Published in final edited form as:

Mol Cell Endocrinol. 2016 February 5; 421: 82–97. doi:10.1016/j.mce.2015.11.031.

Mouse models of adrenocortical tumors

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Abstract

The molecular basis of the organogenesis, homeostasis, and tumorigenesis of the adrenal cortex has been the subject of intense study for many decades. Specifically, characterization of tumor predisposition syndromes with adrenocortical manifestations and molecular profiling of sporadic adrenocortical tumors have led to the discovery of key molecular pathways that promote pathological adrenal growth. However, given the observational nature of such studies, several important questions regarding the molecular pathogenesis of adrenocortical tumors have remained. This review will summarize naturally occurring and genetically engineered mouse models that have provided novel tools to explore the molecular and cellular underpinnings of adrenocortical tumors. New paradigms of cancer initiation, maintenance, and progression that have emerged from this work will be discussed.

Keywords

Adrenal cortex; adrenal tumor model; adrenocortical carcinoma; adrenal hyperplasia; gonadectomy-induced tumorigenesis; spontaneous adrenal tumor

1. Introduction

Adrenal neoplasms are commonly diagnosed endocrine findings (Young, 2007). While the incidence of adrenocortical tumors (ACTs) is relatively high, affecting an estimated 3% to 7% of the population, most are benign adenomas. However, even in non-malignant tumors, hormonal hyperfunction can lead to significant morbidity. In addition to benign adrenocortical adenomas (ACAs), malignant carcinomas may also arise in the adrenal cortex. These rare tumors, adrenocortical carcinomas (ACCs), are highly aggressive and routinely fatal, largely due to the high proportion of patients diagnosed at an advanced stage

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Conflicts of interest

Authors declare no conflicts of interest.

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(Else et al., 2014). Surgical resection is therefore limited to a small cohort of ACC patients, and treatment is otherwise restricted to cytotoxic chemotherapy, radiation, and the adrenolytic drug mitotane. Given the prevalence and severity of ACAs and ACCs, respectively, the treatment and management of adrenal tumors remain a significant public health challenge.

Historically, mouse models have been essential for the study of adrenal tumorigenesis. Beginning in the early 1900s, discoveries in mice containing spontaneous or gonadectomy-induced adrenal tumors started to provide fundamental insights about cell growth and differentiation within the adrenal cortex. Later, new technologies allowed for the development of genetically modified models that focused on specific genes and pathways. These models have been and will continue to be critical for validating and interpreting the large amount of data emerging from the recent “omics” era. Finally, xenograft models in which human tumor tissue (primary or cell line-derived) is grown in immunocompromised mice have become more widely used in the adrenal field. These models are particularly well suited to study the heterogeneous nature of human ACCs. However, we will not address xenograft models further, as they will be discussed in greater detail elsewhere in this issue.

Here, we aim to provide a comprehensive overview of spontaneous and genetically modified mouse models of ACTs, including conditions of adrenal hyperplasia. We discuss the models in the context of the human pathology that they are most closely associated with, even though some models do not fully recapitulate the human disease. We describe key aspects of how each model was generated, the adrenal phenotype observed, and relevant implications for human health. Collectively, these models have provided valuable insights on the growth, differentiation, and transformation of the adrenal cortex, which are essential for the development of novel therapeutic strategies for the treatment of adrenal diseases.

2. Mouse models of adrenocortical hyperplasia

Adrenocortical hyperplasia is a broad term that describes a group of conditions characterized by bilateral adrenal enlargement. Unlike the majority of ACTs, which are unilateral, monoclonal, and sporadic, the bilateral nature of hyperplasia is consistent with a polyclonal origin (Beuschlein et al., 1994, Diaz-Cano et al., 2000). Causes of hyperplasia include inherited genetic syndromes, sporadic/idiopathic forms, and overstimulation of the adrenal cortex by extrinsic factors such as adrenocorticotrophic hormone (ACTH) (Xing et al., 2015). Adrenocortical hyperplasia can be functionally classified as hormonally silent or actively producing steroids. Among the latter group, cortisol, aldosterone, and androgen-secreting forms have been documented (Ghayee et al., 2011, Piaditis et al., 2015, Stratakis et al., 1998). Although adrenocortical hyperplasia encompasses a heterogeneous group of diseases, specific molecular pathways are commonly dysregulated. In particular, most cases of cortisol-secreting hyperplasias have abnormal activation of the protein kinase A (PKA) pathway, while aldosterone-secreting forms are characterized by abnormal calcium-calmodulin dependent kinase (Ca^{2+} -CAMK) signaling (Stratakis, 2013, Zennaro et al., 2015). Physiologically, these molecular pathways are key regulators of cortisol and aldosterone production, respectively. In humans, adrenal hyperplasia can also occur in the context of rare multiple neoplasia syndromes, such as MEN1, Carney complex,

adenomatous polyposis coli, or McCune-Albright syndrome (Lerario et al., 2014). Several transgenic mouse models have been developed to recapitulate different types of human adrenocortical hyperplasia. In the following section, we summarize these models and discuss the molecular aspects of each that led to their adrenal manifestations (Table 1).

2.1 ACTH-dependent hyperplasia

As previously mentioned, chronic overstimulation of the adrenal glands by ACTH results in bilateral adrenal enlargement due to hyperplasia within the adrenal cortex. Among the causes of ACTH-dependent adrenocortical hyperplasia are Cushing's disease (ACTH-producing pituitary adenoma), ectopic ACTH syndrome, and a group of diseases broadly known as congenital adrenal hyperplasia (CAH) (Xing et al., 2015). CAH is characterized by inherited enzymatic defects in steroid hormone biosynthesis that result in cortisol deficiency. This loss of negative feedback on the hypothalamic-pituitary axis results in increased ACTH production, subsequent adrenal enlargement, and increased steroidogenic activity with accumulation of steroidogenic precursors. In humans, the most common form of CAH is 21-hydroxylase deficiency. Although mouse models of ACTH-dependent adrenal hyperplasia exist (Caron et al., 1997, Mullins et al., 2009, Riepe et al., 2005), they are beyond the scope of this review.

2.2 ACTH-independent hyperplasia

2.2.1 Aldosterone-producing hyperplasia—Primary aldosteronism, defined as autonomous aldosterone secretion by the adrenals, is the leading cause of secondary hypertension, with an estimated prevalence of ~10% among hypertensive patients (Zennaro et al., 2015). Causes of primary aldosteronism include aldosterone-producing adenomas (APA), bilateral sporadic adrenal hyperplasia, and familial hyperaldosteronism types I–III. The majority of cases are sporadic, since familial hyperaldosteronism is very rare. Furthermore, it has been demonstrated that abnormal activation of Ca^{2+} -CAMK signaling, which results in transcriptional activation of the aldosterone biosynthetic machinery, is the underlying molecular abnormality in both APAs and hyperplasias. Somatic and germline molecular defects in adrenal cells have been described in several ion-transporting membrane proteins, including potassium channels (*KCNJ5*), voltage-gated calcium channels (*CACNA1D* and *CACNA1H*), and Na^+/K^+ pumps (*ATP1A1* and *ATP2B3*). These defects ultimately lead to increased cytoplasmic Ca^{2+} and abnormal activation of CAMK, causing autonomous secretion of aldosterone and cell proliferation. Mouse models that recapitulate these defects have contributed substantially towards our understanding of how the regulatory mechanisms of calcium homeostasis become disrupted.

TASK1/TASK3 mouse models: TASK1 (KCNK3) and TASK3 (KCNK9) are two-pore domain potassium channels that play an important role in the maintenance of the highly polarized state of the cell membrane of adrenocortical cells. These channels form heterodimers that allow a high transmembrane background potassium conductance, which maintains an electronegative gradient across the cell membrane. The activity of these channels is inhibited by angiotensin-II, which binds to its AT1 receptor and promotes activation of phospholipase C, leading to generation of inositol-triphosphate (IP3) and diacyl-glycerol (Bandulik et al., 2015). The resulting fluctuations in the electronegative

gradient across the membrane then activate voltage-gated calcium channels that lie in the cell membrane, allowing a calcium influx into the cytoplasm. IP₃ also promotes release of calcium from the endoplasmic reticulum. In addition to angiotensin-II, fluctuations in extracellular potassium levels cause membrane depolarization and activation of calcium channels. Increased cytoplasmic calcium activates the CAMK pathway, which ultimately promotes aldosterone production and proliferation (Zennaro et al., 2015).

Several mouse models of *Task1/Task3* inactivation have been generated. Heitzman et al (Heitzmann et al., 2008) characterized the adrenal phenotype of the previously developed whole-body *Task1* knockout (KO), in which the first exon of *Task1* was disrupted by homologous recombination in embryonic stem cells (ESCs) (Aller et al., 2005). These mice exhibited an adrenal zonation defect and severe hyperaldosteronism, characterized by high expression of aldosterone synthase (*Cyp11b2*) in the zona fasciculata (zF), but not in the zona glomerulosa (zG) (Heitzmann et al., 2008). Remarkably, expression of *Cyp11b1* and corticosterone production were not affected. Interestingly, as the animals aged, the zonation defect and hormonal abnormalities persisted only in the females. Compensatory expression of *Task3* and other channels in male mice reversed the abnormal phenotype after puberty, suggesting an effect of the male hypothalamic-pituitary-gonadal (HPG) axis in restoring potassium conductance. Moreover, treatment of the female *Task1* KO mice with testosterone restored normal zonation and reversed their hyperaldosteronism, implicating elevated androgens (or perhaps the resultant down regulation of pituitary luteinizing hormone) in the observed defects. Electrophysiological studies performed on adrenal primary cultures further showed membrane polarization abnormalities, secondary to decreased potassium conductance.

Task3 inactivation (*Task3* KO mice) (Guyon et al., 2009) similarly revealed severe hyperaldosteronism in newborn mice with additional increases in both progesterone and corticosterone, consistent with widespread cortical dysfunction (Bandulik et al., 2013). Increased aldosterone production and increased responsiveness to angiotensin-II were also observed in electrophysiological studies (Guagliardo et al., 2012). However, similar to *Task1* KO mice, adult mice exhibited a much milder phenotype (Guagliardo et al., 2012, Lazarenko et al., 2010), again consistent with compensatory mechanisms of potassium homeostasis. These results suggested that the *Task3* KO model might more closely recapitulate a human condition known as low-renin hypertension, which is thought to be a mild form of primary aldosteronism. Penton et al (Penton et al., 2012) further characterized this model and demonstrated that under a low sodium/high potassium diet, aldosterone secretion was normal. However, under a high sodium/low potassium diet, KO animals failed to suppress aldosterone secretion and exhibited significantly higher aldosterone-to-renin ratios than controls. A significantly decreased membrane resting potential in the glomerulosa cells paralleled this abnormal aldosterone secretion.

Davies et al (Davies et al., 2008) characterized a double *Task1;Task3* whole-body KO mouse in which the second exons of *Task1* and *Task3* were disrupted by homologous recombination in ESCs (Mulkey et al., 2007). Largely in accordance with the observations of the single *Task1* and *Task3* KO mice, the double KO mice exhibited electrophysiological abnormalities consistent with loss of activity of these channels and developed severe

primary aldosteronism. Remarkably, aldosterone secretion was exquisitely sensitive to mild fluctuations in angiotensin-II levels, consistent with the inability to regulate potassium homeostasis in the absence of both *Task1* and *Task3*.

Together, mouse models of *Task1* and *Task3* inactivation recapitulate important functional aspects of human primary aldosteronism. Although hyperplastic or nodular growth is not observed, these mice develop autonomous, renin-independent aldosterone secretion, not suppressible by high sodium intake, and exquisitely sensitive to angiotensin-II. In addition, these models provide strong evidence for the critical role of membrane polarization and dynamic ionic changes in the regulation of aldosterone secretion. Thus, these models have helped to establish a new paradigm in the physiopathology of primary aldosteronism.

2.2.2 Cortisol-producing hyperplasias—ACTH-independent cortisol producing adrenocortical hyperplasias are a rare cause of ACTH-independent Cushing syndrome, collectively responsible for <10% of cases (Bourdeau et al., 2007). Based on the morphological aspects of the adrenal lesions, cortisol-producing hyperplasias can be broadly classified into micronodular and macronodular forms (Stratakis, 2008). A common feature of virtually all forms of cortisol-producing adrenocortical hyperplasia is the abnormal activation of the PKA pathway (Stratakis, 2013). PKA is a serine-threonine kinase that is the main mediator of cAMP signaling in mammals. The PKA holoenzyme is a tetramer constituted by two catalytic and two regulatory subunits. In the adrenal glands and other endocrine organs, the most important catalytic and regulatory subunits are PRKACA and PRKARIA, respectively (de Jossineau et al., 2012). In adrenocortical cells, activation of the ACTH receptor (MC2R) by its cognate ligand increases the activity of a membrane-bound heterotrimeric G protein, which in turn activates adenylate-cyclase, the enzyme that converts ATP to 3'-5'-cAMP. Upon binding of cAMP to its regulatory subunits, the PKA holoenzyme complex dissociates, releasing the catalytic subunits, which in turn phosphorylates the cAMP response element-binding protein (CREB), the transcription factor that mediates ligand-induced gene expression in target tissues. In the adrenal gland, CREB activation participates in the stimulation of steroidogenesis (in particular cortisol production) and in the activation of an ACTH-dependent proliferative response. Once ACTH is no longer present, cAMP is inactivated by phosphodiesterases and the PKA holoenzyme is reassembled, terminating pathway activation. Several molecular defects in PKA components have been described in different forms of adrenal hyperplasia. While inactivating germline mutations of *PRKARIA* and the phosphodiesterases *PDE8E* and *PDE11A* have been described in micronodular hyperplasia, activating mutations in *GNAS* and overexpression of “illicit” receptors (G protein-coupled receptors that do not normally regulate cortisol secretion) are often present in macronodular forms (Stratakis, 2013). Recently, germline *ARMC5* mutations were described in up to 50% of cases of primary adrenocortical macronodular hyperplasia (Alencar et al., 2014, Assie et al., 2013). The exact function of *ARMC5* and how it contributes to the overexpression of illicit receptors is currently unknown, but it is thought to function in part as tumor suppressor gene, since a second somatic hit is usually present.

PRKARIA mouse models: Germline inactivating mutations of *PRKARIA* cause a multiple endocrine neoplasia syndrome (MEN) known as Carney complex (CC), an autosomal dominant syndrome comprised of spotty skin pigmentation, cardiac myxomatosis, endocrine manifestations (pituitary adenomas, adrenocortical micronodular hyperplasia, thyroid and gonadal tumors), and schwannomas (Salpea et al., 2014). Specifically, the adrenal manifestation associated with CC is known as primary pigmented nodular adrenocortical disease (PPNAD). This type of adrenal hyperplasia is characterized by the presence of cortisol-secreting bilateral adrenal micronodules (< 1 cm) that are characteristically pigmented due to accumulation of lipofuscin. Since some CC-associated tumors exhibit LOH of the locus, *PRKARIA* is thought to be a tumor-suppressor gene. More recently, somatic inactivating mutations of *PRKARIA* have also been identified in ACTs (Espiard et al., 2014, Zheng et al., 2015).

Since 2002, several mouse models that disrupt *Prkar1a* by different strategies have been developed, including two conventional whole-body transgenic mice, a tetracycline-regulated antisense RNA, and several tissue-specific KO models. In the conventional KO models, homozygous embryos exhibited early lethality by E8.5–10.5 due to a generalized failure to develop mesodermal structures (Amieux et al., 2002, Kirschner et al., 2005). While heterozygous embryos were viable and developed a spectrum of manifestations that highly overlap CC, no adrenal phenotype was observed. To circumvent the early embryonic lethality seen with homozygous *Prkar1a* inactivation, a tetracycline-induced antisense RNA was developed (Griffin et al., 2004). In this model, the antisense RNA was under the control of a Tet-Off system called rTA/X2AS. In the presence of doxycycline, expression of the antisense RNA was inhibited, thus allowing expression of *Prkar1a*. Importantly, treatment of pregnant female mice with doxycycline rescued the offspring from embryonic lethality. Upon cessation of doxycycline treatment, the antisense RNA was activated and expression levels of *Prkar1a* decreased 70% over baseline. Additional biochemical analyses in the liver and adrenal gland demonstrated significantly increased PKA activity in the rTA/X2AS mice. Several neoplastic and endocrine manifestations were evident in these mice as early as 6 months, including lymphoproliferative disease, mesenchymal tumors, and histological changes in the thyroid and adrenal glands. Adrenocortical manifestations included cortical hyperplasia with pigmented deposits and X-zone persistence in both male (after puberty) and female (after pregnancy) mice, suggesting that abnormal PKA activity may influence adrenal differentiation and that PPNAD lesions might originate from (or involve) fetal precursors. The transgenic animals also exhibited increased corticosterone production, leading to increased body weight and fat. The neoplastic manifestations of this model were similar to human patients, except for hematological malignancies, which are not part of CC. In addition, the phenotype was very similar to *Prkar1a*^{+/-} mice with inclusion of the aforementioned adrenal defects. Similar to CC patients, LOH of the *Prkar1a* locus was detected in most lesions.

Largely in agreement with these observations, Sahut-Barnola et al later developed an adrenal-specific *Prkar1a* KO mouse by crossing *Prkar1a*-floxed mice with *Akr1b7*-Cre mice (AdKO mice) (Sahut-Barnola et al., 2010). As expected, these mice developed hormone excess (ACTH-independent hypercortisolism) and adrenal hyperplasia.

Furthermore, an age-dependent centrifugal expansion of hypertrophic eosinophilic cells was seen, which was first detected by 5 months of age. By 10 months, the entire cortex was occupied and normal zonation was highly disrupted. Immunohistochemically, these cells did not stain for nuclear β -catenin but did express steroidogenic factor 1 (SF1), GATA4, and the X-zone marker 20- α -hydroxysteroid dehydrogenase (HSD), suggesting a fetal origin. Remarkably, in contrast with a normal X-zone cell, AKR1B7 immunostaining was also positive. Moreover, these abnormal cells were not affected by puberty in males or pregnancy in females, and male mice developed a much milder phenotype.

Together, the various mouse models of *Prkar1a* inactivation have provided new insights about the molecular pathogenesis of CC. In particular, these models have validated an important role for PKA pathway activation during tumorigenesis in a dose-dependent manner. Additionally, these models suggest that the expansion of fetal precursor cells may contribute to CC-related adrenocortical hyperplasia.

MEN1 mouse models: Multiple neoplasia type 1 (MEN1) is an autosomal dominant syndrome characterized by development of tumors of the parathyroids, pancreatic islets, and anterior pituitary (Agarwal, 2013). Other manifestations include typical skin lesions, carcinoid tumors, and bilateral adrenocortical macronodular hyperplasia. Germline mutations in *MEN1*, located at 11q13, are present in ~75% of patients. *MEN1* is a classical tumor suppressor gene and inactivation of the second allele (LOH) is invariably present in MEN1-associated tumors. The typical adrenal manifestation of MEN1 is a bilateral enlargement of the adrenal cortex with macronodular features, histologically resembling primary macronodular adrenocortical hyperplasia (PMAH). This manifestation occurs in ~20% of MEN1 patients (Gatta-Cherifi et al., 2012). While these nodules are usually benign, malignant transformation is not an infrequent finding. Additionally, 11q13 LOH has been reported in sporadic ACC (Kjellman et al., 1999), and more recently, *MEN1* has been identified as a significantly mutated gene by molecular profiling studies (Assie et al., 2014). The exact function of the protein encoded by *MEN1*, menin, has been the focus of several recent studies. Menin is a nuclear scaffold protein that regulates gene transcription and chromatin remodeling, interacting with several transcription factors, including JunD, NFKB1 and SMAD3 (Agarwal, 2013). It is currently unknown why endocrine tissues are especially prone to tumor development in MEN1 patients.

Several *Men1* KO mice have been reported, including whole-body and tissue-specific models. However, since no adrenal cortex-specific *Men1* knockouts have been generated, we will discuss adrenocortical manifestations of the whole-body KO models. These models all utilized a similar strategy to target exons 1–8 in ESCs and overall reported very similar findings (Bertolino et al., 2003, Crabtree et al., 2001, Harding et al., 2009, Loffler et al., 2007). Specifically, homozygous mice were not viable and exhibited embryonic lethality by E11.5–E12.5 due to multiple developmental abnormalities, including craniofacial, neural tube, and heart formation defects (Bertolino et al., 2003, Crabtree et al., 2003). In contrast, heterozygous mice were viable and developed a myriad of tumors that highly overlapped with the human phenotype. The spectrum of tumors developed by the mice included pituitary adenomas (reported to have a female predominance), pancreatic islet cell neuroendocrine tumors, extra-pancreatic gastrinomas, parathyroid tumors, thyroid nodules,

Leydig cell tumors, sex cord stromal cell tumors, and adrenocortical lesions. As expected, all tumors exhibited LOH, except for gonadal tumors. Interestingly, in contrast to other mouse models in which the incidence of ACTs is higher in females, some *Men1*^{+/-} models exhibited male predominance. The pathology of tumors in these mice progressed from hyperplasia to adenoma to carcinoma in an age-dependent manner. In the report by Crabtree et al, ACCs were present in 20% of male mice, but not in female mice (Crabtree et al., 2001). Bertolino et al reported enlarged adrenals, adrenocortical nodular hyperplasia, and adenomas in 9% of animals by 8–12 months (Bertolino et al., 2003). By 18 months, 46% of animals developed ACAs and ACCs. Similarly, Loffler et al reported abnormalities in the adrenal glands of 59% of the mice, ranging from bilateral adrenal enlargement to adenomas, but the incidence of adrenal tumors was similar in male and female mice (Loffler et al., 2007). On the other hand, a model developed by Harding et al found ACTs only in male mice (Harding et al., 2009). These tumors stained positive for 3-β-HSD, indicating that they were potentially capable of producing steroid hormones. In fact, one out of four mice in which serum was available for hormone determinations exhibited high corticosterone levels. In contrast, expression of 3-β-HSD was negative in adrenocortical hyperplasia samples. Overall, whole-body *Men1* KO mouse models nicely recapitulate the human MEN1 syndrome, exhibiting a similar spectrum of tumors and including the discussed adrenocortical lesions.

3. Mouse models of gonadectomy-induced adrenocortical neoplasia

Since the 1940s, prepubertal gonadectomy (GDX) has been known to induce ACTs in mice and ferrets. These lesions are malignant sex steroid-producing tumors thought to originate from the progenitor cell niche of the adrenal gland (Bielinska et al., 2006, Johnsen et al., 2006). Thus, GDX-induced adrenal tumor models offer unique insights about the regulation of adrenocortical progenitor cell growth and differentiation. Importantly, GDX uncouples endocrine signaling between the gonads and the hypothalamic-pituitary axis, resulting in elevated levels of luteinizing hormone (LH) and decreased levels of gonadal hormones including circulating inhibin (Bernichtein et al., 2009, Bielinska et al., 2003). As a result, several mouse models have combined GDX with genetic manipulation of inhibin-α (*Inha*), including either whole-body *Inha* KO (*Inha* KO) or transgenic expression of oncogenic SV40 from the *Inha* promoter (*Inha*/TAg) (Table 2).

While the downstream molecular events differ between the strains inherently sensitive to GDX and genetically modified GDX models, they all share a set of common features. Specifically, GDX results in chronic elevation of gonadotropin hormones and subsequent adrenal activation of a gonadal-enriched gene expression program (Looyenga et al., 2006). Most notable perhaps is the activation of the gonadal-specific *Gata4* transcription factor and inactivation of adrenal-specific *Gata6* (Johnsen et al., 2006, Krachulec et al., 2012, Looyenga et al., 2006). Granulosa and theca lineage genes are both represented, including the peptide hormone anti-Müllerian hormone (*Amh*), the hormone receptors LH receptor (*Lhcgr*) and *Amh* receptor (*Amhr2*), and the sex steroid biosynthetic enzymes *Cyp17a1* and P450 aromatase (*P450arom*). In addition to this gene expression profile, GDX-induced tumors also share common histological features. In particular, tumors are composed of spindle-shaped cells, called type “A” cells, and large, lipid-filled type “B” cells (Bielinska et

al., 2003). Type A cells are highly proliferative and form wedge-shaped extensions displacing normal adrenal cells in the adrenal cortex, while highly steroidogenic B cells develop later within patches of A cells and produce sex steroids – especially estrogen (Bielinska et al., 2005, Bielinska et al., 2006, Bielinska et al., 2003, Johnsen et al., 2006).

Together, the molecular and histological features observed in GDX-induced tumor models suggest that these gonadal-like neoplasias likely arise from multipotent adrenal progenitor cells. During development, adrenocortical and gonadal cells arise from a common precursor structure called the adrenogonadal primordium (AGP). Adrenal tumor formation following GDX suggests that the adrenal cortex retains a pluripotent population of progenitor cells with developmental plasticity to adopt a gonadal phenotype (Looyenga et al., 2006). Supporting this notion, recent reports have found that Wilms tumor suppressor gene 1 (WT1) defines a long-lived adrenocortical progenitor population that differentiates into gonadal steroidogenic tissue following GDX (Bandiera et al., 2013). The contribution of progenitor cells to normal adrenal homeostasis and the stress response is critical for adrenal function; thus, GDX models are important for studying the pathological neoplastic growth and lineage specification of adrenal progenitors *in vivo*.

3.1 Gonadectomy (GDX)-induced neoplasias in susceptible mouse strains

The history of GDX-induced ACTs in mice dates back to observations made by Woolley and Little, who first discovered that CE mice were highly susceptible to this type of neoplasia (Woolley et al., 1943). Since then, further studies have identified other susceptible mouse strains that develop adrenal tumors with near complete penetrance following GDX (DBA/2J, CE/J, C3H, NU/J, BALB/c and B6D2F1), as well as resistant strains (C57BL/6 and FVB/N) (Bielinska et al., 2003, Johnsen et al., 2006, Krachulec et al., 2012, Rosner et al., 1966, Woolley, 1945). Further characterization of GDX-induced adrenal neoplasias showed that tumorigenesis, while predicted to result from loss of a gonadal factor and/or increase in pituitary gonadotropins, was also mediated by intrinsic genetic attributes of susceptible lines (Bielinska et al., 2005, Bielinska et al., 2006, Röhrig et al., 2015). One particularly compelling study by Röhrig et al used GFP-labeled ES cells from a resistant strain (C57BL/6) and non-labeled ES cells from a susceptible strain (B6D2F1) to generate chimeric mice. Following GDX, adrenal neoplasms were only derived from GFP negative cells, suggesting that tumorigenesis occurred through a mechanism inherent to the GDX-sensitive strain (Röhrig et al., 2015).

To determine the genetic basis of strain susceptibility, linkage analysis has been performed in susceptible (DBA/2J) and resistant (C57BL/6J) mouse strains. These studies identified a significant quantitative trait locus on chromosome 8 containing the dominant negative Wnt inhibitor, *Sfrp1* (Bernichtein et al., 2009, Bernichtein et al., 2008), which was significantly down regulated in GDX-induced tumors. Corroborating this finding, increased Wnt signaling through mutations in regulatory components of the Wnt pathway is one of the most common genetic aberrations found in human ACC tumors and *SFRP1* expression is significantly repressed in pediatric ACTs (Leal et al., 2011). In conjunction with studies that defined Wnt signaling as an essential component of adrenocortical progenitor cells (Kim et

al., 2008), these observations implicate an important role for Wnt signaling in promoting adrenal tumorigenesis, both in GDX-induced and human tumors.

In addition to a potential role for *Sfrp1*, a polymorphism in *Nr5a1*, the gene encoding SF1, has also been identified in GDX-susceptible mouse strains. As discussed in greater detail later in this review, SF1 is a transcription factor required for adrenal and gonadal development (Luo et al., 1994). Moreover, several lines of evidence have demonstrated that *NR5A1* dosage is critically important for normal adrenal development and homeostasis (Val et al., 2007). Interestingly, the *Nr5a1-A172* allele segregated with mouse strains showing high steroidogenic capacity and resistance to GDX-induced adrenocortical neoplasms (Correa et al., 2012, Frigeri et al., 2002, Schimmer et al., 2002). Furthermore, this allele is associated with XY male-to-female sex reversal in C57BL/6 mice and is hypothesized to impart hypomorphic SF1 activity that compromises the timing and activation of testis differentiation during gonadal development (Correa et al., 2012, Munger et al., 2013). SF1 cooperates with GATA4 to further gonadal development, and human *NR5A1* mutations have been identified that ablate GATA4 interaction leading to incomplete male development and/or sex reversal (Tremblay et al., 2003). This suggests that gonadal differentiation in the adrenal neoplasias of GDX-susceptible mouse strains may in part reflect a dependence on SF1 activity, similar to what is observed in sex determination. Interestingly, *Nr5a1* dosage modulation by the transcription factors WT1 and Cited2 is also required for the initiation of adrenal development (Val et al., 2007). Thus, the sensitivity of adrenal multipotent progenitors to *Nr5a1* dosage may reflect combinatorial transcription factor activities that contribute to the adrenal versus gonadal differentiation phenotype observed in these models.

3.2 Genetically modified GDX models

3.2.1 Inhibin KO models of GDX-induced adrenocortical neoplasia—In addition to GDX-sensitive mouse strains, *Inha* KO mice represent a second model highly prone to GDX-induced adrenal neoplasia. Incidentally, these mice have been maintained in a ‘resistant’ mixed C57BL/6 background, indicating that adrenal-derived inhibin is a strong tumor suppressor in the adrenal gland and that the molecular mechanisms of tumorigenesis may diverge from the described GDX-sensitive lines (Burns et al., 2003, Kumar et al., 1996, Looyenga et al., 2007). Originally generated to study gonadal tumorigenesis, *Inha* KO mice develop gonadal sex cord tumors as early as 4 weeks of age, which is shortly followed by cachexia-like symptoms and death (Matzuk et al., 1994). This model has provided important insights about the relationship between adrenal inhibin and other members of the TGF β superfamily, including SMAD proteins.

Inhibin is a secreted peptide that participates in negative feedback inhibition of the HPG axis. While produced in both the gonad and adrenal cortex, inhibin classically functions as a gonadal hormone that together with gonadal sex steroids maintains normal gonadotropin levels by opposing activin-induced production and secretion of follicle-stimulating hormone (FSH) from the anterior pituitary gland. Removal of the gonads through castration disrupts this axis, leading to increased serum gonadotropins (Cook et al., 2004, Gregory et al., 2004). The adrenal glands of *Inha* KO mice are completely normal until GDX, at which time, gonadal lineage specification, uncontrolled expansion, and ultimate tumor formation occurs

– presumably due to the combination of elevated gonadotropins and loss of adrenal inhibin. Double transgenic mice lacking *Inha* and a functional GnRH receptor (hypogonadal hpg) do not develop gonadal or adrenal tumors and have suppressed LH and FSH levels (Kumar et al., 1996), suggesting that both hormones might be required for tumorigenesis. However, LH was later shown to be the key adrenal tumor-promoting gonadotropin, as loss of FSH alone did not prevent adrenal tumorigenesis (Kumar et al., 1999). Moreover, compound *Inha* KO:LH β -CTP mice, which express LH chimeric protein from the bovine LH β promoter, display 6.7 fold higher LH levels than *Inha* KO mice following GDX. These mice also have earlier onset adrenal tumorigenesis, increased tumor burden, and more rapid lethality compared to *Inha* KO mice (Beuschlein et al., 2003). Together, these studies confirm that LH is the major gonadotropin driving GDX-induced tumorigenesis in the absence of adrenal inhibin.

Inhibin and activin are members of the TGF β superfamily of cell signaling proteins that mediate cellular growth, differentiation, and apoptosis. Inhibin was initially defined as an antagonist of activin-mediated SMAD2/3 protein activation. It was predicted that loss of adrenal inhibin resulted in constitutive activation of signaling downstream of a TGF β superfamily member. This activation was hypothesized to cooperate with LH to mediate adrenal tumorigenesis. However, genetic ablation of *Smad2* in *Inha* KO mice did not alter development of gonadal tumors, suggesting that *Smad2* was not required for tumor progression (Rajanahally et al., 2010). However, phosphorylated SMAD3 was observed in the peripheral adrenal cortex of *Inha* KO mice following GDX (Beuschlein et al., 2003). This observation suggested that in adrenocortical progenitor cells, SMAD3 is a key downstream effector of TGF β activation that mediates tumorigenesis. Supporting this notion, heterozygous deletion of *Smad3* greatly delayed adrenal disease progression and improved survival of *Inha* KO mice (Li et al., 2007, Looyenga et al., 2007). Rather than unopposed activin signaling, further studies determined that elicited SMAD3 activation in *Inha* KO mice resulted from an LH-induced increase in *Tgf- β 2* and *Tgfbr1* expression in the subcapsular cortex, which was antagonized *in vitro* by the addition of inhibin (Looyenga et al., 2010). This mechanism of inhibin-mediated TGF- β 2 signaling was further studied in the Y1 adrenocortical cell line. These studies showed that inhibin assisted in clearance of betaglycan, a TGFBR1 co-receptor, from the cell surface, resulting in desensitization of TGF- β 2 signaling. Thus, these studies collectively support a mechanism in which elevated LH levels following GDX activates SMAD3 signaling through TGF- β 2, ultimately driving uncontrolled expansion of re-specified GATA4 expressing adrenocortical cells into gonadal theca and granulosa lineages (Looyenga et al., 2010).

Further studies have built on this paradigm and focused on the consequences of ectopic SMAD3 activation, particularly its effect on CCND2. This proliferative factor is part of a complex that regulates phosphorylation of retinoblastoma (Rb) protein and subsequent cell cycle progression from G1 to S phase. Previous studies have tied SMAD3 activation to *Ccnd2* expression through promoter transactivation with GATA4 (Anttonen et al., 2014, Sicinski et al., 1996) and stimulation by FSH and activin (Park et al., 2005). Supporting this notion, loss of *Ccnd2* in *Inha* KO mice attenuated tumor development following GDX (Burns et al., 2003). These studies suggest that increased FSH levels following GDX may

induce *Ccnd2* specifically in the peripheral adrenal cortex (where FSH receptors are located), thus supporting neoplasia in this region (Burns et al., 2003).

In summary, studies of *Inha* KO mice have demonstrated a critical role for adrenal inhibin in repressing LH-driven and TGF β 2-dependent expansion of adrenal progenitor cells that have adopted a GATA4 gonadal differentiation program. The associated mechanism of tumorigenesis in *Inha* KO studies is particularly interesting given the alterations in TGF- β signaling observed in human cancers (reviewed in (Elliott et al., 2005, Massagué, 2008).

3.2.2 Inhibin/TAg mouse models of GDX-induced adrenocortical neoplasia—

Similar to *Inha* KO mice, *Inha*/TAg transgenic mouse lines rapidly develop sex cord-stromal tumors and malignant ACTs upon GDX (Beuschlein et al., 2003, Kananen et al., 1996, Matzuk et al., 1994). This mouse model achieves malignant transformation through expression of the oncogenic SV40 T antigen virus under control of the *Inha* promoter. Like *Inha* KO mice, initiation and progression of adrenal neoplasms is gonadotropin-dependent, which has been demonstrated using several mouse lines. First, *Inha*/TAg mice lacking a functional GnRH receptor (hypogonadal hpg) do not develop gonadal or adrenal tumors (Rilianawati et al., 1998). In contrast, *Inha*/TAg mice crossed with LH β -CTP transgenic mice (Risma et al., 1995) develop both adrenal and gonadal tumors (Mikola et al., 2003). Interestingly, this occurs in the absence of GDX in female *Inha*/TAg;LH β -CTP mice. These observations may reflect gender-specific differences in LH levels, as female LH β -CTP mice express higher levels of LH than their male counterparts (Mikola et al., 2003). Taken together, *Inha* KO models and *Inha*/TAg transgenic mice share many aspects of adrenal and gonadal tumorigenesis, suggesting a set of downstream regulatory networks that guides the abnormal timing and expression of gonadal gene programs. Inhibin serves to prevent activation of this gonadal program in multipotent adrenocortical progenitor cells.

3.2.3 GATA factor-related mouse models of GDX-induced adrenocortical neoplasia—

The GATA family of transcription factors, which includes GATA4 and GATA6, are important for development and differentiation of multiple mesendodermal lineages. These factors all contain zinc-finger DNA-binding domains and function as potent inducers of cellular reprogramming and pluripotency (Shu et al., 2015). With respect to the AGP, several studies suggest that GATA4 expression guides gonadal differentiation, while GATA6 drives an adrenal-specific program. This is supported by mice containing SF1 targeted ablation of *Gata6*, which have accumulation of GATA4-expressing gonadal-like cells within the adrenal cortex (Pihlajoki et al., 2013). Furthermore, *Gata4/Gata6* double KO animals develop ectopic adrenal-like function in the testes (Padua et al., 2015). Taken together, these observations support a role for GATA4 and GATA6 in establishing and maintaining gonadal and adrenal cell identity, respectively.

GATA4 appears to modulate ACT progression following GDX (Krachulec et al., 2012). In particular, a GATA4 and WT1-positive cell population within the adrenal capsule has been shown to differentiate into gonadal-like steroidogenic tissues following GDX (Bandiera et al., 2013). To further interrogate this finding, a transgenic mouse model with ectopic *Gata4* expression in the zG driven by a 6.4 kb fragment of the murine *21-hydroxylase* (21-OH, *Cyp21a1*) gene promoter was generated in a ‘non-susceptible’ (C57BL/6) mouse strain

(Chrusciel et al., 2013). Intact female mice showed progressive adrenal neoplasia characterized by non-steroidogenic A cells that are GATA4-positive. Moreover, GDX in these animals triggered formation of ACAs containing both A and B cells, implicating LH and gonadotropin dysregulation in the production of steroidogenic neoplastic cells (Chrusciel et al., 2013). This study also suggested that these neoplasias arise in multipotent progenitor cells residing in the zG.

3.2.4 Summary and perspectives of genetically modified GDX models—

Genetically modified mouse models of GDX-induced adrenocortical neoplasias have been used to delineate a mechanism of adrenal progenitor differentiation towards a gonadal-like differentiation path in response to chronic LH-dependent gonadotropin signaling. Although gonadotropin-dependent ACTs in humans are rare, a subset of ACAs and ACTH-independent primary macronodular hyperplasias are responsive to LH, indicating that adrenocortical cells with the capacity to respond to high levels of gonadotropin stimulation are present in both mice and humans (Christopoulos et al., 2004, Christopoulos et al., 2005). Furthermore, the molecular profile of GDX-induced adrenal neoplasms resembles ovarian thecal metaplasias, where subcapsular wedge-shaped nodules form in the adrenals of post-menopausal women (Fidler, 1977, Jabara et al., 2003, Wont et al., 1971). They are also reminiscent of follicular theca cells from polycystic ovaries (Kaaijk et al., 2000). Both of these conditions are characterized by increased LH release from the pituitary gland. Thus, the differentiation of subcapsular progenitor cells into a gonadal lineage is consistent with the multipotent nature of these cells and the shared AGP origins in both human and murine adrenal tissue.

4. Mouse models of adrenocortical adenoma (ACA) and carcinoma (ACC)

ACAs and ACCs are benign and malignant neoplasms, respectively, that each originates in the adrenal cortex. Although still controversial, increasing evidence suggests that in some cases, adrenal tumors follow a classical hyperplasia-adenoma-carcinoma sequence, similar to what is observed in other solid tumors (Stratakis, 2003, Stratakis, 2014). This potential model for adrenal oncogenesis has important clinical implications, since the majority of adrenal masses are incidentally found ACAs. Thus, a clear understanding of the cellular and molecular context in which ACA and ACCs develop is critical for the treatment and management of adrenal tumors. In the following section, we summarize mouse models associated with ACA and ACC and how they have influenced the current understanding of adrenal tumorigenesis.

4.1 Spontaneous models

Despite the high prevalence of adrenal tumors in humans (Else et al., 2014), spontaneous development of primary ACTs in mice is exceptionally rare. This phenomenon was first observed in the Slye stock of mice in the early 1900s. Pathologist Maud Slye studied over 150,000 mice between 1910 and 1930 to understand the heritability of cancer. In 33,000 autopsies performed on mice following their natural deaths, Slye and her colleagues noted only 4 adrenal neoplasms: 1 ACA and 3 mesothelial tumors (Slye et al., 1921). Furthermore,

in more than 3,000 mammary carcinomas, no adrenal metastases were found despite a high prevalence of lung metastases.

Given these early observations, the 1943 report of a spontaneous ACC arising in a laboratory mouse at the National Cancer Institute was rather surprising. A 24-½ month old strain C female mouse – an inbred albino strain later used to generate BALB/c mice – was found to have a 4mm right adrenal tumor (Dalton et al., 1943). Although there was no evidence of gross metastases, the original tumor was locally invasive and displayed a rapidly advanced growth rate upon transplantation into successive generations of recipient mice. The tumor, which was designated ‘adrenal tumor C-199,’ was consequently classified as a low grade ACC. Cytopathologic testing found that tumor cells contained variable mitochondria and Golgi substance in close contact with the nuclear membrane, which was known to be characteristic of undifferentiated cells that lie between the capsule and zG (Salmon et al., 1941). These observations suggested that adrenal tumor C-199 likely originated in what was then termed the “subcapsular region” of the adrenal cortex (Dalton et al., 1943). Adrenal tumor C-199 was one of the first spontaneous ACCs documented in mice and was successfully transplanted for more than 11 generations.

Shortly after the characterization of adrenal tumor C-199, a mouse strain highly susceptible to ACA was discovered. In a 1946 report, Kirschbaum and his colleagues described their NH strain of mice, in which nearly all female mice over the age of one year developed ACAs (Kirschbaum et al., 1946). Although a high incidence of adrenal tumors had previously been observed in particular strains of gonadectomized animals (Woolley et al., 1943), this was the first report of noncastrate mice exhibiting a strong propensity for adrenal tumorigenesis. While 13 of 14 female mice (92.9%) developed ACAs at one year of age, adrenal tumors were infrequent in male mice. At one year of age, a cohort of 8 male mice showed hyperplasia beneath the capsule but never adenoma formation, and at two years of age, a single male mouse in the stock developed a malignant ACC tumor. This predilection for adrenal tumor formation in female NH mice is particularly interesting given the higher incidence of adrenal tumors found in female patients. Both ACAs and ACCs occur more often in women than men, with respective ratios of 1.7 and 1.5–2.5 (Barzon et al., 1998, Else et al., 2014, Luton et al., 1990), and there is an apparent increase in the diagnosis of ACC during pregnancy (Luton et al., 1990). Taken together, these observations in humans and the NH mouse strain suggest a potential role for sex steroids in adrenal tumor formation. In addition to sex differences, the genetic background of the NH strain is noteworthy. NH mice were described in 1940 as a model of spontaneous mammary tumor formation that maintained relatively high genetic diversity (Strong, 1940). The line was developed as a hybrid between strains that were highly resistant (JK and N strains) and moderately susceptible (CBA strain) to mammary tumorigenesis. Given that the CBA strain was derived from DBA mice (Strong, 1942) containing a known functional polymorphism in *Nr5a1* (Frigeri et al., 2002), it is tempting to speculate that the NH mouse strain may also be prone to adrenal tumors due to alterations in *Nr5a1*. This well studied transcription factor (SF1) is essential for adrenal development (Luo et al., 1994) and its gene dosage is associated with pediatric ACC (Figueiredo et al., 2005, Pinto et al., 2015), which will be discussed in greater detail below.

While the NH strain and adrenal tumor C-199 provided early mouse models of spontaneous ACA and ACC, neither resulted in functional adrenal tumors (i.e. hormone-producing), which occur in a large number of human patients. The first spontaneous, hormone-producing ACT arose in a laboratory mouse in the 1950s. During Operation Greenhouse, the 1951 American nuclear test series, a group of inbred LAF₁ mice were exposed to high levels of irradiation. A small subset of these mice (<1%) later developed adrenal tumors (Cohen et al., 1957), but it remains uncertain whether they were spontaneous or radiation-induced. Regardless, one of the resulting tumors – termed ‘tumor strain 2’ – secreted both mineralocorticoids and glucocorticoids, and displayed a highly malignant phenotype evidenced by numerous pulmonary metastases at necropsy. The original tumor, which measured 12x20mm in size, was taken from the right adrenal of an adult male mouse and was maintained through intramuscular transplantation into recipient LAF₁ mice. Tumor strain 2 grew efficiently in all hosts and maintained its metastatic potential, at least initially. Moreover, tumors sustained steroid production, with recipient animals showing signs of excess mineralocorticoids (atrophy of the zG with hypernatremia and polyuria) and glucocorticoids (atrophy of the zF with depressed eosinophil levels).

As one of the first transplantable, hormone-producing adrenal tumors in mice, tumor strain 2 formed the basis for several studies to follow. Specifically, slices of transplanted tumors were optimized for *in vitro* culture and used to develop an ACTH activity assay (Cohen et al., 1957). Since the tumor slices were highly sensitive to ACTH stimulation, these studies suggested that the original tumor might have originated in the zF. However, ACTH responsiveness and corticosterone synthesis decreased with later transplantations (Bloch et al., 1960). In addition to *in vitro* organ cultures, tumor strain 2 was also employed to test various chemotherapeutic agents *in vivo* (Humphreys et al., 1965). This established the feasibility of drug testing on functional adrenal tumors, but after nearly 15 years of transplantation, tumor strain 2 had lost its ability to metastasize (Humphreys et al., 1965). Together with the changes in steroidogenic potential previously noted, these observations highlight the inherent challenge of genetic drift over long-term serial transplantation. Tumor strain 2 was ultimately adapted for growth in culture by undergoing alternating periods of growth as a monolayer culture and as a tumor in mice (Buonassisi et al., 1962). This process selected for cells with an increased capacity for growth and steroid production, and from this culture-adapted tumor, Yasumura and colleagues cloned an ACTH-responsive cell line named Y1 (Yasumura et al., 1966). Y1 cells are still widely used as an *in vitro* cell line and syngeneic xenograft model (Hantel et al., 2015).

4.2 Genetically modified models

Spontaneous mouse models provided the basis for many early studies on adrenal tumorigenesis and steroid production. However, their low penetrance coupled with the availability of new technologies to efficiently manipulate the mouse genome led to a strong interest in developing genetically modified models of ACA and ACC. Engineered to contain specific genetic alternations that promote *de novo* tumor formation within the adrenal cortex, genetically modified tumor models are largely hypothesis-driven and depend on prior human clinical observations or *in vitro* findings to identify genes and pathways of interest. Once identified, a given gene can be deleted, overexpressed, or mutated within the adrenal cortex

to experimentally define its role in the pathogenesis of adrenal tumors. Here, we describe genetically modified mouse models associated with ACA and ACC that have been previously characterized (Table 3).

4.2.1 Steroidogenic factor-1 (SF1)—SF1 (Ad4BP; NR5A1) is a nuclear receptor transcription factor that was initially identified as a regulator of steroid hydroxylases (Lala et al., 1992, Morohashi et al., 1992). However, studies in humans and mice have since demonstrated that SF1 plays an even larger role in endocrine function and is essential for proper adrenal and gonadal genesis and differentiation. In humans, mutations in the gene encoding SF1, *NR5A1*, often result in adrenal insufficiency and sex reversal (Ozisik et al., 2002) and in mice, *Nr5a1* null animals lack adrenal glands and gonads at birth (Luo et al., 1994). Moreover, gene dosage is critically important for proper SF1 function, which is evident in *Nr5a1*^{+/-} mice. These animals have smaller adrenal glands (Bland et al., 2000), show signs of insufficiency under stress stimuli, and fail to undergo compensatory growth in response to unilateral adrenalectomy (Beuschlein et al., 2002). Conversely, *NR5A1* overexpression is frequently observed in adrenal tumors. In childhood ACTs, 90% of cases display gains of 9q, which is the chromosomal region containing *NR5A1* (Figueiredo et al., 2005, Pinto et al., 2015). Further characterization of these tumors confirmed that 9q amplification is strongly linked with increased *NR5A1* copy number and overexpression of SF1 protein (Figueiredo et al., 2005, Pianovski et al., 2006). In adult ACTs, *NR5A1* copy number variation is far less common (Almeida et al., 2010), but *NR5A1* expression levels are significantly correlated with clinical outcome (Sbiera et al., 2010). Taken together, these observations suggest SF1 may play an important role in adrenal tumorigenesis, particularly in pediatric patients.

As a model of increased *Nr5a1* dosage, lines of transgenic mice with a 500-kb yeast artificial chromosome (YAC) containing the rat *Nr5a1* gene were characterized (Doghman et al., 2007). While the YAC contained 2 additional genes, it is presumed that SF1 activity was the dominant driver of phenotypic changes, in part because the YAC was capable of rescuing adrenal and gonadal development in *Nr5a1*-deficient mice (Karpova et al., 2005). The *Nr5a1* transgenic lines, termed YAC TR mice, contained multiple copies of the *Nr5a1* gene and had significantly elevated SF1 protein levels in their adrenal glands (Doghman et al., 2007). Moreover, YAC TR mice developed ACTs that first appeared as nodular hyperplasias beneath the capsule and subsequently progressed to tumors with complete penetrance. Although these results supported a functional role for SF1 during adrenal tumorigenesis, the morphology of the resulting tumors was strikingly different from that of human tumors. Specifically, YAC TR adrenal tumors expressed *Nr5a1* and gonadal markers (i.e. *Gata4*), lacked certain steroidogenic enzymes (i.e. *p450scc*), and exhibited Stat3 signaling activity (Doghman et al., 2007). Thus, YAC TR adrenal tumors displayed a gonadal phenotype that most closely resembled the gonadectomized mouse models previously discussed.

One possible explanation for the strong phenotypic differences between ACTs in *NR5A1*-amplified human tumors compared to *Nr5a1* transgenic mice is the cellular context. Pediatric ACTs, particularly those diagnosed in the first years of life, have been hypothesized to originate in fetal adrenal cells (Lalli et al., 2015), where the fetal adrenal

enhancer (FAdE) controls *NR5A1* expression (Zubair et al., 2006). Although both adrenal and gonadal cells arise from a common *NR5A1*-positive precursor, FAdE-driven *NR5A1* expression is restricted to the adrenal primordium (Zubair et al., 2006, Zubair et al., 2008). Thus, *NR5A1* overexpression in human pediatric ACTs likely occurs in an adrenal restricted lineage. In contrast, the YAC used to generate *Nr5a1* transgenic mice contained the entire *Nr5a1* locus as well as substantial portions of the 5' and 3' flanking regions (Karpova et al., 2005). Predicted to contain additional enhancer elements, the YAC used in this approach may have resulted in *Nr5a1* overexpression in pluripotent adrenogonadal precursor cells that were capable of adopting a more gonadal phenotype (Looyenga et al., 2006). However, a difference in the lineage in which *NR5A1* was overexpressed is unlikely to fully account for the discrepancy between human and YAC TR tumors, particularly given the phenotype of FAdE-SF1 transgenic mice. These animals contained multiple copies of a transgene engineered to express *Nr5a1* using the fetal enhancer (Zubair et al., 2009). FAdE-SF1 transgenic mice had larger adrenal glands as well as ectopic adrenal tissue in the thorax, but did not develop adrenal tumors. These results suggest that *NR5A1* overexpression alone in fetal adrenal cells is not sufficient for transformation. Rather, *NR5A1* likely cooperates with other pathway alternations, including the germline *TP53* mutations frequently observed in pediatric ACTs (Wasserman et al., 2012), to promote adrenal tumor formation. Although YAC TR and FAdE-SF1 mice do not fully recapitulate *NR5A1*-amplified human tumors, these models clearly demonstrate that SF1 strongly influences steroidogenic cell fate of the adrenal versus gonadal cell.

4.2.2 Wnt/ β -catenin—The Wnt pathway was first linked to adrenal tumorigenesis nearly 15 years ago by two studies that found patients with familial adenomatous polyposis (FAP) were 2–4 times more likely than the general population to develop adrenal masses (Marchesa et al., 1997, Smith et al., 2000). Wnt signaling is highly conserved throughout evolution and is essential for embryonic development and adult homeostasis in several organs (van Amerongen et al., 2009), including the adrenal gland (Kim et al., 2008). Although Wnt signaling encompasses three distinct signal transduction pathways, canonical Wnt/ β -catenin signaling is the most well characterized pathway in adrenal biology, where activation is enriched in the zG (Walczak et al., 2014). Briefly, in the absence of Wnt ligands, β -catenin is continually degraded through the action of a group of proteins termed the destruction complex (reviewed in (Clevers et al., 2012)). Upon ligand binding, activated Wnt receptors on the cell surface disrupt this complex, thereby allowing β -catenin to accumulate in the cytoplasm and subsequently translocate to the nucleus to facilitate gene transcription.

In the previous studies of FAP patients with adrenal tumors, patients were found to possess a germline mutation in one copy of the adenomatous polyposis coli (*APC*) gene. Further genetic analysis of their adrenal tumors showed simultaneous somatic *APC* mutations (Gaujoux et al., 2010), highly suggestive of biallelic *APC* inactivation. Since *APC* is an important component of the β -catenin destruction complex, loss of *APC* in the adrenal tumors of some FAP patients suggested that aberrant Wnt/ β -catenin signaling might be involved in adrenal tumorigenesis. This notion was further supported by DNA microarray profiling of ACTs, which found Wnt target genes significantly up regulated in ACC samples

(Giordano et al., 2003). Later immunohistochemistry studies showed abnormal cytoplasmic and/or nuclear β -catenin staining in 54% of ACTs (with equal frequency in both adenomas and carcinomas) and in the human ACC cell line, H295R (Tissier et al., 2005). Nearly half of the tumors with abnormal β -catenin staining (as well as H295R cells) also contained activating mutations in *CTNNB1*. Finally, H295R cell proliferation was shown to be inhibited in a dose-dependent manner by the Wnt antagonist, PKF115–584 (Doghman et al., 2008). Taken together, these results strongly implicated aberrant Wnt/ β -catenin signaling in adrenal tumorigenesis.

Based on these clinical and *in vitro* observations, two independent mouse models of constitutive β -catenin activation in the adrenal gland were generated. First, *0.5 Akr1b7-Cre* mice, which express Cre recombinase in all steroidogenic cells of the adrenal cortex (Lambert-Langlais et al., 2009), were crossed with *Catnb^{lox(ex3)}* mice (Harada et al., 1999). In this model, loxP sites flank exon 3 of *Ctnnb1*, where critical GSK3 β phosphorylation sites reside to mediate β -catenin turnover. Consequently, *0.5 Akr1b7-Cre*-driven loss of exon 3 resulted in β -catenin stabilization throughout the adrenal cortex (Berthon et al., 2010). Histological analysis of resulting mice, termed *Cat* mice, showed severe defects in adrenal architecture with progressive dysplasia and hyperplasia. By 10 months of age, increased proliferation and accumulation of steroidogenic as well as non-steroidogenic cells was observed within the cortex, suggesting that both direct and indirect mechanisms of adrenal hyperplasia occurred in response to aberrant Wnt signaling. Notably, ectopic β -catenin activation triggered increased differentiation of the zG with concomitant inhibition of zF differentiation, ultimately leading to increased aldosterone production. While these abnormalities in adrenal architecture and function were seen in 10-month-old mice, malignant features were not observed until 17 months of age, when only a fraction of female *Cat* mice (~20%) showed vascular and peritoneal invasion.

In a second approach, ectopic Wnt activation in the adrenal cortex was achieved through conditional loss of *Apc*, similar to what is observed clinically in FAP patients and some sporadic ACCs (Assie et al., 2014). Here, mice harboring an *Apc* allele floxed at exon 14 (*Apc^{loxP/loxP}*) were crossed with mice expressing the Sf1-Cre transgene (Heaton et al., 2012). Importantly, a stochastic Sf1-Cre driver was used, which resulted in Cre-mediated recombination in a subset of adrenocortical cells (Bingham et al., 2006, Kim et al., 2008), thus mimicking the sporadic nature of human ACCs. Compared to *Cat* mice, the resulting APC KO progeny showed similar levels of Wnt target gene activation and also displayed progressive hyperplasia. Furthermore, a subset of APC KO mice (~27%) went on to develop microscopic or macroscopic adenomas at 45 weeks of age and older, but progression to carcinoma was never seen.

The generation of adrenocortical-restricted *Cat* and APC KO mice established new genetic models of adrenal tumorigenesis. While both approaches resulted in aberrant Wnt activation in the adrenal cortex and ultimate tumor formation, notable differences were seen. Specifically, *Cat* mice appeared to develop a more severe phenotype than APC KO mice, including shorter tumor latency, increased invasiveness, and indirect stromal cell defects. However, these more advanced phenotypes were limited to female mice, as male *Cat* mice consistently showed slower progression of disease (Berthon et al., 2010) reminiscent of the

patterns in human patients previously discussed. The observed discrepancies between models might be explained by variations in Cre activity or the different molecular approaches used to stabilize β -catenin. Nevertheless, both models found that tumors induced by aberrant Wnt activation in the adrenal gland had low penetrance and slow overall progression, suggesting that other molecular pathways likely cooperate with Wnt signaling to drive adrenal tumorigenesis.

4.2.3 Insulin-like Growth Factor 2 (IGF2)—One pathway hypothesized to interact with Wnt/ β -catenin signaling during adrenal tumorigenesis is insulin-like growth factor 2 (IGF2). Similar to the Wnt pathway, a possible connection between IGF2 and ACC was first suggested by a familial cancer susceptibility syndrome. Specifically, predisposition to ACC was observed in a portion of pediatric patients with Beckwith-Wiedemann syndrome (BWS) (Henry et al., 1989), which is characterized by alterations at 11p15, an imprinted locus where *IGF2* and several other genes reside (Else, 2012). Based on this association, Gicquel and colleagues evaluated sporadic adult ACTs and found *IGF2* overexpressed in 83% of carcinomas compared to just 11.7% of adenomas (Gicquel et al., 1994). Following this seminal study of 23 human tumors, several large-scale studies followed and confirmed that *IGF2* was overexpressed in 80–90% of human ACCs (de Fraipont et al., 2005, Gicquel et al., 2001, Giordano et al., 2009, Ribeiro et al., 2012). Since 11p15 is an imprinted locus, *IGF2* is normally expressed from the paternal allele. However, in ACC, biallelic expression is frequently achieved through loss of the maternal allele and duplication of the paternal allele (Gicquel et al., 1994).

As the most frequently up regulated gene in ACC, *IGF2* has been extensively studied in adrenal cancer (reviewed in (Drelon et al., 2013, Else et al., 2014, Fottner et al., 2004)). With specific regard to mouse models, several approaches have been employed to elucidate the role of IGF2 during adrenal tumorigenesis. These models result in a 2- to 7-fold increase in basal *Igf2* expression in the adrenal cortex, but surprisingly do not produce adrenal tumors. In the first approach, transgenic mice were generated using the rat phosphoenolpyruvate carboxykinase (*PEPCK*) promoter to drive expression of human *IGF2* (Weber et al., 1999). *PEPCK* activity was observed in all layers of the mouse adrenal cortex beginning around birth (Zimmer et al., 1990), which resulted in postnatal *IGF2* overexpression. Three-month-old male mice had 4- to 6-fold elevated IGF2 serum levels and hyperplastic growth of the zF leading to increased overall adrenal weight (Weber et al., 1999). However, adrenal tumors were not seen in animals up to 18 months of age. Next, the *Igf2/H19* imprinting control region was targeted, which is required for silencing of the maternal allele. By crossing male Sf1-Cre mice (stochastic driver) with female *H19^{loxDMD/loxDMD}* mice (Thorvaldsen et al., 2006), *Igf2* was expressed from both alleles, resulting in an overall 2-fold increase in *Igf2* levels (Heaton et al., 2012). This triggered activation of downstream signaling pathways and mild adrenal dysplasia but no tumor formation, even in mice up to 45 weeks of age. Finally, *Igf2* was overexpressed in the mouse adrenal cortex using regulatory regions from the *Akr1b7* gene (Drelon et al., 2012). These animals, termed AdIgf2 mice, had almost 7-fold higher basal levels of *Igf2* (up to 87-fold higher with ACTH stimulation) and increased infiltration of mesenchymal cells in the adrenal cortex but again no tumor development. Together, these studies strongly argued

against any significant role for IGF2 during malignant transformation of the adrenal gland, despite high IGF2 being a distinguishing feature of human ACCs.

While IGF2 overexpression did not trigger tumor formation in mice, it did have a clear mitogenic effect on the adrenal cortex, suggesting that IGF2 may play a role in tumor maintenance rather than initiation. This type of two-hit scenario became a particularly attractive hypothesis in light of the Wnt pathway data previously discussed. Specifically, Wnt pathway activation is common to both human ACAs and ACCs, while increased IGF2 is predominately an ACC feature (Heaton et al., 2012). Furthermore, β -catenin stabilization in mice initiated tumors that progressed slowly (Drelon et al., 2012, Heaton et al., 2012) while *Igf2* overexpression promoted cell growth but never initiated tumors (Drelon et al., 2012, Heaton et al., 2012, Weber et al., 1999). These observations led to a proposed model of cooperation between the Wnt pathway and IGF2, which was tested in two independent mouse models. First, mice were generated with joint loss of *Apc* and overexpression of *Igf2* by mating the APC KO and *H19^{DMD}* mice previously discussed (Heaton et al., 2012). This resulted in a shift towards a more aggressive tumor phenotype, marked by earlier onset of tumor formation, a higher tumor penetrance and formation of one overt carcinoma. A second model combining the described Cat and AdIgf2 mice confirmed these results and also found a higher tumor Weiss score when both pathway alterations were present (Drelon et al., 2012). However, as just 2 carcinomas were observed between the models, these results supported only a modest level of cooperation between the Wnt pathway and IGF2 during adrenal tumorigenesis.

Collectively, mouse models of *Igf2* overexpression in the adrenal cortex, both alone and in combination with Wnt activation, have failed to validate IGF2 as a significant driver of ACC. With human tumors showing up to 200-fold higher *IGF2* levels (Heaton et al., 2012), it is possible that the level of *Igf2* overexpression achieved in these models was not sufficient to observe an effect. However, there is growing evidence to suggest that while IGF2 overexpression is a common marker of ACC, it alone may not have substantial functional consequences. This is supported by whole-genome transcriptome analyses of human ACC tumors, where unsupervised clustering does not distinguish between low-IGF2 and high-IGF2 tumors (Assie et al., 2012). Furthermore, clinical trials in advanced ACC patients have found little benefit of IGF-IR inhibitors (Fassnacht et al., 2015, Haluska et al., 2010, Lerario et al., 2014). Most recently, a double blind, placebo-controlled phase 3 study observed no difference in the progression free survival or overall survival of patients who received linsitinib (OSI-906), a dual IGF-IR and insulin receptor inhibitor, compared to placebo (Fassnacht et al., 2015). However, a long lasting, partial response was observed in 3 patients (3%). Together with the mouse modeling data, these results have shifted our understanding of IGF2 dysregulation in ACC – IGF2 now appears to be uniquely targetable in only a highly specific subset of tumors. Genetic profiling of sensitive human tumors may facilitate the discovery of the cellular context in which IGF2 functions. Additionally, since most tumors with high IGF2 have dysregulation of the entire 11p15 locus, it is also critical to expand our studies beyond IGF2 to understand the contribution of other genes and micro-RNAs in this region to the pathogenesis of ACC.

4.2.4 TP53—A central role for *TP53* in adrenal tumorigenesis was first recognized in the context of Li Fraumeni syndrome (LFS). This familial cancer syndrome was initially described in 1969 in a group of four families who developed a high frequency of neoplasms (Li et al., 1969). The syndrome manifested itself early and often resulted in multiple primary malignancies, which was highly suggestive of a causative “hereditary or oncogenic agent.” Further study of LFS families found an excess of ACC among the spectrum of tumors that developed (Li et al., 1988). Moreover, after nearly 20 years of study, germline *TP53* mutations were identified as the causative “agent” in the majority of LFS cases (Malkin et al., 1990), thus establishing the link between *TP53* and adrenal cancer. Since this seminal work, germline *TP53* mutations have been found in up to 80% of childhood ACTs, with the largest series to date recently reporting a prevalence of 50% (Wasserman et al., 2015). Importantly, the rate of germline *TP53* mutations in ACC is age-dependent and declines to between 3% and 7% in the adult ACC population (Herrmann et al., 2012, Raymond et al., 2013). A wide spectrum of mutations in *TP53* has been identified that confer variable degrees of functional loss (Wasserman et al., 2015). However, perhaps the most well studied mutation in the context of adrenal tumors is R337H, a low penetrance allele commonly found in southeast Brazil (reviewed in (Custodio et al., 2012)).

Despite the high prevalence of *TP53* mutations in ACC, no direct mouse models of adrenal restricted *TP53* loss have been developed to date. Nevertheless, there are several models that offer insight about the functional consequences of *TP53* loss during adrenal tumorigenesis. One of these models began with characterization of the adrenocortical dysplasia (*Acd*) mouse, which contains a spontaneous, recessively inherited mutation that results in adrenal insufficiency, among other developmental defects (Beamer et al., 1994). Studies of these mice identified a splice donor mutation in the *Acd* gene (homologue of human TPP1), a critical component of the shelterin complex that controls telomere protection (Keegan et al., 2005). Thus, the *Acd* phenotype is caused by telomere dysfunction and subsequent genomic instability. Interestingly, when these mice were crossed to a p53 null background, adrenal size and architecture normalized (Else et al., 2009). However, there was also a significant increase in carcinoma formation, including ACC in 5% of animals. These observations suggest that the adrenal hypoplasia seen in *Acd* mice results from p53-dependent senescence and apoptosis. Moreover, with specific regard to ACC, these results also suggest that release from p53-sensitive checkpoints is a critical step in the process of adrenal tumorigenesis. Recent genomic profiling of human tumors further supports this notion and has found recurrent mutations in several known cell cycle regulators, including *CDKN2A*, *CDK4*, *RBI*, and *CCNE1* (Zheng et al., 2015).

In addition to the *Acd* mouse, two transgenic lines expressing SV40 T-antigen in the adrenal cortex have been generated. SV40 T-antigen is a potent oncogene that acts in part by binding and inactivating p53 (reviewed in (Colvin et al., 2014)). In the first model, the promoter of the human P450 cholesterol side-chain cleavage gene (*P450scc*) was used to express an SV40 construct containing both large T and small t antigens (Mellon et al., 1994). This approach resulted in expression of SV40 T-antigen early in adrenal development. Two female transgenic mice were obtained that both developed adrenal tumors within 3 months of age and steroidogenic cell lines were successfully established

from each tumor. Of note, the tumors produced progesterone and lacked aldosterone synthase, suggesting that these tumors arose early in the differentiation process. These results are consistent with *P450scc* expression previously being detected in adrenal progenitor cells as early as embryonic day 12 (Rogler et al., 1993). Complementary to these studies, a subsequent model was generated using a 0.5 kb region of the *Akr1b7* promoter to express SV40 T-antigen (large T antigen only) in the adrenal gland (Sahut-Barnola et al., 2000). In this model, 2 of 3 surviving founder mice developed adrenal tumors. One female sacrificed at 4 months of age had a unilateral adrenal tumor as well as tumors of the ovary and cervix, and one male mouse sacrificed at 7 months of age had bilateral adrenal tumors. These tumors were subsequently used to generate ACTH-responsive cell lines (ATC1 and ATC7-L), suggesting that the original tumors had zF characteristics consistent with the zF-restricted expression of the *Akr1b7* enzyme. However, it is difficult to know precisely where these tumors arose given that the *Akr1b7* promoter region used in this model was previously shown to be active in all steroidogenic cells of the adrenal cortex (Lambert-Langlais et al., 2009).

Together, the *Acd* mouse and SV40 transgenic models discussed here strongly support a role for p53 during adrenal tumorigenesis. Specifically, these mouse models suggest that evading p53-dependent checkpoints is an important step in the transformation process and that the adrenal gland is particularly sensitive to p53 dysregulation. Since these models induced molecular alternations with consequences extending beyond p53 loss, future models are needed to explore the exact consequences of *TP53* inactivation in the adrenal. To this end, a mouse model bearing a targeted R334H-*TP53* mutation that closely mimics the highly prevalent founder R337H-*TP53* mutation often observed in southeast Brazil has been developed at St. Jude Children's Research Hospital. The mice are currently being characterized in terms of adrenal development and potential tumorigenesis (G. Zambetti, personal communication).

5. Future Models of Adrenocortical Tumors

Current mouse models of ACTs are largely based on the knowledge gained from rare genetic syndromes in which adrenal tumors occur, including Carney complex, MEN1, familial adenomatous polyposis, Beckwith-Wiedemann syndrome, and Li Fraumeni syndrome. As a result, the majority of available models focus on the molecular pathways associated with these diseases (i.e. *PRKA1A*, *MEN1*, the Wnt pathway, the IGF pathway, and *TP53*). However, large-scale genomic profiling efforts over the last decade - and particularly in just the last few years - have expanded our understanding of the genes and pathways that are recurrently altered in adrenal tumors. While these studies confirmed many of the previously known players, they also identified a number of new potential driver genes. These include *ARMC5* mutations in primary macronodular adrenal hyperplasia (Alencar et al., 2014, Assie et al., 2013, Faucz et al., 2014), somatic *ATRX* mutations in pediatric ACC (Pinto et al., 2015), and several new genes in adult ACCs, including *ZNRF3*, *DAXX*, *TERT*, *MED12*, *CDC27*, *SCN7A*, *SDK1*, *KREMEN1*, and *ATM* (Assie et al., 2014, Christofer Juhlin et al., 2014, Ross et al., 2014). With an increasing number of genetic tools available to manipulate the mouse genome, we expect several new mouse models will be available in the near future (Fig. 1). We hope these models will complement the growing

field of xenograft tumor models to more faithfully recapitulate the spectrum and diversity of human ACTs. Moreover, we believe these models will be critical for the development of personalized therapeutic strategies that are needed to treat complex adrenal tumors.

Acknowledgments

National Institutes of Health (NIH) research grants (RO1 DK062027 and RO1 CA134606 to G.D.H.), NIH training grants (Developmental Origins of Metabolic Disorders T32 DK071212 to K.J.B. and Cancer Biology T32 CA009676 to H.A.H.), and CAPES (BEX 8726/13-2 to A.M.L.) supported this work.

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Highlights

- Summary of spontaneous/genetic models of adrenal tumors and related human pathology
- Models associated with rare genetic syndromes and adrenal hyperplasia are discussed
- Gonadectomy promotes adrenal neoplasia and aberrant progenitor cell specification
- Mouse models have studied Wnt, IGF2 and p53 dysregulation in adrenal tumorigenesis
- Novel models are needed to recapitulate the high complexity of human adrenal tumors

Figure 1. Tools available for the generation of future mouse models of adrenocortical tumors (Left) Schematic representation shows the architecture of the normal mouse adrenal gland including the outer adrenal capsule and the inner cortex that is comprised of the zona glomerulosa (zG) and the zona fasciculata (zF). Additionally, the X-zone/fetal zone persists until puberty in male mice and pregnancy in female mice. (Right) Several gene promoters and Cre drivers with zone-specific activity are available to target different regions of the adrenal gland. Reporter strains further allow tracking of specific populations within these potential models.

Table 1

Summary of current genetically modified mouse models of adrenal hyperplasia

Model	Gene	Promoter/Driver	Phenotype	Reference
Task1 KO	<i>Kcnk3</i>	Whole-body KO	Severe hyperaldosteronism. Disruption of normal zonation with ectopic expression of <i>Cyp11b2</i> in the zG.	Heitzmann, et al. (2007)
Task3 KO	<i>Kcnk9</i>	Whole-body KO	Mild autonomous aldosterone production in adult animals. Severe hyperaldosteronism in newborn mice with autonomous corticosterone and progesterone secretion.	Guagliardo, et al. (2012), Bandulik, et al (2013)
Task1; Task3 KO	<i>Kcnk3/Kcnk9</i>	Whole-body KO	Severe hyperaldosteronism. No zonation defect.	Davies, et al. (2008)
Prkar1a ^{2/+}	<i>Prkar1a</i>	ElIA-Cre (ubiquitous)	Spectrum of tumors highly overlapping with human CC. No adrenal phenotype.	Kirschner, et al. (2005)
rTA/X2AS	<i>Prkar1a</i>	Tet-Off system	Spectrum of tumors highly overlapping with human CC. Persistence of the X-zone in males and females. Nodular cortical changes. Autonomous corticosterone secretion.	Griffin, et al. (2004)
AdKO	<i>Prkar1a</i>	0.5 <i>Akr1b7</i> -Cre	Autonomous corticosterone secretion. Expansion of X-zone derived aberrant progenitor-like cells that ultimately occupy the entire cortex.	Sahut-Barnola, et al. (2010)
Men1 ^{+/-}	<i>Men1</i>	Whole-body KO	Spectrum of tumors highly overlapping with human MEN1. Adrenal hyperplasia to adenoma to carcinoma evolution.	Crabtree, et al. (2001), Bertolino, et al. (2003), Loffler, et al. (2007), Harding, et. Al (2009)

Abbreviations: zG, zona glomerulosa; CC, Carney complex; MEN1, Multiple endocrine neoplasia type 1; Prkar1a, Protein kinase cAMPdependent regulatory subunit type 1 alpha; Kcnk3, Potassium channel subfamily K member 3; Kcnk9, Potassium channel subfamily K member 9, beta 1; Akr1b7, aldo-keto reductase family 1, member b7.

Table 2

Summary of current gonadotropin-dependent mouse models of adrenocortical neoplasia

Model	Gene	Promoter	Phenotype	Reference
Inha KO	<i>Inhibin-α</i> (mouse)	Whole-body	Intact: Ovarian and testicular tumors. Cachexia at 6–7 weeks and death at 12 (male) or 17 (female) weeks. GDX: Adrenal tumors (84.8% unilateral, 15.1% bilateral).	Matzuk, et al. (1994)
Inha KO; hpg/hpg	<i>Inhibin-α</i> (mouse) <i>GnRH</i> (mouse)	Whole-body	Intact: Suppression of LH and FSH levels with gonadal hypoplasia and grossly normal adrenal glands. GDX: N.D.	Kumar, et al. (1996)
Inha KO; Fshβ ^{-/-}	<i>Inhibin-α</i> (mouse) <i>Fshβ</i> (mouse)	Whole-body	Intact: No FSH expression. Slower development and progression of gonadal tumors with less cachexia. GDX: N.D.	Kumar, et al. (1999)
Inha KO; LHβ-CTP	<i>Inhibin-α</i> (mouse) <i>LH-β</i> (bovine) and <i>hCG-β</i> (human) chimeria protein	Whole-body <i>LH-α</i> (bovine)	Intact: LH overexpression. Large bilateral ovarian tumors. Death at 6 (female) weeks and adrenal X-zone regression. GDX: Larger unilateral adrenal tumors with earlier onset. Contralateral X-zone regression.	Beuschlein, et al. (2003)
Inha KO; Cyclin D2 ^{-/-}	<i>Inhibin-α</i> (mouse) <i>Ccnd2</i> (mouse)	Whole-body	Intact: Ovarian and testicular tumors with later onset and less aggressive progression. GDX: Prolonged survival. Adrenal tumors (83% unilateral).	Burns, et al. (2003)
Inha KO; Madh3 ^{+/-}	<i>Inhibin-α</i> (mouse) <i>Smad3</i> (mouse)	Whole-body	Intact: Significant attenuation of tumor growth rate in ovarian and testicular tissue. GDX: Prolonged survival with adrenal tumors histologically similar to ovarian tumors.	Looyenga, et al. (2007)
Inha/TA _g	<i>SV40</i> (Large T antigen)	6kb <i>Inhibin-α</i> (mouse)	Intact: Granulosa and Leydig cell tumors at 5–6 months. GDX: Progressive and aggressive adrenocortical tumors.	Kananen, et al. (1996)
Inha/TA _g ; hpg	<i>SV40</i> <i>GnRH</i> (mouse)	6kb <i>Inhibin-α</i> (mouse)	Intact: No tumors. GDX: N.D.	Kananen, et al. (1997) Rilianawat, et al. (1998)
Inha/TA _g ; LHβ-CTP	<i>SV40</i> <i>LH-β</i> (bovine) and <i>hCG-β</i> (human) chimeric protein	6kb <i>Inhibin-α</i> (mouse); <i>LH-α</i> (bovine)	Intact: Granulosa and adrenocortical tumors in female mice. Leydig cell tumors in male mice at 3 months of age. GDX: N.D.	Mikola, et al. (2003)
21-OH-Gata4	<i>Gata4</i>	6.4kb <i>Cyp21a1</i>	Intact: Adrenal neoplasia and subcapsular A cells. GDX: Adrenal neoplasia and subcapsular A and B cells.	Chrusciel, et al. (2013)
Gata6 cKO	<i>Gata6</i> ^{F/F}	Sf1-Cre (Stochastic)	Intact: Thin capsule. Dysplastic subcapsular cells. Ectopic chromaffin cells. X-zone not identifiable (consistent with early regression of the fetal zone). GDX: Subcapsular hyperplasia with A and B cells.	Pihlajoki, et al. (2013)
C57BL/6 Gata4 ^{+/-}	<i>Gata4</i> ^{F/F}	<i>Amhr2</i> -Cre	Intact: No gross pathology reported. GDX: Adrenal neoplasia by 6 months with A and B cells. Impaired adrenal tumorigenesis with <i>Gata4</i> haploinsufficiency.	Krachulec, et al. (2012)

Abbreviations: Inha, inhibin; KO, knockout; GDX, gonadectomy; hpg, hypogonadal mutant mouse; GnRH, gonadotropin releasing hormone; N.D., not determined; Fshβ, follicle-stimulating hormone beta subunit; FSH, follicle-stimulating hormone; LH, luteinizing hormone; hCG-β, human

chorionic gonadotropin beta subunit; Ccnd2, cyclin D2; Madh3, mothers against DPP homolog 3; Smad3, smad family member 3; TAg, tumor antigen; SV40, simian virus 40; Gata4, GATA binding protein 4; Cyp21a1, cytochrome P450, family 21, subfamily a, polypeptide 1; Gata6, GATA binding protein 6; cKO, conditional knockout; Sf1, steroidogenic factor-1; Amhr2, anti-mullerian hormone receptor, type II.

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Table 3

Summary of current genetically modified mouse models of ACA and ACC

Model	Gene	Promoter/Driver	Phenotype	Reference
YAC TR	<i>Nr5a1</i> (rat)	YAC transgene	Nodular hyperplasia with progression to gonadal-like tumors.	Doghman, et al. (2007)
FAdE-SF1	<i>Nr5a1</i> (mouse)	FAdE	Enlarged adrenal glands with ectopic adrenal tissue in the thorax.	Zubair, et al. (2009)
Cat	<i>Ctnnb1</i> (mouse)	0.5 <i>Akr1b7</i> -Cre	Progressive hyperplasia with late adenoma formation and increased aldosterone production. Carcinoma formation in 20% of female mice.	Berthon, et al. (2010)
APC KO	<i>Apc</i> (mouse)	Sf1-Cre (stochastic)	Progressive hyperplasia with late adenoma formation.	Heaton, et al. (2012)
PEPCK-IGF-II	<i>IGF2</i> (human)	<i>PEPCK</i> (rat)	Hyperplasia of the zF with increased overall adrenal weight.	Weber, et al. (1999)
<i>H19^{DMD}</i>	<i>Igf2/H19</i> ICR	Sf1-Cre (stochastic)	Mild adrenal dysplasia.	Heaton, et al. (2012)
AdIgf2	<i>Igf2</i> (mouse)	0.5 <i>Akr1b7</i>	Mesenchyme infiltration.	Drelon, et al. (2012)
APC KO- <i>H19^{DMD}</i>	<i>Apc</i> (mouse) <i>Igf2/H19</i> ICR	Sf1-Cre (stochastic)	Earlier onset hyperplasia and more frequent adenoma formation than APC KO mice. One overt carcinoma.	Heaton, et al. (2012)
Cat;AdIgf2	<i>Ctnnb1</i> (mouse) <i>Igf2</i> (mouse)	0.5 <i>Akr1b7</i>	Progressive hyperplasia with late adenoma formation. Increased Weiss score and proliferation compared to Cat mice.	Drelon, et al. (2012)
<i>Acd^{acd/acd}; p53^{+/-}</i>	<i>Acd</i> (mouse) <i>Tp53</i> (mouse)	N/A	Increased carcinoma formation compared to <i>Acd^{acd/acd}</i> mice. SF1-positive ACC in 5% of animals.	Else, et al. (2009)
<i>P450scc</i> -SV40	SV40 (large T and small t antigens)	<i>P450scc</i> (human)	Steroidogenic tumor formation within 3 months of age.	Mellon, et al. (1994)
AdTAg	SV40 (large T antigens)	0.5 <i>Akr1b7</i>	Formation of ACTH-responsive tumors.	Sahut-Barnola, et al. (2000)

Abbreviations: YAC, yeast artificial chromosome; Nr5a1, nuclear receptor subfamily 5, group A, member 1; Sf1, steroidogenic factor-1; Ctnnb1, catenin (cadherin-associated protein), beta 1; Akr1b7, aldo-keto reductase family 1, member b7; APC, adenomatous polyposis coli; KO, knockout; PEPCK, phosphoenolpyruvate carboxykinase; IGF2, insulin-like growth factor 2; ICR, imprinting control region; Acd, adrenocortical dysplasia; TP53, tumor protein P53; SV40, simian virus 40; P450scc, P450 cholesterol side-chain cleavage gene; TAg, tumor antigen.