 years more than 70% of female carriers of *PRKAR1A* defects had manifested with PPNAD, whereas only 45% of males had clinical evidence of this disease. Indeed, Cushing syndrome, ACA, and ACC, in particular, are more frequent in females. *PRKAR1A* expression is not known to be dependent on, or affected by gender in humans or mice; however, adrenal lesions primary developed in female mice with *R1α* defects (Sahut-Barnola et al., 2010; Griffin et al., 2004a; Griffin et al., 2004b), other mouse models show adrenal tumor development in females predominantly (Berthon et al., 2010).

2.2. Identifying new genes for PPNAD, iMAD, and related micronodular hyperplasias

The database analysis showed that very few young children with corticotropin (ACTH)-independent iMAD and no other tumors have Carney complex or *PRKAR1A* mutations (Bertherat et al., 2009; Horvath et al., 2010a). A handful of these patients had *PDE11A* or *PDE8B* defects, as we reported previously (Horvath et al., 2006a; Horvath et al., 2008a; Horvath et al., 2008b; Carney et al., 2010). Thus, there are as yet unidentified molecular cause(s) of iMAD (Figure 1). Since we have shown cAMP-signaling alterations in most cortisol-producing hyperplasias (Bimpaki et al., 2009; Bourdeau et al., 2006), our hypothesis is that the defects in these patients are in related molecules.

iMAD with or without *PDE11A* or *PDE8B* mutations (Carney et al., 2010) looks different from PBAD in infantile McCune-Albright syndrome due to *GNAS* mutations (Carney et al., 2011) that we studied and recently reported (Figure 2). In all instances, what is becoming clear from these descriptive studies is that in all micronodular forms of BAH that we have studied so far, we see persistence of cells that are probably derived from fetal adrenal precursors (Figure 2). The successful characterization of fetal cells giving rise to PPNAD and iMAD is important in the future since so far the evidence derived for this phenomenon is only histopathological (Carney et al., 2010; Carney et al., 2011). Mouse studies have provided more evidence for adrenal TSPCs being the culprit, and will be more extensively discussed below.

2.3. PRKAR1A and PKA activity; the role of the other PKA subunits

The PKA holoenzyme is a tetramer comprised of a dimer of two regulatory (R) subunits bound each to a catalytic (C) subunit (Amieux and McKnight, 2002). Four major R subunits (R1α, R1β, R2α, and R2β) coded by the *PRKAR1A*, *-R1B*, *-2A* and *-2B* genes, respectively) and four C subunits (Cα, Cβ, Cγ, and PRKX coded by the *PRKACA*, *-CB*, *-CG* and *PRKX* genes, respectively) have been identified (McKnight et al., 1998). Type I PKA (PKA-I) contains either R subunit R1α or R1β in its structure; PKA-II contains either R subunit R2α or R2β. Heterotetramers occasionally also form (Skalhegg and Tasken, 2000). cAMP binding to the R subunits of PKA releases the C subunits (Taskén et al., 1997), which allows them to phosphorylate cytoplasmic or nuclear targets such as cAMP response element (CRE)-binding (CREB) protein, resulting in activation of transcription of CRE-containing genes (Nesterova and Stratakis, 2007). The PKA system has a substantial capacity of self-

regulation (Amieux and McKnight, 2002; Bossis and Stratakis, 2004): for example, overexpression of Ca or $\text{C}\beta$ in cell culture results in significant compensation by an increase in $\text{R1}\alpha$ subunit protein (Nesterova et al., 2008). The cellular localization of PKA has a pivotal role in determining which substrates are phosphorylated and it is controlled by the scaffolding A-kinase anchor proteins (AKAPs), which bind to the R subunits (Martin et al., 2007). We showed that *PRKAR1A* haploinsufficiency is all that is needed for increased C subunit activity (Meoli et al., 2008; Nesterova et al., 2008; Robinson-White et al., 2006a; Robinson-White et al., 2006b), however, the addition of LOH in affected tissues (and, thus, complete loss of $\text{R1}\alpha$ protein) leads to further and substantial increase in PKA activity (Nesterova and Stratakis, 2007; Mavrikakis et al., 2006). *PRKAR1A* mutations that do not lead to NMD and are expressed at the protein level have more or less the same effect: in one way or another, they lead to release of the inhibitory control of $\text{R1}\alpha$ on the C subunits (Greene et al., 2008; Meoli et al., 2008). The central role of C subunits in PKA-related disease pathogenesis is also shown in animal models (Tsang et al., 2010; Sahut-Barnola et al., 2010; Kirschner et al., 2005; Amieux and McKnight, 2002; Skålhegg et al., 2002; Yin et al., 2011), and is suggested by recently discovered genetic defects in *PRKAR1A* that increase binding to the Ca (Linglart et al., 2011). The role of the X-chromosome located and $\text{R1}\alpha$ -binding PRKX catalytic subunit (Diskar et al., 2010; Li et al., 2005) remains unknown.

2.4. The role of the PDEs

We identified mutations in cAMP-binding PDEs, as predisposing to BAHs and possibly other adrenal tumors (Horvath et al., 2006a; Horvath et al., 2008a; Horvath et al., 2008b; Libe et al., 2008; Rothenbuhler et al., 2012; Vezzosi et al., 2012).

PDE11A-inactivating, protein-truncating mutations appear to be present rarely in the population but two functional *PDE11A* missense substitutions, R804H and R867G, are present at much higher rates of 2.4% and 3%, respectively; additional sequence changes (mostly missense variants) are present in various frequencies. *PDE11A* variants appear to affect the phenotype of PPNAD and increase the incidence of testicular lesions when co-inherited with a *PRKAR1A* mutation (Libe et al., 2011). *PDE11A* functional variants are also more frequent among patients with testicular (Horvath et al., 2009) and prostate cancer (Faucz et al., 2011), two tissues where *PDE11A* is expressed highly. Thus, *PDE11A* gene defects may have wider implications that go beyond a predisposition to adrenocortical diseases.

Since our description of *PDE8B* mutations in iMAD (Horvath et al., 2008a; Horvath et al., 2008b) and now in MMAD and other ACTs (Rothenbuhler et al., 2012), there has been considerable interest in this enzyme which is the PDE with the highest affinity for cAMP (Conti and Beavo, 2007; Gamanuma et al., 2003), *PDE8B* is also the highest expressed cAMP-specific PDE in the adrenal cortex (Horvath et al., 2008b), and is present at high levels in a number of other endocrine tissues (Horvath et al., 2008b). *PDE8B* has been implicated in the regulation of thyroid-stimulating hormone (TSH) levels and insulin release in humans (Arnaud-Lopez et al., 2008; Horvath et al., 2010b; Dov et al., 2008). Recently, Cushing syndrome due to an iMAD-like phenotype was described in a previously established mouse model of *Pde8b* KO confirming the primary role of *PDE8B* in adrenocortical hyperplasias (Tsai et al., 2011; Shimizu-Albergine et al., 2012).

2.5. Identifying the genetic defects in other disorders associated with adrenal tumors; Carney Triad

We studied families that had gastrointestinal neoplasms (GIST), PGL and/or PHEO, and occasionally ACTs (mostly adenomas or MMAD/AIMAH); the trait was inherited in an

autosomal dominant manner (Carney and Stratakis, 2002). We referred to this condition as the dyad (or syndrome) of “paraganglioma and gastric stromal tumors” (Carney and Stratakis, 2002; Perry et al., 2006), but it is also now known as the “Carney-Stratakis syndrome or dyad” (CSS) (Daum et al., 2006; (<http://www.ncbi.nlm.nih.gov/omim;entry#606864>, Stratakis and Carney 2009; Vaughan et al., 2011; Wang et al., 2011), and appears to be distinct from Carney Triad. In CSS, we found germline mutations of the *SDHB*, *SDHC* and *SDHD* genes, or *SDHx* collectively (McWhinney et al., 2007; Pasini et al., 2008), these defects were known to be involved in inherited PGL and PHEOs (King et al., 2011a; Raygada et al., 2011) but were not known to cause GISTs. The patients or their tumors (so called “wild-type” or WT GISTs) did not have mutations of the *KIT* or platelet-derived growth factor receptor-alpha (*PDGFRα*), genes that have been associated with GIST development (Matyakhina et al., 2007; Pasini et al., 2007). *SDHx* mutations are now found in up to 12% of all patients with WT GIST (Janeway et al., 2011). In addition, *SDHB* mutations were studied in pediatric PHEO and PGL patients in a series of studies that have changed the way children with these tumors are approached (Timmers et al., 2008; Lodish et al., 2010; Pasini et al., 2008; King et al., 2011a; King et al., 2011b). It is possible that *SDHx* mutations may be present in other endocrine (and non-endocrine tumors): in a family with PHEO and PGLs due to an *SDHD* defect, we discovered a growth hormone (GH)-producing pituitary tumor that had LOH for *SDHD* and deficient mitochondrial complex-II activity (Xekouki et al., 2012a); this observation has led to an ongoing investigation of a series of families with pituitary tumors and PGLs or PHEOs uncovering what is essentially a new syndromic association (data not shown). Since we showed that SDHB immunostaining may be used as a diagnostic marker for all lesions with possible *SDHx* mutations (Gaal et al., 2011), this method is now being used as a screening tool for a number of lesions and syndromic associations.

Our efforts to identify the genetic defect involved in adrenal lesions associated with Carney Triad have not been successful so far. *SDHx* mutations are not present in other adrenal tumors (unpublished data) or in Carney Triad, as we already mentioned. Thus, SDH does not appear to be involved in MMAD/AIMAH as FH is, despite that (a) these two enzymes work in tandem in the Krebs cycle; and (b) other evidence supporting that hypoxia signaling pathways may be important in MMAD/AIMAH (Matyakhina et al., 2005; Almeida et al., 2011b). We have formed an international consortium to identify the genetic defect(s) in Carney Triad and this work is ongoing. The SOMATICS software for the mining of a genome-wide SNP dosage analysis of multiple primary tumors from a few patients with Carney Triad was a product of this work (Assié et al., 2008).

3. Mouse studies

Findings in mice have been very rewarding in the understanding of cAMP/PKA defects (Almeida et al., 2010; Tsang et al., 2010; Almeida et al., 2011a). Several tissue-specific KOs (neural crest, heart, pituitary, adrenal, thyroid) from the original *Prkar1a*^{loxP/lop} line have now either been published or are near completion (Jones et al., 2010b; Yin et al., 2008a; Sahut-Barnola et al., 2010; Jones et al., 2010a; Yin et al., 2008b, 2008c).

Prkar1a mouse studies

cAMP-dependent PKA abnormalities are the first cell biological effect of R1α haploinsufficiency (Kirschner et al., 2005; Griffin et al., 2004a, 2004b; Amieux and McKnight 2002; Nesterova et al., 2008; Robinson-White et al., 2006a, 2006b; Mavrikakis et al., 2006). Increased kinase activity in response to cAMP was found in all human and mouse tissues or cells carrying R1α defects but how this phenomenon was mediated: was it PKA-type imbalance (type I vs. type II PKA), decreased binding to the catalytic subunits (and if yes, which one), or both that were essential for tumor formation?

3.1. The *Prkaca*^{+/-} x *Prkar1a*^{+/-} mice

Molecular investigations such as the study of the *RIα*Δ 184–236 (*RIα*del6 mutant) in human cell lines indicated that *Ca* inhibition was the main role of *RIα* subunit (Meoli et al., 2008), consistent with more recent data (Linglart et al., 2011; Willis et al., 2011). Thus, we hypothesized that reduction of *Ca* would lead to reduced PKA activity and fewer tumors in *Prkar1a*^{+/-} mice. Two tissue-specific *Ca* variants, *Ca*1 and *Ca*S, are encoded by alternative use of 2 exons located 5' to exon 2 in the *Ca* gene: *Ca*1 is ubiquitously expressed and *Ca*S is seen only in sperm (Vetter et al., 2011). There are numerous splice variants of the *Cβ* gene but only the *Cβ*1 variant is ubiquitously expressed, while the *Cβ*2 variant is mostly associated with lymphoid, and the *Cβ*3 and *Cβ*4 variants with neural tissues; *Cγ*'s presence is limited (Howe et al., 2002; Zhang and Daakka., 2011). Thus, PKA activity in most tissues (other than brain, kidney and spleen where there is *Cβ* activity) is *Ca*-dependent (Skålhegg et al., 2002).

We obtained the *Prkaca*^{+/-} mice from Dr. S. McKnight's laboratory (Skålhegg et al., 2002); the *Prkaca*^{-/-} mouse is viable only in small numbers and in backgrounds other than C57BL/6. The few *Prkaca*^{-/-} mice that survive neonatal death are small (weigh about 65% of their wild-type littermates) and infertile (Skålhegg et al., 2002). A cross between *Prkaca*^{-/-} and *Prkar1a*^{+/-} showed that absence of *Ca* can abrogate some of the early heart development effects of *RIα*'s absence (at E7.5) (Amieux and McKnight 2002); *Prkaca*^{-/-} *Prkar1a*^{+/-} embryos still died at E9.5 (Amieux and McKnight 2002). None of the *Prkar1a*^{+/-} *Prkaca*^{+/-} mice showed schwannomas or thyroid tumors (Tsang et al., 2010) seen in the *Prkar1a*^{+/-} mice (Kirschner et al., 2005). However, multiple bone lesions developed in the *Prkar1a*^{+/-} *Prkaca*^{+/-} mice: they first appeared at 4–5 months of age; 90% of *Prkar1a*^{+/-} *Prkaca*^{+/-} mice exhibited these lesions by 6 months, and 100% by 9 months. *Prkar1a*^{+/-} *Prkaca*^{+/-} mice not only developed these lesions earlier but also showed an increased number of lesions when compared to *Prkar1a*^{+/-} mice. Osteochondromyxoma, cartilaginous metaplasia, chondromas and osteochondrodysplasia were observed in up to 1/3 of the long bones (femur, tibia) and in most of the vertebral bodies (up to 23% of the spinal column and 100% of the caudal vertebrae) (Tsang et al., 2010).

The bone lesions were reminiscent of fibrous dysplasia (FD) (Riminucci et al., 1997): Osteoblast-like cells lined along the trabecular bone in younger *Prkar1a*^{+/-} *Prkaca*^{+/-} mice (at 3–4 months of age), and then gradually, with advancing age, filled the marrow with loosely arranged collagenous connective tissue and fibroblastoid cells (Figure 3). Progression included an increase in active fibroblasts arranged in a uniform array parallel to the axis of the vertebrae. Cells always expanded from the same location in the diaphyseal region: immediately under the growth plate, from the endosteal surface of the proximal periosteum and nearby trabecular bone. Abundant amounts of loosely arranged collagenous connective tissue filled the marrow spaces. Lesions from *Prkar1a*^{+/-} *Prkaca*^{+/-} mice were also more cellular and contained more, albeit irregular, cartilage or bone islands. Despite active proliferation and new bone formation there was an overall undermineralization in both *Prkar1a*^{+/-} and *Prkar1a*^{+/-} *Prkaca*^{+/-} that was further pronounced in the latter. A previously undocumented observation was that the single heterozygote, *Prkaca*^{+/-} mice showed an overall gain in bone formation that was derived from primarily cortical bone; on the other hand, trabecular bone in *Prkaca*^{+/-} mice was decreased. In 6-month-old *Prkar1a*^{+/-} *Prkaca*^{+/-} animals, Raman microspectroscopy (RMS) showed that the unaffected bones had the expected lamellar/fine-fibered bone at the midline and woven bone at other sites; in affected bones, these patterns were replaced by mineralized material that had an intermediate, mixed organization. Cells from the lesions were of the osteoblast lineage, arrested at a partially differentiated stage, or bone stromal cells (BSC), as in other FD-like lesions (Riminucci et al., 2006; Bianco et al., 1998). They expressed alkaline phosphatase and *Runx2*, osteocalcin (weakly), and they were negative for osteopontin. Cells lining newly formed bone were

more mature looking, almost fully differentiated osteoblasts. Osteoclasts were also activated in the lesions. Despite the absence of one *prkaca* allele, there was an overall increase in PKA activity in bone tumors, mediated mainly by PKA-II. Tumor cells showed an induction in the expression of *Prkx* and *Cβ1* and a reduction in *Cβ2* when compared with normal bone tissue. When tumors from *Prkar1a*^{+/-} mice were compared to those of *Prkar1a*^{+/-}*Prkaca*^{+/-} animals, the latter had a higher expression of *Cβ2*, as well. The gene signature of the tumor cells showed high levels of mesenchymal markers, like *n-cadherin*, *vimentin*, *snail1*, *twist*, *mmp2*, *mmp9*, *tgfb1* and *coll1a1*. What appeared to be the most upregulated molecular pathway in BSCs was that of the *Wnt* signaling with increased expression of *brachyury* (the *T* gene), *Wnt* ligands (*Wnt3a*, *Wnt7a*, *Wnt8a*) and down-regulation of *Wnt* inhibitors, such as *Dkk1*. Increased *Wnt* signaling was consistent with our previous data (Almeida and Stratakis, 2011; Almeida et al., 2011b; Almeida et al., 2012), including somatic β -catenin (*CTTNB1*) mutations in patients with germline *PRKAR1A* defects (Tissier et al., 2005; Gaujoux et al., 2008; Tadjine et al., 2008) and data from others (Berthon et al., 2012).

3.2. The *Prkar1a* adrenal-specific KO (AdKO) mouse

A mouse was produced by the group of Dr. Martinez in France with targeted *Prkar1a* gene inactivation in adrenocortical cells by mating the *Prkar1a* floxed mice with the *Akr1b7-Cre* expressing mouse line (that allowed for specific gene ablation in adrenal steroidogenic cells). Adrenal cortex-specific *Prkar1a* KO mice (AdKO) developed Cushing syndrome (Sahut-Barnola et al., 2010) due to abnormal adrenocortical cell differentiation and hyperplasia.

Hyperplasia in the AdKO mouse was shown to be caused by improper maintenance and subsequent expansion of fetal adrenocortical cells in adult adrenal glands (Sahut-Barnola et al., 2010; de Joussineau et al., 2012), consistent with the observations in iMAD and PBAD (Carney et al., 201; Carney et al., 2011) and the BSC expansion and bone tumors in *Prkar1a*^{+/-}*Prkaca*^{+/-} mice (Tsang et al., 2010). Pointing to the significance of steroidogenic studies in adrenal hyperplasias (where we now obtain LC/MS/MS data), the AdKO mouse adrenals produced cortisol (in addition to corticosterone) due to continuing expression of the usually exclusively fetal (in the rodent adrenal) *Cyp17* (Sahut-Barnola et al., 2010).

3.3. The *Prkar1a*^{+/-} x *Trp53*^{+/-}, *Prkar1a*^{+/-} x *Rb1*^{+/-} mice, and *Prkar1a*^{+/-} mice treated with chemicals

We performed these studies to (1) test our hypothesis that *Prkar1a* deficiency is a generic (albeit relatively weak) tumorigenic signal; and (2) identify signaling pathways that are altered in tumors that are *Prkar1a*-deficient regardless of other alterations.

The data (Almeida et al., 2010) showed that not only *Prkar1a* loss increases tumors in almost all tissues where lesions form due to other defects (like in *Tp53* or *Rb1*) (Figure 8), but also that these lesions shared increased *Wnt* signaling which emerged, once again, as a mediator of PKA signaling-induced tumorigenicity (Almeida and Stratakis, 2011; Almeida et al., 2010), albeit probably preceded by other molecular events (see below).

3.4. The role of cyclooxygenase 2 (COX2) and prostaglandin E2 (PGE2) in mouse bone tumor cell proliferation

Patients with neonatal onset multisystemic inflammatory disease (NOMID) due to inflammasome defects develop FD-like bone lesions, almost identical to those of *Prkar1a*^{+/-}*Prkaca*^{+/-} mice (Hill et al., 2007). We studied NOMID bone lesions and found PKA activation (Figure 4) and high PRKACB expression (Almeida et al., 2011a). *Prkar1a*^{+/-}*Prkaca*^{+/-} bone tumors demonstrated high expression of caspase-1 and interleukin 1 β (IL1B) (Almeida et al., 2011a); activation of IL1B is the end-result of inflammasome

defects leading to NOMID (Feldmann et al., 2002). COX2 stimulation and PGE2 overproduction by IL1B are well known (Park et al., 2006), we then found high PGE2 levels in *Prkar1a*^{+/-}*Prkaca*^{+/-} bone tumors.

The PKA-induced activation of the inflammasome in mice with PKA defects was the first such demonstration. It also led us to recognize that high PGE2 was most likely the reason for the higher cAMP levels in the bone lesions of these mice (Tsang et al., 2010; Kirschner et al., 2005; Almeida et al., 2011a). COX2 activation and high PGE2 provide yet another connection to Wnt signaling (Kleiveland et al., 2008; Goessling et al., 2009; Grösch et al., 2001; Maier et al., 2005; Takahashi-Yanaga and Kahn, 2010), PGE2-driven cAMP levels (Zhang and Daaka, 2011; Biddulph et al., 2000; Regan, 2008; Blackwell et al., 2010; Qian et al., 2011; Chun et al., 2010) create a local feedback loop within the bone microenvironment and may explain the similarities of these lesions with FD. They also opened a new window: COX2 inhibitors may be used for treating PKA-induced/FD-like lesions in humans (Takahashi-Yanaga and Kahn, 2010).

3.5. The *Prkar1a*^{+/-} x *Prkar2a*^{-/-} and *Prkar1a*^{+/-} x *Prkar2b*^{-/-} mice

Since R1β is primarily expressed in brain (*Prkar1b*^{-/-} mice have nociceptive pain and long-term potentiation defects and depression) (Brandon et al., 1995), and none of our studies so far have suggested its involvement in tumor formation, we did not pursue it. The *Prkar2a*^{-/-} mouse has a normal early phenotype; R1α appeared to compensate for total PKA activity but long-term follow-up data are lacking (Burton et al., 1997; Rao et al., 2004). The *Prkar2b*^{-/-} mouse was lean, with resistance to diet-induced obesity and abnormal behaviors (Cummings et al., 1996; Czyzyk et al., 2008). R1α is overexpressed at the protein level in *Prkar2b*^{-/-} mice which consequently have higher PKA-I activity and are not known to develop any tumors (Cummings et al., 1996). Work on the crosses of these two mouse models with *Prkar1a*^{+/-} that was suggested previously is ongoing, including investigation of the metabolic phenotypes.

4. Conclusions

Data in the last few years have pointed to the clinical and molecular heterogeneity of adrenal hyperplasias and related tumors. The identification of *PRKARIA*, *PDE11A*, *PDE8B* mutations in adrenocortical lesions and of defects of mitochondrial oxidation leading to a variety of tumors represent significant advances. New phenotypes and their genetic defects were recognized, and the complexity of signaling pathways involved in their pathogenesis was revealed. However, in a host of pathologies, from iMAD to Carney Triad, the responsible genes are yet to be identified. Perhaps the most significant advance was our recent understanding from the study of human tissues and the use of animal models that at least certain endocrine tumors, such as those initiated by *PRKARIA* defects in the adrenal and the bone, may be due to the abnormal proliferation of pluripotential cells. These cells reside in tissues and although they share features with fetal stem cells (Tsang et al., 2010; Almeida et al., 2011a; de Joussineau et al., 2012), their origin and how they give rise to tumors (Figure 5) in a variety of tissues are debated (Ricci-Vitiani et al., 2007; Nishimura et al., 2005; Grachtchouk et al., 2011; Liu et al., 2011; Wicha et al., 2006; Borovski et al., 2011). Both *Wnt* and *Hedgehog* signaling and other pathways are essential for tumor stem cell maintenance and/or proliferation (Liu et al., 2006; Klaus and Birchmeier, 2008). The identification of new genetic defects, the elucidation of the role of other PKA subunits and PDEs, and the focus on these pluripotential cells represent a natural continuation of our studies in the future.

Abbreviations

BAH	bilateral adrenal hyperplasia
MMAD/AIMAH	massive macronodular adrenocortical disease/ACTH-independent adrenocortical hyperplasia
ACC	adrenal cortical carcinoma
ACA	adrenal cortical adenoma
ACT	adrenal cortical tumor
PRKAR1A	regulatory subunit of cAMP-dependent PKA
PPNAD	primary pigmented nodular adrenocortical disease
PDE	phosphodiesterase
iMAD	isolated micronodular adrenocortical disease
TSPCs	tissue-specific pluripotential cells
GIST	gastrointestinal stromal tumors
SDH	succinate dehydrogenase
GRA	glucocorticoid-remediable aldosteronism
HLRC	hereditary leiomyomatosis and renal cancer
LOH	Loss-of-heterozygosity
CREB	cAMP response element (CRE)-binding protein
NOMID	neonatal onset multisystemic inflammatory disease

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Highlights

- cAMP signaling affects cell growth, development, and tumor formation in cAMP-sensitive tissues
- primary bilateral adrenal hyperplasias are genetically heterogeneous; most have defects in cAMP
- Tissue-specific pluripotential cells may initiate tumor formation in cAMP-sensitive tissues

Figure 1.

Three iMADs from 3 different patients; none was inherited, there were no *PRKARIA*, *PDE11A*, *PDE8B*, or *GNAS* defects, and they showed varying degrees of hyperplasia, pigmentation and nodularity.

Figure 2.

PBAD due to a *GNAS* mutation (Carney et al., 2011): the arrow points to hyperplastic cortex. Histology, to the right, shows variegated appearance with alternating areas of hyperplasia interspersed with areas of cortical atrophy. PBAD appears to be forming from expansion of fetal islets that are supposed to involute as the adult cortex forms.

Figure 3. Osteoblast-like cells started filling the marrow in young animals (ovoid); they always expanded from the same location in the diaphyseal region, immediately under the growth plate, from the proximal periosteum and nearby trabecular bone.

Figure 4.

A. High expression of pre-osteoblastic transcription factor Ets1 in *Prkar1a^{+/}Prkaca^{+/}* bone tumors. PKA catalytic subunits increased Ets1 expression; Ets1 activates caspase 1 (casp1) and other components of the inflammasome. **B.** Increased casp1 expression in *Prkar1a^{+/}Prkaca^{+/}* bone tumors against those of the *Prkar1a^{+/}* mice; IL1B was also highly expressed in *Prkar1a^{+/}Prkaca^{+/}* bone tumors, especially against bone tissue from the *Prkaca^{+/}* mice. **C.** Increased PGE-2 levels in the cells from bone tumors from the *Prkar1a^{+/}Prkaca^{+/}* and the *Prkar1a^{+/}* mice, against MC3T3 osteosarcoma cells. **D.** Effect of Ac-YVAD-CMK, a selective and irreversible casp1 inhibitor, on the proliferation of MC3T3 osteosarcoma cells and those from bone lesions from *Prkar1a^{+/}Prkaca^{+/}* and *Prkar1a^{+/}* mice; the proliferation of *Prkar1a^{+/}Prkaca^{+/}* cells was significantly inhibited. PGE2 and cAMP levels also decreased (data not shown) in response to the casp1 inhibitor.

Figure 5.

Pluripotent cell-derived endocrine (and other) tumors, as some cancers, may be formed (lower panel) by stem cells or tissue-specific pluripotent cells (TSPCs). These cells differentiate and mature during life (upper panel), in fetal and then adult tissues, respectively, leaving always a small population of cells (light green color) within the organs/glands that are there retaining regenerative/healing capacity; it is possible that only the elderly tissues (last collection of cells in upper panel) do not retain substantial number of TSPCs and that may also be organ-specific. However, at any time, these pluripotent cells may become the origin of a benign (blue) or malignant (red) neoplasm, shown by the multiple arrows, as a result of genetic or epigenetic alterations. In other words, TSPC-derived lesions may originate in early life (lower panel) without these cells ever differentiating (lower panel) or could form later in life from the residual pluripotent cells in mature tissues (upper panel).