ADRENAL CORTEX: FROM PHYSIOLOGY TO DISEASE

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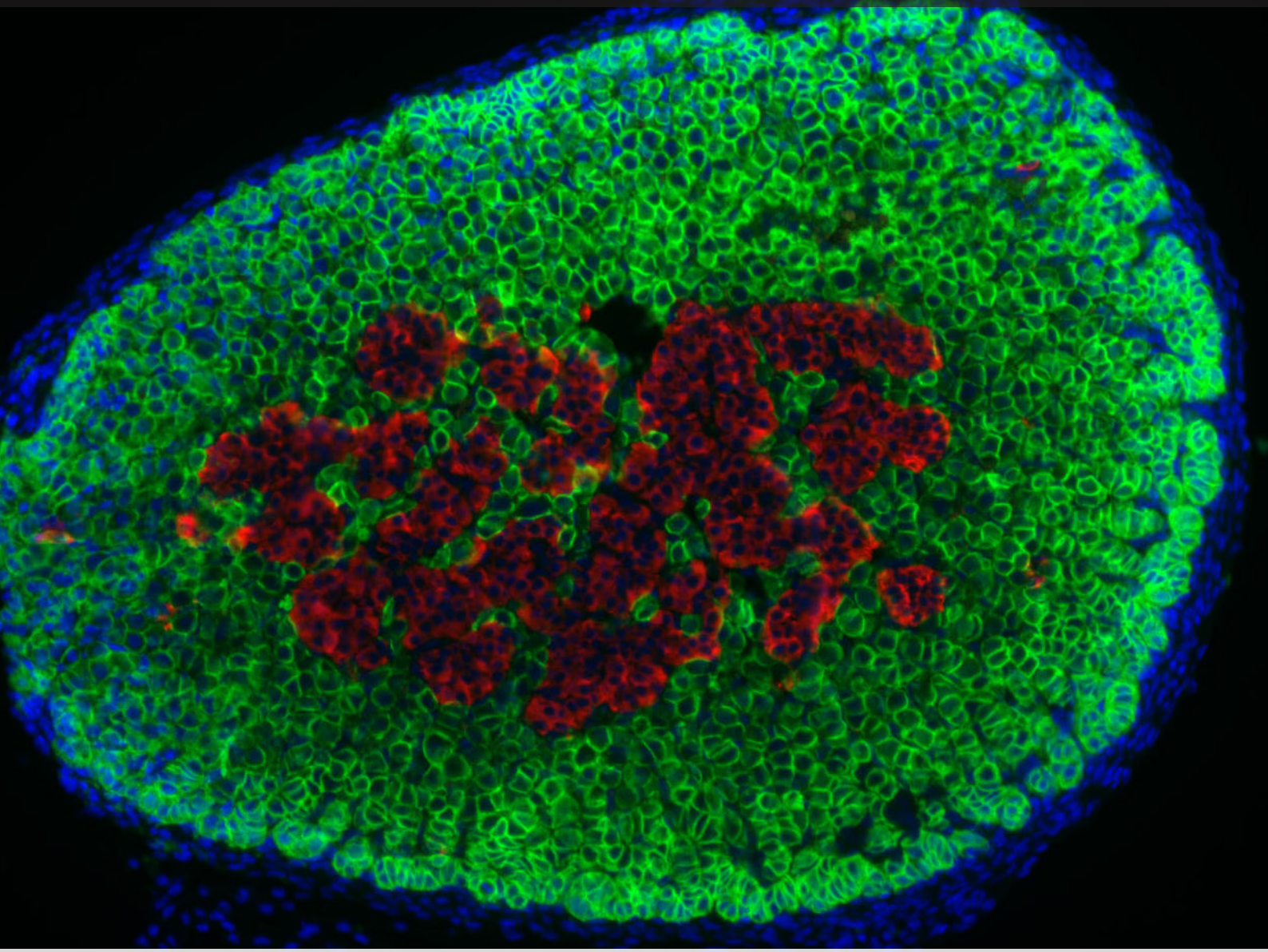

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ADRENAL CORTEX: FROM PHYSIOLOGY TO DISEASE

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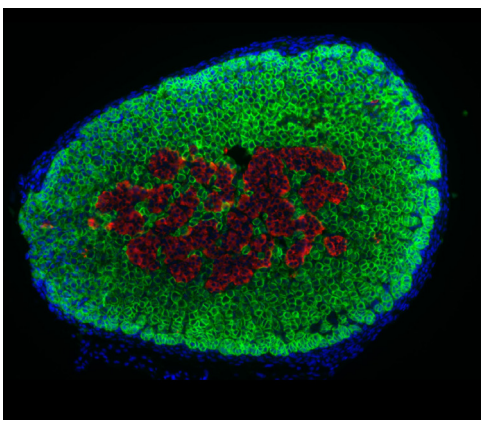

One day post-partum mouse adrenal gland from a mTmG,Sf1:Cre cross. Nuclei were stained with Hoechst (blue), GFP (marking Sf1:Cre recombined cells) in green and Tyrosine Hydroxylase (chromaffin cells) in red.

Image by Isabelle Barnola.

The adrenal gland plays essential roles in the control of body homeostasis, stress and immune responses. The adrenal cortex represents up to 90% of the gland and is specialised in the production of mineralocorticoids, glucocorticoids and adrenal androgens. This production is tightly coordinated and results from a unique zonal organisation. Although our knowledge of the molecular mechanisms controlling adrenal steroidogenesis is quite extensive, for decades, the mechanisms of adrenal cortex development, cellular homeostasis and renewal have remained elusive. The advent of new high-throughput technologies and sophisticated genetic approaches has brought tremendous progress in our understanding of how the adrenal cortex achieves and maintains its particular organisation. The aim of this Frontiers in Endocrinology Topic is to provide readers with a snapshot of our current knowledge on adrenal physiology and how deregulations of these processes result in adrenal diseases. This includes but is not limited to, basic research on adrenal development, cell lineage identification, progenitor cells, tissue renewal, control of differentiation and zonation and clinical research on the identification of disease-related genes.

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EDITORIAL
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Editorial: Adrenal Cortex: From Physiology to Disease

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Keywords: adrenal, development, physiology, zonation, disease, benign tumour, cancer, insufficiency

The Editorial on the Research Topic

Adrenal cortex: from physiology to disease

The adrenal gland plays essential roles in the control of body homeostasis, stress, and immune responses. The adrenal cortex represents up to 90% of the gland and is specialized in the production of adrenal steroids. The coordinate production of these steroids relies on adrenal cortex zonation, which corresponds to the establishment of distinct concentric functional zones in the perinatal period: outermost zona glomerulosa synthesizes mineralocorticoids, zona fasciculata produces glucocorticoids, and innermost zona reticularis synthesizes both glucocorticoids and adrenal androgens.

This zonal organization has to be maintained throughout the life of the individual, despite constant centripetal tissue renewal. The review by Pihlajoki et al. summarizes the latest findings on the mechanisms of adrenal cortex renewal, which relies on outer cortex progenitors recruitment and lineage conversion along cell migration within the cortex. This paper also provides a comprehensive overview of the hormones, signaling pathways, and transcription factors that control these processes to allow for on-demand adaptation of cortical function and maintenance of adrenal homeostasis.

Defects in adrenal development and maintenance are associated with adrenal insufficiency, a life threatening condition for which lifelong hormonal replacement therapies can be challenging. The review by Ruiz-Babot and colleagues sheds light on novel developments in the field of adrenal replacement, including pluripotent cell reprograming and the use of encapsulating devices with semi-permeable membranes to avoid immune rejection of grafts. These promising approaches could pave the way for future clinical management of adrenal-insufficiency patients.

While adrenal insufficiency is clinically problematic, the opposite situation in which adrenal steroid production is increased also raises significant clinical concerns. Hypercortisolism results in Cushing's syndrome associated with central obesity, arterial hypertension, immunosuppression, and depression. Hyperaldosteronism is associated with high blood pressure and profound cardiovascular and renal alterations, which result in increased risk of cardiovascular failure. These highly morbid syndromes are the consequence of either benign hyperplasia and tumors or adrenocortical cancer (ACC).

The review by Boulkroun and colleagues establishes the molecular bases of normal control of aldosterone production and elaborates on recent next-generation sequencing (NGS) analyses that allowed identification of mutations in potassium and calcium channels as key players in the development of hyperaldosteronism. Even if these mutations can explain increased aldosterone secretion, they are unlikely to account for tumor growth. Boulkroun et al. summarize data showing that deregulated cell growth in aldosterone-producing adenomas is likely to result from WNT and SHH signaling pathway activation in these tumors.

Deregulated protein kinase A (PKA) signaling is a common theme in adrenal tumors associated with ACTH-independent hypercortisolism. These include primary pigmented adrenocortical disease (PPNAD), adrenal adenomas, bilateral macronodular adrenal hyperplasia (BMAH), and adrenal

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cancer. The review by Berthon et al. provides in-depth insight into the genetic causes of deregulated PKA signaling, including inactivating *PRKAR1A* mutations in PPNAD and activating *PRKACA* mutations in cortisol-producing adenomas. Interestingly, mutations in either *PRKAR1A* or *PRKACA* were not found in BMAH. The review by Drougat et al. emphasizes the discovery of mutations in *ARMC5* as a likely cause of these particular benign adrenal tumors and elaborates on potential pathogenic mechanisms. Lefebvre et al. shed another light on BMAH by focusing on the paracrine regulation of cortisol secretion. They gather data showing that cortisol secretion is stimulated by the release of a number of factors either produced by non-steroidogenic cells within the cortex (mast, chromaffin, and endothelial cells) or by a subset of aberrantly differentiated steroidogenic cells that can release serotonin or even ACTH within the hyperplastic tissue. They further suggest that aberrant ACTH production and expression of ectopic receptors, such as the receptors of LH, GIP, and 5-HT7, may be the result of aberrant differentiation of gonadal-like cells, triggered by driver mutations, such as ARMC5 inactivation.

Beyond steroid hormone excess, which is also observed in about 40–60% of patients, ACC still represents a major therapeutic challenge. The review by Libé and colleagues provides an overview of current diagnosis and treatment of ACC, which emphasizes the need for novel therapeutic targets in a cancer with dismal prognosis. The review by Drougat and colleagues provides insight into the role of mutations targeting the WNT signaling pathway (essentially activating mutations of *CTNNB1* and deletions of *ZNRF3*) and their pathogenic role in ACC. This paper on adult ACC is nicely complemented by Lalli and Figueiredo's review that focuses on pediatric ACC. These are rare tumors that generally occur in the context of *TP53* alterations, in particular the specific R337H mutation found with high frequency in Southern Brazil. The authors present evidence that these tumors are likely to derive from the fetal adrenal and discuss the common and divergent alterations found in pediatric and adult ACC, which highlights the lack of effective prognosis markers in the former. Deregulation of miRNA production is a common theme in most cancers. Nadia Cherradi provides a comprehensive overview of miRNA deregulation in ACC and shows that they can provide novel insight into the pathogenesis of ACC and may constitute interesting therapeutic targets. This review also highlights the usefulness of circulating miRNAs as novel non-invasive diagnostic and prognostic biomarkers in ACC. It further elaborates on an exciting aspect of miRNAs biology that involves their circulation within ACC cell-derived exosomes, which would allow communication with tumor microenvironment.

We hope that you will find this topic inspiring and that it will shed light on exciting aspects of adrenal physiology and disease.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Adrenocortical zonation, renewal, and remodeling

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The adrenal cortex is divided into concentric zones. In humans the major cortical zones are the zona glomerulosa, zona fasciculata, and zona reticularis. The adrenal cortex is a dynamic organ in which senescent cells are replaced by newly differentiated ones. This constant renewal facilitates organ remodeling in response to physiological demand for steroids. Cortical zones can reversibly expand, contract, or alter their biochemical profiles to accommodate needs. Pools of stem/progenitor cells in the adrenal capsule, subcapsular region, and juxtamedullary region can differentiate to repopulate or expand zones. Some of these pools appear to be activated only during specific developmental windows or in response to extreme physiological demand. Senescent cells can also be replenished through direct lineage conversion; for example, cells in the zona glomerulosa can transform into cells of the zona fasciculata. Adrenocortical cell differentiation, renewal, and function are regulated by a variety of endocrine/paracrine factors including adrenocorticotropin, angiotensin II, insulin-related growth hormones, luteinizing hormone, activin, and inhibin. Additionally, zonation and regeneration of the adrenal cortex are controlled by developmental signaling pathways, such as the sonic hedgehog, delta-like homolog 1, fibroblast growth factor, and WNT/β-catenin pathways. The mechanisms involved in adrenocortical remodeling are complex and redundant so as to fulfill the offsetting goals of organ homeostasis and stress adaptation.

Keywords: adrenal cortex, hormone, plasticity, stem cell, steroid, steroidogenesis

INTRODUCTION

The adrenal cortex is a major source of steroid hormones, which are synthesized from cholesterol through the sequential actions of a series of cytochrome P450 (CYP) enzymes and hydroxysteroid dehydrogenases (HSDs) (**Figure 1**) (1). Anatomically and functionally distinct zones in the adrenal cortex synthesize specific steroid hormones in response to endocrine and paracrine signals. The regulation of adrenocortical development and homeostasis has been the subject of intensive investigation over the past decade (2–4). This review article summarizes recent advances in our understanding of adrenocortical zonation, renewal, and remodeling. Animal models useful for studies of adrenocortical biology, such as the mouse, rat, and ferret, are highlighted.

ADRENOCORTICAL ZONATION IN HUMANS AND ANIMAL MODELS

The adrenal cortex of humans is composed of three concentric layers: the zona glomerulosa (zG), zona fasciculata (zF), and zona reticularis (zR) [reviewed in Ref. (2)]. The outermost layer, the zG, functions as part of the renin-angiotensin-aldosterone system (RAAS). In response to angiotensin II (Ang II) or elevated plasma potassium ion (K^+) concentrations, zG cells secrete aldosterone, a mineralocorticoid that induces the retention of sodium ion (Na^+) and water and the excretion of K^+ by the kidney. Cells in the zG express the Ang II receptor (AT1R) and aldosterone synthase (CYP11B2). At the ultrastructural level, zG cells are typified by numerous mitochondria with lamelliform cristae and a few cytoplasmic lipid droplets (**Figure 2A**). Cells in the zF produce glucocorticoids as part of the hypothalamic-pituitary-adrenal (HPA) axis. zF cells respond to adrenocorticotropic hormone (ACTH) via its receptor (MC2R) and the accessory protein MRAP. Cells in the zF are organized in cord-like structures, or fascicles, that are surrounded by fenestrated capillaries. Cells in this zone contain numerous mitochondria with tubulovesicular cristae, many cytoplasmic lipid droplets, and prominent smooth endoplasmic reticulum (**Figure 2B**) (5, 6). The innermost layer of the cortex, the zR, secretes the weak androgen dehydroepiandrosterone (DHEA) and its sulfated form DHEA-S (1). Cells of the zR resemble those of the zF but contain fewer lipid droplets and more lysosomes and vacuoles (6). The adrenal gland is covered by a fibrous capsule that serves as both a support structure and a reservoir of stem/progenitor cells for the cortex (see Section "Adrenocortical Stem Cells") (7).

Species differ in their adrenocortical zonation patterns (8) (**Figure 3**). In the mouse and rat, the adrenal cortex contains zG and zF, but there is no recognizable zR. The adrenal cortex of the young mouse contains an additional, ephemeral layer known as the X-zone (9, 10). The function of the X-zone remains controversial, but it may be involved in progesterone catabolism (11). The rat adrenal cortex contains a less prominent layer, the undifferentiated zone (zU), located between the zG and zF (12). The zU has been implicated in adrenocortical homeostasis and remodeling (see Section "Delta-like Homologue 1 Pathway") (12, 13). Cells in the inner aspect of the zU express MC2R and cholesterol side-chain

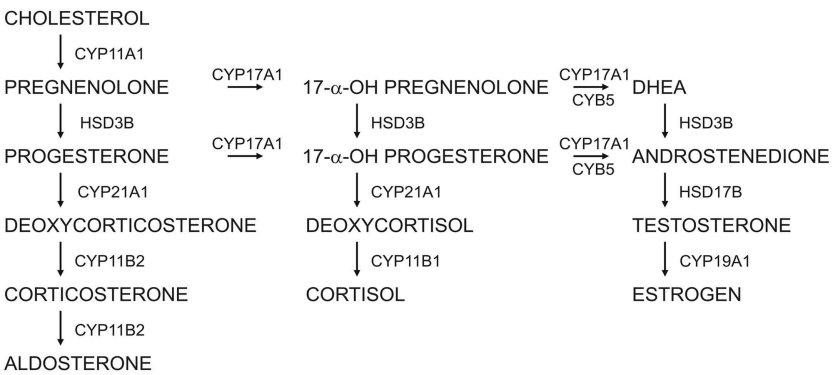

FIGURE 1 | Steroidogenic pathways in the human adrenal cortex and gonads.

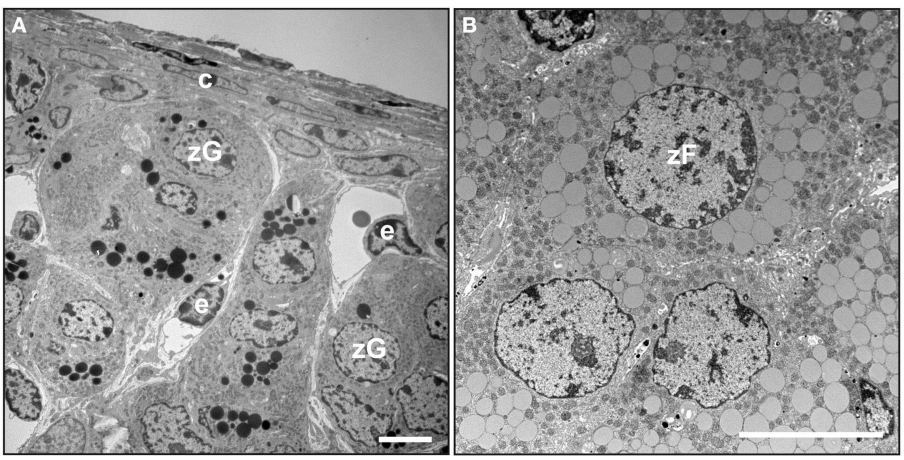

FIGURE 2 | Electron microscopy of mouse adrenal cortex. Adrenal glands from a 4-month-old female mouse were fixed in Karnovsky's solution, postfixed in 2% OsO_4, dehydrated, and then embedded in epon. Thin sections were stained with uranyl acetate plus lead citrate and examined by transmission electron microscopy. **(A)** Adrenal capsule and zona glomerulosa. **(B)** Zona fasciculata. Abbreviations: c, capsule; e, endothelial cell; zF, zona fasciculata cell; zG, zona glomerulosa cell. Bars, 4 μm.

cleavage enzyme (CYP11A1), which catalyzes the first reaction in steroidogenesis. The inner zU lacks expression of markers of the zG (*Cyp11b2*) or zF (steroid 11β-hydroxylase; *Cyp11b1*) (14). Thus, the inner zU may represent a transitional population of cells committed to the steroidogenic phenotype. An analogous layer, the zona intermedia (zI), is present in the adrenal glands of ferrets (15). Recently, the spiny mouse (genus *Acomys*) has attracted attention as a novel model for the study of adrenocortical development and function. In contrast to the laboratory mouse (genus *Mus*), the adrenal cortex of the spiny mouse contains the zR and secretes both cortisol and DHEA (16). In this respect the adrenal gland of the spiny mouse mimics that of humans.

Species also vary in the repertoire of steroidogenic enzymes and cofactors expressed in the adrenal cortex, and these differences impact function (**Figure 3**). Two factors that are differentially expressed among species are 17α-hydroxylase/17,20 lyase (CYP17A1) and cytochrome b_5 (CYB_5). CYP17A1, a bifunctional enzyme, catalyzes the 17α-hydroxylation reaction required for cortisol synthesis and the 17,20-lyase reaction required for the androgen production (1). The lyase activity is enhanced by allosteric interactions with CYB_5 (1). Cells in the zF and zR of humans and ferrets have 17α-hydroxylase activity, so cortisol is the principal glucocorticoid secreted by the adrenal gland of these organisms (8). In humans the adrenal cortex begins to produce DHEA and DHEA-S at adrenarche, contemporaneous with increased expression of *CYB5* in the zR (1). The adrenal glands of ferrets produce only limited amounts of androgens due to low CYB_5 expression (8, 17). Cells in the adrenal cortex of adult mice and rats lack CYP17A1, so corticosterone is the principal glucocorticoid secreted, and adrenal androgens are not produced (8). The relative strengths and weaknesses of established and emerging animal models are summarized in **Table 1**.

ADRENOCORTICAL RENEWAL AND REMODELING

The adult adrenal cortex is a dynamic tissue. Cells lost through senescence or injury are continually replenished through cell

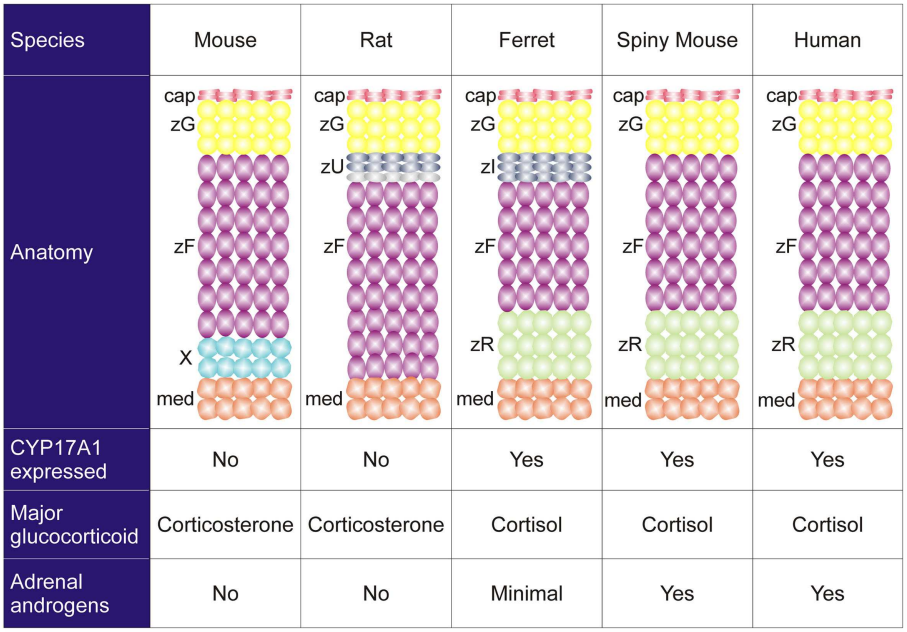

FIGURE 3 | Comparative anatomy and physiology of the adrenal cortex. The undifferentiated zone of the rat adrenal is subdivided into outer (dark gray) and inner (light gray) zones that differ in marker expression and function (see the text). Abbreviations: cap, capsule; med, medulla; X, X-zone; zF, zona fasciculata; zG, zona glomerulosa; zI, zona intermedia; zR, zona reticularis; zU, undifferentiated zone.

Table 1 | Advantages and disadvantages of various animal models for studies of adrenocortical zonation and remodeling.

	Mouse	Rat	Spiny mouse	Ferret
Advantages	• Genetically and epigenetically tractable • Well suited for transplantation experiments • Gonadectomy triggers the accumulation of gonadal-like cells in the adrenal cortex (see Section "LH Signaling")	• Well suited for pharmacological studies (see Section "Adrenocortical Renewal and Remodeling") • Adrenal enucleation experiments are feasible (see Section "Adrenocortical Renewal and Remodeling")	• Adrenal gland is anatomically and functionally similar to that of humans	• Well characterized neuroendocrine physiology • Gonadectomy triggers the accumulation of gonadal-like cells in the adrenal cortex (see Section "LH Signaling")
Disadvantages	• Lacks zR and does not produce androgens	• Lacks zR and does not produce androgens	• Not widely available • Not standardized with regard to genotype	• Not standardized with regard to genotype

division and differentiation (2, 4). In the adult adrenal gland, most cell proliferation occurs near the periphery of the cortex, as shown by bromodeoxyuridine and [^{3}H]thymidine labeling experiments [reviewed in Ref. (3)]. The remarkable regenerative capacity of the organ is evidenced by rat adrenal enucleation experiments, wherein the gland is incised and squeezed so as to extrude the cortex. Within weeks a new adrenal cortex regenerates from the remaining capsule and adherent subcapsular cells [reviewed in Ref. (18)].

Constant cellular turnover in the adrenal cortex facilitates rapid organ remodeling in response to physiological demand for steroids. Zones can reversibly enlarge, shrink, or alter their biochemical profiles to accommodate physiological needs or in response to experimental manipulations (**Table 2**). For example, administration of captopril, an inhibitor of the RAAS, leads to contraction of the zG in rats [reviewed in Ref. (2)].

OVERVIEW OF ADRENOCORTICAL DEVELOPMENT

Embryogenesis and early postnatal development provide a contextual framework for understanding the mechanisms involved in adrenocortical zonation and homeostasis. Although structurally and functionally distinct, the adrenal cortex, ovary, and testis arise from a common progenitor, the adrenocortical primordium (AGP). The AGP is derived from a specialized region

Table 2 | Triggers of zonal remodeling in the adrenal cortex.

Zone (species)	Physiological or experimental trigger	Effect	Reference
zG (rat)	↓ [Na^+] or ↑ [K^+] in diet	Expands the zone, increasing aldosterone production	(2)
	↑ [Na^+] or ↓[K^+] in diet	Contracts the zone, decreasing aldosterone production	
zF (rat)	ACTH	Expands the zone, increasing glucocorticoid production	(2)
	Dexamethasone	Contracts the zone, decreasing glucocorticoid production	
zR (primates)	Adrenarche in humans and chimpanzees	Increases the expression of CYB_5, enhancing DHEA production	(19)
	Social status in marmosets	Adult females develop a functional zR in a reversible manner dependent on social status	(20)
	Cortisol in human adrenocortical cells	Stimulates DHEA production through competitive inhibition of 3βHSD2 activity	(21)
X-zone (mouse)	Puberty in males or first pregnancy in females	Induces regression of the zone	(22)
	Activin	Induces regression of the zone	(23)
	Gonadectomy	Delays regression of the zone or induces growth of a secondary zone	(22, 23)

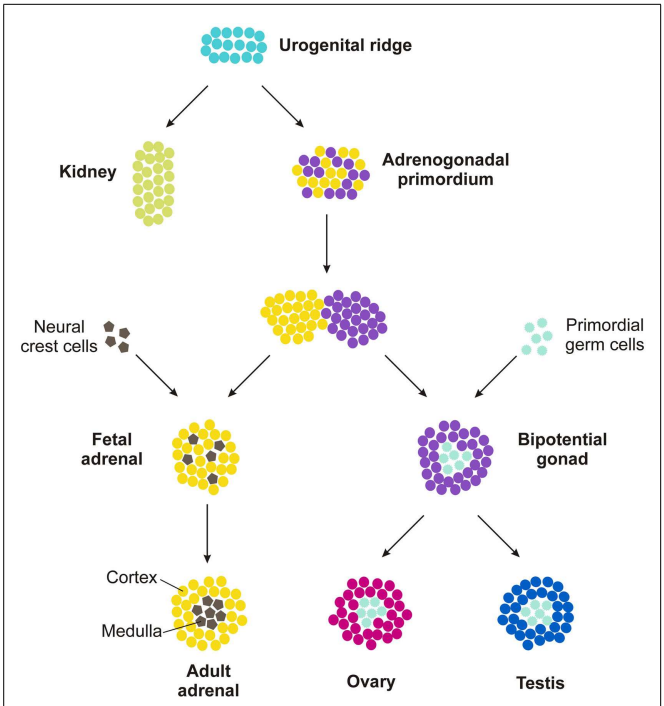

FIGURE 4 | Development of the adrenal gland and gonads.

of celomic epithelium known as the urogenital ridge (**Figure 4**), which also gives rise to the kidney and progenitors of definitive hematopoiesis. Cells in the AGP co-express the transcription factor genes Wilms tumor suppressor-1 (*Wt1*), GATA-binding protein 4 (*Gata4*), and steroidogenic factor-1 (*Sf1*, also called *AdBP4* or *Nr5a1*) [reviewed in Ref. (2, 24, 25)]. As development proceeds, progenitors of the adrenal cortex and the gonad separate and activate different transcriptional programs. Adrenal progenitor cells in the AGP migrate dorsomedially into subjacent mesenchyme, upregulate expression of *Sf1*, and downregulate expression of *Wt1* and *Gata4* (25, 26). In contrast, gonadal progenitor cells in the AGP migrate dorsolaterally and maintain expression of *Sf1*, *Wt1*, and *Gata4*. Adrenal precursors combine with neural-crest derived sympathoblasts, the precursors of chromaffin cells in the medulla, to form the adrenal anlagen. Gonadal progenitors combine with primordial germ cells to form the bipotential gonad. Subsequently, the nascent adrenal glands become enveloped by capsule cells, which are derived from both surrounding mesenchyme and fetal adrenal cells that previously expressed *Sf1* [reviewed in Ref. (27)].

In rodents, zonal patterns of steroidogenic enzyme expression first become evident during embryonic development [reviewed in Ref. (24)]. In mice, expression of *Cyp11a1* is first detectable in the nascent adrenal at embryonic day (E) 11.5–12.5 (26, 28), and there is a concurrent increase in the level of endogenous biotin (29). Expression of the zF marker *Cyp11b1* begins at E13.5, whereas expression of the zG markers Ang II receptor type 1 (*At1b*) and *Cyp11b2* appears in the periphery of the cortex just before birth, and *Cyp11b2* and *Cyp11b1* expression domains are mutually exclusive at this stage (30–32).

By the eighth week of gestation in humans, the fetal adrenal cortex contains two morphologically distinct layers: an inner fetal zone (Fz) and an outer definitive zone (Dz) (33). The Fz is thick and contains large, eosinophilic cells, whereas the Dz is thin and contains small, basophilic cells. Functionally, the Fz resembles the adult zR. The Fz expresses *CYP17A1* and *CYB5* and produces large amounts of DHEA and DHEA-S, which are converted by the sequential actions of the liver and placenta into estrogens. A third cortical zone, termed the transitional zone (Tz), becomes evident shortly thereafter. The Tz produces cortisol, and an early burst of cortisol production during the ninth week of gestation, coinciding with a transient increase in expression of 3β-hydroxysteroid

dehydrogenase type 2 (*HSD3B2*), is thought to safeguard female sexual development by suppressing the fetal HPA axis and thereby inhibiting adrenal androgen production (34). At birth, the adrenal gland is almost as large as the kidney, but the size of the organ decreases dramatically over first 2 weeks of neonatal life; the Fz involutes via apoptosis, and there is a concomitant reduction in adrenal androgen production (1). The mouse X-zone, a remnant of the fetal adrenal that regresses postnatally (9), is thought to be the analog of the human Fz. Postnatally, the human Dz differentiates into the anatomically and functionally distinct zones of the adult cortex.

ADRENOCORTICAL STEM CELLS

The adrenal cortex contains stem/progenitor cells that can divide and differentiate to replenish senescing cells and maintain or expand zones (**Table 3**) [reviewed in Ref. (4)]. In one long-standing model of adrenal zonation, the cell migration model, stem/progenitor cells in the periphery of the adrenal cortex differentiate and migrate centripetally to repopulate the gland before undergoing apoptosis in the juxtamedullary region (35). Aspects of this model have been validated through lineage tracing analyses (24, 30, 36), but recent studies indicate that the regulation of zonation is more complex than originally appreciated [reviewed in Ref. (13)]. It is now clear that distinct pools of stem/progenitor cells exist in the adrenal capsule, subjacent cortex, juxtamedullary region, and other sites (**Table 3**). Some of these pools appear to be activated only during specific developmental windows or in response to extreme physiological demand. Under certain experimental conditions, adrenocortical zones can be replenished by centrifugal migration (37, 38). For example, stem/progenitor cells in the juxtamedullary region can proliferate, differentiate, and centrifugally repopulate the cortex with fetal-like cells, as is seen in gonadectomy (GDX)-induced secondary X-zone formation and in a genetic model of dysregulated cAMP production (37, 39, 40). The mechanisms that govern centripetal and centrifugal migration are not well understood. Whether centrifugal migration operates under basal conditions is unknown.

ADRENOCORTICAL CELL PLASTICITY

Cell plasticity is another mechanism for replenishing adrenocortical cells lost to senescence or injury. Plasticity refers to the ability of cells to adopt an alternate functional identity in response to cues from the hormonal milieu and cellular microenvironment. One form of plasticity entails trans-differentiation, the direct conversion of one differentiated cell into a differentiated cell of another lineage (42). A second form of plasticity involves de-differentiation, wherein a differentiated cell reverts to a less differentiated cell within the same tissue lineage (42). Interconversion of differentiated cells, either through trans- or de-differentiation, provides an alternative to regeneration via mobilization of stem/progenitor cells. Such functional redundancy ensures organ homeostasis and an optimal adaptation to stress (13).

The plasticity of differentiated adrenocortical cells was elegantly demonstrated in fate mapping studies by Freedman et al. (36), who used *Cyp11b2*-Cre to permanently mark zG cells and their descendants with green fluorescent protein (GFP). By tracing the fate of GFP^+ cells, the investigators showed that adrenocortical zonation is orchestrated in part by direct lineage conversion of zG cells into zF cells (**Figure 5**). To show that zG-to-zF conversion participates in adrenocortical remodeling, Freedman et al. treated adult mice with glucocorticoids to inhibit the HPA axis (36). Glucocorticoid treatment caused contraction of the zF and loss of GFP^+ cells in this zone. Following withdrawal of exogenous glucocorticoids, zG-to-zF conversion resumed and the zF expanded. Remarkably, when conversion of zG to zF cells was abrogated through conditional deletion of the *Sf1* gene in $CYP11B2^+$ cells, a functional zF still formed, implying the existence of alternate routes for differentiation of zF cells. These alternative sources for zF cells remain the subject of active investigation. Collectively, these results support a model in which differentiated cells undergo lineage conversion during adrenocortical renewal and remodeling.

Table 3 | Stem/progenitor cell populations that give rise to steroidogenic and non-steroidogenic cells in the adrenal cortex.

Stem/progenitor population	Location	Comments	Reference
$WT1^+$ progenitors	Capsule	Under basal conditions, $WT1^+$ capsule cells give rise to steroidogenic cells in the adrenal cortex. GDX triggers their differentiation into gonadal-like tissue	(25)
$GLI1^+$ progenitors	Capsule	In response to SHH, $GLI1^+$ progenitors migrate into the cortex and differentiate into steroidogenic cells	(27, 30, 41)
$TCF21^+$ progenitors	Capsule	$TCF21^+$ capsular cells give rise to non-steroidogenic stromal cells in the adrenal cortex	(27)
SHH^+ progenitors	Subcapsular region	These progenitors give rise to steroidogenic cells in the zF and zG but not capsule cells	(27, 30, 41)
Fetal adrenal-like progenitors	Juxtamedullary region	These progenitors, normally dormant in the adult, can become activated following certain experimental manipulations and migrate centrifugally	(37, 39, 40)

These progenitor populations, defined by fate mapping studies and related techniques, are not mutually exclusive. For example, $WT1^+$ progenitors have been shown to co-express Gli1 and Tcf21. Some of these progenitors give rise to differentiated cells only during specific developmental windows or in response to experimental manipulation.

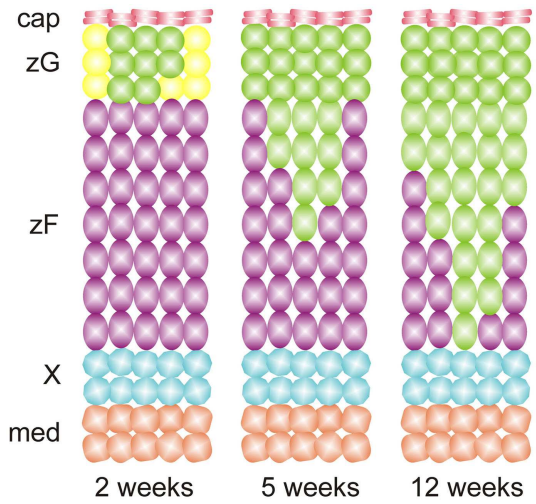

FIGURE 5 | Adrenocortical zonation during postnatal mouse development results from lineage conversion of zG cells into zF cells, as evidenced by fate mapping using *Cyp11b2*-cre and a GFP reporter. Recombination of the reporter in zG leads to expression of GFP (green cells). The resultant cells migrate inward and differentiate into zF cells. Abbreviations: cap, capsule; med, medulla; X, X-zone; zF, zona fasciculata; zG, zona glomerulosa.

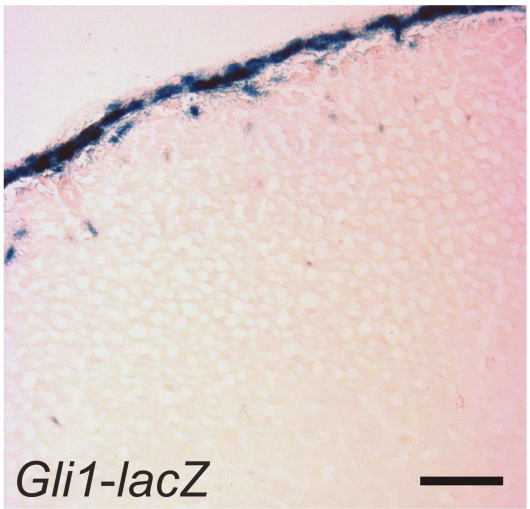

FIGURE 6 | GLI1$^+$ cells in the adrenal capsule. An adrenal gland from a 1-month-old female *Gli1-lacZ* mouse was whole mount stained with X-gal, cryosectioned, and counterstained with eosin. Bar, 50 μm.

DEVELOPMENTAL SIGNALING PATHWAYS IMPLICATED IN ADRENOCORTICAL ZONATION, RENEWAL, OR REMODELING

Developmental signaling pathways control cell pluripotency, differentiation, and patterning in various tissues. As detailed below, some of these signaling pathways play key roles during the exponential growth phase of adrenal cortex development (12, 24, 43, 44). Additionally, these pathways regulate renewal and remodeling in the adult organism.

HEDGEHOG PATHWAY

The hedgehog family of morphogens comprises sonic hedgehog (SHH), Indian hedgehog, and desert hedgehog. Each of these ligands binds to Patched-1 (PTCH1), a transmembrane receptor that is expressed on target cells (45). In the absence of hedgehog binding, PTCH1 inhibits the G protein-coupled receptor Smoothened (SMO) [reviewed in Ref. (2, 46)]. As a result, the zinc finger transcription factors GLI2 and GLI3 are proteolytically digested and lose their activation domains (47). The resultant truncated forms of GLI2 and GLI3 repress transcription. Binding of hedgehog ligands to PTCH1 relieves the inhibition it exerts on SMO, thereby preventing the proteolytic processing of the GLI factors. Full-length GLI2 and GLI3 act as transcriptional activators. The related transcriptional activator, GLI1, is not expressed in the absence of hedgehog ligand, but is upregulated by activation of the pathway. Consequently *Gli1* expression serves as a useful marker for active hedgehog signaling (48).

SHH, the only member of the hedgehog family produced in the adrenal cortex, is secreted by subcapsular cells that express *Sf1* but not the terminal enzymes required for corticoid synthesis (30, 41, 49). Capsular cells, which do not express *Sf1*, respond to SHH by expressing *Gli1* (**Figure 6**). Some of these GLI1$^+$ capsule cells migrate centripetally into the cortex, lose responsiveness to SHH, and become steroidogenic, as evidenced by upregulation of *Sf1* and differentiation markers characteristic of the zG (*Cyp11b2*) or zF (*Cyp11b1*) (**Table 2**). GLI1$^+$ progenitor cells efficiently contribute to steroidogenic lineages during the exponential phase of cortical growth in embryo, fetus, and newborn mouse (30). In the adult mouse, GLI1$^+$ progenitors contribute to the cortex with low efficiency, but the pathway can be activated in the adult following experimental manipulations such as dexamethasone-induced cortical atrophy. Conditional deletion of *Shh* in steroidogenic cells of the mouse adrenal results in cortical hypoplasia and capsular thinning, but does not cause major alterations in zonation (30, 41, 49).

DELTA-LIKE HOMOLOG 1 PATHWAY

A related signaling protein implicated in adrenocortical homeostasis is Delta-like homolog 1 (DLK1). This factor, also known as preadipocyte factor-1 (PREF-1), is a transmembrane protein related to the Notch family of signaling molecules. DLK1 was originally identified as an important regulator of the undifferentiated state in preadipocytes (50). Cleavage of the extracellular domain of DLK1 by TNF-α converting enzyme produces a biologically active soluble peptide that inhibits the differentiation of preadipocytes into mature adipocytes (50). Subsequent studies showed that DLK1 controls the quiescence of stem/progenitor cells in not only adipose tissue but also other tissue types, including the adrenal cortex (12, 50).

Adrenal enucleation experiments have shown that *Dlk1* expression is downregulated and not re-established until zonation of the cortex is complete, suggesting that DLK1 is a negative regulator of adrenocortical differentiation (51). *Dlk1* is co-expressed with *Shh* in the outer zU of the rat (**Figure 7**) (12). Soluble DLK1, like SHH, modulates *Gli1* expression in nearby capsule cells. In addition to being co-expressed, *Dlk1* and *Shh* are coordinately regulated (12).

Both genes are downregulated in the adrenals of mice fed a low Na^+ diet. Conversely, *Dlk1* and *Shh* are upregulated in the adrenals of mice treated with captopril. These findings suggest that DLK1 and SHH may act together to fine tune the activation of signal receiving cells in the adrenal capsule of the rat. The expression pattern of *Dlk1* differs between rats and mice; in mice *Dlk1* is expressed in the adrenal capsule rather than the underlying cortex. Nevertheless, indirect evidence suggests that in mice, as in rats, DLK1 may negatively regulate the differentiation of $GLI1^+$ capsular progenitor cells (43).

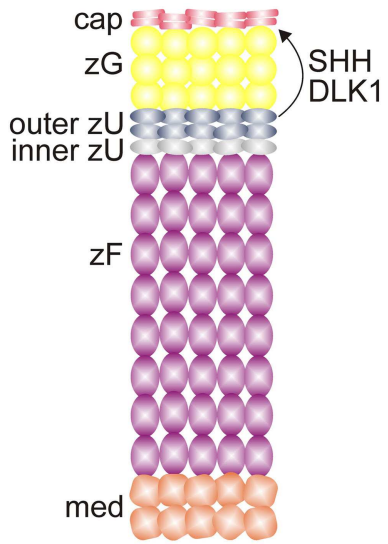

FIGURE 7 | *SHH* and *DLK1* are co-expressed in the outer zU of the rat adrenal cortex and may act in concert to regulate stem/progenitor cells in the adrenal capsule. Abbreviations: cap, capsule; DLK1, delta-like homolog-1; med, medulla; SHH, sonic hedgehog; X, X-zone; zF, zona fasciculata; zG, zona glomerulosa; zU, undifferentiated zone.

FIBROBLAST GROWTH FACTOR PATHWAY

Mouse genetic studies have implicated the FGF signaling pathway in adrenocortical development and maintenance [reviewed in Ref. (2, 43)]. The FGF family comprises a large group of extracellular ligands that signal through a family of tyrosine kinase receptors, the FGF receptors (FGFRs). In mammals, the FGFR family consists of four genes, FGFR1-4, which undergo alternative splicing to generate an array of receptors that differ in ligand affinities (52). In the presence of heparin, FGFs bind to their cognate receptors, promoting receptor dimerization and autophosphorylation. This in turn stimulates downstream signaling pathways, including the phosphatidylinositol 3-kinase (PI3K), Janus kinase and signal transducer and activator of transcription (JAK–STAT), and mitogen-activated protein kinase (MAPK) pathways. FGF signaling is essential for proper patterning of the embryo, and this pathway participates in stem cell maintenance (53). Factors in the FGF pathway are expressed in both the adrenal capsule and cortex, as summarized in **Table 4**.

WNT/β-CATENIN SIGNALING

β-catenin exists in two pools: a cytoskeletal pool controls the interaction of cadherin complexes with adherens junctions, while a cytoplasmic pool participates in canonical WNT signaling, acting as a co-activator for transcription factors of the TCF/LEF family [reviewed in Ref. (2)]. Transcriptionally active β-catenin has been demonstrated in the AGP, the adrenal primordium, and adrenal subcapsular cells of the fetus and adult (61) (**Figure 8**). WNT/β-catenin signaling is thought to maintain the undifferentiated state of adrenocortical stem/progenitor cells (7, 62). Targeted mutagenesis of β-catenin in $SF1^+$ cells causes late onset adrenal hypoplasia, presumed to be the result of stem/progenitor cell pool

Table 4 | FGF ligands and receptors implicated in adrenocortical cell development and homeostasis.

	Protein	Location	Comments	Reference
Ligands	FGF1	Cortex	This isoform activates FGFR2 IIIb	(43)
	FGF2	Capsule	FGF2, which activates FGFR1 IIIc, acts as a mitogen for adrenocortical cells both in culture and in gland regeneration experiments and has been shown to bind specifically to cells from the zG	(43, 54–58)
	FGF9	Capsule	This isoform activates FGFR1 IIIc	(43)
Receptors	FGFR1 IIIc	Capsule and cortex	This FGFR isoform is expressed in both capsule and cortex, although its precise role in adrenocortical development is unknown	(43)
	FGFR2 IIIb	Cortex	Like SHH and β-catenin, this FGFR isoform is expressed in the subcapsular region; embryos with a global *Fgfr2 IIIb* deletion have hypoplastic adrenal glands, impaired steroidogenesis, and thickened adrenal capsules with increased *Gli1* expression	(43, 59)
	FGFR2 IIIc	Cortex	Like SHH and β-catenin, this FGFR isoform is expressed in clusters of cells in the subcapsular region. Deletion of both FGFR2 isoforms in steroidogenic tissues leads to hypoplastic adrenals	(43, 60)
	FGFR3 IIIc	Cortex	This isoform is expressed in cortex, although its precise role in adrenocortical development is unknown	(43)

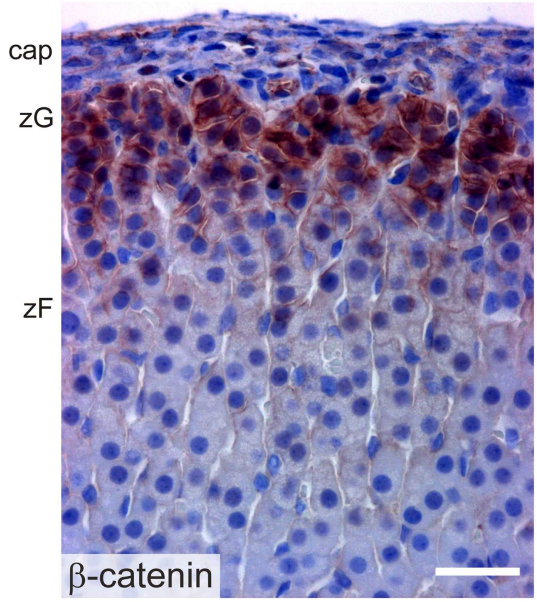

FIGURE 8 | Immunoperoxidase staining of β-catenin in the adrenal cortex of a 2-month-old female mouse. Abbreviations: cap, capsule; zF, zona fasciculata; zG, zona glomerulosa; Bar, 30 μm.

depletion (61). On the other hand, constitutive activation of β-catenin in steroidogenic cells expressing aldo-keto reductase family 1, member B7 (*Akr1b7*) causes abnormal accumulation of undifferentiated cells in the capsule and subcapsule and a concomitant increase in *Shh* mRNA expression (40).

Regulation of the WNT/β-catenin pathway is complex and entails not only a family of WNT ligands but also multiple receptors, co-receptors, decoy receptors, and other modulators (**Table 5**). This complexity allows fine tuning of the response to morphogen gradients. Stem cell self-renewal mechanisms are frequently co-opted to drive oncogenesis, and WNT signaling is the pathway most frequently mutated in adrenocortical carcinomas (63) (**Table 5**).

In addition to its proposed role in stem cell maintenance and recruitment, the WNT/β-catenin pathway has been implicated in tissue patterning in the adult organism. For example, proper zonation of the liver requires restriction of WNT/β-catenin signaling to hepatocytes near the central vein (64). In an analogous fashion, restriction of WNT signaling to the periphery of the adrenal cortex is thought to direct zonation in this tissue. Constitutive activation of β-catenin signaling in the mouse zF using *Akr1b7*-cre triggers the ectopic expression of the zG marker *Cyp11b2* and increased production of aldosterone (40, 65). Moreover, studies have shown that β-catenin directly regulates the expression of genes critical for zG function, including *At1r* and *Cyp11b2* (66).

Recent studies have shown that proper differentiation of zF cells requires suppression of WNT/β-catenin signaling (67). *In vitro* treatment of a zF cell line (ATCL7) with a chemical inducer of canonical WNT signaling (BIO) resulted in down-regulation of genes essential for zF function, including *Mc2r*, *Cyp11a1*, and *Cyp11b1* (68). Promoter analyses suggested that the molecular basis for this repression may involve the displacement of SF1 from steroidogenic gene promoters by β-catenin (68). These experiments also identified CCDC80 as a novel secreted inhibitor of zF steroidogenesis. Collectively these studies suggest that coordinated regulation of WNT/β-catenin signaling is critical for adrenocortical patterning; WNT/β-catenin signaling must be active for zG determination and must be extinguished for zF determination.

OTHER SIGNALING PATHWAYS IMPLICATED IN ADRENOCORTICAL GROWTH AND REMODELING

Adrenocortical growth and homeostasis are controlled by a diverse array of endocrine/paracrine factors, including ACTH, Ang II, and insulin-related growth factors (IGFs) (15, 24). Hormones traditionally associated with reproductive function, including luteinizing hormone (LH), activin, inhibin, and prolactin, also influence the differentiation and function of adrenocortical cells [reviewed in Ref. (15)].

cAMP SIGNALING

Many of the hormones that regulate adrenocortical cell proliferation bind to G-protein coupled receptors on the surface of cells [reviewed in Ref. (38)]. Activation of these receptors stimulates adenylate cyclase, resulting in cAMP production. cAMP binds to the regulatory subunits of PKA, allowing the catalytic subunits of protein kinase A (PKA) to phosphorylate downstream effectors, including transcription factors that enhance expression of steroidogenic genes (38).

Inactivating mutations in the protein kinase-A regulatory subunit gene (*PRKAR1A*) lead to excessive cAMP production. Such mutations cause Carney complex, a syndrome associated with pituitary-independent Cushing syndrome and adrenocortical neoplasia. Conditional deletion of *Prkar1a* in the adrenal cortex of mice (using *Akr1b7*-cre) leads to disrupted stem/progenitor cell differentiation, excess cell proliferation, and impaired apoptosis in the adrenal cortex (37). This resistance to apoptosis is mediated in part by crosstalk between the PKA and mammalian target of rapamycin (mTOR) pathways (39). As these mice age, a new zone composed of cells that express *Cyp17a1* and secrete cortisol appears in the inner aspect of the cortex. This ectopic X-like zone is thought to arise from normally dormant stem/progenitor cells in the juxtamedullary region (37, 38). These studies and others (38) indicate that normal adrenocortical cell differentiation and proliferation require proper regulation of PKA activity.

IGF SIGNALING

This pathway has been implicated in growth and differentiation of adrenocortical cells. The IGF family consists of two ligands, IGF1 and IGF2, which bind to the receptor tyrosine kinase IGF1R and promote mitosis/survival via signaling through the MAPK and PI3K pathways (76, 77). *IGF1* and *IGF2* are expressed at comparable levels in the adult adrenal cortex, whereas *IGF2* is highly and preferentially expressed in the fetal adrenal cortex. IGF1R is enriched in the subcapsular region (78). The activity of IGFs is modulated by a family of six IGF-binding proteins (IGFBPs), which can bind and either stimulate or inhibit the activity of IGFs (76).

Table 5 | Factors implicated in WNT/β-catenin signaling in the adrenal cortex.

Factor	Function	Adrenocortical phenotypes	Reference
WNT4	Ligand that activates signaling	*Wnt4*$^{-/-}$ mice have impaired zG differentiation and decreased aldosterone production	(69)
Frizzled (FZD)	Receptor for WNTs		(70)
LDL receptor-related proteins 5 and 6 (LRP5/6)	Co-receptors for WNTs		(44)
R-spondin-3 (RSPO3)	Ligand that potentiates WNT signaling		(71)
Leucine-rich repeat containing G protein-coupled receptor 5 (LGR5)	Receptor for RSPO3; inhibits the activity of ZNRF3		(72)
Zinc and ring finger 3 (ZNRF3)	E3 ubiquitin ligase that inhibits signaling by promoting the degradation of FZD/LRP	Somatic mutations in *ZNRF3* are common in human adrenocortical carcinomas	(73)
Secreted frizzled related proteins (SFRP1/2)	Decoy receptors that inhibit signaling by sequestering WNT ligands away from activating receptors	The *Sfrp1* locus has been linked to GDX-induced adrenocortical neoplasia in the mouse; decreased expression of *SFRP2* is associated with aldosterone-producing adenoma development	(66, 74)
Dickkopf-3 (DKK3)	Inhibits signaling by interacting with LRPs	*Dkk3* expression is greater in the zG than in other zones. Genetic studies indicate that *Dkk3* regulates aldosterone biosynthesis	(70, 75)
Kringle containing transmembrane protein 1 (KREMEN1)	Inhibits signaling by binding DKK3 and LRPs and inducing internalization of FZD	Somatic mutations in *KREMEN1* are common in human adrenocortical carcinomas	(63)

Mice deficient in both the *Igf1r* and the insulin receptor (*Insr*) genes exhibit adrenal agenesis and male-to-female sex reversal (79). The AGP of the double knockout mice contains half the number of SF1^{+} cells found in wild-type mice. These data indicate that IGF signaling is pivotal for adrenocortical cell specification. Additionally, IGFs have been shown to enhance basal and ACTH-induced steroidogenesis in fetal and adult adrenocortical cells (80).

TRANSFORMING GROWTH FACTOR β SIGNALING

The Transforming growth factor β (TGF-β) signaling pathway has been implicated in the maintenance and differentiation of stem/progenitor cells (81). The TGF-β superfamily consists of a diverse array of ligands. Two members of this family, activin and inhibin, are expressed in the fetal and adult adrenal cortex, and have been shown to regulate the growth, function, and survival of adrenocortical cells. Activin signaling is mediated by type I and type II receptors, which are integral membrane receptor serine/threonine kinases. Intracellular SMAD proteins transduce signals from these receptors to the nucleus (81). Activin has been shown to inhibit adrenocortical cell growth, enhance apoptosis of X-zone cells, and modulate steroidogenesis (23, 82, 83). By binding beta-glycan and ActRIA, inhibin blocks activin binding to the type II receptor and subsequent recruitment of the signaling type I receptor (83).

Following GDX, ovarian-like tissue accumulates in the adrenal cortex of *Inha*$^{-/-}$ mice in an LH dependent manner (23, 84, 85). The loss of *Inha* results in constitutive TGF-β2 activation in adrenocortical progenitor cells, with subsequent expansion of cells that express *Gata4* and other gonadal-like markers. Thus, *Inha* impacts cell fate decisions (adrenal *vs.* gonadal) in adrenal cortex.

LH SIGNALING

This glycoprotein hormone is composed of a common gonadotropin α-subunit and hormone-specific β-subunit. LH is secreted from the pituitary in response to gonadotropin releasing hormone (GnRH). LH binds to G-protein–coupled surface receptor, LHCGR, present on gonadal steroidogenic cells and activates downstream signals, including the cAMP/PKA, MAPK, and PI3K pathways (15). This in turn leads to enhanced expression of steroidogenic enzyme genes, resulting in increased production of sex steroids. Activation of LHCGR also has pleiotropic effects on cell growth and differentiation.

Cells in the adrenal glands express LHCGR and can respond to surges in LH, as evidenced by the phenomenon of GDX-induced adrenocortical neoplasia (71). Following GDX, gonadal-like neoplasms accumulate in the subcapsular region of the adrenal cortex of certain strains of mice. This phenomenon is thought to reflect LH-induced metaplasia of stem/progenitor cells in the adrenal cortex, although the term "neoplasia" is used more often than "metaplasia" to describe the process, because with time these lesions can evolve into frank adenomas or carcinomas. The neoplastic cells express gonadal-like markers (e.g., *Lhcgr*, *Gata4*, and *Cyp17a1*) and secrete sex steroids (86). This phenomenon occurs

in other species such as ferrets and goats [reviewed in Ref. (71)]. Moreover, adrenocortical tumors with histologic features resembling luteinized ovarian stroma ("thecal metaplasia") have been reported, albeit rarely, in postmenopausal women and men with acquired testicular atrophy. Genetic and pharmacologic experiments using mice or ferrets support the premise that LH has a central role in GDX-induced adrenocortical neoplasia [reviewed in Ref. (15, 71)]. The formation of ectopic gonadal-like tissue in the adrenal gland can be viewed as an extreme example of adrenocortical remodeling in response to GDX (13, 25).

TRANSCRIPTION FACTORS IMPLICATED IN RENEWAL AND REMODELING

SF1

SF1 is a master regulator of adrenocortical development and the prototype of steroidogenic transcription factors. SF1 regulates a wide array of genes required for steroidogenic cell function (87, 88). Traditionally, SF1 has been classified as an orphan nuclear receptor, but recent studies have shown that certain phospholipids and sphingolipids bind and regulate this transcription factor [reviewed in Ref. (89)]. For example, the activity of SF1 can be modulated by phosphorylation of the 3-position of the inositol head group of phosphatidylinositol-4,5-bisphosphate $PI(4,5)P_2$ while this phospholipid is bound to SF1 (90). Thus, it is hypothesized that multiple bioactive lipids function as ligands for SF1 and differentially regulate SF1 activity in a context-dependent manner (89).

Sf1$^{-/-}$ mice exhibit degeneration of the AGP due to apoptosis, which results in agenesis of both the adrenal glands and gonads (91). Similarly, targeted mutagenesis of transcription factors that activate *Sf1* expression, such as *Wt1*, *Pbx1*, and *Cited*, severely impairs adrenal gland development [reviewed in Ref. (25, 26, 92)]. *Sf1*$^{\pm}$ mice have small adrenal glands, reduced corticosterone production in response to stress, and impaired compensatory growth response following unilateral adrenalectomy (91, 93). Individuals with mutations in the DNA-binding domain of SF1 exhibit primary adrenal failure and gonadal dysgenesis. In addition to regulating steroidogenesis, this transcription factor has been implicated in the control of other fundamental cellular processes including glycolysis (87, 88).

Mice harboring multiple copies of *Sf1*, mimicking the amplification of *Sf1* seen in childhood adrenocortical carcinoma (94, 95), develop adrenocortical neoplasms that express gonadal-like markers. This suggests that SF1 can influence cell fate determination. Intriguingly, genetic ablation of the SF1 target gene *Vnn1*, encoding the gonadal-like marker Vanin-1, has been shown to reduce the severity of neoplastic lesions in the *Sf1* transgenic mice (96). Similarly, mice in which the endogenous *Sf1* gene of the mouse has been replaced with a mutant lacking a key SUMOylation site exhibit abnormal cell fate specification in steroidogenic tissues, including ectopic expression of gonadal markers (97). The mutant mice also exhibit persistence of the X-zone (97).

DOSAGE-SENSITIVE SEX REVERSAL, ADRENAL HYPOPLASIA CRITICAL REGION ON CHROMOSOME X (DAX1)

The activity of SF1 is modulated by *Dax1* (also called *Nr0b1*), an X-linked gene that encodes a repressor of steroidogenic gene expression (98). In response to ACTH, SF1-positive subcapsular progenitors downregulate *Dax1* and differentiate into adrenocorticoid-producing cells. DAX1 deficiency in humans and mice leads to excessive differentiation of subcapsular progenitors and eventual depletion of the stem/progenitor cell compartment (99, 100). Cytomegaly, a hallmark of adrenal dysfunction associated with *Dax1* deficiency (98, 99, 101), is thought to be a compensatory response to a reduced number of cortical cells or to progenitor cell exhaustion (100).

TCF21

TCF21 (also known as POD1) is a basic helix-loop-helix transcription factor functions as a repressor of *Sf1* (102). *Tcf21* is expressed in the adrenal capsule of adult mice (103), and adrenal glands from *Tcf21*$^{-/-}$ mice exhibit ectopic expression of *Sf1* in the capsule (103). As mentioned previously, some capsule cells are derived from progenitors in the fetal adrenal cortex, and it has been proposed that TCF21 downregulates *Sf1* expression in these cells upon recruitment into the capsule (27). Lineage tracing studies have shown that TCF21^{+} capsular cells give rise to non-steroidogenic stromal cells in the adrenal cortex, but not to steroidogenic cells (27). Collectively these studies suggest that TCF21^{+} cells in the adrenal capsule participate in adrenocortical homeostasis.

WT1

Fate mapping studies of WT1^{+} cells have identified long-lived progenitor population in the adrenal capsule characterized by expression of *Wt1* and *Gata4*, markers of the AGP (25, 104). Under basal conditions these AGP-like cells give rise to normal adrenocortical cells (**Figure 9**). GDX activates these WT1^{+} progenitors and drives their differentiation into gonadal-like steroidogenic tissue. Hence, WT1^{+} capsular cells represent a reserve stem/progenitor cell population with AGP-like features that can be mobilized in response to extreme physiological demand (i.e., the hormonal changes associated with GDX).

In the mouse embryo *Wt1* repression is necessary for proper expression of *Sf1* and differentiation of stem/progenitor cells into adrenocortical cells (25, 104). Ectopic expression of a transcriptionally active isoform of WT1 in SF1^{+} progenitors causes adrenocortical hypoplasia, increased expression of *Gata4*, *Gli1*, and *Tcf21*, and contraction of the X-zone. WT1 directly regulates the expression of *Gli1* in adrenal tissue suggesting that ectopic expression of *Wt1* prevents differentiation into SF1^{+} adrenocortical steroidogenic cells by maintaining cells in a GLI1^{+} progenitor state.

GATA BINDING PROTEIN-6 (GATA6)

This transcription factor is expressed in the adrenal cortex of the fetal mouse (105). Postnatally, adrenal expression of *Gata6* is limited to capsular and subcapsular cells (106). Targeted deletion of *Gata6* in SF1^{+} cells results in a pleiotropic adrenal phenotype that includes a thin adrenal cortex, cytomegaly, blunted corticoid production, ectopic chromaffin cells, and aberrant expression of gonadal-like markers (106). Thus, GATA6 is thought to limit the differentiation of adrenal stem/progenitor cells into gonadal-like cells.

Gata6 mutant mice also exhibit abnormal adrenocortical zonation: virgin females lack an X-zone, and castrate males lack a

secondary X-zone (**Figures 10A,B**) (106). *Gata6* is not expressed in the X-zone of postnatal wild-type mice, arguing that the effect of *Gata6* ablation on X-zone development is either a non-cell autonomous phenomenon or that it occurs in fetal adrenal cells that co-express *Gata6* and *Sf1*-cre (106). Recently, Sergei Tevosian's laboratory reported that *Gata4*/*Gata6* double knockout mice generated with *Sf1*-cre exhibit severe adrenal hypoplasia; female double knockout mice die from adrenocortical insufficiency, whereas their male counterparts survive due to heterotopic glucocorticoid production by cells in the testes (107).

Circumstantial evidence from other organ systems suggests that GATA6 may modulate developmental signaling pathways in the adrenal cortex. In epithelial cells of the lung and intestine, GATA6 interacts with the WNT/β-catenin and TGF-β signaling pathways to regulate the balance between stem/progenitor cell expansion and differentiation (108–113). Hindlimb buds express *Gata6* in an anterior-posterior gradient, and conditional deletion of *Gata6* in limb bud mesenchyme of mice leads to ectopic expression of *Shh* and its target gene *Gli1*. The mutant mice develop hindlimb preaxial polydactyly. Conversely, enforced expression of *Gata6* in the limb bud represses expression of Shh and results in hypomorphic limbs. In an analogous fashion, GATA6 may repress transcription of *Shh* and *Gli1* in the adrenal cortex. Consistent with this notion, *Gli1* has been shown to be upregulated in the adrenal glands of gonadectomized *Gata6*$^{flox/flox}$;*Sf1*-cre mice (106).

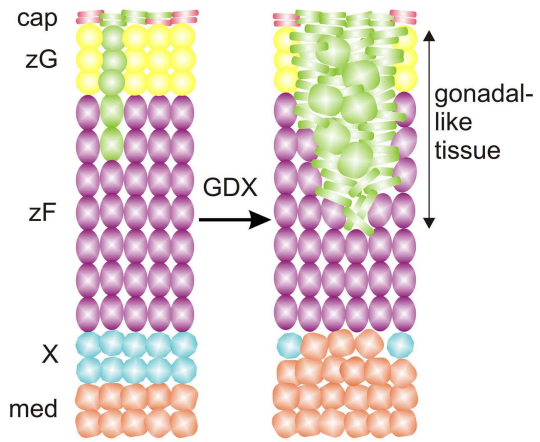

FIGURE 9 | WT1 marks a population of AGP-like progenitors within the adrenal capsule of the mouse. Under basal conditons, AGP-like cells give rise to normal steroidogenic cells in the cortex, as evidenced by lineage tracing analysis with a GFP reporter. Gonadectomy (GDX) triggers the differentiation of AGP-like cells into wedges of gonadal-like steroidogenic tissue. Secretion of sex steroids and other hormones by the ectopic gonadal tissue causes regression of the subjacent X-zone. Abbreviations: cap, capsule; med, medulla; X, X-zone; zF, zona fasciculata; zG, zona glomerulosa.

SUMMARY AND PERSPECTIVES

The continual remodeling of the zones of the adrenal cortex requires the precise control of cell growth and differentiation. The process involves distinct pools of stem/progenitor cells in the capsule, subcapsule, and elsewhere. Direct lineage conversion of mature steroidogenic cells is also integral to adrenocortical zonation and remodeling. The pathways involved are complex and redundant so as to fulfill the offsetting goals of organ homeostasis and stress adaptation. Disruption of these pathways can lead to neoplasia.

Although much has been learned about the regulation of adrenocortical homeostasis and regeneration, there are still many unanswered questions. It has proven difficult to isolate and characterize adrenocortical stem cell populations, and we do not know how these populations vary with age. Nor do we understand the relative contributions of the hedgehog, DLK1, FGF, and WNT/β-catenin signaling pathways to adrenocortical differentiation, or how these pathways interface with classic endocrine signaling systems, such as the RAAS and the HPA axis. The positional cues that mediate differentiation during centripetal (or centrifugal) migration also remain enigmatic. In other epithelial organs (e.g., liver, intestine, and lung) the development of *in vitro* systems, such as organoid cultures and induced pluripotent stem cell models, has helped to elucidate the regulation of differentiation (114). To date, there has been little progress in the development of *in vitro* models to study adrenocortical differentiation. Hopefully, such techniques will emerge in the coming years and help drive the field forward.

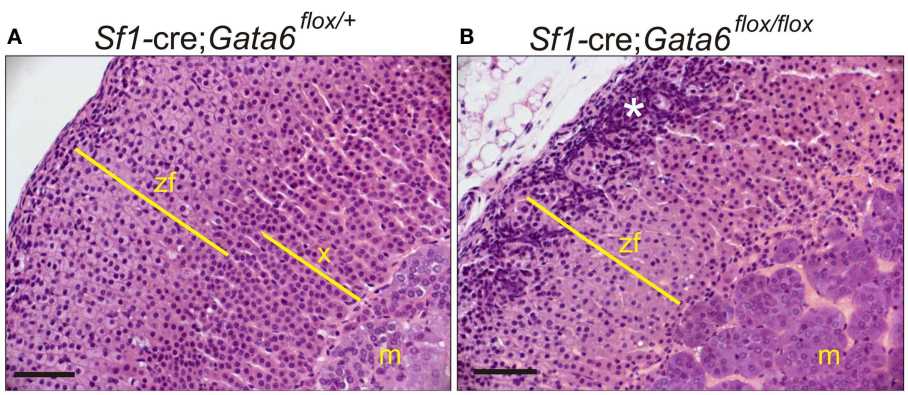

FIGURE 10 | GATA6 is required for formation of a secondary X-zone. **(A,B)** 3-week-old *Sf1-cre; Gata6*$^{flox/+}$ or *Sf1-cre; Gata6*$^{flox/flox}$ mice were orchiectomized. Adrenal tissue was harvested 1 month later, and paraffin sections were stained with H&E. Note the absence of a secondary X-zone in the mutant mice. The asterisk highlights gonadal-like cells in the subcapsular region. Bar, 50 μm.

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New directions for the treatment of adrenal insufficiency

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Adrenal disease, whether primary, caused by defects in the hypothalamic–pituitary–adrenal (HPA) axis, or secondary, caused by defects outside the HPA axis, usually results in adrenal insufficiency, which requires lifelong daily replacement of corticosteroids. However, this kind of therapy is far from ideal as physiological demand for steroids varies considerably throughout the day and increases during periods of stress. The development of alternative curative strategies is therefore needed. In this review, we describe the latest technologies aimed at either isolating or generating *de novo* cells that could be used for novel, regenerative medicine application in the adrenocortical field.

Keywords: regeneration, adrenal cortex, stem cells, transplantation, encapsulation, steroidogenesis, zonation, SF1

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The adrenal cortex is the primary site of steroid synthesis, producing glucocorticoids under the control of the hypothalamic–pituitary–adrenal (HPA) axis and mineralocorticoids under the control of the renin–angiotensin system. Glucocorticoids affect carbohydrate metabolism and mediate the mammalian stress response, while mineralocorticoids control blood volume and salt homeostasis. For this reason, the adrenal cortex is essential for life.

The adrenal cortex originates in a group of mesoderm-derived cells lying between the urogenital ridge and the dorsal aorta, forming the precursors of the adrenal glands and the gonads, the adrenogonadal primordium (AGP) (1). At around embryonic day (e)9.0 in the mouse, these cells begin to express the transcription factor steroidogenic factor 1 (SF1), which is essential for both adrenal and gonadal development (2). SF1 not only binds to response elements in the promoter regions of steroidogenic genes to positively regulate their transcription but can also be considered a true effector of cell fate as it starts a genetic program driving embryonic mesenchymal cells toward a steroidogenic phenotype/lineage (3, 4); its absolute requirement for steroidogenesis has been recently demonstrated *in vivo* (5). Other than SF1, at least four additional transcription factors have been shown to be key determinants of adrenal cortex development: Wilms tumor 1 (WT1), a zinc finger protein, and CBP/p300-interacting transactivator 2 (CITED2), which are both expressed at early stages in the urogenital ridge and synergistically promote adrenal development through induction of SF1 transcription (6, 7); pre-B-cell leukemia transcription factor 1 (PBX1), a homeodomain protein, which has been proposed to facilitate the access of important developmental factors (such as SF1) to chromatin to induce differentiation (8, 9); dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX1) (10), whose mutations are associated with a variety of developmentally relevant conditions in the adrenal and gonads (11), such as adrenal hypoplasia congenita. Moreover, multiple pathways have been implicated in the fine-tuned regulation of adrenal cortex development and function [reviewed in Ref. (12–15)]. The contribution of each of these signaling pathways has been studied by employing either *in vitro* systems or animal models, such as genetic lineage tracing (16, 17). Not surprisingly, the alteration of some of these pathways, such as the WNT pathway, results in adrenal cancer [reviewed in Ref. (18)].

The adult gland is divided into at least three distinct zones arranged concentrically around the medulla; the zona glomerulosa (ZG, which synthesizes mineralocorticoids), which lies just underneath the capsule, the zona fasciculata (ZF, which synthesizes glucocorticoids), and then the zona reticularis (which synthesizes adrenal androgens in humans and primates). Zonation occurs around birth [for review, see Ref. (12)].

Primary adrenal insufficiency is due to a number of adrenal disorders, and management of these patients can be challenging (19). Current treatments entail lifelong replacement with exogenous steroids. None suitably mimic the diurnal pattern of cortisol noted in healthy individuals, and objective variables to measure quality of replacement are lacking. Fine-tuning of replacement leaves only a narrow margin for improvement. Under-replacement can result in severe impairment of well-being and incipient crisis. Conversely, chronic over-replacement can lead to substantial morbidity including obesity, osteoporosis, and impaired glucose tolerance. Furthermore, despite attention to detail with regards to replacement regimens, overall standardized mortality ratio for patients with adrenal failure is ~2.1.

Transplantation of Adrenal Glands and Cells

Adrenal autotransplantation in humans is poorly characterized. A few surgeons tested the potential benefits of adrenal autotransplantation in patients with Cushing's disease since the early 1960s. At that time, replacement therapy with synthetic steroids was becoming the gold standard for patients with Cushing's disease but they hypothesized that autotransplantation would be an effective alternative to lifelong therapy (20–26). Adrenals were mainly transplanted in the thigh, because it is an easily accessible site should the transplant become not viable and need removal. The degree of autotransplant survival was, however, questionable, at least in those cases where follow-up is available (27). More recently, a successful mother-to-daughter allograft has been reported in a pediatric patient who developed adrenal insufficiency following fulminant meningococcemia (28). Allotransplantations of whole adrenal–kidney or adrenal–kidney–pancreas have also been described in clinical reports (29, 30); however, these multiple organ transplants are only feasible and/or recommended when the recipients have severe co-morbidities.

The refinement of allotransplantation and xenotransplantation has been the focus of more recent research, where a variety of animal models have been employed. Hornsby and colleagues performed initial work, demonstrating that transplantation of human adrenocortical cells into adrenalectomized severe combined immunodeficiency (scid) mice could be an effective technique for the treatment of adrenal insufficiency (31). They also developed a model, in which they showed that co-transplantation in a polycarbonate cylinder of clonal or primary bovine adrenocortical cells with 3T3 fibroblasts into scid mice could rescue them from developing adrenal insufficiency (32, 33). 3T3 cells were either treated with mitomycin C or lethally irradiated (to prevent proliferation upon implantation) and were overexpressing FGF1 (to aid vascularization).

Through their work, they also showed that transplanted adrenocortical cells (of either human or bovine origin) could survive and form a functional, vascularized tissue that was able to replace the host animal's organ. The newly formed tissue was able to produce and secrete the necessary amounts of cortisol, albeit aldosterone was only produced by primary bovine adrenocortical cells, but not from clonal cells (32). One possible reason for this might be that bovine adrenocortical cells preparations, albeit derived from the ZF, could contain some ZG cells or that some ZF cells could transdifferentiate to a ZG phenotype. It was later shown, however, that ZF cells are incapable of giving rise to ZG cells (34). In the same study, Teebken and Scheumann showed that transplanting ZG cells can give rise to both ZG and ZF, thus making ZG cells more suitable for transplantation (34). This is presumable because the harvest of ZG cells might result in the inclusion of progenitor cells with bi-potency, given their location in the subcapsular region close to fully mature ZG cells in mouse (16, 35) and rat (36, 37). More recently, using mouse cell fate mapping and gene-deletion tools, Freedman and colleagues demonstrated that ZF cells indeed derive from fully differentiated ZG cells through lineage conversion (38). Similar results were observed during adrenal autotransplantation in the spleen of adrenalectomized rats (39).

Furthermore, genetic modifications such as immortalization of bovine primary adrenocortical cultures with telomerase reverse transcriptase (hTERT) have been shown to be very effective in xenotransplantation procedures. This technique aids the expansion of adrenocortical cells *in vitro* leading to improved efficacy (40, 41). Although hTERT is known to be a key-tumorigenic factor, when expressed on its own it did not alter the properties of the donor's adrenocortical cell (42).

Possible Platforms for Cell Transplantation

Other groups looked into harnessing the properties of the adrenal cortex extracellular matrix (ECM). It is well established that the ECM plays a significant role in organ growth and development, as well as function, by providing structural support and by regulating cell signaling and cell-to-cell interactions (43). The ECM of the adrenal cortex is quite complex, mainly composed of collagen IV, fibronectin, and laminin and it was postulated that different ECM components might affect the biological activity of cortical cells (44). In fact, primary cultures of human fetal adrenal cells seeded on collagen IV or laminin were found to be more proliferative, as opposed to fibronectin, which enhanced cell apoptosis. In addition, collagen increased cell sensitivity to ACTH, whereas fibronectin and laminin had the opposite effect (45).

Further work to assess the function of implanted cells seeded on a collagen matrix, using a mouse model with adrenal insufficiency, determined that treatment involving staged bilateral adrenalectomy of the animals and implantation of collagen sponges each time was the most beneficial. This protocol lead to a 100% survival rate (up from 42% in mice with only one implant and 0% in animal receiving the graft without cells) and reversal of adrenal insufficiency, supported by restoration of corticosterone levels and the expression of adrenal markers by the transplanted cells (46).

Some researchers focused on the use of the native organ's own decellularized ECM to act as a scaffold in transplantation procedures to regenerate various organs, including the heart, liver, and lungs (47). *In vitro* studies, using porcine decellularized adrenal ECM as a scaffold for human fetal adrenal cells, resulted in successful cell attachment and a good cell function, the latter assessed by cell proliferation and cortisol secretion (48). Therefore, decellularized adrenal ECM could represent an improved scaffold for transplanted cells, providing a native three-dimensional structure that would support growth and incorporation of transplanted cells into the native environment to form a fully functional adrenal tissue.

Stem Cells and Reprograming Strategies

An ever-increasing number of translational scientists are striving to take advantage of the remarkable properties of stem cells that are not only offering an unparalleled opportunity to study human biology and disease processes *in vitro* but also to translate basic scientific discoveries to therapeutic applications. The development of cell therapy has, however, been overlooked to date in field of endocrinology, with the exception of the worldwide effort to generate functional pancreatic beta cells to cure type-I diabetes. Indeed, the Californian company Viacyte has been granted FDA approval to launch the first clinical evaluation of a stem-cell-derived islet replacement therapy for the treatment of patients with type-I diabetes in 2015 (http://viacyte.com/clinical/clinical-trials/). Other clinical trials are in the pipeline, a culmination of decades of intense *in vitro* and animal studies carried out by dozens of laboratories (49). Despite the fact that the morbidity of diabetes vs. adrenal insufficiency is clearly not comparable, conceivably the search for alternative treatments for adrenal insufficiency has been neglected, except for a few important discoveries (see below).

Cellular reprograming describes the process where a fully differentiated, specialized cell type is induced to transform into a different cell type that it would not otherwise become under normal physiological conditions. Sir John Gurdon of Britain and Shinya Yamanaka of Japan were awarded the Nobel Prize in 2012 for their groundbreaking discoveries in the field. Gurdon's research showed that it was possible to reverse the specialization of cells. By transferring a nucleus from a frog's intestinal cell into a frog's egg cell that had its nucleus removed, he was able to obtain a tadpole. Building on Gurdon's work, Yamanaka published a paper in 2006 demonstrating that mature murine cells can become immature stem cells (called inducible pluripotent stem cells, IPSCs) by expressing genes encoding four transcription factors (50). IPSCs can then be differentiated to several tissues using specific cocktails of growth factors/cytokines/chemical compounds. Yamanaka's breakthrough opened the door to studying tissue-specific diseases and developing diagnosis and treatments. Reprograming is not only achieved through the generation of IPSCs but also through direct reprograming (also known as lineage conversion). Lineage conversion, which is usually achieved by forced expression of lineage-determining factors, is a recently developed and attractive alternative to obtain cells of a given lineage [reviewed in Ref. (51, 52)]. Lineage conversion into several clinically relevant cell types might also prove to be a safer alternative to IPSCs; in fact, genetic (copy number variation, chromosome duplication) and epigenetic variations, which have been described in IPSCs lines and that have raised a number of questions regarding the functional relevance as well as patient safety in potential translational applications, are uncommon during lineage conversion (51).

In recent years, several studies have shown the possibility of obtaining cells with steroidogenic properties resembling adrenocortical cells from murine and human cell sources (**Table 1**). Pioneering studies in mouse embryonic stem cells (ESCs) showed that the ectopically stable expression of SF1 in the presence of cAMP resulted in a dramatic change of cell morphology and subsequent upregulation of *Cyp11a1* together with an induction of steroidogenesis. However, these cells were incapable of producing different steroid hormones *de novo*, since only progesterone was detected after treatment with 20α-hydroxycholesterol, a freely diffusible form of cholesterol (53).

TABLE 1 | Details of published studies on adrenocortical or adrenogonwadal reprograming.

PMID	Article	Cells	Origin	Methodology
9199334	Crawford PA, et al. *Mol Cell Biol* (1997)	Embryonic stem cells (ESC)	Mouse (RW4 129/SvJ)	Stable transfection of SF1
15569155	Gondo S, et al. *Genes Cells* (2004)	Bone marrow stem cells (BMCs)	Mouse [C57BL/6Tg14 (act-EGFP)osbY01]	Adenovirus SF1
16728492	Yazawa T, et al. *Endocrinology* (2006)	Bone marrow stem cells (BMCs)	Human (hMSChTERT-E6/E7)	Stable transfection of SF1
17975261	Tanaka T, et al. *J Mol Endocrinol* (2007)	Bone marrow stem cells (BMCs)	Human (commercial cell line)	Adenovirus SF1
18566117	Gondo S, et al. *Endocrinology* (2008)	Adipose mesenchymal cells (AMCs)	Mouse (C57BL/6J) (B6)	Adenovirus SF1
19359379	Yazawa T, et al. *Endocrinology* (2009)	Bone marrow stem cells (BMCs)	Human (hMSChTERT-E6/E7)	Retrovirus SF1/LRH-1
20133449	Yazawa T, et al. *Mol Endocrinol* (2010)	Umbilical cord blood (UCB-MSCs)	Human (umbilical cord blood)	Retrovirus SF1
21129436	Yazawa T, et al. *Mol Cell Endocrinol* (2011)	Embryonic stem cells (ESC)	Mouse (EBRTcH3)	Retrovirus (inducible SF1)
21610156	Jadhav U, et al. *Endocrinology* (2011)	Embryonic stem cells (ESC)	Mouse (R1 ES cell line)	Stable transfection of SF1
21764617	Mazilu JK, et al. *Mol Genet Metab* (2011)	Mesoderm-derived cells	Human	Adenoviral SF1/Dax1/Cited2/ Pbx1/WT1
22324479	Wei X, et al. *Cell Prolif* (2012)	Umbilical cord mesenchymal stem cells (UC-MSCs)	Human (umbilical cord)	Adenovirus SF1
22778223	Sonoyama T, et al. *Endocrinology* (2012)	Embryonic Stem cell (ESC) iPS (from fibroblasts)	Human (H9 and KhES1) human (201B7)	Mesoderm diff. and nucleofection SF1

In 2004, Gondo and colleagues described the capacity of long-term cultured mouse bone marrow cells (BMCs) to differentiate into a steroidogenic lineage using adenoviral-mediated SF1 overexpression (54). In this study, the authors were able to detect several steroidogenic enzymes and quantify the levels of all steroid hormones except aldosterone; further study showed that the cells failed to express *Cyp11b2*. Interestingly, reprogramed cells were responsive to ACTH, resulting in enhanced steroidogenic enzyme upregulation and hormone production. Despite the improvement in the steroidogenic profiling compared with the previous study, these cells showed a mixed pattern of adrenal and gonadal phenotypes. Moreover, only supraphysiological concentrations of ACTH were able to significantly induce progesterone and deoxycortisone secretion. It also raises the question of the function of MC2R in BMCs physiology, as the authors found the receptor to be expressed in non-reprogramed cells.

The same group later obtained steroidogenic cells from mouse adipose tissue-derived mesenchymal cells (AMCs), using a similar strategy (55). Remarkably, when the steroidogenic gene expression profile and hormone production of AMCs and BMCs were compared, AMCs showed an enhanced cortisol/testosterone ratio compared with BMCs. This enrichment of the adrenocortical vs. gonadal phenotype was further potentiated by treatment with all-trans retinoic acid (ATRA), suggesting that the tissue/cell source and the culture conditions are essential to determine the resultant phenotype during reprograming.

The first studies describing the generation of steroidogenic cells from human origin were from Miyamoto's research group. In this work, human mesenchymal stem cells (hMSCs) stably expressing SF1 (as well as hTERT, E6, and E7) were able to upregulate steroidogenic enzymes and produce both adrenal and gonadal steroids after treatment with cAMP. Immunohistochemical analysis of rat GFP^+ BMCs injected into rat testes showed upregulation of steroidogenic enzymes in the engrafted cells (56). Taken together, these studies demonstrated that MSCs have the ability to differentiate into steroidogenic cells both *in vivo* and *in vitro*.

Since then, several groups have succeeded in differentiating human cells to a steroidogenic phenotype. Tanaka et al. efficiently reprogramed human BMCs upon overexpression of SF1 with adenovirus (57) and more recently this was achieved by retroviral overexpression of LRH-1 [like SF1, a member of the NR5A nuclear receptor family (58)]. However, it is likely that LRH-1 has a more prominent role in gonadal steroidogenesis *in vivo*, since LRH-1 is barely detectable in the adrenal cortex but highly expressed in the gonads, while SF1 is abundantly expressed in both adrenals and gonads (59, 60), and actually at higher levels in cells of the AGP destined to form the adrenal cortex (7).

Human steroidogenic-like cells were also successfully reprogramed from umbilical cord blood mesenchymal stem cells (UCB-MSCs) (61) and umbilical cord Wharton's jelly-derived MSC (UC-MSCs) (62) through retroviral or adenoviral overexpression of SF1. In the latter study, the authors compared UC-MSCs with BM-MSCs and concluded that UCS-MSCs are the better cell sources since, after reprograming, these cells had a higher proliferative potential, expressed higher levels of steroidogenic enzymes, secreted more steroid hormones, and had a significantly higher cell viability.

Recently, several groups have changed their focus onto ESCs, as the first attempts to reprogram these cells were only partially successful (53). Yazawa et al. reported that to efficiently reprogram mouse ESCs (mESCs) to a steroidogenic phenotype, an initial differentiation to a mesenchymal lineage is needed (63). Once differentiation was achieved (using pulse exposures to ATRA and plating cells into collagen IV-coated dishes), cells became steroidogenic upon overexpression of SF1, resulting in an upregulation of steroidogenic enzymes as well as steroid hormone production. Interestingly, the gene expression profile of these cells was similar to that of the zona fasciculata. Alternatively, Jadhav and Jameson provided evidence that steroidogenic cells can be produced from mESCs using different protocols, the most efficient involving the withdrawal of leukemia inhibitory factor from the ESC medium followed by treatment with cAMP. Using this protocol, steroid-producing reprogramed ESCs appear to acquire a gonadal-like cell type lineage (64). Again, these works highlight the importance of the reprograming strategy/protocol to obtain a specific adrenal/gonadal-like cell type.

Sonoyama et al. demonstrated the capacity of human ES cells (hESCs) to become steroidogenic (65). Using a similar approach as previously reported (63, 64), after differentiation of hESCs to a mesodermal lineage (in this case using a GSK3β inhibitor) and upon overexpression of SF1 and cAMP treatment, hESCs showed overexpression of steroidogenic enzymes as well as hormone production. The authors also obtained the first steroidogenic cells reprogramed from an IPSC line (201B7, obtained from human fibroblasts).

Despite none of the strategies described above have been tested in *in vivo* models of adrenal insufficiency (i.e., adrenalectomized animals), they provide strong evidence that cells can be reprogramed to a steroidogenic phenotype through overexpression of SF1 and cAMP treatment. The choice of the most appropriate source of cells as substrates for reprograming is still debated and might differ depending on downstream applications. Differences in species, cell/tissue source, cellular development stage, epigenetic landscape, SF1/transcription factors dosage (66), regulatory feedbacks of activators/repressors, culture conditions, and timings of reprograming might affect the final phenotype of the reprogramed cells. In this regard, one cannot rule out the possibility that in order to generate fully functional adrenocortical cells, other transcriptions factors might be needed. Factors, such as Dax1, Pbx1, Cited2, WT1, or WNT4, known to be associated with adrenal and gonadal development have been used in reprograming strategies with minor effects on steroidogenic outcome (57, 58, 67). However, given the importance of dosage and regulatory feedback between these key transcription factors, reprograming strategies using a combination of them at specific dosages might be indispensible to obtain cells with a steroidogenic pattern resembling the one found *in vivo*.

There are still several hurdles to overcome to efficiently reprogram human cells to be used for personalized cell-based therapies in patients with adrenal insufficiency in clinics: (1) the conditions to obtain cells with restricted adrenocortical-specific gene expression and hormone production are far from optimized (see above). (2) In most of the protocols, upon overexpression of SF1, cells terminally differentiate with a consequent growth

arrest. This makes it difficult to prepare the large amount of cells needed for cell therapy. The use of inducible vectors should allow the expression of SF1 (and/or other factors) at a desired culture time-point when there are enough cells for clinical purposes. (3) The methodologies used until now are all based on the overexpression of SF1 exogenously, either episomally or virally. Optimization of the culture conditions in a gene delivery-free model of reprograming might help to avoid the

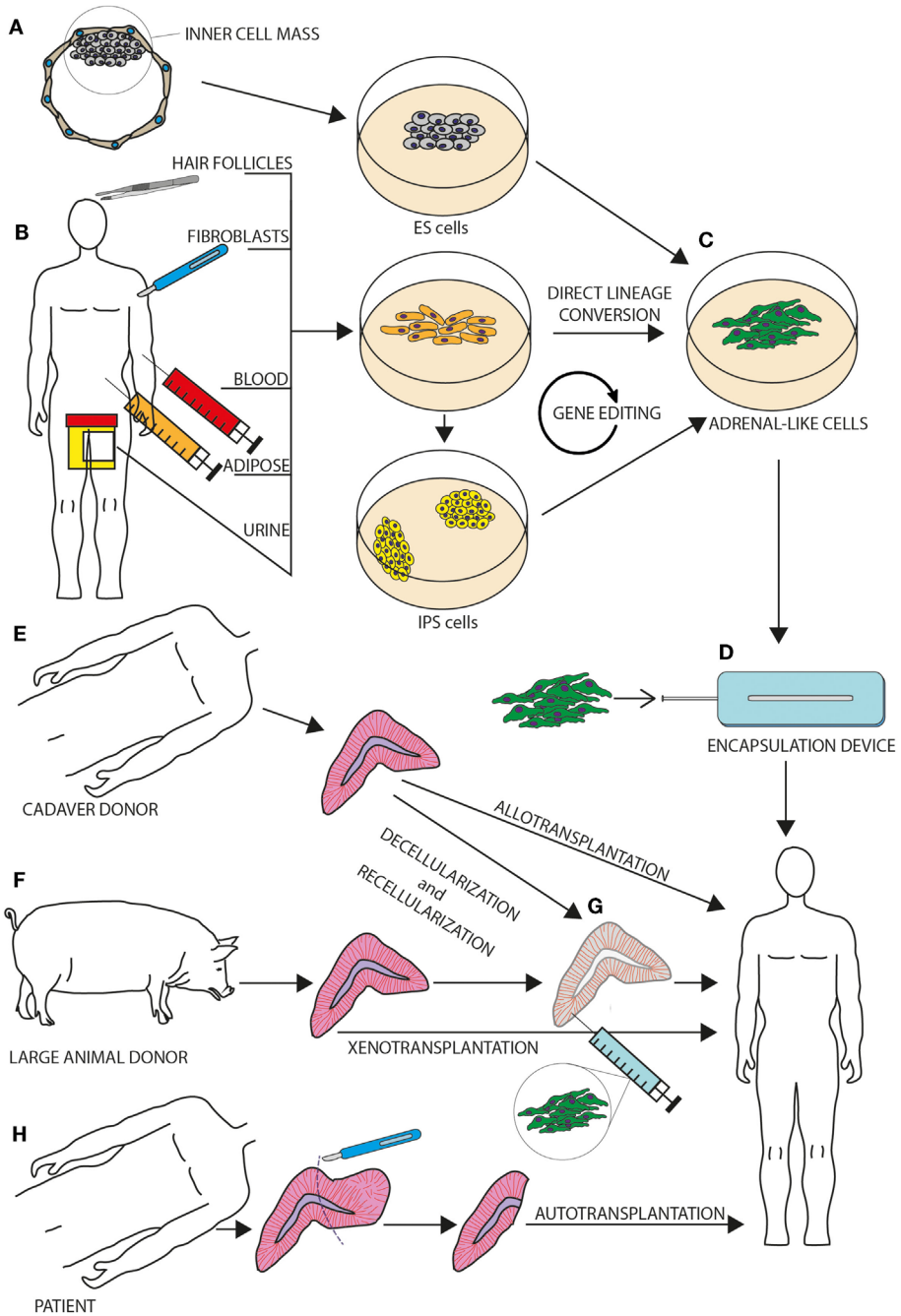

FIGURE 1 | This figure outlines novel and tested strategies for the treatment of adrenal insufficiency. Human embryonic stem (ES) cells **(A)** as well as somatic cells (such as fibroblasts, hair follicle dermal papillae, adipose tissue-derived stem cells, and urine-derived stem cells) established from human donors **(B)** can be cultured *in vitro* and induced to acquire an adrenocortical phenotype **(C)** through specific differentiation protocols. A gene-editing step can be included in case of monogenic disorders. Reprogramed cells that have successfully acquired an adrenocortical phenotype could then be implanted back into a donor, either inside an encapsulation device **(D)** or inside a decellularized adrenal of human or large-animal origin **(G)**. While autotransplantation **(H)** has been trailed in humans in pioneering surgery during the 1960s (see text) and allotransplantation **(E)** has been a poorly tested option, xenotransplantation **(F)** has never been tested in humans.

safety concerns about these cells. The use of excisable vectors together with the new CRISPR-dCasVP64 technology to activate specific transgenes without the use of transcription factors (68) might reduce the safety concerns of this method of obtaining reprogramed steroidogenic cells *in vitro*.

Recent Breakthroughs in Overcoming Rejection and the Treatment of Monogenic Diseases

A major factor limiting the application of stem-cell therapy in patients transplanted with ECs-derived adrenocortical cells or even autologous IPSCs-derived adrenocortical cells (i.e., in those individuals affected by autoimmune Addison's) is the recipient's need to adhere to lifelong immunosuppression. An effort going back a couple of decades in the field of biomaterials is providing us with the technology, which can make an impact in the clinical setting today; in fact, several encapsulation devices (endowed with excellent biocompatibility) have been developed and are undergoing clinical testing in patients with type-I diabetes. Animal studies (49) and published preliminary human studies (69) have demonstrated that the device's semipermeable membranes can tightly immune isolate transplanted cells while allowing diffusion of nutrients, such as glucose. Therefore, encapsulating adrenocortical cells is a strategy that should prevent rejection of the grafted tissue, whichever the source of it.

In recent times, a fast-paced development of gene-editing technologies using different approaches has made it possible to correct known disease-causing mutations without leaving any footprint (70). This technology could be successfully applicable to monogenic conditions causing adrenal insufficiency (such as congenital adrenal hyperplasia or familial glucocorticoid deficiency). For example, cultures of reprogrammable cell sources such as skin fibroblasts or urine-derived cells could be established and the mutated gene reverted to a wild-type status via gene editing; after successful reprograming to an adrenocortical phenotype (via IPSCs generation or via lineage conversion), cells could be reimplanted back into the donor, housed either inside an encapsulation device or a decellularized adrenal gland obtained from cadaver or large animal.

Potential future strategies, as well as pioneering past and present attempts to cure adrenal insufficiency are outlined in **Figure 1**.

In conclusion, while autotransplantation has long been discarded, allotransplantation is currently being considered only in specific clinical settings and xenotransplantation has not reached the bedside, stem-cell biology has granted great promise for tissue engineering and regenerative medicine. Treatments employing stem cells offer the potential to successfully treat patients with new modalities in the future, by essentially regenerating and replacing non-functional tissues or organs. A permanent collaborative and multidisciplinary effort carried out by translational scientists with expertise in stem cells, bioengineering, and material science is undeniably necessary to fully develop alternative treatments for adrenal insufficiency that have true clinical relevance.

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Molecular and cellular mechanisms of aldosterone producing adenoma development

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Primary aldosteronism (PA) is the most common form of secondary hypertension with an estimated prevalence of ~10% in referred patients. PA occurs as a result of a dysregulation of the normal mechanisms controlling adrenal aldosterone production. It is characterized by hypertension with low plasma renin and elevated aldosterone and often associated with hypokalemia. The two major causes of PA are unilateral aldosterone producing adenoma (APA) and bilateral adrenal hyperplasia, accounting together for ~95% of cases. In addition to the well-characterized effect of excess mineralocorticoids on blood pressure, high levels of aldosterone also have cardiovascular, renal, and metabolic consequences. Hence, long-term consequences of PA include increased risk of coronary artery disease, myocardial infarction, heart failure, and atrial fibrillation. Despite recent progress in the management of patients with PA, critical issues related to diagnosis, subtype differentiation, and treatment of non-surgically correctable forms still persist. A better understanding of the pathogenic mechanisms of the disease should lead to the identification of more reliable diagnostic and prognostic biomarkers for a more sensitive and specific screening and new therapeutic options. In this review, we will summarize our current knowledge on the molecular and cellular mechanisms of APA development. On one hand, we will discuss how various animal models have improved our understanding of the pathophysiology of excess aldosterone production. On the other hand, we will summarize the major advances made during the last few years in the genetics of APA due to transcriptomic studies and whole exome sequencing. The identification of recurrent and somatic mutations in genes coding for ion channels (*KCNJ5* and *CACNA1D*) and ATPases (*ATP1A1* and *ATP2B3*) allowed highlighting the central role of calcium signaling in autonomous aldosterone production by the adrenal.

Keywords: primary aldosteronism, aldosterone producing adenoma, somatic mutations, potassium channels, calcium channels, ATPase, wnt/β-catenin pathway, shh signaling pathway

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Background

Aldosterone is synthesized from cholesterol by a series of specific enzymatic reactions in the zona glomerulosa of the adrenal cortex; the final steps are catalyzed by the aldosterone synthase (encoded by *CYP11B2*). Aldosterone production from the adrenal cortex is tightly controlled to maintain electrolyte and fluid homeostasis; the two principal secretagogues are the renin/angiotensin system and the

extracellular concentration of potassium (K^+). The stimulation by angiotensin II or K^+ results in depolarization of the zona glomerulosa cell membrane and opening of voltage-gated calcium (Ca^{2+}) channels, leading to an increase of intracellular Ca^{2+} concentration. Angiotensin II, by its binding to the angiotensin II type I receptor (AT1R), also acts by increasing inositol triphosphate formation leading to the release of Ca^{2+} from the endoplasmic reticulum. Activation of the calcium signaling pathway triggers a phosphorylation cascade, involving calmodulin and calmodulin-dependent kinase I/IV, leading to the activation of specific transcription factors (NURR1, NGF1B, CREB) that bind to the promoter region and positively regulate the transcription of *CYP11B2* leading to an increase in aldosterone biosynthesis (**Figure 1**) (1). Hence, the activation of hormone synthesis is Ca^{2+} dependent, and the regulatory mechanism involves Ca^{2+} mediated processes.

Deregulation of the mechanisms regulating aldosterone biosynthesis results in primary aldosteronism (PA), the most common form of secondary hypertension with an estimated prevalence of about 10% in referred patients and 4% in primary care (2) and as high as 20% in patients with resistant hypertension (3). PA is characterized by hypertension with elevated plasma aldosterone and low plasma renin levels, and often associated with hypokalemia. The two major causes of PA are unilateral aldosterone producing adenoma (APA) and bilateral adrenal hyperplasia (BAH), accounting together for ~95% of cases. The early detection of PA has an important impact on clinical outcome and survival given the major cardiovascular adverse effect of aldosterone excess, which is independent of blood pressure (BP). Patients with PA have been reported to exhibit more severe left ventricular hypertrophy and diastolic dysfunction than patients with essential hypertension and a high prevalence of myocardial infarction, stroke, and atrial fibrillation (4, 5). Despite the publications in 2008 of guidelines for the management of PA, there remain a few critical issues related to diagnosis, subtype differentiation, and treatment of non-surgically correctable forms (6). A better understanding of the pathogenic mechanisms of the disease should lead to the identification of more reliable diagnostic and prognostic biomarkers for a more sensitive and specific screening and new therapeutic options.

During the last few years, major advances have been made in understanding the genetic basis of APA, with the identification of mutations in genes coding for ion channels [*KCNJ5*, coding for the G protein-activated inward rectifier potassium channel

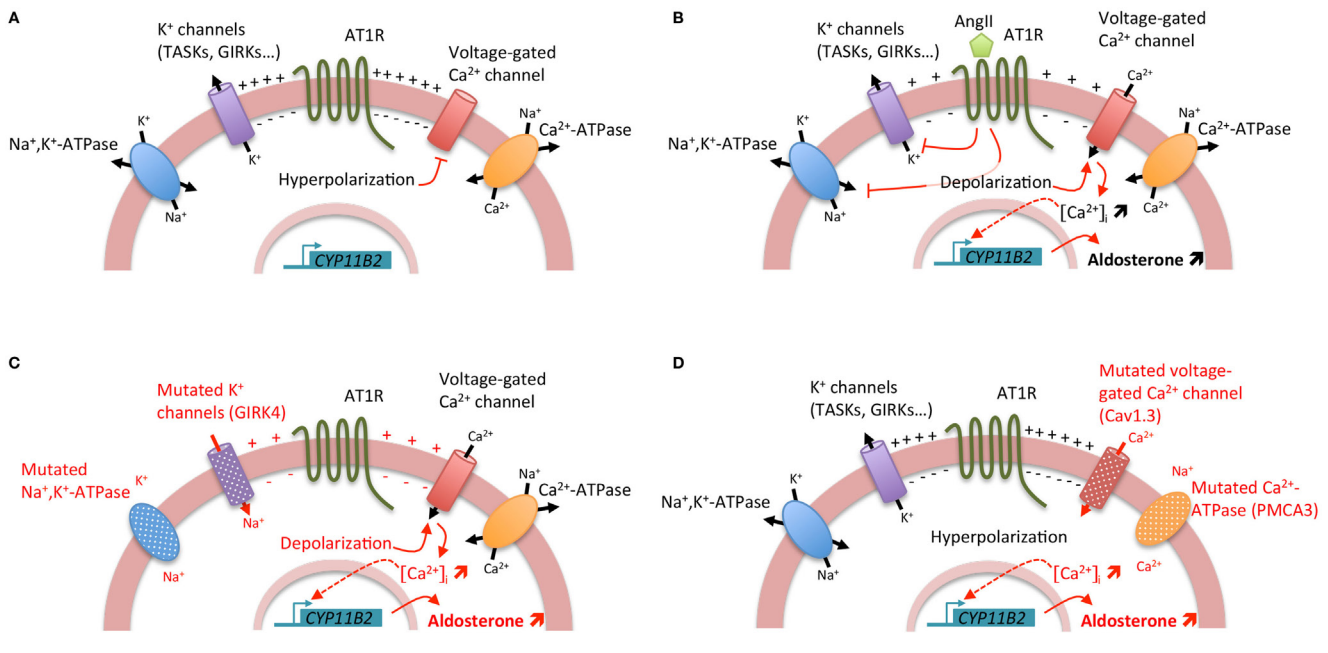

FIGURE 1 | Regulation of aldosterone biosynthesis in normal and pathological conditions. **(A)** Under resting conditions, zona glomerulosa cells exhibit a strongly negative membrane potential (−80 mV) due to the expression of a large number of potassium channels. **(B)** Stimulation of aldosterone biosynthesis by AngII. The binding of AngII to the AngII type I receptor (AT1R) induces a cascade of events leading to the zona glomerulosa cell depolarization and the increase of intracellular Ca^{2+} concentration. The inhibition of potassium channels and Na^+, K^+-ATPase by AngII results in zona glomerulosa cell depolarization, opening of voltage-gated Ca^{2+} channels, and increase of intracellular Ca^{2+} concentration. Furthermore, activation of AT1R leads also to the increase of inositol triphosphate formation and consequently to the release of Ca^{2+} from the endoplasmic reticulum. Activation of the calcium signaling pathway triggers a phosphorylation cascade, involving calmodulin and calmodulin-dependent kinase I/IV, leading to the activation of specific transcription factors that bind to the promoter region and positively regulate the transcription of *CYP11B2* leading to an increase in aldosterone biosynthesis. **(C)** Genetic alterations in *KCNJ5* (coding for the potassium channel GIRK4) and *ATP1A1* (encoding the α1 subunit of the Na^+, K^+-ATPase) genes lead to cell membrane depolarization triggering opening of voltage-gated Ca^{2+} channels and consequently positive regulation of *CYP11B2*. **(D)** Genetic alterations in *ATP2B3* (coding for the plasma membrane Ca^{2+} ATPase, PMCA3) and *CACNA1D* (encoding the Cav1.3 subunit of the L-type voltage-gated Ca^{2+} channel) genes lead directly to the increase of intracellular Ca^{2+} concentration by affecting calcium recycling and influx, resulting in positive regulation of CYP11B2.

4 (GIRK4) (7) and *CACNA1D*, encoding the Cav1.3 channel (calcium channel, voltage-dependent, L type, alpha 1d subunit) (8, 9)] and ATPases [*ATP1A1*, coding for the α1 subunit of the Na^+/K^+-ATPase (9, 10) and *ATP2B3* encoding the plasma membrane Ca^{2+}-ATPase, type 3 (10)] in more than 50% of APA. Interestingly, all these mutations lead to the activation of calcium signaling, the major trigger for aldosterone production (**Figure 1**). However, if the role of these mutations in regulating aldosterone production has been clearly established, their implication in proliferation and APA formation are still matter of debate (11).

In this review, we will summarize our current knowledge on the molecular and cellular mechanism of APA development. We will discuss how various animal models have improved our understanding of the pathophysiology of excess aldosterone production. We will also summarize the major advances made during the last few years in the comprehension of the genetic basis of APA formation using omics approaches, highlighting the major role of the ionic equilibrium and regulation of cell membrane potential in autonomous aldosterone overproduction.

Ionic Equilibrium and Membrane Potential Regulation

The regulation of cell membrane potential of the zona glomerulosa is crucial to maintain the cell in a hyperpolarized state in the absence of a secretagogue stimulus. The zona glomerulosa cell membrane is selectively permeable to K^+, giving it the characteristics of a K^+ electrode over a wide range of extracellular K^+ concentrations, due to the expression of a large number of potassium channels. However, their major role in the development of APA was highlighted only recently by the identification of somatic and germline mutations in genes coding for proteins involved in ionic equilibrium and membrane potential regulation but also by the establishment and analysis of mouse models in which the expression of specific potassium channels was invalidated.

Alteration of Ionic Equilibrium in APA

In 2011, by a whole exome sequencing approach, few recurrent somatic *KCNJ5* mutations were identified (7). These mutations (p.Gly151Arg and p.Leu168Arg) are located near or within the selectivity filter of the channel GIRK4. Additional mutations in or surrounding the selectivity filter have been identified, including p.Gly151Glu, p.Thr158Ala, p.Glu141Gln, p.Ile157Ser, delIle157, InsThr149 (12–16). All these mutations result in a significant decrease in K^+ selectivity and greater influx of Na^+ into the cell, resulting in chronic cell depolarization followed by opening of voltage-dependent calcium channels and activation of calcium signaling and aldosterone production (11, 17). Germline *KCNJ5* mutations were also identified as the causative event of Familial hyperaldosteronism type III (FH-III). FH-III was first described in 2008 in a father and two daughters with early-onset severe arterial hypertension resistant to medical treatment and hypokalemia (18). To control BP, a bilateral adrenalectomy was required for all three individuals; histology revealed massive hyperplasia of the adrenal cortex (18). Further exome sequencing performed on APA allowed the identification of other somatic mutations in genes coding for ATPases, namely *ATP1A1* (9, 10) and *ATP2B3* (10) and the Cav1.3 calcium channel, *CACNA1D* (8, 9). Whereas mutations in *KCNJ5* and *ATP1A1* affect adrenal zona glomerulosa cell membrane potential and intracellular ionic homeostasis, with chronic depolarization leading to opening of voltage-dependent calcium channels and activation of calcium signaling and aldosterone production (7, 9–11), mutations in *ATP2B3* and *CACNA1D* modify directly intracellular calcium equilibrium, also leading to an activation of calcium signaling and aldosterone production (**Figure 1**) (8–10).

Prevalence of Somatic Mutations and Genotype/Phenotype Correlations

The prevalence of somatic mutations in APA has been extensively investigated in many studies (9, 10, 14, 19–22). *KCNJ5* mutations are the most frequent genetic abnormalities reported in APA with a prevalence of ~40% in Caucasian population, and as high as 70% in series from Japan (21, 23). The mutations affecting *ATP1A1* and *ATP2B3* genes are less frequent with a reported prevalence of 5.3 and 1.7, respectively (9, 10, 20). Mutations in the *CACNA1D* gene are the second most frequent genetic alterations observed in APA with a prevalence comprised between 5 and 9.3% (8, 9, 20). Interestingly, whereas mutations in *KCNJ5*, *ATP1A1*, and *ATP2B3* are located in specific "hot spots," a large number of mutations were reported in different exons of the *CACNA1D* gene, affecting more frequently segment M4 and M6 of the protein, implying the necessity of a large genotyping of *CACNA1D* in APA. Different studies established correlations between clinical and biological parameters and the mutational status of the tumor (10, 19, 20). Hence, patients with *KCNJ5* mutations were more frequently female and diagnosed younger than patients harboring *CACNA1D* mutations and non-carriers (20); and *CACNA1D* mutations associated with smaller adenoma size (9, 20). Some studies reported also association between the mutational status and cellular composition of the adenoma. APA harboring *KCNJ5* mutations would be composed essentially of zona fasciculata-like cells whereas those carrying *CACNA1D* mutations of a majority of zona glomerulosa-like cells (9), although this association was not replicated in all series (20). The exploration of the relationship between adrenal cortex remodeling and *KCNJ5* mutations revealed the absence of association between the *KCNJ5* mutational status and the nodulation score in the peritumoral tissue, the vascularization and the presence of zona glomerulosa hyperplasia in the peritumoral cortex, suggesting that *KCNJ5* mutations are not likely to be responsible for a specific microenvironment propitious to promote adrenal cortex remodeling and APA formation (24).

Lessons from Potassium Channel Knock-Out Mouse Models

Though the role of all these mutations in abnormal aldosterone secretion has been clearly established, their impact in adenoma formation still remains unclear. Indeed, whereas in HAC15 cells, the overexpression of GIRK4 carrying the p.Thr158Ala mutation was responsible for a significant increase in aldosterone production, it induced, in parallel, a decrease in cell proliferation, independently of intracellular Ca^{2+} concentration (11). Likewise, the overexpression of p.Glu151Arg or p.Glu151Gln in HEK293T cells resulted in rapid Na^+-dependent lethality (15). More extensively, a still open question is to know whether a modification in the ionic

equilibrium and the regulation of the cell membrane potential are also able to promote adenoma formation. Response elements came some years ago with the investigation of mouse models in which TASK1 and/or TASK3 potassium channels were invalidated to determine the contributions of TASK channels to background K^+ currents in adrenal zona glomerulosa cells and test their role in the control of aldosterone production (25, 26). TASK1 and TASK3 are two-pore domain K^+ channels (K2P) that contribute largely to the very high background conductance of zona glomerulosa cells, making of zona glomerulosa cells highly sensitive sensor for plasma K^+ concentration. They clamp the cell membrane to hyperpolarized voltages, restraining the production of aldosterone in absence of stimulus. In mouse adrenal cortex, whereas TASK1 expression is found throughout the zona glomerulosa and fasciculata, TASK3 expression is restricted to zona glomerulosa (25). Deletions of task1 and task3, respectively, lead to the development of hyperaldosteronism or low-renin hypertension (25–28) In task1$^{-/-}$ mice, hyperaldosteronism was due to aberrant functional zonation of the adrenal cortex, with intense *cyp11b2* expression being localized in zona fasciculata instead of the zona glomerulosa. Interestingly, young task1$^{-/-}$ mice exhibited PA both in males and females; after puberty, this phenotype was only observed in females. Hyperaldosteronism was modulated by sexual hormones, being corrected by testosterone administration in task1$^{-/-}$ females and triggered by castration in males (26), suggesting that after puberty other factors, including task3 potassium channels, could substitute for the absence of task1 and promote compensatory mechanisms in male task1$^{-/-}$ mice (26). Deletion of task3 in mice leads to low-renin salt-sensitive hypertension, with suppressed plasma renin and aldosterone secretion that is not suppressible by increasing salt intake (27). Primary cultures of adrenocortical cells of these mice were strongly depolarized when compared with wild-type mice, and in fresh adrenal slices, calcium signaling was abnormal in zona glomerulosa cells (28). Finally, deletion of both task1 and task3 results in a marked depolarization of the zona glomerulosa cell membrane potential and a mild hyperaldosteronism with plasma aldosterone levels stimulated by a low-sodium diet but not suppressed by a high-sodium diet and partially responsive to AngII blockade (25). Interestingly, invalidation of these different potassium channels leads to hyperaldosteronism due to abnormal depolarization of the zona glomerulosa cell membrane resulting in increased intracellular Ca^{2+} concentration and stimulation of aldosterone biosynthesis; however formation of adrenal tumors has never been observed in these models indicating that other mechanisms are required to promote increased cell proliferation in APA. Although the invalidation of task1 and task3 in mice resulted in hyperaldosteronism or low-renin hypertension, to date no mutation in *KCNK3* or *KCNK9* genes has been reported in APA. However a reduced expression of TASK2, encoded by *KCNK5*, has been recently described in APA compared with normal adrenal (29), and the expression in H295R cells of a TASK2 dominant-negative mutant resulted in increased aldosterone production and *CYP11B2* and *StAR* expression. Comparison of gene expression profiles of adrenal glands of task1$^{-/-}$ female and male mice allowed the identification of a cluster of genes closely associated with hyperaldosteronism (30), among them dickkopf3 (Dkk3), a member of the dickkopf family of Wnt signaling modulators. Inactivation of dkk3 in task1$^{-/-}$ mice resulted in the extension of the phenotype of hyperaldosteronism to male animals, without inducing abnormal zonation of the adrenal cortex (30). Interestingly, the expression of Dkk3 was found to be frequently downregulated in almost any cancer entity and emerged as a potential key player in tumor suppression (31). These results suggest that the Wnt/β-catenin pathway could play a role in the development of APA.

Activation of Sonic HedgeHog and Wnt/β-Catenin Pathway: Common Features of APA

The role of specific mutations of channels and ATPases in affecting aldosterone biosynthesis is now clearly established, whereas the question of the mechanism responsible for abnormal proliferation leading to adenoma formation is still open. In 2011, Lifton suggested that *KCNJ5* mutations could be responsible for both autonomous aldosterone production and abnormal cell proliferation (7); however it has been rapidly shown that cells expressing mutated *KCNJ5* channels were less proliferative (11), raising the questions as to the events leading to abnormal cell proliferation and adenoma formation? Two specific pathways are known to play a crucial role in adrenal development: the Sonic HedgeHog and the Wnt/β-catenin pathways.

Wnt Signaling Pathway in Proliferation and/or Aldosterone Biosynthesis

The Canonical and Non-Canonical Wnt/β-Catenin Pathway

Wnt signaling has been shown to be a key signaling pathway in both normal adrenal development and tumorigenesis. The "canonical Wnt signaling pathway" acts through the regulation of the amount of the transcriptional regulator β-catenin, which controls the expression of specific genes involved in development. In the absence of Wnt, β-catenin is a part of the axin complex consisting of adenomatous polyposis coli (APC), axin, glycogen synthase kinase-3β (GSK-3β), and casein kinase-1β (CK-1β). CK-1β and GSK-3β sequentially phosphorylate β-catenin in its N-terminal part resulting in its ubiquitination and degradation by the proteasome, thus preventing β-catenin from translocation to the nucleus and activation of specific Wnt target genes. The Wnt/β-catenin activation occurs through the binding of Wnt ligand to its cell surface receptor consisting of a frizzled receptor and its co-receptor, the low-density lipoprotein receptor related protein (LRP) 6 or LRP5. Activation of the receptor leads, through an unknown mechanism, to the phosphorylation of the disheveled (Dvl) protein, which prevents GSK-3β from phosphorylating specific substrates such as axin, APC, and β-catenin. Hence, the binding of Wnt ligands to their receptor results in the inhibition of β-catenin phosphorylation, dissociation from the axin complex, accumulation in the cytoplasm and translocation to the nucleus where it serves as a transcriptional coactivator of transcription factors of the T-cell factor (TCF)/lymphocyte enhancer factor (LEF) family. TCF/LEF target genes are involved in regulating cell proliferation, stem cells maintenance, and differentiation. To increase the complexity of the system, Wnt signaling independent of β-catenin has been described as "non-canonical Wnt signaling pathway." It implicates small GTPases/jun N-terminal kinase

(JNK) and intracellular calcium signaling (32). Finally, the activation of the Wnt pathway can be antagonized by specific natural molecules including secreted Frizzled-related proteins (sFrps) and Dickkopk (Dkk) family members. SFrps and Dkk are secreted proteins acting on different components of the Wnt signaling pathway. sFrps display a high sequence homology with the Wnt binding site of Frizzled allowing sFRP proteins to directly bind to Wnts, thus functioning as Wnt antagonists for both "canonical" and "non-canonical" pathways (33), whereas Dkk members are not only able to inhibit the Wnt coreceptors LRP5 and 6 but also to bind with high affinity to the transmembrane proteins Kremen 1 and 2, which also modulate Wnt signaling (33, 34).

Role of Wnt/β-Catenin Pathway in Adrenal Function

The Wnt/β-catenin pathway plays an important role in embryonic development, stem cell maintenance, and differentiation in many tissues. During the two last decades, the role of Wnt/β-catenin in adrenal development has been highlighted by the exploration of different mouse models in which expression of different components of the pathway were disrupted (35–37) or constitutively activated (38). The first element indicating a role of this pathway in adrenal is the localization of some of its components (i.e., β-catenin, wnt4, dkk3, sfrp1…) specifically in the subcapsular zone and in zona glomerulosa (39, 40). The loss of Wnt4 was associated with abnormal differentiation of the definitive zone of the adrenal cortex and aberrant migration of adrenocortical cells into the developing gonad (35) and with a decrease of the number of zona glomerulosa cells which results in a decrease of aldosterone production (36). Interestingly, the expression of Wnt4 mRNA has been reported to be higher in APA than in normal adult adrenocortical cells (41). Overexpression of WNT4 in human adrenocortical cells resulted in an increase of aldosterone biosynthesis, whereas DKK3 had an inhibitory effect, suggesting that Wnt/β-catenin pathway could be also involved in glomerulosa specific functions (42).

Modulation of β-Catenin Expression or Activation in the Adrenal Gland

The disruption of β-catenin specifically in adrenocortical cells, through the use of a sf-1 (steroidogenic factor-1)-Cre mouse, resulted in complete adrenal aplasia or defects in maintenance of the adult cortex resulting in depletion of adrenocortical cells (37). Inversely, the constitutive activation of β-catenin in the adrenal cortex resulted in profound adrenocortical zonation defects characterized by an ectopic activation of the zona glomerulosa differentiation program and inhibition of orthotopic zona fasciculata differentiation. Interestingly, at the age of 10 months, these mice develop hyperaldosteronism (38) similarly to mice expressing a defective APC allele (43), suggesting that constitutive activation of the Wnt/β-catenin pathway could play a role in the development of APA. In human adrenal, while β-catenin expression was found in the entire cortex, its activated form was restricted to zona glomerulosa cells (44), suggesting that restriction of β-catenin activation to sub-capsular regions and in zona glomerulosa is necessary for the development of functional zonation in the human adrenal cortex. Studies in human adrenocortical cells have indicated that Wnt signaling molecules may also have multiple actions on steroidogenesis, particularly in regulating aldosterone biosynthesis (36, 45). All these results suggest that aberrant Wnt signaling may be driving the development of APA. Recently, the activation of Wnt/β-catenin has been reported in two-thirds of APA (44, 46). Whereas activating mutations of the β-catenin are found in a wide variety of human cancers including adrenocortical tumors and adrenocortical adenoma, only few mutations were reported in APA (8, 47) strongly suggesting that the activation of β-catenin was not associated with the presence of mutation (47–49). Moreover, in adrenocortical carcinoma, the activation of β-catenin was associated with a poor prognosis (49), whereas in APA it was not associated with specific tumor characteristics. Thus Wnt/β-catenin activation may play distinct roles in APA compared to adrenal cortex carcinoma, contributing to aldosterone hypersecretion rather than to autonomous cell proliferation (46). The activation of β-catenin was not only associated with an increased expression of specific target genes, i.e., *AXIN2* and *LEF1*, but also with down regulation of SFRP2, a member of the SFRP family of Wnt signaling inhibitors (46). Interestingly, s*frp2* knockout mice exhibit an increase in plasma aldosterone concentration, associated with ectopic expression of *cyp11b2* in adrenal cortex, similarly to what observed in mice expressing the constitutive active form of β-catenin in adrenal cortex (46).

Shh Signaling Pathway in APA

Similarly to the Wnt/β-catenin signaling pathway, Sonic HedgeHog signaling (shh) is essential for adrenal gland development and maintenance. Shh encodes a secreted signal that belongs to the Hedgehog family. The activation of shh signaling occurs through its binding to a receptor complex formed by the twelve transmembrane domain protein patched-1 (PTCH1) and the G-protein coupled receptor Smoothened (SMO). In the presence of Shh, SMO is released from PTCH1 inhibition and activates the transcription factors GLI1, GLI2, and GLI3 (50). In rodent adult adrenals, Shh is expressed exclusively in the subcapsular region of the cortex in cells also expressing sf1, indicating their commitment to steroidogenic cells (51–53). Similarly, in human adult adrenals, the expression of SHH was found to be restricted to a few numbers of cells of the subcapsular region, where stem/progenitor cells are supposed to be localized (44, 54). Mice invalidated for Shh, specifically in Sf1 positive cells, exhibit reduced proliferation of capsular cells and a significant reduction of adrenocortex thickness and adrenal size but no modification of adrenal zonation. Moreover, the remaining adrenal cortex was able to synthetize steroids, indicating that shh is essential for expansion of the adrenal cortex but not for zonation and differentiation (55). Interestingly, the expression of Shh was found in APA as well as in the entire hyperplasic zona glomerulosa, with a similar pattern of expression than *CYP11B2* and Dab2 (44). The activation of the SHH signaling pathway in APA was confirmed by transcriptomic analysis (44). These results suggest that APA have acquired some characteristics of stem/precursor cells or, alternatively, that reexpression of fetal markers from the definitive zone in the adrenal cortex could underlie excessive proliferation and APA formation. Remarkably, the antagonism of hedgehog signaling has been shown to inhibit the proliferation of H295R cells (56) and to decrease cell viability (57). Moreover, the inhibition of shh signaling pathway results in the inhibition of wnt/β-catenin signaling (57). Interestingly, the activation of shh

signaling pathway was found to be increased in adult adrenocortical carcinoma (57) as well as in non-producing adenoma (56), suggesting a role in tumor formation or development.

Clock Genes in the Control of Aldosterone Production

Many physiological functions such as metabolism, BP, and renal function are regulated by the circadian clock (58–60). Up to 10% of the transcriptome has been estimated to be under the control of the circadian clock and a number of diseases are associated with clock gene disorders (61). The circadian timing system is organized in central and peripheral clocks. The central circadian clock is composed of specialized neurons in suprachiasmatic nuclei in the hypothalamus and is synchronized to the daily light/dark cycle through the retino-hypothalamic tract (62). The peripheral circadian clocks, found in most peripheral tissues, are synchronized to geophysical time through a wide range of master clock-dependent stimuli (62, 63). Four canonical proteins are the components of the circadian time clocks: period (Per)1–3, cryptochrome (Cry)1–2, Bmal1, and Clock. Clock and Bmal1 form a heterodimer that interacts with E-boxes to transcriptionally upregulate clock-controlled genes, which include Per and Cry (62, 64). Cry proteins act as potent transcriptional repressors that downregulate the transcription of E-box (CACGTG) enhancer-containing clock genes (including Per- and Cry-encoding genes) as well as a wide variety of clock-controlled genes (65, 66).

Different circadian mutant mice models show abnormalities in BP regulation and/or plasma aldosterone concentration. BP is decreased in *clock* knockout mice, accompanied by changes in circadian rhythms of urinary sodium and potassium excretion, and loss of the circadian rhythmicity of plasma aldosterone (67). A mouse model carrying a conditional allele of the circadian clock gene Bmal1 and expressing Cre recombinase under the endogenous Renin promoter (Bmal1lox/lox/Ren1dCre) loose the BMAL1 protein expression in the renin-secreting granular cells of the juxtaglomerular apparatus. These mice exhibit decreased BP, increased urine volume, changes in the circadian rhythm of urinary sodium excretion, and significantly reduced plasma aldosterone (63). Mice lacking the core clock components Cry1 and Cry2 (referred as Cry-null mice) show disrupted rhythmic behavior, physiology, and metabolism (68, 69). Interestingly, Cry-null mice exhibit salt-sensitive hypertension due to increased aldosterone production by the adrenal gland (70). Investigation of steroidogenic alterations in Cry-null mice showed chronic overexpression of *Hsd3b6* mRNA and chronically enhanced 3β-hydroxysteroid dehydrogenase activity in adrenal cortex. *Hsd3b6* encodes a dehydrogenase-isomerase specifically expressed in zona glomerulosa, which catalyzes the conversion of pregnenolone into progesterone, an enzymatic reaction required for aldosterone biosynthesis. The inactivation of Cry genes leads to chronically enhanced mineralocorticoid production, which, in turn, renders BP salt sensitive (70). On the other hand, it has been previously shown that Per1 and Cry2 modulate opposing actions on Per1 target gene expression in some tissues (71). Remarkably, Per1 knockout mice exhibit lower BP when compared to wild-type mice (60). To verify the hypothesis that Per1 plays a role in the regulation of aldosterone levels, Richards et al. have performed RNA silencing and pharmacological blockade of Per1 nuclear entry in the NCI-H295R human adrenal cell line, demonstrating that *Hsd3b6* expression is decreased after Per1 knockdown *in vitro* (72). In addition, they have demonstrated that Per1 heterozygous mice exhibited lower plasma aldosterone levels and reduced *Hsd3b6* mRNA expression *in vivo*, with a significant blunted circadian expression of this gene (72). In the human adrenal, two 3β-hydroxysteroid dehydrogenase isoform are expressed, namely *HSD3B1* and *HSD3B2* (70). In the adrenal cortex, expression of *HSD3B1* is specific to the zona glomerulosa (70), suggesting its potential involvement in adrenal zona glomerulosa pathophysiology. Both HSD3B1 and HSD3B2 are found to be express in APA; and whereas HSD3B2 expression was higher than that of HSD3B1 in APA, only the level of HSD3B1 expression was correlated with plasmatic aldosterone concentration and CYP11B2 expression in APA, suggesting that HSD3B1 may contribute to autonomous aldosterone production in APA (73).

Conclusion

Despite major advances performed these last years in our understanding of the pathophysiology of APA development, the natural history of APA formation is still a matter of debate. Our current knowledge is not enough advanced to explain the mechanisms involved in APA formation. However, the identification, in about 50% of APA, of recurrent somatic mutations in genes coding for ionic channels and ATPases has elucidated the mechanism responsible for the autonomous aldosterone production. On the other hand, activation of the Wnt/β-catenin pathway or reexpression of stem/precursor cell markers, i.e., shh, could explain the abnormal proliferation leading to the formation of an adenoma. It is possible that APA formation is the result of the combination of two events: (1) the activation of signaling pathways such as wnt/β-catenin or shh pathways driving abnormal cell proliferation and creating a favorable environment for (2) the occurrence of recurrent somatic mutations responsible for autonomous aldosterone production.

Although it was suggested that genetic alterations leading to abnormal calcium signaling are sufficient for both abnormal proliferation and inappropriate aldosterone production in APA, there are some evidences suggesting that mutations in potassium and calcium channel and ATPases may not be sufficient for promoting cell proliferation and tumor formation. It could be speculated that some groups of cells start to abnormally proliferate creating a propitious environment for the emergence of specific mutations affecting ionic channels and ATPases leading to increased aldosterone production. Further mechanistic insight may come from specific mouse models developing a phenotype of hyperaldosteronism in the context of an APA.

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PRKACA: the catalytic subunit of protein kinase A and adrenocortical tumors

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Cyclic-AMP (cAMP)-dependent protein kinase (PKA) is the main effector of cAMP signaling in all tissues. Inactivating mutations of the *PRKAR1A* gene, coding for the type 1A regulatory subunit of PKA, are responsible for Carney complex and primary pigmented nodular adrenocortical disease (PPNAD). *PRKAR1A* inactivation and PKA dysregulation have been implicated in various types of adrenocortical pathologies associated with ACTH-independent Cushing syndrome (AICS) from PPNAD to adrenocortical adenomas and cancer, and other forms of bilateral adrenocortical hyperplasias (BAH). More recently, mutations of *PRKACA*, the gene coding for the catalytic subunit C alpha (Cα), were also identified in the pathogenesis of adrenocortical tumors. *PRKACA* copy number gain was found in the germline of several patients with cortisol-producing BAH, whereas the somatic Leu206Arg (c.617A>C) recurrent *PRKACA* mutation was found in as many as half of all adrenocortical adenomas associated with AICS. *In vitro* analysis demonstrated that this mutation led to constitutive Cα activity, unregulated by its main partners, the PKA regulatory subunits. In this review, we summarize the current understanding of the involvement of *PRKACA* in adrenocortical tumorigenesis, and our understanding of PKA's role in adrenocortical lesions. We also discuss potential therapeutic advances that can be made through targeting of *PRKACA* and the PKA pathway.

Keywords: adrenal cortex, adenoma, *PRKACA*, PKA, Cushing syndrome

Introduction

The adrenal cortex is divided into three concentric zones: the outermost zone named *zona glomerulosa*, the centrally located *zona fasciculata* and the innermost, *zona reticularis* involved in the production of mineralocorticoids, glucocorticoids, and androgens, respectively (Blake et al., 2008; Mcnicol, 2013). Thus, adrenal dysfunction leads to several hormonal syndromes due to the hypo- or hyper-secretion of one or more adrenal hormones.

In this review, we focus on Cushing's syndrome (CS) resulting from overproduction of cortisol from adrenocortical tumors (ACT). CS leads to central obesity and metabolic abnormalities and several other manifestations including moon face, buffalo hump, striae, and opportunistic infections (Newell-Price et al., 2006). Severe and prolonged hypercortisolism could lead to increased morbidity and mortality, due to sepsis, cardiovascular, and other complications (Plotz et al., 1952; Arnaldi et al., 2012). Hypersecretion of cortisol can be due to either an excess of pituitary or ectopic adrenocorticotropin hormone (ACTH) secretion or adrenocortical tumors

(ACT) secreting cortisol autonomously; the latter form of CS is known as "ACTH-independent CS" (AICS).

Cortisol-producing ACTs include bilateral adrenocortical hyperplasias (BAH), adrenocortical adenomas (ACA) and cancer (ACC). BAHs account for 10–15% of AICS and are classified in two subtypes: macronodular (nodules >1 cm) and micronodular (nodules <1 cm) (Lacroix, 2009; Duan et al., 2014). Macronodular hyperplasia, previously known as massive macronodular adrenocortical disease (MMAD) or ACTH-independent macronodular adrenocortical hyperplasia (AIMAH), has been recently renamed as primary macronodular hyperplasia (PMAH) after the discovery of intra-adrenal ACTH production (Lacroix, 2013; Louiset et al., 2013). PMAH is typically diagnosed in the fifth and sixth decade of life; subclinical CS is common in this disease (Duan et al., 2014), despite the fact that macroscopically, the adrenal glands are massively enlarged with a combined weight reach from 60 to 200 g.

Micronodular hyperplasias include a pigmented form named primary pigmented nodular adrenocortical disease (PPNAD), which is typically diagnosed at a younger age. PPNAD is the most common endocrine lesion of Carney complex (CNC), occurring in more than 60% of CNC patients (Almeida and Stratakis, 2010). Grossly, PPNAD is associated with normal to slightly enlarged adrenal glands (4.3–17 g) with a large number of yellow to brown-black micro-nodules (0.1–0.3 mm in size) due to lipofuscin accumulation (responsible for the pigmentation) (Stratakis and Boikos, 2007; Stratakis, 2008).

Unilateral ACTs, ACAs account for 90% of adrenal CS (Newell-Price et al., 2006; Bertagna et al., 2009). Clinically, these tumors arise at any age, with a slight female predominance. The presentation ranges from subclinical to overt CS. Macroscopically, the average ACA ranges in size from 1.5 cm to 6 cm and weighs between 10 and 40 g. In contrast to ACAs, ACCs are rare and account for few cases of adrenal CS (Wajchenberg et al., 2000). They arise sporadically, mostly around the fourth and fifth decade of life; ACCs typically weigh more than 100 g, tend to be adherent to other tissues, or invade adjacent structures.

cAMP Signaling, *PRKAR1A* Defects, and Adrenocortical Tumors

In normal physiology, cAMP signaling plays an essential role in the regulation of cortisol secretion under the control of the hypothalamic-pituitary-adrenal axis (**Figure 1**). Hypothalamic corticotropin-releasing hormone (CRH) secretion stimulates ACTH secretion from the pituitary gland, both acting through their respective G-protein coupled receptor (GPCR). ACTH binds to the melanocortin-2 receptor (MC2R) in *zona fasciculata* cells, leading to the activation of adenylate cyclase, which ensures the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP activates its main intracellular mediators, EPAC (Exchange Protein Activated by cAMP), and a serine/threonine kinase called cAMP-dependent protein kinase A (PKA) (Bossis and Stratakis, 2004). The protein kinase A (PKA) holoenzyme is a heterotetramer that consists of two regulatory subunits each binding to one catalytic subunit (Bossis and Stratakis, 2004). Four regulatory (RIα, RIβ, RIIα, and RIIβ) and four catalytic (Cα, Cβ, Cγ, and Prkx) subunits have been described (Almeida and Stratakis, 2011). In order to activate PKA cAMP interacts with the regulatory subunit of PKA leading to a conformational change permitting the release of the catalytic subunits. The free catalytic subunits phosphorylate downstream targets such as cAMP response element-binding protein (CREB), which induce the transcription of target genes, such as those involved in cortisol synthesis (**Figure 1**) (Christenson et al., 1999; Manna et al., 2009). The intracellular cAMP is hydrolysed by specific phosphodiesterases (PDEs), and the two regulatory and catalytic subunits of PKA are reassembled in order to return to their inactive state (Stratakis and Boikos, 2007).

Several lines of evidence support cAMP's role in the development of cortisol-producing ACTs (Stratakis, 2014a). In McCune-Albright syndrome (MAS), which is caused by mutations in the *GNAS* gene that encodes the stimulatory subunit α of the G protein (Weinstein et al., 1991), ACAs or, more frequently, BAH are common. In CNC, inactivating mutations of the *PRKAR1A* gene (encoding the RIα subunit of PKA) (Kirschner et al., 2000a,b) lead to PPNAD. Mutations of the *PRKAR1A* gene have also been identified in sporadic cases of PPNAD (not associated with CNC), as well as in ACAs (Groussin et al., 2002; Bertherat et al., 2003). In addition, a number of *in vitro* and transgenic mouse studies have demonstrated that *PRKAR1A* is an adrenocortical tumor suppressor gene and its inactivation leads to ACTH-independent cortisol secretion (Sahut-Barnola et al., 2010; Almeida and Stratakis, 2011).

PRKACA Genetic Defects Lead to Tumors of the Adrenal Cortex

In an initial cohort of 10 cortisol-producing ACAs associated with overt AICS, the Leu206Arg (c.617A>C) *PRKACA* recurrent mutation was identified in 70% of these cases; with one ACA having another *PRKACA* defect, Leu199_Cys200insTrp (Beuschlein et al., 2014). Both of these mutations affect residues that are highly conserved across species from invertebrates to humans suggesting the major role played by these amino acids is in protein function (Beuschlein et al., 2014; Goh et al., 2014). Dalmazi and collaborators have also identified two additional mutations: the insertion Cys200_Gly201insVal (c.600_601insGTG) and the missense Ser213Arg (c.639C>G) associated with 12 base pair duplication Leu212_Lys214insIleIleLeuArg (c.638_640insATTATCCTGAGG), in respectively 13.4% (3/22) and 4.5% (1/22) of their cortisol-producing ACA cohort (Di Dalmazi et al., 2014b). Recently, four independent projects of exome sequencing of cortisol-producing ACA development (Cao et al., 2014; Goh et al., 2014; Kubota et al., 2014; Sato et al., 2014) led to confirmation of *PRKACA*'s role in the pathogenesis of this neoplasm. The Leu206Arg variant has been identified at a frequency that ranges from 14.2 to 65.5% of cortisol-producing ACA depending on the studies (Beuschlein et al., 2014; Cao et al., 2014; Di Dalmazi et al., 2014a; Goh et al., 2014; Nakajima et al., 2014; Sato et al., 2014). No *PRKACA* mutations were found

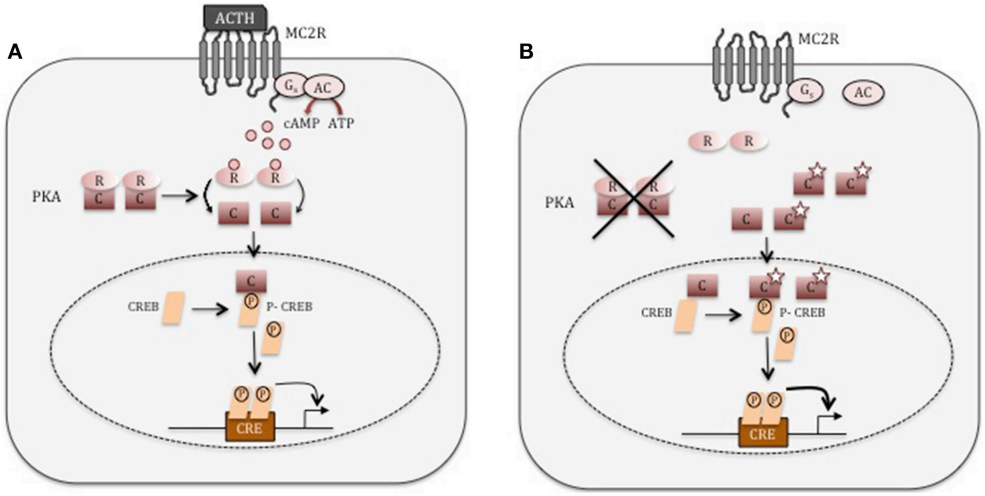

FIGURE 1 | cAMP signaling. (A) In normal adrenocortical cells, ACTH binds to its G-coupled receptor, MC2R. This leads to the activation of adenylate cyclase (AC), which convert ATP into cAMP. cAMP then binds the regulatory (R) subunit of PKA, inducing the release of the catalytic subunit (C). The catalytic subunit phosphorylates its downstream target such as CREB, which in turn induces the expression of genes involved in cortisol synthesis. **(B)** In adrenocortical adenoma cells producing cortisol autonomously with *PRKACA* mutations (star), the catalytic (C) subunit of PKA is unable to interact with the regulatory subunit (R). The unregulated *PRKACA* may now mediate its serine-threonine kinase activity without any restrains.

either in 1600 in-house exomes or in the 1000 Genomes Project data set or in blood of patients harboring *PRKACA* mutations in tumors. The Leu206Arg substitution likely alters the function of the Cα subunit at the heterozygote state, since both the wild type and mutant alleles were expressed in the tumor tissue (Beuschlein et al., 2014; Goh et al., 2014).

Functional Analysis of *PRKACA* Mutations

PRKACA encodes the most highly expressed catalytic PKA isoform in the human adrenal and the functional consequences of two mutant variants have been predicted using different modeling approaches based on mouse PKA crystal structure (Beuschlein et al., 2014; Cao et al., 2014; Goh et al., 2014; Sato et al., 2014). In the absence of cAMP, the regulatory subunit fits into a highly conserved hydrophobic cleft in the catalytic subunit formed by Leu206 and Leu199. Therefore, the substitution from the small hydrophobic leucine to a large positively charge hydrophilic arginine in position 206 should abolish the interaction between the catalytic and regulatory subunit leading then to cAMP-independent PKA activation (**Figure 1**) (Beuschlein et al., 2014; Cao et al., 2014; Goh et al., 2014; Sato et al., 2014). Similar consequences are predicted for each one of the pathogenic *PRKACA* variants that have been identified so far in ACAs (Di Dalmazi et al., 2014a).

The activating effect of the novel *PRKACA* mutations in ACA predicted by what is known about the structural biology of the PKA tetramer has been validated by *in vitro* experiments in HEK293 cells for the most frequent variant, Leu206Arg (Beuschlein et al., 2014; Cao et al., 2014; Goh et al., 2014; Sato et al., 2014). The expression of Leu206Arg *PRKACA* increases the PKA activity and the level of CREB phosphorylation at Ser133 in basal conditions compared to the wild type *PRKACA* in two independent studies; the Leu206Arg did not interfere with the catalytic activity (Beuschlein et al., 2014; Cao et al., 2014; Goh et al., 2014). In contrast with cells transfected with the wild-type sequence, the Leu206Arg is not responsive to cAMP stimulation and its activity is not reduced by the co-expression with excess wild-type regulatory subunit (Beuschlein et al., 2014). The absence of interaction between this variant and the regulatory subunit was confirmed by FRET and co-immunoprecipitation experiments (Beuschlein et al., 2014; Goh et al., 2014; Sato et al., 2014). Altogether, these results demonstrated that the Leu206Arg mutant protein is constitutively active. Consistent with these conclusions, basal PKA activity in ACA with *PRKACA* mutations compared to those without mutations is increased (Beuschlein et al., 2014; Cao et al., 2014). Similarly, Goh and collaborators demonstrated by immunohistochemistry a higher staining of the phosphorylation of CREB at Ser133 in 8 *PRKACA* mutant ACA vs. 5 ACA without identified mutations (Goh et al., 2014). However, Sato and collaborators did not find any differences in phosphorylation level of CREB by Western blot (Sato et al., 2014).

Clinical Phenotype Associated with *PRKACA* Mutations

In total, the *PRKACA* gene has now been sequenced in 854 ACTs and no mutations have been found in cortisol-producing ACCs, non-secreting ACAs, androgen-secreting ACAs, aldosterone-producing ACAs, and adrenal oncocytomas. Thus, the overall frequency of the *PRKACA* hotspot mutation is 38.2% and it has been identified in cortisol-producing adenomas only (**Table 1**). One study described a predominance of *PRKACA* mutations in females (Cao et al., 2014). Patients harboring tumors with *PRKACA* mutations were diagnosed with CS at younger ages (45.3 ± 13.5 vs. 52.5 ± 11.9 years) (Goh et al., 2014). In five studies including both overt and subclinical CS (Beuschlein et al., 2014; Di Dalmazi et al., 2014b; Goh et al., 2014; Nakajima et al.,

TABLE 1 | Frequency of copy number gain (CNG) including *PRKACA* gene and *PRKACA* mutations in adrenocortical tumors.

References	Cortisol-producing ACA	Cortisol-producing ACA with over CS	Cortisol-producing BAH	Cortisol-producing ACC	Non-secreting ACA	Aldosterone-producing ACA	Androgen-producing ACA	Adrenocortical oncocytoma
Beuschlein et al., 2014	22.2% mutations (22/99)	37% mutations (22/59[a])	1.75% CNG (5/35[b])	0% (0/42)	0% (0/20)	0% (0/20)	–	–
Cao et al., 2014	65.5% p.Leu206Arg (57/87)	NA	0% (0/13 PMAH)	0% (0/16)	–	–	–	0% (0/3)
Goh et al., 2014	23.6% p.Leu206Arg (57/87)	35% p.Leu206Arg (10/28)	–	0% (0/8)	–	–	–	–
Di Dalmazi et al., 2014b	32.3% mutations (22/68[c])	34.3% mutations (22/64[c])	0% (0/8)	0% (0/5)	–	–	–	–
Nakajima et al., 2014	14.2% p.Leu206Arg (3/21)	23% p.Leu206Arg (3/13)	–	–	0% (0/32)	–	0% (0/4)	–
Sato et al., 2014	52.3% p.Leu206Arg (34/65)	57.1% p.Leu206Arg (32/56)	–	–	–	–	–	–
Total	38.2% (151/395)	40%(89/220)	8.9% CNG (5/56)	0% (0/71)	0% (0/52)	0% (0/53)	0% (0/4)	0% (0/3)

[a] *21/59 harboring p.Leu206Arg and 1/59 with Leu199_Cys200insTrp.*

[b] *31 PPNAD + 2 iMAD+ 2 PMAH.*

[c] *18/22 harboring p.Leu206Arg: 3/22 Cys200_Gly201insVal; 1/22 Ser213Arg+Leu212_Lys214insIle-Ile-Leu-Arg*

2014; Sato et al., 2014), *PRKACA* mutations are significantly associated with overt CS and higher serum cortisol level after 1 mg of dexamethasone, increased urinary free cortisol and midnight cortisol levels compared to patients without mutations (Beuschlein et al., 2014). These results highlight a direct link between *PRKACA* mutations and cortisol production, which is expected knowing the physiological function of PKA. In accordance with this observation, no *PRKACA* mutations have been found in subclinical CS patients in three independent studies (Beuschlein et al., 2014; Di Dalmazi et al., 2014b; Nakajima et al., 2014). However, Gao et al. and Sato et al. have found 11% (3/27) and 22% (2/9) of *PRKACA* mutations, respectively, at position 206 in subclinical CS patients (**Table 1**) (Goh et al., 2014; Sato et al., 2014). The term subclinical CS is used to describe cortisol-secreting tumors in patients without any typical symptoms of CS. However, its usage can vary between investigators and countries; this may explain the differences observed between these studies. Interestingly, in transcriptomic data from 25 wild-type ACA and 11 mutant ACA, 232 genes are differentially expressed and pathway analysis demonstrates an enrichment in "biosynthesis and metabolism of steroid and cholesterol" and "response to chemical stimulus" (Cao et al., 2014). This is in accordance with the essential role of PKA in the control of cortisol secretion.

PRKACA and other Defects in ACAs

Altogether these results demonstrate that *PRKACA* mutations constitutively activate PKA leading to cortisol-producing adenomas, thereby suggesting that *PRKACA* is a main contributor to adrenocortical tumorigenesis. By whole-exome sequencing analysis, even if the number of exonic mutations is generally low, *PRKACA* is not the only oncogene mutated in cortisol-producing tumors. Activating mutations have also been identified in *CTNNB1* and *GNAS* genes at lower frequency (Cao et al., 2014; Goh et al., 2014; Sato et al., 2014). Importantly, *PRKACA* mutations were never found in association with other mutations. This suggests that the identified activating mutations may be mutually exclusive confirming the driver role played by Cα subunits in the development of cortisol-producing ACA. Interestingly, Goh and collaborators sequenced both ACA and ACC and were able to divide the ACA into two groups based on genetic results (Goh et al., 2014). Five out of 22 ACA are genetically closer to 3 ACC even if their Weiss score is 0 or 1, without any histological evidence of carcinoma. These findings are consistent with a progressive model of tumors forming in the adrenal cortex in the sequence hyperplasia-adenoma-carcinoma (Berthon et al., 2010; Stratakis, 2014b). However, the second ACA group, which included the ACA with *PRKACA* mutations, appears to have a distinct tumorigenesis mechanism. This is supported by the observation of three studies that tumors with mutations in *PRKACA* were significantly smaller than the non-mutant ones (Di Dalmazi et al., 2014b; Goh et al., 2014; Sato et al., 2014). Similarly, the weight of the sporadic ACT harboring *PRKAR1A* mutations is lower (11.2 ± 0.8 vs. 23.4 ± 12.05 g) (Bertherat et al., 2003). Therefore, PKA activation through constitutive activation of Cα subunit or RIα loss-of-function drastically increases cortisol secretion but has a limited impact

on cell proliferation and tumor growth. Most recently, genomic duplication of the locus of *PRKACB* encoding for the Cβ catalytic subunit have also been described in a patient with CNC without CS (Forlino et al., 2014). It is possible that this reflects different roles of the two main catalytic subunits of PKA, with regards to their function in the adrenal cortex.

PRKACA Copy Number Gain and Bilateral Adrenocortical Hyperplasia

Comparative genomic hybridization of 35 BAH with overt CS demonstrated copy number gain at chr19p locus that included *PRKACA* gene in 5 patients (Beuschlein et al., 2014; Stratakis, 2014a; Carney et al., 2015). The defect was present in the germline and there were no *PRKACA* coding sequence mutations. Two patients, a mother and a son, included in this study the same duplication and both presented with BAH (Beuschlein et al., 2014; Carney et al., 2015); this is the only case of inheritance in the cohort. Other duplications at this locus have not been found in 24 cortisol-producing ACAs (Carney et al., 2015). Interestingly, the histological phenotype of these five patients has been published and 3 of them looked like PPNAD. This phenotype is comparable with PKA activation through *PRKAR1A* inactivation or PDEs mutations causing PPNAD (Almeida and Stratakis, 2010). However, the two remaining patients did not have PPNAD but diffuse adrenal cortex hyperplasia with nodules (Carney et al., 2015). This demonstrates that the same genetic alteration can lead to different histological phenotypes. It also demonstrates that differences in *PRKACA* gains and, thus, functional "dosage" have different effects on the histology of the adrenal cortex (Stratakis, 2014a). Whereas somatic mutations that lead to overactivity cause ACAs (Stratakis, 2014a), germline defects cause BAH depending on genetic dosage (Lodish et al., 2015).

Is Cα a new Therapeutic Target for Cushing Syndrome?

PRKACA mutations have been described in almost 40% of cortisol-producing ACA and are therefore, the most frequent genetic alteration in these tumors (**Table 1**). Beyond the importance of PKA in adrenal, its involvement in genetic diseases like CNC and cancers has been demonstrated (Caretta and Mucignat-Caretta, 2011). The ability to inhibit its constitutive activation through chemical components is a major challenge due to its critical role on cell function. Two inhibitors H89 and KT5720 have successfully decreased PKA activity induced by the transfection of Leu206Arg variant in HEK293 cells (Sato et al., 2014). These two inhibitors have been extensively used to better understand the role of PKA, however, their lack of specificity has been well established (Lochner and Moolman, 2006; Murray, 2008). Most of the inhibitors targeted the ATP-binding site but this was problematic due to the high percentage of identity of this domain among the Ser/Thr kinases family responsible to the low specificity of these inhibitors (Sapio et al., 2014). Moreover, these inhibitors cannot be used for activating *PRKACA* mutations. Better understanding of PKA function permits the development of substrate-competitive inhibitors, which would be more specific as there is diverse substrate-binding domain. The PKA inhibitor (PKI) is an endogenous thermostable peptide that interacts with the catalytic domain and is able to inhibit Leu206Arg variant *in vitro* (Cao et al., 2014). However, its main disadvantage preventing its use for clinical application is its weak permeability and its susceptibility to proteases. The discovery of new PKA inhibitors is desirable and can be helpful in the treatment of cortisol-producing ACA but also others cancers.

Conclusions

The discovery of somatic mutations in *PRKACA* is one additional proof of the central role of cAMP-PKA pathway in the development of cortisol-producing ACA. The high frequency of mutations (approximately 40%) and the even higher presence of PKA function alterations (Bimpaki et al., 2009), suggest that perhaps other components of the pathway may also be found mutated in the future. However, as *PRKACA* is ubiquitously expressed, its mutations may also be found in other tissues with PKA-dependent tumorigenesis. Future analysis and use of animal models will provide useful information to help answer this, and other, questions.

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Novel insights into the genetics and pathophysiology of adrenocortical tumors

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Adrenocortical tumors (ACTs) are typically unilateral and can be classified as benign adrenocortical adenomas (ACAs) or malignant adrenocortical cancers (ACCs). In rare cases, tumors may occur in both adrenal glands as micronodular hyperplasia (primary pigmented nodular adrenal dysplasia) or as macronodular hyperplasia (primary bilateral macronodular adrenal hyperplasia, PBMAH). The study of certain tumor predisposition syndromes has improved our understanding of sporadic ACTs. Most ACAs are associated with abnormalities of the cAMP signaling pathway, whereas most ACCs are linked to alterations in IGF2, TP53, or the Wnt/βcatenin pathways. Over the past year, single-nucleotide polymorphism array technology and next-generation sequencing have identified novel genetic alterations in ACTs that shed new light on the molecular mechanisms of oncogenesis. Among these are somatic mutations of PKA catalytic subunit alpha gene (*PRKACA*) in ACA, germline, and somatic mutations of armadillo repeat containing 5 gene (*ARMC5*) in primary bilateral macronodular adrenal hyperplasia and somatic alterations of the E3 ubiquitin ligase gene *ZNRF3* in ACC. This review focuses on the recent discoveries and their diagnostic, prognostic, and therapeutic implications.

Keywords: adrenocortical adenoma, hyperplasia, adrenocortical carcinoma, PRKACA, ARMC5, ZNRF3

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Introduction

The pathogenic mechanisms underlying adrenocortical tumors (ACTs) are complex and heterogeneous. The most common ACT is benign, unilateral, non-secreting (adrenocortical adenomas, ACAs-NS), and often discovered incidentally. ACTs exist in the bilateral form but are much less frequent. The symptoms due to ACT are caused by steroid excess (Cushing's syndrome) in the case of secreting benign tumors. The aggressive and deadly forms of ACT are adrenocortical cancers (ACCs) but have an overall low incidence of appearance. The clinical consequences of ACC can be due to steroid oversecretion, tumor growth, or metastasis. ACCs are rare and show heterogeneity in malignancy, in levels of hormone secretion, and in tumor progression. It is also difficult to predict evolution and prognosis although these cancers are globally associated to poor outcome.

Till now, the majority of genetic and molecular alterations of benign tumors has been closely linked to abnormalities in the cAMP signaling pathway. Somatic and germline mutations were identified in actors of the cAMP pathway as the *PRKAR1A* gene (*regulatory subunit of the cAMP-dependent protein kinase A*) (1, 2), *GNAS* gene (α *subunit of the stimulatory G protein*) (3), and the *PDE11A*/*8B* genes (*cAMP-degrading phosphodiesterase 11A and 8B,* respectively) (4, 5). Other alterations modulating the cAMP/PKA pathway activity that stimulates steroidogenesis are present

in ACA. For example, ectopic expression of the gastric inhibitory polypeptide receptor (GIPR) in the human adrenal gland causes significant hypercortisolemia after meal ingestion and leads to Cushing's syndrome (6, 7). Ectopic expression of other receptors belonging to binding G protein-coupled receptors classes such as vasopressin, serotonin, and catecholamine receptors have been described in the bilateral hyperplasias of the adrenal cortex and cortisol-secreting adenomas (ACA-S) (8, 9). In contrast to ACA, ACCs have been related to alterations in various pathways such as IGF2, TP53, or Wnt/βcatenin. Initially, progress in identifying genes involved in sporadic ACT came mainly from the study of rare familial cases (10–12): *TP53* tumor suppressor gene and its predisposition's locus on chromosome 17p13.1 involved in Li–Fraumeni syndrome; the imprinted gene encoding the insulin-like growth factor IGF2, located on chromosome 11p15.5 and associated with Beckwith–Wiedemann syndrome, germline *PRKAR1A* mutations identified in Carney complex. Moreover, somatic mutations in the *CTNNB1* gene have been reported in both benign and malignant ACTs (13). However, alterations in these several genes are identified only in subgroups of ACA and ACC. Over the last 5 years, the development of high-throughput sequencing has revealed several frequent alterations in genes not previously described, underlying new insights in the pathogenesis of benign and malignant forms of ACT. For example, a hotspot somatic mutation in the PKA catalytic subunit alpha gene (*PRKACA*) has been identified in ACA (14), germline, and somatic mutations of armadillo repeat containing 5 gene (*ARMC5*) have been described in patients with primary bilateral macronodular adrenal hyperplasia (PBMAH) (15), and somatic alterations in the E3 ubiquitin ligase gene *ZNRF3* were recently identified in ACC (16). In this review, we aim to give an overview of recent advances in the genetics of ACT, focusing on the latest driver genes identified, and therefore improving our understanding of the pathophysiology of these tumors.

Adrenocortical Adenomas

Prior to the introduction of next-generation sequencing, mutations in some genes such as *GNAS* or *PRKAR1A* had been reported in ACA-S. Activating mutations of the *GNAS* alpha subunit (17) and *PRKAR1A*-inactivating mutations (18) promote the cAMP pathway activation. *CTNNB1*-activating mutations had been found in ACA-NS and ACA-S but their prevalence was higher among ACA-NS (13, 19, 20). However, these mutations accounted for only a subset of ACA. Recently, Beuschlein and collaborators identified a hotspot mutation in *PRKACA* gene through whole-exome sequencing in ACA-S (14). The somatic mutation, p.L206R/c.617A > G was present in more than one-third of the examined tumors. This result was confirmed by four other groups, which has reported the same recurrent mutation in the *PRKACA* gene (21–24). This mutation occurs in the C-terminus of the activation segment in the $p + 1$ loop of PRKACA protein (**Figure 1A**). This region is a specific binding site for the interaction between catalytic and regulatory subunits of PKA (25). The p.L206R point mutation results in the introduction of a voluminous and positively charged amino acid that inhibits the formation of stable complexes between subunits of PKA (23, 24, 26). This mutation prevents the interaction of the catalytic subunit of PKA with the regulatory subunit, resulting in an increased phosphorylation of substrates and finally, in an excessive steroidogenic activity (**Figure 2A**). The consequence of this lack of interaction has been shown for both RIA (*PRKAR1A*) and RIIB (*PRKAR2B*) regulatory subunits (26). L206R mutation of *PRKACA* in ACA-S was associated with more severe phenotypes (Cushing's syndrome) (14). Another mutation in the *PRKACA* gene, Leu199_Cys200insTrp, identified only in one study, has the same effect on the stability of the PKA complex (14, 26) (**Figures 1A** and **2A**).

Adrenal cortex and cortisol-secreting adenomas are characterized by a high occurrence of *PRKACA*-activating mutations. However, other mutations in *GNAS* and *CTNNB1* genes are found in some ACA-S without *PRKACA* mutations and are mutually exclusive (21, 23). The hotspot mutation in the *PRKACA* gene seems to be sufficient to alter the endocrine and proliferative systems in ACA-S and represents the main genetic risk factor associated with this type of tumor (14, 21–24).

Primary Bilateral Macronodular Adrenal Hyperplasia

Primary bilateral macronodular adrenal hyperplasia described first in 1964 is a rare type of bilateral ACTs leading to adrenal Cushing's syndrome (33). PBMAH are often revealed incidentally during radiological examinations or by the presence of overt Cushing's syndrome. Both adrenal glands are enlarged massively with the presence of numerous macronodules. This adrenal disorder is usually diagnosed in patients aged between 40 and 60. In addition to ectopic expression of G protein-coupled receptors, it has been described in PBMAH an abnormal expression of paracrine factors (34–36). For instance, recently, an ACTH production by adrenocortical cells was reported in a large series of PBMAH, which can play a role in cortisol hypersecretion (36). Despite the fact that most cases of PBMAH appeared to be sporadic, some familial cases were reported, supporting the idea of a germline hereditary factor. Mutations or variants of some genes involved in the cAMP signaling pathway have been identified as in *GNAS*, *PDE11A*, and *PDE8B* genes but are only present in a limited fraction of PBMAH cases.

Combining single-nucleotide polymorphism (SNP) array and whole-genome sequencing, the first gene predisposing to PBMAH in adults has been recently identified (15). The most frequent somatic chromosome alteration in PBMAH was a loss of heterozygosity (LOH) at 16p and, the most frequent mutation identified was in *ARMC5* gene, located at 16p11.2. *ARMC5* alterations were detected in tumors obtained from 18 of 33 patients who had undergone surgery (55%). In all cases, both alleles of *ARMC5* carried alteration: one germline and the other somatic. For some cases with an *ARMC5* germline mutation, different nodules from one or both adrenal glands were analyzed. In each case, the same germline mutation was detected in all nodules and associated with a nodule-specific second somatic *ARMC5* alteration (LOH, nonsense or missense mutation). The discovery of *ARMC5* alterations establishes the first direct genetic link to PBMAH. The pattern of mutations suggests a "two-hit" model of a tumor suppressor gene, responsible for a hereditary predisposition syndrome. Subsequent studies confirm the recurrent mutation of

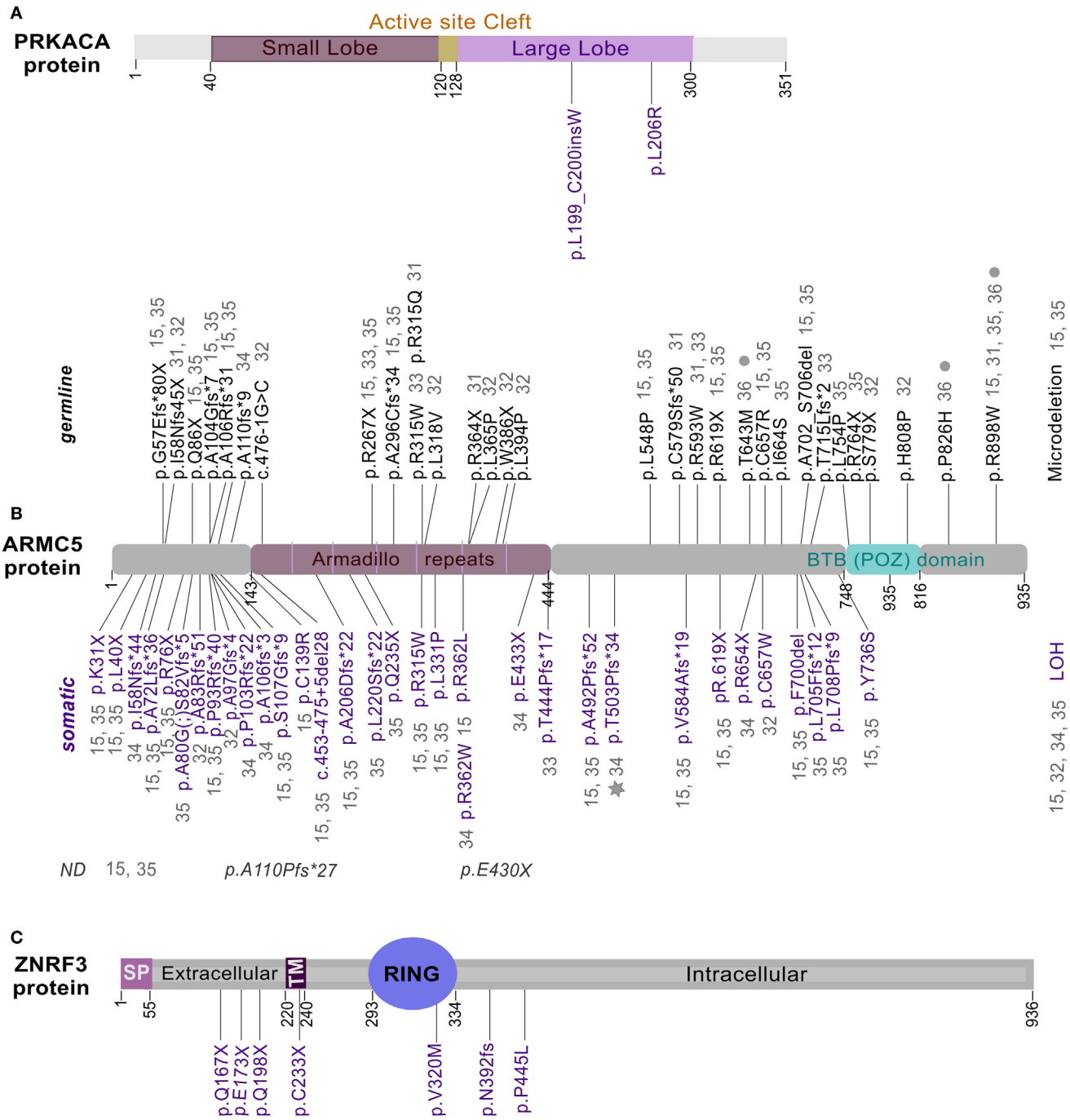

FIGURE 1 | Location of mutations in PRKACA, ARMC5, and ZNRF3. Full length of PRKACA **(A)**, ARMC5 **(B)**, and ZNRF3 **(C)** proteins were represented with mutations identified in ACA-S (14, 21–24), PBMAH (15, 27–32), and ACC (16), respectively. Somatic mutations are shown in purple, while germline mutations in black. Gray star and filled gray circles represent mutations identified in a meningioma (31) and in patients with hyperaldosteronism (32), respectively. ND, germline or somatic status not determined.

ARMC5 in family members with PBMAH (27–30). In these various studies, the percentage of *ARMC5* mutations reaches 25% in index cases of PBMAH. Recently, the high frequency of alterations in the *ARMC5* gene has been confirmed in a large cohort of 98 patients with PBMAH, including operated and non-operated patients (31). Up to now, these recent studies identified – in patients with PBMAH – in addition to LOH and a microdeletion, a total of 61 different mutations in *ARMC5*: 27 germinal, 30 somatic, two which

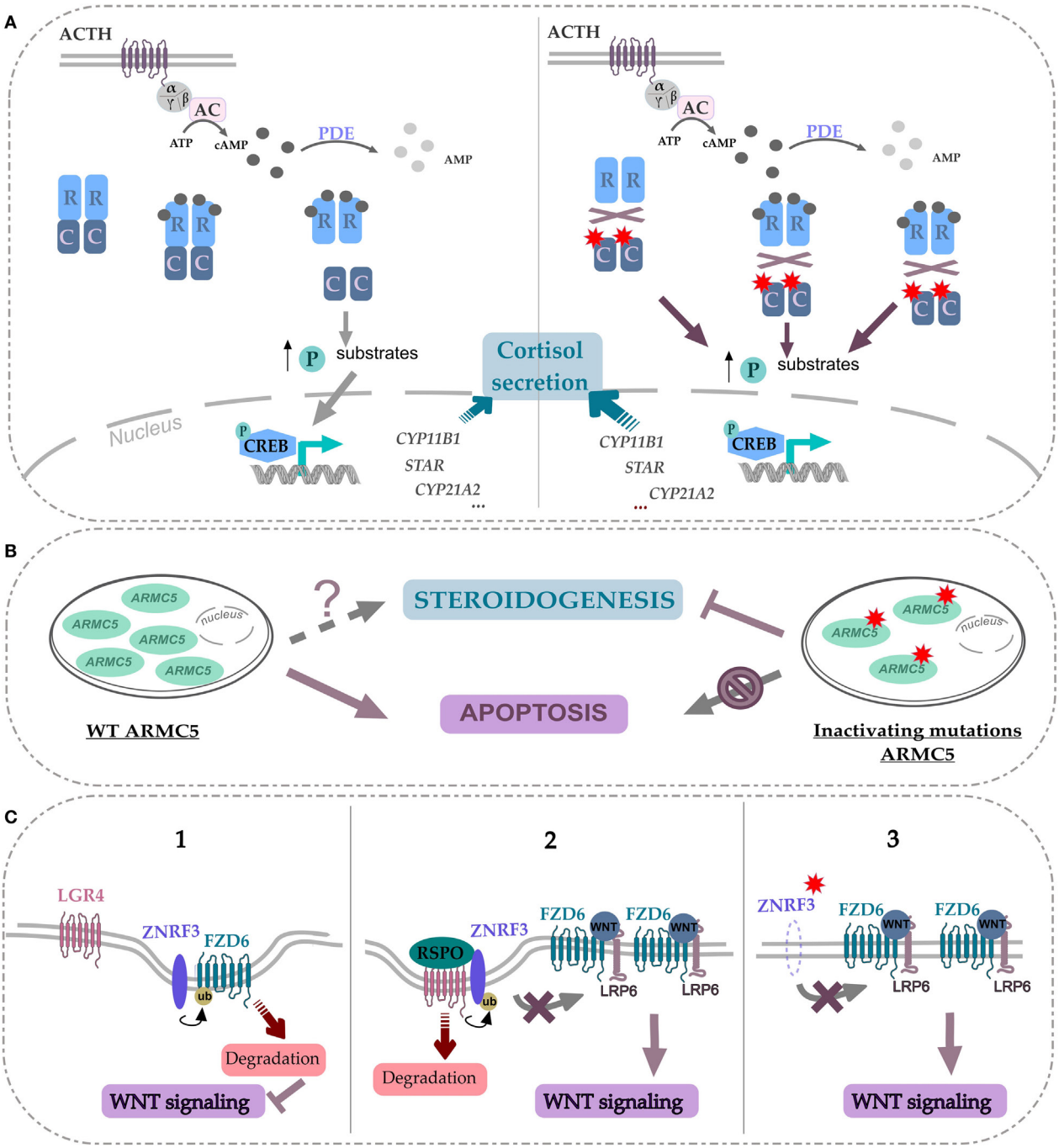

FIGURE 2 | Effects of recently identified drivers in signaling pathways involved in adrenal tumorigenesis. **(A)** Alterations in the PKA pathway caused by PRKACA$^{p.L206R}$ and PRKACA^{Leu199_Cys200insTrp} mutations in adrenocortical adenomas. Under normal conditions, the catalytic and regulatory subunits of PKA form a heterotetramer. In response to ACTH stimulation, the catalytic subunits are released from the complex in an active form. The free catalytic subunits phosphorylate their substrates and thereby increase the transcription of target genes. These target genes are mainly involved in the synthesis of cortisol and other steroids. The p.L206R and Leu199_Cys200insTrp mutations in the *PRKACA* gene alter its interaction with regulatory subunits of PKA leading to constitutive activation of PKAcα and increased steroidogenesis. **(B)** ARMC5, a new indirect or direct regulator of steroidogenic and apoptosis processes. ARMC5 inactivating mutations induce a decreased steroidogenic capacity and a protection against cell death. **(C)** ZNRF3 a negative feedback regulator of the Wnt/β-catenin signaling pathway. 1, In the absence of R-spondin (RPSO), ZNRF3 induces the poly-ubiquitination of Frizzled receptors (FZD) that promote their degradation. 2, In the presence of RSPO, ZNRF3 interacts with the complex RSPO/LGR4, which results in the turnover of ZNRF3 and thus the stability of FZD and the stimulation of Wnt/β-catenin signaling. 3, In ACC, ZNRF3 alterations can produce similar effects of RSPO stimulation leading to an activation of Wnt/β-catenin signaling. Red stars represent mutated or altered proteins.

have been identified at somatic and germline levels in different cases (p.R315W and p.R619X) and two without status available (**Figure 1B**). All these mutations can be found all along the protein in different domains. Two of the germline mutations are found in several index cases and in at least three studies suggesting a founder effect: p.R267X and p.R898W (15, 27–32).

The *ARMC5* encodes a protein of 935 amino acids and the peptide sequence reveals two distinctive domains: ARM domain in the N-terminal and a BTB/POZ in the C-terminal (Bric-a-Brac, Tramtrack, Broad-complex/Pox virus, and Zinc finger) (**Figure 1B**). These domains are highly conserved through evolution and have been shown to be involved in mediating protein-protein interactions, but targeted proteins recognition by these domains is not understood yet. The mechanism of ARMC5 action is unknown because no study has ever been focused on its biological function, and no diseases have been associated with the *ARMC5* gene until now. Recent functional study on *ARMC5* gene, performed in the human adrenocortical cells H295R, showed that *ARMC5* gene silencing alters the expression of genes involved in steroidogenesis leading to a global decreased of cortisol secretion (15) (**Figure 2B**). These data are consistent with previous expression-profile studies (37, 38). It is therefore likely that, despite the reduced secretory capacity of each cell, the overall production of cortisol was increased because of the large adrenal mass. All data describing *ARMC5* mutations show that patients suffering from PBMAH have a phenotype more severe than patients without *ARMC5* mutation (15, 31). Patients with ARMC5 mutations present with larger tumor volumes, increased numbers of tumor nodules, and more severe hypercortisolism (31). Recently, *ARMC5* mutations have been associated with another steroid hypersecretion. Indeed, six patients of 56 (10.7%) with primary hyperaldosteronism had germline mutations in the *ARMC5* gene. Among these six patients, two suffered from PBMAH (32).

The genomic and functional data indicate that *ARMC5* has a role of tumor suppressor gene because two inactivating mutations seem necessary to develop PBMAH and human cells (H295R and HeLa) transfected with non-mutated ARMC5 resulted in cell death (**Figure 2B**). In contrast, this effect was not observed with missense mutations. This suggests that ARMC5 plays a significant role in cell apoptosis (15, 31).

Bilateral adrenalectomy is considered as the single treatment of choice for PBMAH, the finding of *ARMC5* gene is promising for the discovery of new therapeutic perspectives. Interestingly, a somatic mutation in *ARMC5* gene has also been found in a meningioma in patients with an *ARMC5* germline mutation and a PBMAH (30). These data suggest that genetic alterations of the *ARMC5* gene may cause the development of different associated tumors with PBMAH. With the recent advances in the genetic methods, it is possible to imagine that future studies will reveal cases with *ARMC5* mutations in other types of tumors without PBMAH. Now, it is necessary to better know the functional role of the ARMC5 protein in order to understand the impact of these mutations on the initiation and/or development of PBMAH.

Mutations in the *DOT1L* (DOT1-like histone H3K79 methyltransferase) and *HDAC9* (histone deacetylase 9) genes have also been found in patients with PBMAH. Unlike *ARMC5* mutations, their frequency is lower and appeared only in two and one cases, respectively. These new mutations seem to define a little subgroup of PBMAH without *ARMC5* mutations (21). DOT1L and HDAC9 are methyltransferase and histone deacetylase, respectively; these two nuclear proteins are involved in the transcriptional regulation. Further investigations will help to delineate the importance of these three genes in the adrenal function. In regard to the high frequency (20%) of mutations in *ARMC5* gene in all index cases analyzed, its systematic genetic screening appears to be important for patients with PBMAH or Cushing syndrome. This screening can be used for early detection of PBMAH in family members with no clinical evidence.

Adrenocortical Cancer

ACC is a rare and highly aggressive endocrine tumor that affects one to two persons per 1 million of the population per year (39). The prognosis of ACC is very poor, with a 5-year survival rate under 35% in most series (40–43). Currently, surgery is the only curative therapy available. Medical treatments, including the adrenolytic drug mitotane and cytotoxic chemotherapy, show only limited therapeutic potential (44). The rarity of ACC is a limiting factor in the progress to understand the pathophysiology of this tumor. Up to now, somatic inactivating mutations of the tumor suppressor gene *TP53* and activating mutations of the proto-oncogene β-*catenin* (*CTNNB1*) were the most frequent mutations identified in ACC (13, 42, 45, 46).

Recently, a cohort of 122 ACC, from the European Network for the Study of Adrenal Tumors (ENSAT), was analyzed by SNP array. Fifty-five of these 122 ACC have also been analyzed by a combination of other genomic approaches, including exome sequencing, DNA methylation, mRNA expression arrays, and miRNA sequencing. Candidate driver genes were validated by targeted sequencing in all tumors. This work confirmed recurrent alterations in the known drivers *CTNNB1* and *TP53* and revealed new genes not previously reported to be altered in ACC. Strikingly, *ZNRF3* (Zinc and ring finger protein 3) was the most frequently altered gene (21%). In a majority of cases, homozygous deletions of *ZNRF3* were observed but few somatic inactivating mutations and two missense mutations were also identified (16) (**Figure 1C**).

ZNRF3 and its homolog *RNF43* (ring finger protein 43) encode proteins with E3 ubiquitin ligase activity that have recently been described as cell-surface transmembrane E3 ubiquitin ligases, acting as negative feedback regulators of Wnt/β-catenin signaling. ZNRF3 and RNF43 contain a signal peptide, an extracellular domain for R-spondin (RSPO)-binding, a single transmenbrane helix, a cytoplasmic really interesting new gene (RING) finger domain, and a C-terminal tail. It has been demonstrated that ZNRF3/RNF43 are associated with the Wnt receptors (Frizzled, FZD), which results in a multi-ubiquitination of lysines in the intracellular domain of FZD and then their internalization and degradation in lysosomes (47, 48). RSPO are secreted proteins known to potentiate the Wnt signaling. Various membrane proteins have been reported to bind RSPO, including FZD and LRP6, LGR4/5/6, Kremen, Syndecan, and ZNRF3/RNF43 (49). Several models of RSPO signaling have been proposed. Recently, published data indicate that the ZNRF3/RNF43-mediated membrane clearance of FZD is reversed upon addition of RSPO (47, 49, 50). Once bound to its receptor (LGR5), RSPO are believed to decoy ZNRF3, thus permitting strong β-catenin signaling (**Figure 2C**).

It has been shown that ZNRF3 protein expression is down regulated in gastric adenocarcinoma tissues compared with adjacent normal gastric tissues (51). Recurrent deletion of three regions in chromosome 22 was identified in osteoblastoma, one of these regions contains *ZNRF3* (52). Moreover, the deletion of *ZNRF3* and *RNF43* in the intestinal epithelium in mouse induces the development of adenoma with an increased nuclear β-catenin and an increased expression of Wnt/β-catenin target genes (48).

Interestingly alterations of *ZNRF3* and *CTNNB1* are completely exclusive in ACC (16), suggesting that *ZNRF3* alterations might play a crucial role in tumorigenesis by activating also the Wnt/β-catenin signaling pathway. Taken together, 37% of ACC samples harbored an alteration affecting the Wnt pathway. These data strongly suggest that in ACC, *ZNRF3* is a tumor suppressor gene related to the Wnt pathway. ACC with altered *ZNRF3* showed transcriptional activation of β-catenin targets, but this activation was weaker than in *CTNNB1*-mutated tumors (16). However, till now, ACCs are the cancers described with the most frequent *ZNRF3* alterations, suggesting a specific mechanism of tumorigenesis into the adrenal cortex tissue. Future functional studies are needed to investigate its role in adrenocortical cells.

Conclusion

Analyses of inherited syndromes associated with an increased risk adrenocortical tumorigenesis, coupled with recent advances in sequencing technology, have improved our understanding of ACT. Recent advances in genomic tools, especially sequencing technologies, have yielded new findings in three types of ACT. Alterations in genes not previously reported were identified: somatic mutations of *PRKACA* gene in ACA, germline and somatic mutations of *ARMC5* gene in PBMAH, and somatic alterations of *ZNRF3* gene in ACC.

It would be worth pursuing functional studies on these genes in order to understand the impact of these alterations on the initiation and/or development of ACT. The identification of signaling pathways playing a major role in ACT development would help to develop new targeted therapies, which are dramatically needed for the management of patients harboring these tumors, especially for ACC.

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Cell-to-cell communication in bilateral macronodular adrenal hyperplasia causing hypercortisolism

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It has been well established that, in the human adrenal gland, cortisol secretion is not only controlled by circulating corticotropin but is also influenced by a wide variety of bioactive signals, including conventional neurotransmitters and neuropeptides, released within the cortex by various cell types such as chromaffin cells, neurons, cells of the immune system, adipocytes, and endothelial cells. These different types of cells are present in bilateral macronodular adrenal hyperplasia (BMAH), a rare etiology of primary adrenal Cushing's syndrome, where they appear intermingled with adrenocortical cells in the hyperplastic cortex. In addition, the genetic events, which cause the disease, favor abnormal adrenal differentiation that results in illicit expression of paracrine regulatory factors and their receptors in adrenocortical cells. All these defects constitute the molecular basis for aberrant autocrine/paracrine regulatory mechanisms, which are likely to play a role in the pathophysiology of BMAH-associated hypercortisolism. The present review summarizes the current knowledge on this topic as well as the therapeutic perspectives offered by this new pathophysiological concept.

Keywords: Cushing's syndrome, catecholamine, serotonin, ACTH, vasopressin, endothelin, leptin, illegitimate receptor

INTRODUCTION

Chronic hypercortisolism results in a series of symptoms, including central obesity, skin changes, and arterial hypertension, known as Cushing's syndrome. In 15–20% of cases, Cushing's syndrome is the consequence of primary adrenal cortisol hypersecretion by bilateral adrenal hyperplasias or unilateral adrenocortical tumors. Bilateral macronodular adrenal hyperplasia (BMAH) is a rare cause of primary adrenal hypercortisolism representing <1% of all cases of Cushing's syndrome (1). In this condition, cortisol hypersecretion by the enlarged adrenal glands leads to suppression of pituitary ACTH secretion. Consequently, the disease has long been named ACTH-independent macronodular adrenal hyperplasia (AIMAH). BMAH appears to be more frequent in women and hypercortisolism is usually diagnosed during the fifth and sixth decades (2, 3). In most patients with BMAH, hypercortisolism is moderate, contrasting with the important adrenal hypertrophy. The great majority of the published cases are sporadic but familial cases of the disease have also been reported (4). It should also be noticed that the extensive use of abdominal imaging, including computerized tomography (CT) scan and magnetic resonance imaging (MRI), has led to a marked increase in incidentally discovered BMAH (5). In this situation, BMAH is frequently associated with subclinical hypercortisolism (6).

At pathological examination, BMAH is characterized by an important increase in adrenal mass, which can reach 10–100 times the normal weight of the glands (7). The adrenal cortex is disorganized by the presence of large lipid-rich macronodules (8). There is no internodular atrophy and the nodules are usually not pigmented (9). At the microscopic level, the macronodules appear to be composed of two types of steroidogenic cells, i.e., large lipid-loaded cells, which are called spongiocytes, and small compact cells (7). Interestingly, these cell types display marked differences in steroidogenic enzyme expression. In fact, 17-hydroxylase is primarily detected in compact cells whereas 3β-hydroxysteroid dehydrogenase is principally expressed by spongiocyte cells (7, 10). This unequal repartition of steroidogenic enzymes among adrenocortical cells may result in relatively inefficient steroidogenesis, likely explaining the discrepancy between the major enlargement of the adrenal glands and the moderate intensity of hypercortisolism generally observed in patients with BMAH.

The pathophysiology of BMAH has long remained unknown. The bilaterality of the adrenal lesions suggested the occurrence of a pathogenic event affecting adrenal gland development during early embryogenesis. In fact, it is now known that BMAH is a genetically determined disease. Various mutational events can favor the development of the disease. The affected genes include the multiple endocrine neoplasia type 1 (*MEN1*), familial adenomatous polyposis (*APC*), phosphodiesterase 11A (*PDE11A*), G-protein αS subunit (*GNAS*), melanocortin type 2 receptor (*MC2R*), fumarate hydratase (*FH*), type A endothelin receptor (*EDNRA*), and protein kinase A catalytic subunit alpha (*PRKACA*) genes (6, 11–13). More recently, it has been shown that more than

50% of patients with BMAH carry mutations of the *ARMC5* gene, which behave as a tumor suppressor gene in the adrenal glands (14). In addition, *ARMC5* mutations may promote the development of a new multiple neoplasia syndrome associating BMAH and meningiomas (15).

The mechanisms involved in the pathogenesis of BMAH-associated cortisol hypersecretion are also better understood. It is indeed well established that, in BMAH tissues, cortisol secretion is stimulated by abnormally expressed membrane receptors, called illicit or illegitimate receptors, which supply the absence of pituitary ACTH (16). Several of these receptors are activated by circulating hormones, such as glucose-dependent insulinotropic peptide (GIP), luteinizing hormone (LH), and glucagon, while others bind paracrine regulatory signals released in the adrenal gland (16–21). More recently, it has been shown that, in addition to membrane G-protein-coupled receptors, BMAH tissues can abnormally express paracrine factors leading to formation of abnormal intraadrenal stimulatory loops, which seem to play an important role in cortisol hypersecretion (22–24). These illicit regulatory processes, which can be regarded as a pathological amplification of the paracrine systems physiologically occurring in the normal adrenal gland. In fact, it has been well established that the secretory activity of the normal adrenal cortex is influenced by various bioactive signals released in the vicinity of adrenocortical cells by chromaffin cells, neurons, cells of the immune system, adipocytes, and endothelial cells (25–27). The present review summarizes the current knowledge on the paracrine regulation of cortisol secretion in BMAHs from which emerges the new pathophysiological concept of paracrinopathy.

SEROTONERGIC PATHWAYS IN BMAH

In the normal adrenal gland, serotonin (5-hydroxytryptamine, 5-HT) is produced by perivascular mast cells (MC), which are primarily located in the subcapsular region of the cortex (28). The regulation of 5-HT release in the adrenal tissue is unknown but it is possible that 5-HT may be secreted in response to activation of the sympathetic system since adrenal MC have been shown to establish connections with cortical nerve endings (29). After its release, 5-HT is able to stimulate corticosteroid secretion through activation of 5-HT_4 receptors positively coupled to adenylyl cyclase and calcium influx (28, 30, 31). It is not excluded that 5-HT may also influence corticosteroidogenesis through indirect mechanisms such as modulation of adrenal blood flow and/or production of cytokines by adrenocortical cells, as observed in rat (32, 33). *In vitro* studies have shown that adrenal 5-HT efficiently stimulates aldosterone secretion but only weakly activates cortisol production (31, 34). These differential actions on mineralo- and glucocorticoid synthesis likely result from the following observations: 5-HT is released by MC in the immediate vicinity of aldosterone-producing cells, and the 5-HT_4 receptor is intensely expressed in zona glomerulosa but much more modestly in zona fasciculata (35, 36). In addition to its effect on the secretory activity of adrenocortical cells, adrenal 5-HT can be locally metabolized into inactive compounds such as 5-hydroxyindolacetic acid and 5-hydroxytryptophol (28, 34). This catabolic process is catalyzed by monoamine oxidase type A, which is mainly expressed by chromaffin cells (34).

In agreement with the data obtained *in vitro*, clinical studies have shown that administration of 5-HT_4 receptor agonists, like zacopride and cisapride, to healthy volunteers induces a significant increase in plasma aldosterone levels without affecting plasma cortisol concentrations (30, 37–40). Interestingly, the stimulatory action of cisapride on aldosterone secretion was found to be additive with that of angiotensin II (38).

The physiological role of the serotonergic control of corticosteroid production remains unknown. However, several studies have shown that BMAH tissues exhibit several alterations in the adrenal serotonergic pathway, which tend to reinforce its stimulatory action on cortisol secretion. First, whereas MC represent the unique source of 5-HT in the normal adrenal, immunohistochemical studies have shown abnormal synthesis of 5-HT in a subpopulation of steroidogenic cells (22). Second, in some patients with BMAH, administration of the 5-HT_4 receptor agonists, cisapride and metoclopramide, is followed by an abnormal elevation of plasma cortisol levels, suggesting an increased sensitivity of the adrenal hyperplastic tissue to 5-HT and 5-HT_4 receptor agonists (19, 22, 41–44). In agreement with this hypothesis, *in vitro* studies conducted on tissue explants derived from BMAH previously responsive *in vivo* to 5-HT_4 receptor agonists showed an increased potency and/or efficacy of 5-HT to stimulate cortisol production, in comparison with normal adrenal samples (22). Collectively, these data suggest that 5-HT exerts an intraadrenal stimulatory tone to stimulate cortisol secretion and is thus involved in the pathogenesis of BMAH-associated hypercortisolism. Consistently, 5-HT_4 receptor antagonists were able to decrease cortisol secretion from perifused BMAH explants (36). Surprisingly, in some BMAH tissues, 5-HT was found to paradoxically inhibit cortisol secretion (45). This unexpected effect, which may counteract the influence of other stimulatory signals and may thus be beneficial by limiting the amplitude of cortisol hypersecretion, could result from abnormal coupling of eutopic 5-HT_4 receptors to transduction pathways or illicit expression of 5-HT receptors negatively coupled to adenylyl cyclase such as the 5-HT_1 and 5-HT_5 types (46).

Clinical studies, by showing illicit cortisol responses to 5-HT_4 receptor agonists in patients with BMAH, indicated that the effect of 5-HT on hyperplastic tissues was, at least in part, mediated by the eutopic 5-HT_4 receptor. As expected, several groups reported an overexpression of the 5-HT_4 receptor mRNA in BMAH tissues (42, 44, 47). Interestingly, the expression profile of 5-HT_4 mRNA splicing variants seems to be different in BMAH samples from that observed in the normal adrenal (42). Immunohistochemical studies showed an ectopic distribution of the 5-HT_4 receptor, which was visualized with high intensity in groups of cells localized in hyperplastic macronodules of the zona fasciculata (36). This result was consistent with the abnormal response of cortisol to 5-HT and 5-HT_4 receptor agonists observed both *in vivo* and *in vitro*. However, in some BMAH tissues, the stimulatory effect of 5-HT on cortisol production was not modified by 5-HT_4 receptor antagonists, indicating that the corticotropic action of the indolamine was mediated by other receptor types. Consistently, 5-HT was found to exert its biological effect on these tissues through activation of the 5-HT_7 receptor (48). 5-HT_7 receptor immunoreactivity could be visualized at the plasma membrane of adrenocortical cells throughout BMAH tissues, at variance with

the normal adrenal gland in which the 5-HT_7 receptor is exclusively detected in artery walls (48). Transcriptomic analyses have also shown an overexpression of the 5-HT_{2B} receptor in BMAH (49). However, the pathophysiological significance of this observation remains unclear since it is not known whether the 5-HT_{2B} receptor is expressed in adrenocortical cells or in blood vessels, as shown in various tissues (50).

In physiological conditions, 5-HT activates glucocorticoid synthesis through activation of the cAMP/PKA pathway (28, 51, 52). As expected, the stimulatory action of 5-HT on cortisol secretion by BMAH tissues was found to be suppressed by the PKA inhibitor H89 (48). These data are consistent with the observation that both the eutopic 5-HT_4 receptor and the ectopic 5-HT_7 receptor, which mediate the corticotropic effect of 5-HT in BMAHs, are positively coupled with adenylyl cyclase (46). However, the influence of 5-HT on steroidogenic enzyme expression in BMAH tissues remains currently unknown.

To summarize, in comparison with the normal adrenal gland, BMAH display molecular and cellular defects, which tend to reinforce the stimulatory effect of the intraadrenal serotonergic tone on cortisol production. These pathological findings include illicit synthesis of 5-HT in adrenocortical cells and aberrant expression of the 5-HT_4 and 5-HT_7 receptors. It thus appears likely that the enhancement of 5-HT paracrine pathways in BMAH tissues is involved in cortisol hypersecretion.

INTRAADRENAL PRODUCTION OF ACTH

It has been shown in several mammalian species including man, that adrenomedullary chromaffin cells stimulate the secretory activity of adrenocortical cells through a paracrine mode of communication involving diverse bioactive signals (53). In particular, it has been shown that chromaffin cells are able to express the gene encoding the precursor of ACTH proopiomelanocortin (POMC) and to synthesize detectable amounts of ACTH (54, 55). The presence of chromaffin ACTH-producing cells has been observed in BMAH tissues as early as 2001 (56). A few years later, several groups reported illicit expression of POMC and synthesis of ACTH in adrenocortical cells in isolated cases of BMAH (57–60). More recently, the presence and role of ACTH was systematically investigated in a large series of 30 cases of BMAH (24). The tissues were found to express POMC mRNA at variable levels. The presence of proconvertase 1, a protease involved in the processing of POMC into ACTH, was also detected in a subpopulation of adrenal cells suggesting that POMC could be converted into ACTH in the hyperplastic tissues. In fact, immunohistochemical studies revealed the presence of ACTH immunoreactivity in chromaffin cells of the adrenal medulla and, as previously noticed, in some adrenocortical cells either isolated or arranged in small clusters disseminated in the tissues. Adrenocortical ACTH-positive cells exhibit the usual characteristics of steroidogenic cells, i.e., loaded with numerous lipid inclusions, and express several markers of steroidogenic differentiation including steroidogenic factor 1 (SF1), the HDL-cholesterol receptor SRB1 (scavenger receptor B1), and 17-hydroxylase. Thus, they represent a subcategory of adrenocortical steroidogenic cells that abnormally express ACTH. The ectopic synthesis of ACTH in these cells is not the consequence of abnormal corticotropic-like differentiation as indicated by the lack of significant T-pit [a transduction factor which drives pituitary corticotrophs differentiation (61)] expression in the tissues (24). The presence of ACTH in adrenocortical cells may rather be regarded as an additional trait of the previously reported neuroendocrine differentiation of the hyperplastic tissues (21, 22, 48). Interestingly, ACTH-positive cells were also labeled by antibodies directed against the Leydig cell marker insulin-like 3 (INSL3) indicating that ACTH synthesis may result from illicit gonadal-like differentiation of some adrenocortical cells (24). This observation is consistent with the data obtained from older studies showing that testicular Leydig cells and ovarian granulosa cells are able to express POMC and synthesize ACTH (62, 63). The expression of gonadal markers in the adrenal hyperplastic tissues is also reliable with previous reports of BMAH-associated with androgens or estrogens overproduction (8, 64–66). As the adrenal glands and gonads derive from a same tissue precursor, the adrenogonadal primordium, it is likely that the presence of gonadal-like cells in the adrenal tissues may result from abnormal differentiation and/or separation of the adrenogonadal primordium during early embryogenesis explaining the bilaterality of the lesions.

In vitro studies revealed that ACTH is released by BMAH tissues in a pulsatile way, consistently with former clinical studies showing a pulsatile mode of cortisol secretion in patients with BMAH (67). The ectopic secretion of ACTH by the adrenal glands could also be observed *in vivo* in two patients through adrenal vein catheterization (24). In fact, adrenal vein sampling demonstrated a significant ACTH concentration gradient between the adrenal versus peripheral veins as well as inferior petrosal sinus in one of the two patients (24, 36). All these results suggested that ACTH produced by intraadrenal gonadal-like cells may stimulate cortisol secretion in BMAH tissues, supplying therefore pituitary ACTH, which is suppressed by cortisol excess. This assumption could be assessed by the following observations: ACTH and cortisol levels were positively correlated in culture medium during perifusion of BMAH samples; basal plasma cortisol concentrations measured in the patients were positively correlated with both the levels of *POMC* mRNA and the ACTH histological score in the tissues; the ACTH receptor (MC2R) antagonists corticostatin and ACTH (7–38) significantly inhibited the production of cortisol *in vitro* by BMAH explants (24). Interestingly, MC2R antagonists also markedly reduced the amplitude of cortisol pulses indicating that oscillations in glucocorticoid production are determined by ACTH-secreting cells (**Figure 1**). Although globally underexpressed (47), MC2R was upregulated by ACTH in BMAH tissues, as previously established in the normal adrenal gland (68). MC2R mRNA levels were indeed positively correlated with POMC mRNA rates and MC2R immunoreactivity was primarily observed in the vicinity of ACTH-positive cells, which were also found to express the receptor (24). Thus, it seems that intraadrenal ACTH may exert autocrine actions in BMAH. The regulation of ACTH production by BMAHs has also been investigated by using the same *in vitro* approach. Dexamethasone and the glucocorticoid receptor antagonist RU486 failed to influence ACTH release indicating that, at variance with pituitary ACTH, intraadrenal ACTH is not regulated by cortisol (24). Conversely, it was observed that several ligands of illicit membrane receptors, i.e., 5-HT, LH/hCG, and GIP, stimulate ACTH release from BMAH explants by increasing

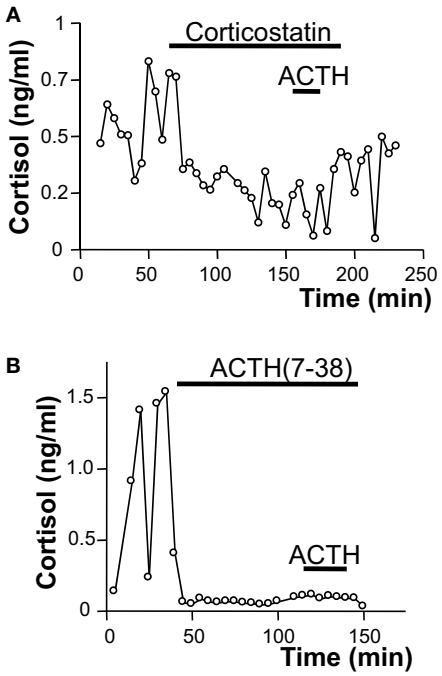

FIGURE 1 | Role of intraadrenal ACTH secretion in the control of cortisol production by bilateral macronodular adrenal hyperplasia (BMAH) associated with Cushing's syndrome. Spontaneous and ACTH-induced cortisol secretions by perifused BMAH explants were inhibited by application of the melanocortin type 2 receptor (MC2R) antagonists corticostatin [2×10^{-7} M; **(A)**] and corticotropin (7–38) [10^{-7} M; **(B)**]. Corticostatin and corticotropin (7–38) significantly reduced the amplitude of cortisol pulses.

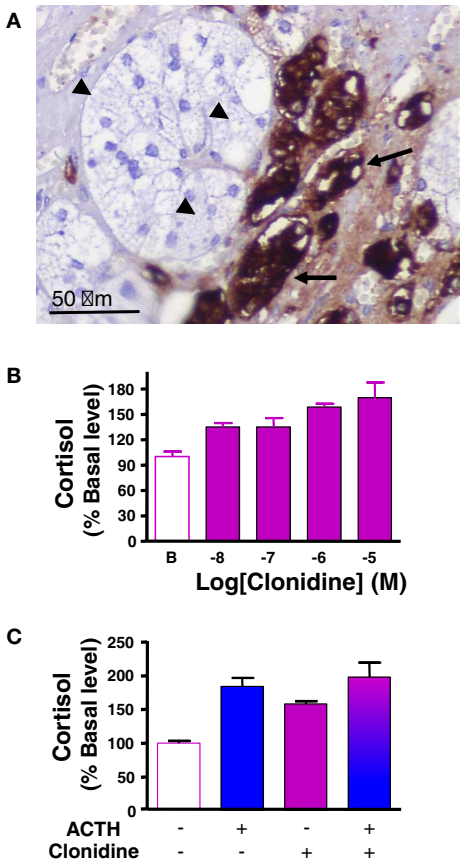

FIGURE 2 | Catecholaminergic pathway in bilateral macronodular adrenal hyperplasia (BMAH) associated with Cushing's syndrome. **(A)** Chromogranin A-positive chromaffin cells (arrows), which represent the main source of catecholamines in the normal adrenal gland, were in close contact with steroidogenic cells (arrow heads) in BMAH tissue. **(B)** Clonidine, an α2 receptor agonist, dose-dependently stimulated cortisol secretion by cultured adrenocortical cells derived from a BMAH tissue. Adapted from Ref. (47). **(C)** The maximum cortisol responses of cultured BMAH adrenocortical cells to high concentrations of ACTH (10^{-10} M) and clonidine (10^{-6} M) were not additive, suggesting that α2 and MC2R receptors are coupled to a common transduction pathway.

pulse amplitude without affecting pulse frequency (24). This unexpected finding suggested that activation of membrane receptors may stimulate cortisol production via two mechanisms including a direct effect on corticosteroidogenesis, as previously shown in BMAH cell culture (22), and an indirect action via ACTH secretion (24). In agreement with this hypothesis, it was observed that MC2R antagonists reduce the amplitude of the cortisol response to GIP. It seems therefore that intraadrenal ACTH is a common intermediate and amplifier of the action of several illicit membrane receptors in BMAH tissues.

CATECHOLAMINERGIC PATHWAY IN BMAH

The catecholamines adrenaline and noradrenaline are secreted by adrenal chromaffin cells under control of splanchnic nerve and proinflammatory cytokines. It has been hypothesized that catecholamines released by chromaffin cells present at the corticomedullary junction and in the cortex, may influence steroid production by adrenocortical cells, in particular during stress and inflammation (25, 27, 69). In support of this hypothesis, *in vitro* experiments have demonstrated that adrenaline and noradrenaline are able to modulate glucocorticoid production in frog and bovine adrenocortical cells (70, 71). However, there is no clear evidence for catecholamine responsiveness in human adrenal, since noradrenaline did not affect *in vitro* cortisol secretion by human normal adrenocortical cells (72). By contrast, abnormal catecholaminergic control of steroidogenesis has been documented in some patients with macronodular adrenal hyperplasia-associated with Cushing's syndrome. Indeed, immunohistochemical studies have revealed the presence of clusters of chromogranin A-immunopositive chromaffin cells in the vicinity of steroidogenic cells, indicating paracrine interactions between the two cell types in hyperplastic tissues (24) (**Figure 2A**). In addition, abnormal elevations of plasma cortisol have been detected in patients placed in physiological conditions associated with increases in endogenous catecholamine, such as upright posture or insulin-induced hypoglycemia (22, 73). Moreover, increases in circulating cortisol levels provoked by administration of isoproterenol, a β-adrenergic receptor agonist, as well as decreases in plasma cortisol concentrations in response to infusion of propranolol, a β blocker, have given evidence for illicit β-adrenergic control of steroidogenesis

(73–76). Aberrant expression of β adrenergic receptors in BMAH tissues has been confirmed by binding, RT-PCR, and functional *in vitro* experiments (22, 59, 73, 77). In particular, hypersensitivities to salbutamol and isoproterenol, two β2 receptor agonists, have been observed on cultured cells derived from BMAH tissues (22, 59). Our group has also demonstrated, by using molecular and cellular biological approaches, the occurrence of illegitimate α2-adrenergic receptors in BMAHs (47). In particular, *in vivo* and *in vitro* experiments have revealed that administration of the α2 receptor agonist clonidine stimulated cortisol synthesis in one BMAH case (47) (**Figure 2B**). Pharmacological studies have shown that the positive effect of clonidine on cortisol production resulted from activation of α2 receptors positively coupled to the adenylyl cyclase/PKA pathway (47). The absence of additive effects of high concentrations of ACTH and clonidine on cortisol production is consistent with a common transduction pathway for α2 and MC2R receptors (**Figure 2C**). Altogether, these data indicate that, in some BMAH tissues, the presence of chromaffin cells intermingled with steroidogenic cells expressing illegitimate β- or α2-adrenergic receptors, give rise to a positive adrenergic regulatory loop, which likely contributes to the pathogenesis of hypercortisolism.

VASOPRESSINERGIC PATHWAY IN BMAH

Arginine vasopressin (AVP) is known to activate glucocorticoid production through a dual action on the hypothalamic–pituitary–adrenal axis. AVP released by hypothalamic neurons is a potent stimulator of ACTH production by pituitary corticotrophs via vasopressin type 1b (V_{1b}) receptors (78, 79). In addition, AVP can be released by adrenomedullary chromaffin cells and act as a paracrine modulator of glucocorticoid production through activation of type 1a receptors (V_{1a}) positively coupled to phospholipase C (78, 79). However, the physiological role of intraadrenal AVP is not known and *in vivo* administration of AVP or its analogs to dexamethasone-pretreated healthy volunteers has no influence on plasma cortisol levels (80, 81). Surprisingly, abnormal plasma cortisol responses to AVP have been observed in patients with BMAH-associated hypercortisolism. AVP-induced increase in cortisol levels was observed in response to injection of AVP analogs or hypertonic saline test, which increases endogenous AVP release, in the absence of any significant variation of plasma ACTH concentration (41, 80, 82). The enhanced sensitivity of adrenocortical cells to AVP has been confirmed *in vitro* by perifusion and cell culture experiments (22, 59, 82). RT-PCR and pharmacological studies have revealed that some BMAH tissues overexpress the eutopic V_{1a} receptor subtype (23, 83, 84) and/or abnormally synthesize ectopic V_{1b} and V_2 receptors (23, 85, 86). Involvement of AVP and V_{1a} receptors in hypercortisolism has been confirmed in a patient with an AVP-sensitive BMAH in whom oral administration of a non-peptidic V_{1a} antagonist significantly decreased urinary cortisol level (82).

It is conceivable that circulating AVP may control cortisol secretion in patients with BMAH expressing illicit vasopressin receptors. However, basal plasma AVP levels (around 10^{-12} M) are much lower than the minimal effective dose of AVP (around 10^{-10} M) to stimulate cortisol release by BMAH tissues *in vitro* (23). It seems therefore more likely that illegitimate adrenal AVP receptors are predominantly activated by locally produced AVP through a paracrine mechanism similar to that observed in the normal adrenal gland. In this respect, BMAH tissues have been shown to contain two types of AVP producing cells, identified as chromaffin and steroidogenic cells, the latter clearly representing an ectopic source of the nonapeptide (22). Collectively, these data indicate that a vasopressinergic loop, resulting from aberrant intraadrenal AVP production and overexpression of functional V_{1a}/V_2 receptors, is involved in the pathophysiology of cortisol excess in some patients with BMAH.

OTHER PARACRINE REGULATORY MECHANISMS

Like the kidneys, the adrenal gland is surrounded by adipose tissue, which may release numerous bioactive substances capable of influencing the secretory activity of steroidogenic cells. Among them, leptin has been shown to dose-dependently inhibit ACTH-induced cortisol secretion through activation of the leptin receptor and repression of *CYP17* expression in adrenocortical cells (87, 88). Thus, it seems that leptin produced by the periadrenal adipose tissue may act as a metabolic signal to exert a negative control on cortisol production. Interestingly, BMAH tissues have been shown to contain clusters of adipocytes sometimes arranged in lipomatous islets (**Figure 3A**), suggesting that the paracrine control of cortisol secretion by leptin could be reinforced in comparison with the normal adrenal gland (21). However, at variance with the physiological process, leptin was found to paradoxically stimulate cortisol release in some BMAH tissues and thus participate in the pathophysiology of hypercortisolism (89) (**Figures 3B,C**). This illicit cortisol response to leptin may result from abnormal coupling of leptin receptors to transduction pathways.

Finally, the adrenal cortex is a richly vascularized organ so that each adrenocortical cell is in close contact with at least one capillary (90). As expected, endothelin and the endothelin-converting enzyme were detected at both mRNA and protein levels in the adrenocortical tissue (91). In addition, adrenocortical cells were found to express the endothelin types A (ETA) and B (ETB) receptors and endothelin-1 is able to stimulate both aldosterone and cortisol production by normal adrenocortical cells (92, 93). Although several studies indicate that endothelin may play a role in the pathophysiology of aldosterone-secreting neoplasms, it is not known whether this peptide may be involved in BMAH-associated hypercortisolism. However, a mutation of the *EDNRA* gene, which encodes the ETA receptor, has been found in a familial case of BMAH suggesting that a defect in the adrenal endothelin pathway may favor the development of adrenal hyperplasia and hypercortisolism (12).

INTEGRATIVE PATHOPHYSIOLOGY OF BMAH-ASSOCIATED HYPERCORTISOLISM

The studies recently published have brought important new insights into the comprehension of the pathophysiology of BMAH, which will undoubtedly stimulate the research on the disease and other adrenal disorders. In particular, it is now unquestionable that BMAH is a genetically determined condition, *ARMC5* being a major susceptibility gene of the disease. However, the mechanisms by which *ARMC5* favors the development of hyperplasia and hypercortisolism are still unknown. In particular, the

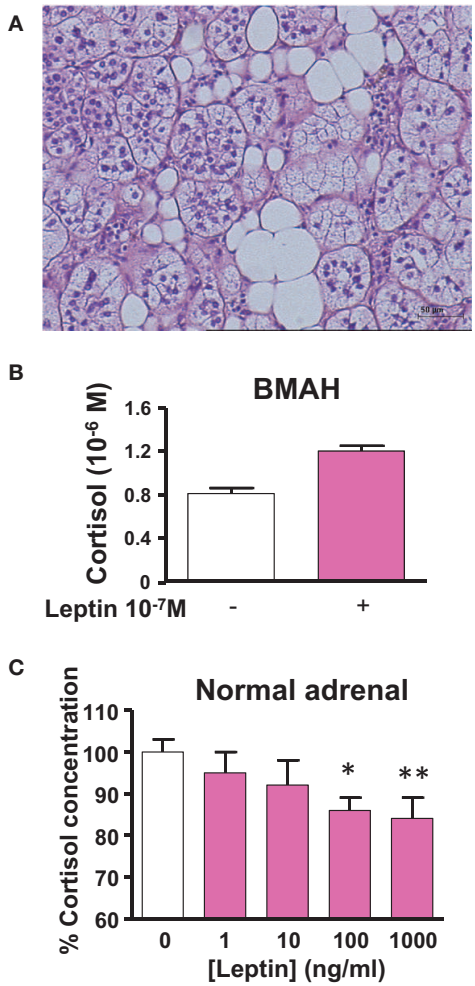

FIGURE 3 | Leptin pathway in bilateral macronodular adrenal hyperplasia (BMAH) associated with Cushing's syndrome. **(A)** Islets of adipocytes in the vicinity of steroidogenic cells in BMAH tissue. **(B)** Abnormal stimulatory effect of leptin (10^{-7} M) on cortisol secretion by cultured adrenocortical cells derived from a BMAH tissue in a patient with Cushing's syndrome ($p = 0.06$). Adapted from Ref. (89). **(C)** Leptin reduces ACTH-induced cortisol secretion by cultured normal adrenocortical cells in a dose-dependent manner (*$p < 0.01$; **$p < 0.001$). Adapted from Ref. (87).

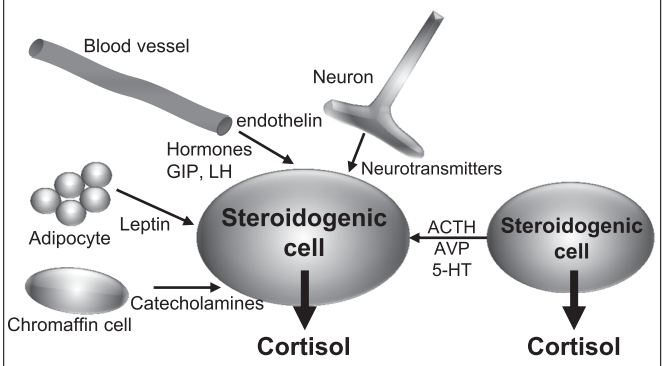

FIGURE 4 | Schematic representation of endocrine and paracrine controls of cortisol secretion in bilateral macronodular adrenal hyperplasia (BMAH) associated with Cushing's syndrome. AVP, vasopressin; 5-HT, serotonin; GIP, glucose-dependent insulinotropic peptide; LH, luteinizing hormone.

pathophysiological processes linking *ARMC5* mutations and the initiation of illicit paracrine regulatory loops will have to be identified. However, all the data summarized in the present review suggest the following sequence of pathogenic events. First, it is likely that the causative mutations of the disease alter adrenal embryogenesis leading to the abnormal presence of gonadal-like cells in the adrenal areas. Progressive expression of POMC and ACTH by these cells then results in adrenocortical hyperplasia and hypercortisolism via activation of the cAMP/pKA pathway by the MC2R. Illicit expression of some membrane receptors may be regarded as a witness of the gonadal-like differentiation of the tissues. This is particularly the case for the LH, GIP, and 5-HT_7 receptors, which are known to be physiologically expressed in the gonads (94, 95). On the other hand, it is conceivable that local production of ACTH may also result into overexpression of membrane receptors and their ligands. This hypothesis appears particularly relevant for the regulation of BMAH tissues by 5-HT. Indeed, an increase in 5-HT_4 mRNA levels has been noticed in adrenal glands removed from patients with ACTH-dependent (Cushing's disease) hypercortisolism in comparison with normal adrenals (47). Intraadrenal ACTH may also be responsible for the unusual expression pattern of 5-HT_4 isoforms in BMAH tissues since recent studies have shown that ACTH globally alters mRNA splicing in adrenocortical cells (96). In addition, important insights have been provided by studies conducted on animal models. In rats, chronic stress, which stimulates ACTH release by the pituitary corticotrophs, induces a significant increase in the expression of the eutopic adrenal 5-HT receptor, which is the 5-HT_7 receptor, as well as abnormal synthesis of 5-HT in clusters of adrenocortical cells (97). The illicit serotonergic loop observed in human BMAH tissues may therefore be regarded as an abnormal activation of a physiological mechanism, which is probably aimed at potentiating the glucocorticoid response to stress. This process may be driven by intraadrenal ACTH and subsequent activation of PKA, which can also be stimulated in BMAH tissues by somatic and/or germline mutations such as those affecting the *PDE11A* and *PRKACA* genes (13, 98) or cAMP-coupled illicit membrane receptors like the LH, GIP, and 5-HT_7 receptors (16, 48). Collectively, these data suggest that intraadrenal paracrine regulatory loops may be regarded as valuable targets for new pharmacological treatments of BMAH-associated hypercortisolism (**Figure 4**). Especially, inhibition of the action of locally produced ACTH, which seems to represent a common intermediate to the influence of several types of abnormally expressed membrane receptors in BMAH tissues, may be a particularly efficient strategy. MC2R antagonists, which are currently under clinical development for the treatment of hypercortisolism associated to Cushing's disease, will have thus to be evaluated in patients with primary adrenal Cushing's syndrome due to BMAH.

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Adrenocortical carcinoma (ACC): diagnosis, prognosis, and treatment

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Adrenocortical carticnoma (ACC) is a rare malignancy with an incidence of 0.7–2.0 cases/million habitants/year. The diagnosis of malignancy relies on careful investigations of clinical, biological, and imaging features before surgery and pathological examination after tumor removal. Most patients present with steroid hormone excess or abdominal mass effects, but 15% of patients with ACC is initially diagnosed incidentally. After the diagnosis, in order to assess the ACC prognosis and establish an adequate basis for treatment decisions different tools are proposed. The stage classification proposed by the European Network for the Study of Adrenal Tumors (ENSAT) is recommended. Pathology reports define the Weiss score, the resection status and the proliferative index, including the mitotic count and the Ki67 index. As far as the treatment is concerned, in case of tumor limited to the adrenal gland, the complete resection of the tumor is the first option. Most patients benefit from adjuvant mitotane treatment. In metastatic disease, mitotane is the cornerstone of initial treatment, and cytotoxic drugs should be added in case of progression. Recently, the First International Randomized (FIRM-ACT) Trial in metastatic ACC reported the association between mitotane and etoposide/doxorubicin/cisplatin (EDP) as the new standard in first line treatment of ACC. In last years, new targeted therapies, including the IGF-1 receptor inhibitors, have been investigated, but their efficacy remains limited. Thus, new treatment concepts are urgently needed. The ongoing "omic approaches" and next-generation sequencing will improve our understanding of the pathogenesis and hopefully will lead to better therapies.

Keywords: adrenocortical carcinoma (ACC), ENS@T staging, prognosis, mitotane, target therapy

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Introduction

Adrenocortical carticnoma (ACC) is a rare malignancy with an incidence of 0.7–2.0 cases/million habitants/year. It occurs at any age, with two peak incidence: the first one in the first decade and the second one between 40 and 50 years. Women are most frequently affected (55–60%) (Kebebew et al., 2006).

Molecular Oncogenesis and the Epigenetic Aspects

In the past, progress in identifying genes involved in ACC came mainly from the study of familial diseases (Else et al., 2014). ACC were frequently associated to the Li–Fraumeni syndrome, due to germline TP53 mutations and the Beckwith–Wiedemann syndrome, due to alterations of the insulin-like growth factor IGF2. At somatic level, inactivating mutations of TP53 and activating mutations of the proto-oncogene β-catenin (CTNNB1) were the most frequent

mutations identified in ACC. Recently, thanks to genomic approaches, including exome sequencing, not only TP53 and CTNNB1 were confirmed as implicated in ACC tumorigenesis, but also ZNRF3 (Zinc and ring finger protein 3) was the most frequently altered gene (21%) (Assié et al., 2014a). Interestingly, ZNRF, as CTNNBI, belong to the WNT pathway and it seems that the mutations in the two genes are mutually exclusive.

Moreover, by comparative genomic hybridization (CGH), chromosomal gains at 5, 7, 12, 16, 19, and 20 and losses at 13 and 22 were observed in ACC. Concerning the epigenetic changes, a specific CpG island methylator phenotype was identified in ACC associated to the hypermethylation of the promoters of specific genes as H19, PLAGL1, G0S2, and NDRG2. In addition, some studies identified a significant up-regulation of miR-483 associated to a downrefulation of miR-195 and miR-335 in ACC (Assié et al., 2014b).

Diagnosis

Endocrine Work-up

The diagnosis of malignancy relies on careful investigations of clinical, biological, and imaging features before surgery and pathological examination after tumor removal. Most patients (40–60%) present steroid hormone excess (glucocorticoids, mineralocorticoids, androgens) or abdominal mass effects (30%), but 15–20% of patients with ACC are initially diagnosed incidentally (Else et al., 2014).

The European Network for the Study of Adrenal Tumors (ENS@T) suggests a pre-operative hormonal workup for suspected ACC (www.ensat.org). In particular, the assessment of basal cortisol, ACTH, dehydroepiandrostenedione sulfate, 17-hydroxyprogesterone, testosterone, androstenedione, and estradiol as well as a dexamethasone suppression test and urinary free cortisol are recommended. In the last years, it seems more evident that some ACC, previously considered as non-secreting, in fact can secrete some urine steroid metabolites and recently urine steroid metobolomic analysis have been introduced in routine use (Arlt et al., 2012).

Imaging

Traditional and functional imagings are able to diagnose correctly an adrenal mass as ACC in most of the cases. The risk for ACC increases with tumor size, with the index of suspicion increasing for tumors >4 cm (sensitivity, 97%; specificity, 52%) and >6 cm (sensitivity, 91%; specificity, 80%) (Sturgeon et al., 2006). Unfortunately, masses from 1 to 4 cm in diameter are diagnostically challenging. Generally, most of the ACC are large, heterogeneous with irregular margins. Necrosis, hemorrhage or calcification can be associated.

Currently, no single imaging method can characterize a localized adrenal mass as ACC. Regarding traditional imaging, abdominal computed tomography (CT) scan is mandatory in suspicion of ACC: many studies have established a threshold of ≤10 Hounsfield Unit (HU) in unenhanced CT for the diagnosis of benign lesion. When the basal density is >10 UH, the contrast media washout is helpful to discriminate the benign adrenal lesions from the ACC. An absolute washout >50% suggests a benign adrenal lesion. As well as CT scan is fundamental to define the disease staging (Ilias et al., 2007; Zhang et al., 2010; Young, 2011), all patients with ACC must perform a chest CT scan in order to detect pulmonary metastases before surgery.

The state of art of the Magnetic Resonance Imaging (MRI) is less known. In case of suspicion of ACC, when the CT scan cannot perfectly characterize the adrenal lesion, three major characteristics of MRI are helpful in the ACC diagnosis: the presence of isointense to hypointense signal on T1-weighted images, a hyperintense signal on T2-weighted images and an heterogeneous signal drop on chemical shift (Elsayes et al., 2004; Bharwani et al., 2011).

Regarding functional imaging, ACC showed high 18F-fluorodeoxyglucose (FDG) uptake (Boland et al., 2011; Deandreis et al., 2014) with a cut-off value > 1.45 for adrenal to liver maximum standardized uptake value (SUV), as reported in a series of 77 patients with surgical proven diagnosis of adrenal adenoma or ACC (Groussin et al., 2009). As the chest and abdominal CT scan, FDG-PET is important for disease staging and prognosis (Leboulleux et al., 2006), but its routine use still needs validation.

In recent years, a new tracer, the metomidate ([^{11}C]MTO) can be useful to prove the adrenocortical origin because it specifically binds to adrenocortical CYP11B enzymes, which catalyze the final steps of steroid synthesis. In a study of 11 patients, ACC showed a higher tracer uptake at [^{11}C]MTO-PET compared to normal adrenal gland and liver (Hahner et al., 2008).

Pathology

The pathological assessment is the key to the final diagnosis of ACC, but it remains challenging. First, as the ACC can be non-secreting tumor, the adrenocortical origin of the mass must be established. The determination of steroidogenic factor 1 (SF-1) expression has proved as the most valid marker (Duregon et al., 2009; Sbiera et al., 2009). Second, multiple parameters (macroscopic and microscopic) have to be evaluated in order to discriminate benign from malignant tumor.

Macroscopy revelead that ACC are usually large, heterogeneous, with a surface ranges from brown to orange to yellow depending on the lipid content of their cells. Necrosis is almost always present. Importantly, the presence of a tumoral invasion at different levels, as the tumor capsule, the extra-adrenal soft tissue or direct invasion of lymphatic channels, blood vessels are the key features of ACC.

Microscopically, the Weiss score is still the best validated score. It is composed of nine items (three concerning the architecture, three the nucleus, and three the presence of any type of invasion) and the presence of one item scores 1. The sum of the positive items defines the final score. It is established that a Weiss score ≥ 3 define an ACC, whereas scores between 0 and 2 defines the adrenal adenoma, even if sometimes a Weiss score of 2 can be suspicious (Weiss, 1984).

The major problem is the reproducibility of this score and in particular the inter-individual reproducibility. Recently, the practice of the Weiss score through virtual microscopy has been improved by the 12 pathologists of the French network for Adrenal Cancer COMETE (Tissier et al., 2012).

Proliferation index, as Ki67 immunomarker or mitotic count, can help to define the diagnosis and prognosis of ACC. It is well-established that ACC generally showed a Ki67 $\geq$ 5%. Recent studies have been demonstrated that Ki67 is a powerful prognostic marker in both localized and metastatic ACC to guide treatment decision (Berruti et al., 2010; Libé et al., 2014; Beuschlein et al., 2015). Moreover, a mitotic count >20 mitoses/50 HPF defines a "high grade ACC" with a worst prognosis compared to "low grade ACC" with $\leq$ 20 mitoses/50 HPF (Miller et al., 2010).

Staging

Tumor staging is a widely used tool to assess prognosis in patients with cancer. For ACC, the tumor–node–metastasis (TNM) classification proposed by ENS@T (**Table 1**) is recommended (Fassnacht et al., 2009). This staging system, defines stage I and stage II as strictly localized tumors with a size of $\leq$5 or >5 cm, respectively. Stage III ACC are characterized by infiltration in surrounding tissue, positive regional lymph nodes or a tumor thrombus in the vena cava and/or renal vein, whereas stage IV is defined by the presence of distant metastasis. The high prognostic potential of the ENS@T staging system has been established in the large cohort of the German ACC registry (Beuschlein et al., 2015) and has been confirmed in the independent SEER cohort (Lughezzani et al., 2010) which demonstrates its superiority to the staging system published by the Union Internationale Contre Le Cancer (UICC).

Prognosis

Three major criteria are mandatory in order to define the disease free survival for the localized ACC (stage I, II, and some III) and the overall survival for stage IV ACC: (1) staging; (2) resection status "R"; (3) Grading (proliferation index, as Ki67% and mitotic count).

Staging

As mentioned above, staging is mandatory to assess prognosis. Five-year stage-dependent survival is 66–82% for stage I, 58–64% for stage II, 24–50% for stage III, and 0–17% for stage IV, according to different series (Icard et al., 2001; Fassnacht et al., 2009; Lughezzani et al., 2010; Kerkhofs et al., 2013).

TABLE 1 | ENS@T classification.

ENS@T stage	
I	T1, N0, M0
II	T2, N0, M0
III	T3–T4, N1
IV	T1–T4, N0–N1, M1

T1, tumor $\leq$ 5 cm; T2, tumor > 5 cm; T3, histologically proven tumor invasion of surrounding tissue; T4, tumor invasion of adjacent organs or venous tumor thrombus in vena cava or renal vein. Venous tumor thrombus is only a criterion in the ENSAT classification.

N0, negative lymph nodes; N1, positive lymph nodes.

M0, absence of distant metastases; M1, presence of distant metastases.

Resection Status "R"

In localized ACC, surgery is the single most important intervention and the complete resection (R0) correlates with a better prognosis (Bilimoria et al., 2008). In fact, an incomplete microscopic resection (R1), an incomplete macroscopic resection (R2) or unknown resection (Rx) are associated with the worst overall survival of 20 and 15%, respectively (Bilimoria et al., 2008).

Grading

Proliferation index, as Ki67 and mitotic count help to assess the ACC prognosis. Very recently, a large European study in localized ACC identified Ki67 as the single most important factor predicting recurrence in patients following R0 resection (Beuschlein et al., 2015). Thus, evaluation of Ki67 indices should be introduced as standard grading in all pathology reports of ACC patients (Beuschlein et al., 2015). More recently, in a large European study on stage IV ACC, the tumor grading, as the association of the Ki67 and the Weiss score, has been considered as an important prognostic parameter of overall survival (Libé et al., 2014), confirming the data on the mitotic count showed in a previous French series (Assie et al., 2007).

Molecular Markers

Molecular markers issued form genomic and epigenomic analyses are emerging and need to be confronted to the previous mentioned criteria. Hypermethylation status, miRNA profile or driver genes mutations, as TP53, ZNRF3, β-catenin constitute valuable candidates that could integrate a future clinico-molecular prognostic classification of ACC patients (Assié et al., 2014a).

Treatment

Currently, the only curative approach to ACCs is complete tumor resection. Adjuvant therapies aim to decrease the risk of recurrence. These two approaches address mainly to localized ACCs (stages I, II, some III), also called "ACC amenable to radical resection." For "unresectable or metastatic ACC" all therapy must be considered palliative, even in some cases (only two tumoral organs, included adrenal), surgery can be considered as an option.

The **Figure 1** showed the current treatment flow-chart for patients with "Localized ACC" and for those with "Metastatic ACC."

Localized ACC

Surgical Treatment

For "localized ACC" ("ACC amenable to radical resection"), complete surgical resection (R0) is the treatment of choice. Appropriate preoperative evaluation and operative planning by a surgeon experienced in the resection of ACC (>10 adrenalectomy/year/surgeon) is of the most importance to assure optimal outcome. Different key questions concern the optimal surgical approach: (1) open adrenalectomy (OA) vs. laparoscopic

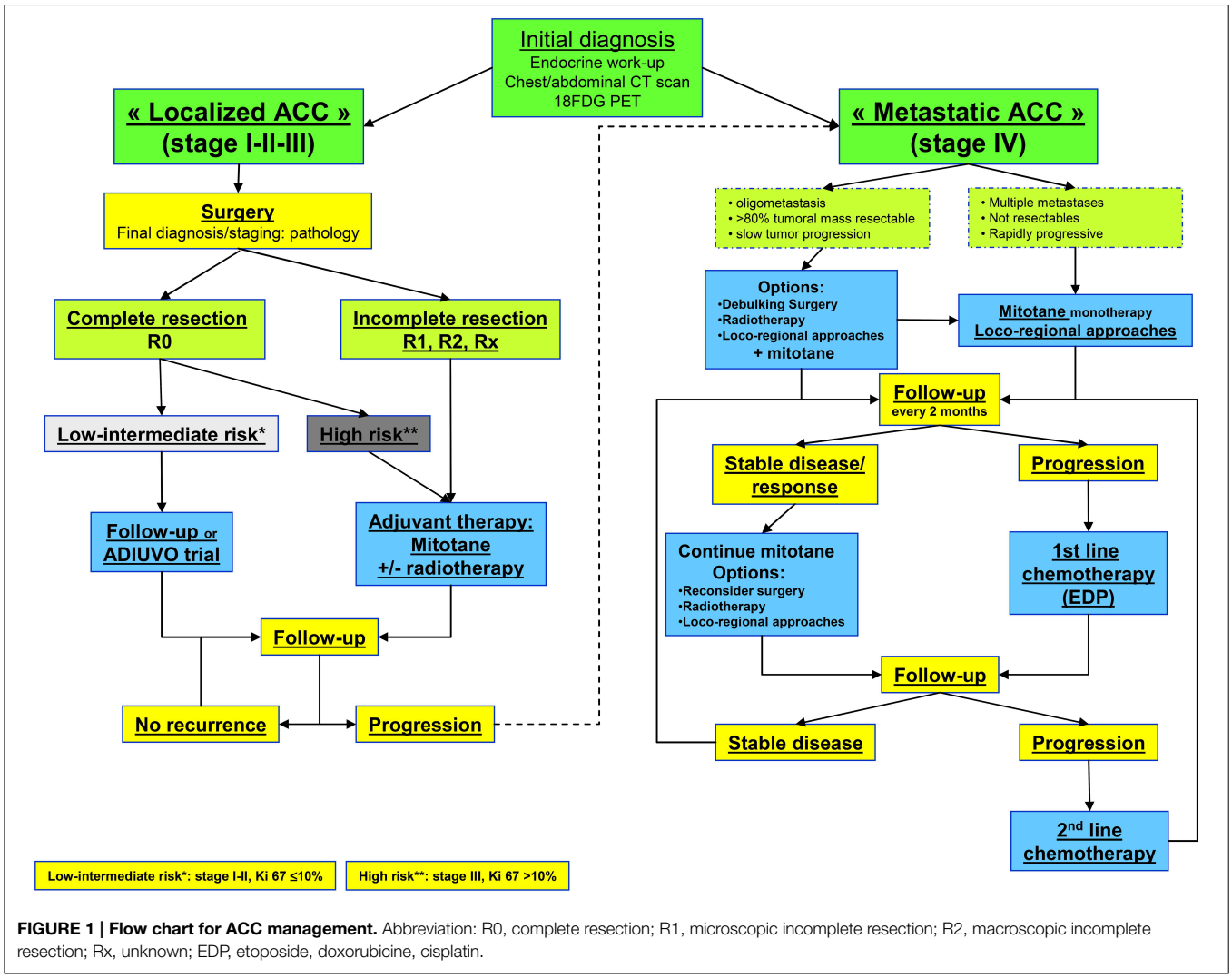

FIGURE 1 | Flow chart for ACC management. Abbreviation: R0, complete resection; R1, microscopic incomplete resection; R2, macroscopic incomplete resection; Rx, unknown; EDP, etoposide, doxorubicine, cisplatin.

adrenalectomy (LA) (2) lymph nodes dissection (LND) (3) large surgery to the adjacent organs (in bloc surgery).

The choice of the best surgical approach (OA vs. LA) remains controversial. OA should still be regarded as standard treatment for ACC, mainly in the case of an infiltrating tumor or suspected lymph nodes (presumable stage III), and LA should be performed only in selected cases (tumor < 5 cm, absence of higher FDG PET uptake, experimented surgeon).

Up to now, published data comparing the efficacy (and safety) of LA vs. OA for ACC are not definitive, as all the series are retrospectives, with limited number of patients, no follow-up and many biais. Indeed, in two studies has been reported a recurrence rate of 86% in the OA group and 100% in the LA group (with local recurrence and peritoneal carcinomatosis) (Gonzalez et al., 2005; Grubbs et al., 2010). These data has been confirmed by Leboulleux et al. (2010), which showed that peritoneal carcinomatosis occurred in only 25% of patients treated by OA, compared to 60% in LA group. In contrast, other studies reported evidence that LA may be comparable to OA in patients with stage 1 and 2 ACC, in terms of recurrence-free survival. A case-control study from the German ACC Registry Group reported no difference in overall or disease-free survival, tumor capsule violation, or peritoneal carcinomatosis among 117 patients undergoing OA and 35 patients undergoing LA for stage 1–3 ACCs less than 10 cm. However, many patients in the OA group had stage 3 disease and only four patients (11%) undergoing LA were found to have stage 3 disease, potentially introducing a bias toward more advanced disease in the OA group, and 37% of all patients had no data regarding margin status (Brix et al., 2010).

Although no standard management has been established concerning the extent of the first surgery, and in particular the LND, a recent retrospective study, suggests that it might improve both diagnostic accuracy and therapeutic outcome, with a significant reduced risk of tumor recurrence for LND patients (Reibetanz et al., 2011).

As far as, the extension of surgery is concerned, it seems to be little benefit of systematically ipsilateral nephrectomy in the

absence of gross local invasion (Gaujoux et al., 2011). However, in order to achieve the complete resection R0, it is mandatory, in case of large tumor and suspicious of organ adjacent invasion or infiltration, to perform a "in bloc" resection, including tumor thrombus embolectomy (Gaujoux and Brennan, 2012).

Adjuvant Therapy

The natural history of recurrence after surgery remains uncertain, but even in case of complete resection, the rate of local recurrence remains important and ranges between 19 and 34%, on the basis of tumor stage. For this reason, adjuvant therapy can be associated after surgery and include mitotane and tumoral bed irradiation.

Mitotane

Mitotane is a derivate of the insecticide dichlorodiphenyltrichloroethane (DDD), with adrenolytic and citotoxic activity: in particular, mitotane metabolites inhibit several enzymes in the adrenocortical steroidogenensis pathway, mainly at the level of the cholesterol side-chain cleavage enzymes CYP11A1 and CYP11B1.

In terms of adjuvant therapy in "localized ACC" after surgery, a large retrospective analysis comparing two independent cohorts (Terzolo et al., 2007) demonstrated that patients with adjuvant mitotane had a significantly improved recurrence-free survival. As this is a retrospective study, it remains a matter to discussion. This is particularly true for patients with presumably low or intermediate risk of recurrence (defined by R0 resection, absence of metastases and Ki67 $< 10\%$) (Berruti et al., 2010). For these patients, a prospective international randomized trial (ADIUVO: https://www.epiclin.it/adiuvo) comparing treatment with mitotane vs. a "watch and see" strategy, can be proposed.

Mitotane Therapy Management

The mechanism of mitotane action and pharmacokinetics data are poorly understood. In fact, the variability of individual plasma levels reached by a given dosage is high and it remains unclear which enzyme metabolize mitotane in human, although there is the first evidence that CYP2B6 might be involved. The dose is initiated at 1 g twice daily and increases every 4–7 days by 0.5–1 g/day until a daily dose of 6.0 g/day is reached. Moreover, two different regimens ("high dose" and "low dose") have been proposed, but no significant difference in mitotane levels and adverse events has been described (Kerkhofs et al., 2010). Several studies demonstrated that a mitotane plasma level ≥ 14 mg/l is required for clinical efficacy and is associated to a better overall survival (Hermsen et al., 2011). Moreover, the same study demonstrated that even mitotane level > 8 mg/l seems to be associated to a better outcome (Hermsen et al., 2011). Mitotane comes with significant toxicity, like dizziness, vertigo, central nervous system disturbances and gastro-intestinal symptoms. Moreover, as the adrenolytic action of mitotane, all patients develop an adrenal insufficiency, which has to be replaced with a high dosage of hydrocortisone. In fact mitotane induces the cytochrome P450 3A4 (CYP3A4) leading to lower the blood levels of many drugs (including the steroids, anti-hypertensives, antibiotics) (Kroiss et al., 2011a).

Radiotherapy

In order to reduce the risk of local recurrence, external radiation therapy of the tumor bed can be an option. In the literature, three retrospective studies with a little number of patients tempt to solve the questions: two of them showed a benefit in preventing local recurrence, but none of them demonstrates an advantage in term of overall survival (Fassnacht et al., 2006; Sabolch et al., 2011; Habra et al., 2013). Currently, an adjuvant therapy is recommended only in case of a particularly high risk for local recurrence (R1 resection) (Berruti et al., 2010).

Metastatic ACC

In metastatic disease, different parameters had to be considered: the tumoral volume, the number of metastatic organs, the progression slopes. Debulking surgery is only of benefit in patients with a tumoral mass respectable, a limited number of tumoral organs (≤ 2), with a slight progression and in case of severe hormone excess that cannot be controlled otherwise. Instead, medical therapy should be initiated as soon as the diagnosis is established.

Mitotane remains the only drug approved by the U.S food and drug Administration (FDA) and European Medicine Executive Agency (EMEA) for treatment of "metastatic ACC." An overview collecting different studies showed that the objective response rate is at best 24% (De Francia et al., 2012).

Recently, the First International Randomized trial in Locally Advanced and metastatic Adrenocortical Carcinoma treatment (FIRM-ACT) trial included 304 patients with metastatic ACC and compared the association of mitotane with etoposide-cisplatin-doxorubicine (M-EDP) with mitotane-streptozotocin (M-Sz) as a first-line or second-line treatment. It was shown that the M-EDP was associated with a better progression-free survival and objective response rate compared to M-Sz (5.0 vs. 2.1 months, 23.2 vs. 9.2%, respectively), although no significant difference was demonstrated on overall survival (Fassnacht et al., 2012). Based on these data, M-EDP is considered as first-line therapy for patients requiring cytotoxic treatment.

For patients failing M-EDP, it has been proposed, as second line chemotherapy, the combination of gemcitabine and capecitabine, leading a disease stabilization for at least 6 months in 29% of patients (Sperone et al., 2009).

Other Treatments

Loco-regional Approaches

In case of metastatic ACC or ACC recurrence local treatment modalities, such as radiofrequency ablation (RFA) or transarterial chemoembolization (TACE) are recommended.

None of these methods has been explored in clinical trials. However, both methods are an alternative to surgery, when surgery is not desired or contro-indicated, or in order to control the disease locally. RFA has been successfully employed in the palliative setting, rendering patients free of liver metastasis (Ripley et al., 2011). TACE, localized chemoembolization, is based on a selective embolization with injection of a high intratumor levels of cytotoxic substances with a minimum of

systemic effects. Predictors of response were a size of <3 cm and high lipidol uptake (Soga et al., 2009).

Targeted Therapy

Up to now, current treatments fail in many patients with metastatic ACC and different molecular target therapies have been tested. The first trial targeted the epidermal growth factor receptor (EGFR) but the combination of erlotinib and gemcitabine failed to give an objective response (Quinkler et al., 2008). The vascular endothelial growth factor (VEGF) is another potential target, as highly expressed in ACC. In a trial with bevacizumab, a humanized anti-VEGF monoclonal antibody, a progression disease was demonstrated in all of the 10 patients enrolled (Wortmann et al., 2010). Similarly, a multitirosine – kinase inhibitor, sorafenib in combination with paclitaxel did not demonstrate any efficacy in a cohort of 25 patients (Berruti et al., 2012). Only the sunitinib, a multi-TKI, demonstrated in a cohort of 35 patients 14% of stable disease. In this trial concomitant administration of mitotane diminished plasma levels of sunitinib and its active metabolite (Kroiss et al., 2011b).

Recently, drugs targeting IGF-2 seemed to be very promising., as IGF-2 is the most-up regulated gene in ACC. Recently, a phase 2 study used a IMCA12 (cixutumab), a fully humanized IGF-1R antibody showed a lack of efficacy in a cohort of 19 patients (Lerario et al., 2014). In another study, the association of cituximab with temsirolomus, an inhibitor of mammalian targets of IGF-1R signaling, led to a stable disease in 42% of the patients (Naing et al., 2013).

The disappointing results of a huge phase 3 trial "GALACCTIC" with a highly specific IGF-1R inhibitor linstinib (OSI-906) in a cohort of 138 metastatic ACC have been recently published: the progression-free and overall survival did not differ between the "OSI-906" and placebo groups (Fassnacht et al., 2015).

Finally, like in the disease heterogeneity, it appears that using one single agent is not sufficient to induce an objective response. Trials with new targeted substances are under study and alternative combination therapy may be promising.

Recently, as [123I]IMTO single-photon emission CT imaging showed high tracer uptake in issue of adrenocortical origin, [131I]IMTO might represent a suitable compound for targeted radionuclide therapy. [131I]IMTO treatment in 11 patients with advanced ACC resulted in median progression-free survival for 1 month in 6 patients who responded to therapy (Hahner et al., 2012).

Follow-up

The follow-up management is not well-standardized yet, but, as ACC is an aggressive malignant tumor, patients should be followed every 3 months during and after initial treatment. Only after a recurrence free-time of 2–3 years the surveillance intervals may be increased to 6 months until a completion of follow-up for a total of 5 years. After 5 years of disease-free, the surveillance can be proposed every 1–2 years, because, although rare, some patients can relapse tardily.

Patients should undergo a complete physical examination, hormonal investigations and a complete imaging work-up, including chest and abdominal CT scan. A [18F] FDG-PET may also be considered, even if it's not considered mandatory in the follow-up of ACC patients.

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Pediatric adrenocortical tumors: what they can tell us on adrenal development and comparison with adult adrenal tumors

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Adrenocortical tumors (ACT) in children are very rare and are most frequently diagnosed in the context of the Li-Fraumeni syndrome, a multiple cancer syndrome linked to germline mutations of the tumor suppressor gene *TP53* with loss of heterozygosity in the tumors. A peak of children ACT incidence is present in the states of southern Brazil, where they are linked to the high prevalence in the population of a specific *TP53* mutation (R337H). Children ACT have specific features distinguishing them from adult tumors in their pathogenetic mechanisms, genomic profiles, and prognosis. Epidemiological and molecular evidence suggests that in most cases they are derived from the fetal adrenal.

Keywords: cancer, adrenal glands, adrenal gland neoplasms, adrenal cortex, genetic

DYNAMICS OF HUMAN ADRENOCORTICAL MORPHOLOGY AND HORMONE SECRETION DURING DEVELOPMENT AND POSTNATAL LIFE

The adrenal gland is a continuously evolving endocrine organ from the developmental to the elder age. In humans, adrenal gland development begins at 3–4 weeks of gestation by a condensation of the coelomic epithelium lining the abdominal cavity, followed at 4–6 weeks of gestation by proliferation and migration of coelomic epithelial cells, and subsequent differentiation of fetal adrenal cortical cells into two distinct zones (inner fetal zone and outer definitive zone) at 8–10 weeks of gestation, while neural crest-derived cells start to infiltrate the gland at 7–8 weeks of gestation to give origin to adrenomedullary cells (1). Starting from around the ninth week of gestation, the embryonal adrenal is surrounded by the adrenal capsule formed by mesenchymal cells. Fetal adrenal cells, which are large and rich in lipids, express the steroidogenic enzyme CYP17, which enables them to produce high levels of DHEA and its sulfoconjugate DHEAS, which play a key role for the maintenance of pregnancy, being metabolized into estrogens by the placenta (1, 2). By the end of the second trimester of gestation, a distinct zone (transitional zone) differentiates between the definitive and fetal zones, which express *HSD3B2*, this way starting cortisol synthesis in the fetus. Close to birth, *HSD3B2* is expressed in the definitive zone, which acquires the capacity to synthesize the mineralocorticoid hormone aldosterone. Cell proliferation in the fetal adrenal is mainly localized in the outer definitive zone, followed by centripetal migration and differentiation into fetal zone cells, which subsequently die from apoptosis in the center of the gland. This streaming process of adrenocortical cell differentiation continues during the whole life, as shown by studies in the mouse (3–5).

Starting shortly after birth, a rapid, dramatic remodeling of adrenal cortex structure takes place, with massive shrinkage of the gland due to apoptosis of the fetal zone and progressive differentiation of the *glomerulosa*, *fasciculata*, and *reticularis* zona, which are the hallmark of the adult adrenal (1). Defects in this process may cause the cytomegalic form of adrenal hypoplasia congenita, a syndrome of adrenal insufficiency due to altered postnatal adrenocortical differentiation due to mutations in the *NR0B1* (*DAX-1*) gene [reviewed in Ref. (6)]. Studies in pre-term neonates have shown that parturition itself is the cause for fetal adrenal involution (7), suggesting a crosstalk between placenta and fetal adrenal in reciprocal maintenance. Remarkably, postnatal adrenal remodeling also takes place in the mouse adrenal cortex, where an inner zone adjacent to the medulla termed zone X, that lineage tracing experiments have shown to be derived from the fetal adrenal (8), regresses after puberty in males and after the first pregnancy in females.

After being suppressed following the regression of the fetal zone, adrenal production of DHEA/DHEAS starts to progressively

increase again by around 8 years of age. This phenomenon is termed adrenarche and is concomitant with full differentiation of the *reticularis* zone, which expresses *CYP17* but not *HSD3B2*. Moreover, in the *zona reticularis*, CYP17 has an increased ratio of 17,20-lyase to 17α hydroxylase activity (which favors DHEA production) compared to the *zona fasciculata*, probably due to increased serine phosphorylation and increased abundance of cytochrome b5 (CYB5), which allosterically stimulates 17,20-lyase activity of CYP17 (9). DHEA/DHEAS levels continue to increase until adulthood and then progressively decline (adrenopause) reaching pre-adrenarche levels by the ninth decade, correlating with progressive atrophy of the *zona reticularis* (10).

ADRENOCORTICAL TUMORS IN CHILDREN AND ADULTS: SIMILARITIES AND DIFFERENCES

Adrenocortical tumors (ACT) are among the most common neoplasms in humans and are frequently detected by hazard during diagnostic procedures for other medical issues (incidentalomas), in the great majority of cases remaining clinically silent and having a completely benign prognosis. In contrast, adrenocortical malignancies (adrenocortical carcinomas or ACC) are very rare, with a general incidence of 0.7–2 cases/million/year, with a maximum between 40 and 50 years of age and a higher frequency in women than in men (11). They become clinically evident with signs and symptoms due to hormone excess (Cushing's syndrome, androgen excess) and/or local symptoms (pain, abdominal discomfort). The prognosis of ACC is still poor, with an average 5-year overall survival around 40%, which is influenced to a great extent by tumor stage at diagnosis. Some histopathological parameters (Weiss score $\geq$3, Ki-67 index $>$10%) also have negative prognostic value (11).

Adrenocortical tumors in children under 15 years of age are even rarer. Their worldwide incidence has been estimated at 0.3/million/year with a bimodal peak under the age of 5 and after 10 years and they also affect girls more frequently than boys. The main reason why ACT in children become clinically evident is virilization, which may be associated to Cushing's syndrome. Overall survival at 5 years after diagnosis in children with ACT is better than in adult patients, approximating 50%. Favorable prognostic factors are younger age ($<$4 years), stage I at diagnosis, tumor weight $\leq$200 g, volume $<$200 cm^3, and presence of virilization alone (12). It is noteworthy that in children ACT, the Weiss score is not a reliable system to assess malignancy (13–15) (**Table 1**).

Childhood malignancies have long been associated to congenital defects (16), which suggest that they may be considered as a degeneration of normal developmental processes. Children ACT are a typical example since they can be found in the context of two genetically determined syndromes, Beckwith-Wiedemann and Li-Fraumeni.

(1) Adrenocortical hyperplasia and neoplasms of variable malignancy are common in Beckwith-Wiedemann syndrome, a systemic overgrowth syndrome caused by genetic defects as uniparental disomy in the 11p15 chromosomal region (17), which cause overexpression of the IGF2 growth factor in the great majority of cases. Loss of heterozygosity (LOH) of the 11p15 region is a systematic finding, not related to prognosis (18–20), in children ACT, leading to *IGF2* overexpression from the paternal allele. Similarly, *IGF2* is expressed at high levels in the fetal adrenal where it has an important role to regulate proliferation and steroid production (1). Conversely, *IGF2* overexpression and abnormalities in the 11p15 region are a marker of malignancy in ACT of adults (21, 22). In mouse models, *Igf2* overexpression in the adrenal induces tissue hyperplasia but is not able to induce malignant tumorigenesis *per se* (23, 24).

(2) Adrenocortical tumors are a distinctive feature of Li-Fraumeni syndrome (LFS), a multiple cancer syndrome due to germline mutations in the *TP53* tumor suppressor gene [(25); reviewed in Ref. (26)] encoding p53, a transcription factor that has a pivotal role in preserving genome integrity and activating apoptosis of cells bearing irreparable DNA damage (27). It has been shown that in LFS, excessive DNA copy number variation exists in the patients' germline, which may predispose to cancer (28). Due to its rarity and its characteristic association with LFS, discovery of an ACT in a child is an absolute indication for researching *TP53* mutations in the proband and in his/her parents as well indicative for genetic counseling. Conversely, germline *TP53* mutations are much less common in adults with ACC (29, 30) (**Table 1**). The high incidence of children ACT in LFS suggests that normal p53 function is required for the physiological process of postnatal fetal adrenal regression (**Figure 1**). In the absence of p53, genetic alterations may accumulate in the adrenal driving proliferation [such as *NR5A1* overexpression, (31, 32); see below section on Whole Genome Studies in Children and Adult ACT Reveal Important Drivers for Tumorigenesis and LOH of 11p15 leading to *IGF2* overexpression (18–20)] of specific cellular clones. This increased proliferative capacity may favor the emergence of further genetic alterations ultimately leading to clonal expansion and tumorigenesis [reviewed in Ref. (33)].

In classical LFS, due to *TP53* mutations that completely abolish protein function, the lifetime incidence of cancer in carriers is close to 100%. However, low-penetrance mutated *TP53* alleles exist that can increase the risk of developing cancer only in a fraction of carriers (34). A remarkable example of that situation exists in southern Brazil. In that geographical region, children ACT prevalence is at least 15-fold higher than in the rest of the world (10). This is related to a specific germline *TP53* mutation (R337H) (35, 36), whose prevalence is very high (0.3%) in the population but whose penetrance to produce ACT in children has been estimated at only about 2% (37). However, the *TP53* R337H mutation has also been reported to be associated to other cancers in the Li-Fraumeni spectrum (38–41) and so its overall penetrance is still unknown. R337 is a conserved arginine residue in the C-terminal tetramerization domain of p53 whose mutation to histidine destabilizes p53 tetramer formation in conditions of elevated temperature and pH (42). It has been shown that a founder effect is responsible for the spreading of the *TP53* R337H mutation in the population of southern Brazil (43, 44). An about 0.5 Mb identical by descent haplotype in 17p13 encompassing the *TP53* gene carrying the R337H mutation is

Table 1 | Distinctive features and common characteristics of ACT in children and adults.

	Children ACT	Adult ACC	References
Peak age at diagnosis	3–4 years	40–50 years; peak extending into the seventh decade	(10, 11, 26)
Clinical presentation	Most often virilization; may be associated with Cushing's syndrome	Cushing's syndrome or hypertension; may be associated with virilization	(10, 11)
Prevalence	Worldwide: 0.3 cases/million/year; southern Brazil: 3.4–4.2 cases/million/year	0.7–2/million/year for ACC	(10, 11)
Most common genomic alterations	11p15 LOH; 9q34 gain; 4q34 loss	Complex pattern	(46, 70–74, 86–92)
Genetic syndromes			
Overall LFS	>50%	Sporadic germline *TP53* mutations	(26, 29, 30)
Endemic germline *TP53* R337H (Brazil)	>93%	<20%	(10)
Beckwith-Wiedemann syndrome	Yes	Uncommon	(17, 47)
FAP	Uncommon	Yes	(47, 48)
MEN1	Uncommon	Yes	(47, 49)
Lynch syndrome	Uncommon	Yes	(47, 50)
NF1	Uncommon	Yes	(47, 51)
Prognostic relevance of			
Pathological (Weiss) score	Low	High	(13–15)
Ki-67 index	Unknown	High	(11)
Prognostic relevance of			
TP53 mutations	No (germline)	Yes (somatic)	(26, 29, 30)
IGF2 overexpression	No	Yes	(18–22)
NOV down-regulation	No	Yes	(19, 52)
SF-1 overexpression	No	Yes	(31, 78, 81, 82)
HLA class II down-regulation	Possible	No	(19, 22)
DLGAP5-PINK1 expression	No	Yes	(54, 56)
BUB1B-PINK1 expression	No	Yes	(54, 56)
Molecular pathways involved			
IGF2	Yes	Yes	(18–22)
p53/Rb	Yes (*TP53* mutations)	Yes (*TP53/CDKN2A/RB1* mutations)	(26, 57, 66, 85)
Beta-catenin	Yes (*CTNNB1* mutations)	Yes (*CTNNB1/ZNRF3* mutations)	(57, 66, 85)
Chromatin remodeling	Yes (*ATRX* mutations)	Yes (*MEN1/DAXX/ATRX/MED12/TERT* mutations)	(66, 85)

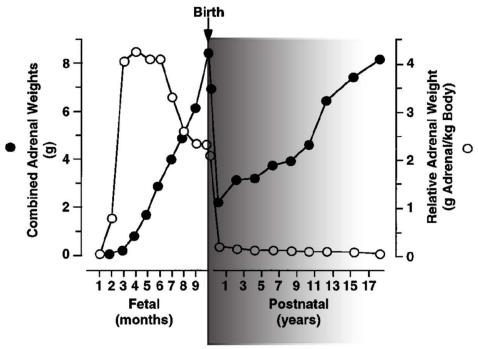
conserved in all carriers of the mutation (45, 46). A newborn screening and surveillance program of the *TP53* R337H mutation carriers in the state of Paraná has proven to be successful to detect ACT in children at an early stage and to treat it with better therapeutic results compared to children who did not undergo surveillance (37).

Apart from rare cases of germline *TP53* mutations, as mentioned before (29, 30), ACC in adults may also be associated to other hereditary conditions in some uncommon cases [reviewed in Ref. (47)]: familial adenomatous polyposis (FAP) (48), multiple endocrine neoplasia type1 (MEN1) (49), Lynch syndrome (50), and neurofibromatosis type 1 (NF1) (51) (**Table 1**).

GENOME-WIDE STUDIES IN CHILDREN AND ADULT ACT

DISTINCT PATTERNS OF CODING GENES EXPRESSION IN CHILDREN VS. ADULT ACT

Children ACT can be readily differentiated from age-matched normal adrenals by unsupervised clustering based on their gene expression profiles (19). As reported before, *IGF2* is the single gene that is most highly up-regulated in children ACT, while genes in the 11p15 region expressed from the maternal allele (*KCNQ1*, *CDKN1C*) are among the most strongly down-regulated transcripts. These data are consistent with the systematic LOH of 11p15 in those tumors, with conservation of the paternal allele and loss of the maternal allele (18, 20). Genes belonging to growth factor receptor and mitogen-activated kinase pathways are also dysregulated in children ACT. This suggests that those signaling pathways may be targets for therapeutic intervention. Furthermore, *HSD3B2*, a steroidogenic enzyme involved in the synthesis of aldosterone and cortisol and expressed in the *glomerulosa* and *reticularis* zones of the adult adrenal cortex and its transcriptional regulators *NR4A1* and *NR4A2* are strongly down-regulated in children ACT, lending further support to the hypothesis of their derivation from the fetal adrenal. This is also suggested by the finding that global gene expression profiles of children ACT are significantly correlated with those present in the fetal adrenal. Another



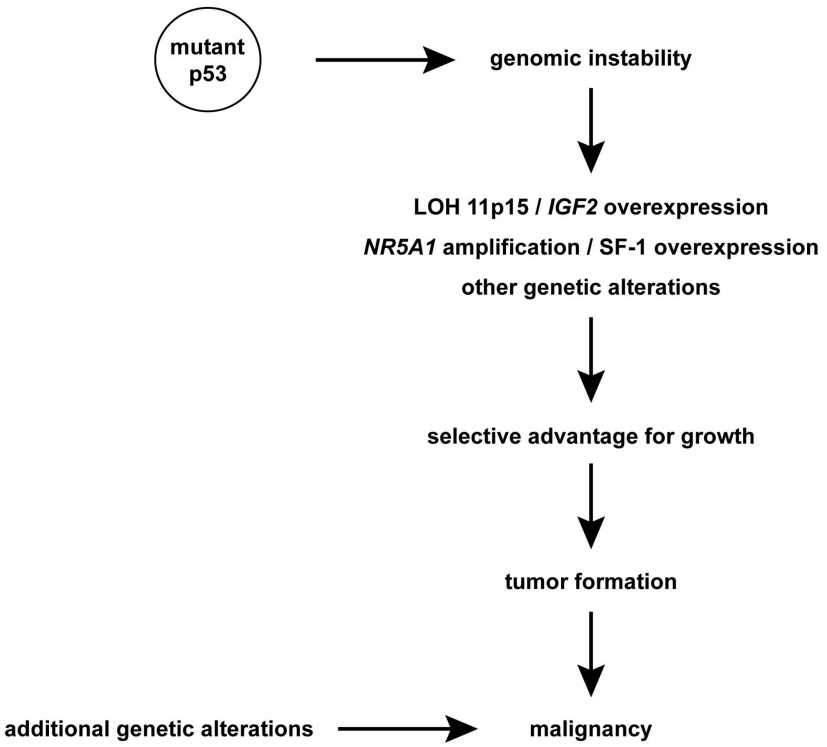


FIGURE 1 | A hypothesis for *TP53* role during physiological adrenocortical remodeling in early postnatal life and pathogenesis of children ACT. A window of sensitivity (with an early peak; shaded in grey) of human adrenal to defective p53 function exists during the first years after birth when its physiological involution takes place. Mutant p53 may favor genomic instability, which in some cells may cause LOH of 11p15 and gain/amplification of *NR5A1*, which confer those cells a selective advantage for growth and lead to tumor formation. Additional genetic alterations arising in this mutation-prone background may cause malignancy. Combined adrenal weight is indicated with black circles. Relative adrenal weight in relationship to body weight is indicated with white circles. Adapted in part from Ref. (1) with permission from Endocrine Society Press.

strongly down-regulated gene in children ACT is *NOV*, encoding a secreted multimodular protein that has a pro-apoptotic function on adrenocortical cancer cells (52). In the study by West et al. (19), a set of 52 differentially expressed genes between adrenocortical adenomas and carcinomas (as distinguished by histological parameters) was identified. It is noteworthy that those included some transcripts encoding HLA class II molecules. Down-regulation of class II expression may represent a mechanism to escape immune surveillance, which could contribute to malignancy. Since malignancy markers are dramatically lacking for children ACT, it will be very important to confirm those data in larger series of patients. However, a recent immunohistochemical study failed to detect consistent HLA class II immunoreactivity in children ACT, both benign and malignant (53).

Unsupervised clustering of gene expression profiles of adult ACT allowed to distinguish two groups, termed C1 and C2 in the study by de Reyniès et al. (54). The C1 group could be further subdivided into C1A and C1B, which correspond to unfavorable and favorable outcome, respectively. Those results were confirmed by another independent study (55), which also confirmed that *IGF2* overexpression is associated to malignancy in adult ACC. From gene expression data, de Reyniès and collaborators identified both a signature for malignancy based on the analysis of the expression of two genes (*DLGAP5/PINK1*) and a two-gene (*BUB1B/PINK1*)

molecular predictor of overall survival for patients with ACC (54). Remarkably, those molecular markers were confirmed to be valid prognostic indicators in adult but not in children ACT in a study on patients from southern Brazil (56) (**Table 1**). Further studies showed that tumors classified in the C1A group could be further divided into two subgroups each one bearing either *TP53* or *CTNNB1* (beta-catenin) mutations and in a third subgroup with no other known mutation (57). The importance of the activation of the beta-catenin pathway for adrenocortical tumorigenesis is also shown by studies in mouse models [(23, 24, 58); reviewed in Ref. (59)].

microRNA SETS DIFFERENTIALLY EXPRESSED IN CHILDREN AND ADULT ACT

In the only study published to date investigating miRNA expression profiles in children ACT, a distinct subgroup of miRNA was found to be differentially expressed in tumor samples compared to age-matched normal adrenal cortex (60). This subgroup included *miR-99a* and *miR-100*, which are down-regulated in children ACT and are able to down-regulate expression of IGF-1R (the receptor for IGF2), mTOR, and its associated protein raptor in adrenocortical cell lines. These proteins are up-regulated in children ACT and their pharmacological blockade is able to significantly decrease adrenocortical cancer cell proliferation (60–63). These results show that *miR-99a* and *miR-100* have an important role in children ACT by the modulation of growth factor signaling through the IGF-1R–mTOR pathway.

On the other hand, several studies reported data on miRNA expression profiles in adult ACT. Those studies show in general only limited overlap [reviewed in Ref. (64)]. Nevertheless, most studies detected overexpression in ACC of *miR-483-5p* and/or *-3p*, whose gene is situated in an intron of *IGF2* and may have an independent oncogenic function (65). *miR-483-3p* was also found up-regulated in children ACT in the study by Doghman et al. (60). Other miRNAs that display similar differential regulation in children and adult ACT are *miR-503* (up-regulated), *miR-195*, *miR-214*, and *miR-375* (down-regulated). A recently published integrative analysis of genomic alterations in adult ACC (66) found up-regulation of miRNAs belonging to the *miR-506-514* cluster on chromosome Xq27 and down-regulation of the expression of the *DLK1-MEG3* miRNA cluster on chromosome 14q in one subgroup of samples with favorable prognosis (C1B; see below section on Whole Genome Studies in Children and Adult ACT Reveal Important Drivers for Tumorigenesis). There is of great interest for the potential use of circulating miRNAs as biomarkers of malignancy in ACC (67–69).

WHOLE GENOME STUDIES IN CHILDREN AND ADULT ACT REVEAL IMPORTANT DRIVERS FOR TUMORIGENESIS

The first studies analyzing children ACT genome copy number alterations by comparative genomic hybridization (CGH) reported patterns of recurrent gains and losses (70–72). In particular, one of the most common alterations found in almost all cases of children ACT investigated was the gain/amplification of 9q34. Gains in this region were also reported in some studies of chromosomal alterations in adult ACT (73, 74). In close proximity to this chromosomal region (9q33) is situated the gene (*NR5A1*) encoding the transcription factor SF-1, a master regulator of adrenocortical and gonadal development [reviewed in Ref. (75, 76)]. Further studies showed that the *NR5A1* gene is amplified and the SF-1 protein is overexpressed in the large majority of Brazilian children ACT (31, 77, 78). Interestingly, the SF-1 protein was overexpressed even in cases lacking gene amplification (31, 78), suggesting that mechanisms in addition to copy number gain may also account for SF-1 overexpression. The dosage-dependent effect of SF-1 in boosting adrenocortical cell proliferation was shown by studies in human cell lines and in transgenic mice (32) by regulation of a fairly large set of dosage-dependent target genes far exceeding its classical steroidogenic targets [(79); reviewed in Ref. (80)]. In children ACT, SF-1 overexpression appears to be a widespread finding, with no relationship with malignancy [(31, 78); see **Figure 1**]. Conversely, SF-1 overexpression in adult ACT is less common than in children (78) and is an unfavorable prognostic marker (81, 82) (**Table 1**). Remarkably, SF-1 transcriptional regulatory activity can be pharmacologically targeted leading to a decrease of adrenocortical cancer cell proliferation (83), suggesting that this transcription factor may represent a novel therapeutic target in ACT.

A subsequent SNP array study on both Brazilian and non-Brazilian ACT cases precisely defined recurrent genomic alterations in children ACT (46), the most frequent being loss of 4q34, gain of 9q33-q34 and 19p, and LOH of the whole chromosome 17 (harboring *TP53*) and 11p15 (harboring *IGF2*). Remarkably, a number of focal deletions were detected at 4q34, defining a common deleted region surrounding the non-coding RNA *LINC00290* gene. It is also noteworthy that the extent of the peak region of gain in 9q33-q34 suggests that other genes lying in a telomeric position with respect to *NR5A1* may also be important for ACT pathogenesis. In addition, focal amplifications and homozygous deletions comprising well-known oncogenes (*MYC, MDM2, PDGFRA, KIT, MCL1, BCL2L1*) and tumor suppressors (*TP53, RB1, RPH3AL*) were identified. Although genomic profiles in non-Brazilian tumors with a mutated *TP53* (other than R337H) were similar to Brazilian tumors, those with a wild-type *TP53* displayed distinct genomic alterations, harboring significantly fewer rearrangements. Remarkably, 50% of *TP53* wild-type tumors investigated in this study displayed as sole rearrangement a copy-neutral LOH of the imprinted region at 11p15, providing further evidence for a major role of this region in ACT development.

The landscape of genomic alterations in a worldwide series of children ACT enrolled at IPACTR (84) has been more precisely defined by a very recent study integrating whole exome, whole genome, and RNA-sequencing data (85). This work confirmed LOH in the 11p15 region in the large majority of cases and systematic overexpression of *IGF2*, together with frequent *TP53* mutations, widespread 9q copy number gain, and 4q34 loss. By comparing the mutant allele fraction of SNV in copy-neutral LOH regions to allelic imbalance values, it was possible to establish that in most cases copy-neutral LOH of chromosomes 11p and 17 occurred early during tumorigenesis, suggesting that those events drive tumor formation. Additional recurrent genetic alterations in children ACT were somatic mutations in the *ATRX* (a DNA helicase) and *CTNNB1* genes. Intriguingly, some tumors

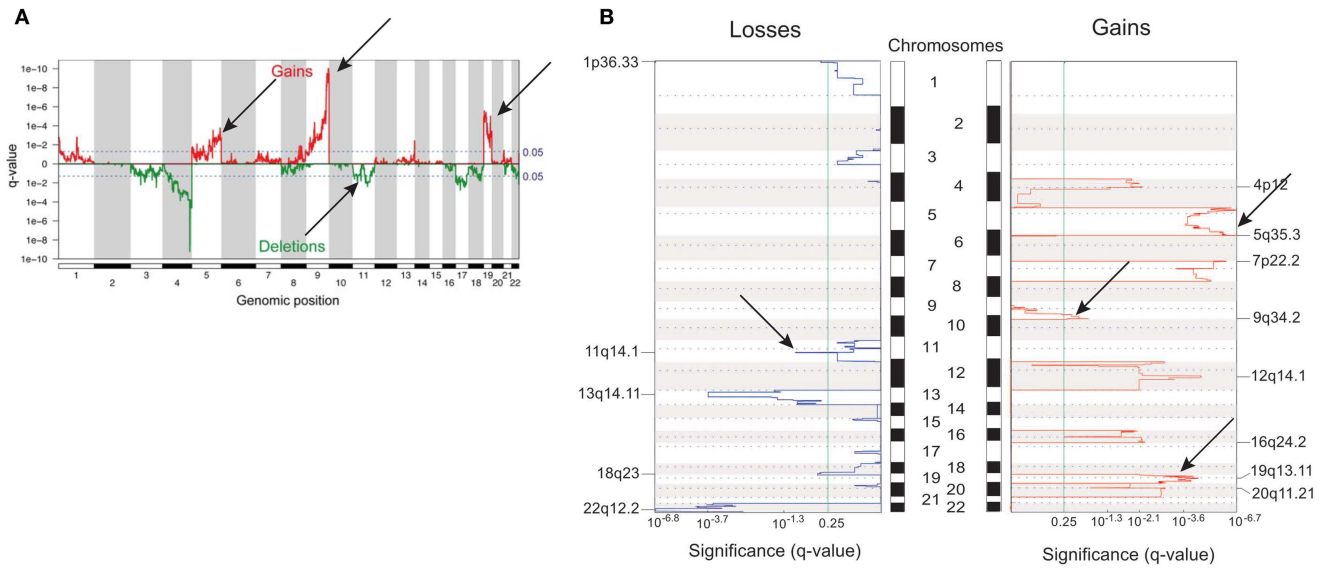


FIGURE 2 | Differences and similarities in genomic profiles between children and adult ACT. **(A)** Genomic alterations in children ACT. **(B)** Genomic alterations in adult ACC. Common regions of gains (chr. 5, 9q, 19) and losses (chr. 11) are indicated with arrows. Adapted from Ref. (46, 91) with permission from Endocrine Society Press.

bore integration of human herpesvirus-6 (HHV6) in the telomeric region of chromosome 11p. A poor outcome was predicted by concomitant *TP53*/*ATRX* mutations and associated genomic abnormalities, including massive structural variations and a high background mutation rate (**Table 1**).

In adult ACT, earlier CGH studies showed a significantly increased prevalence of genomic imbalances in carcinomas compared to adenomas and sometimes contrasting patterns of gain and losses (73, 74, 86–88). CGH array studies evidenced a set of chromosomal aberrations in ACC associated with survival in a fashion dependent on their accumulation (89). Carcinomas were confirmed to harbor a higher number of chromosomal alterations than adenomas (90–92). Recently, activating mutations of the PKA catalytic subunit were shown to be associated with cortisol-secreting adrenocortical adenomas in adults (93–96). In general, gains had a higher impact than losses on gene expression profiles (91). A comparison between genome alterations in children ACT and adult ACC is shown in **Figure 2**. Methylome studies were also performed in adult ACC (97–99). According to their DNA methylation levels, malignant tumors could be divided into two groups, one displaying low and the other one elevated levels of methylation in CpG islands (CpG island methylator phenotype, CIMP). This hypermethylated tumors group could in turn be subdivided into two subgroups (CIMP-high and CIMP-low), which had prognostic relevance, with the CIMP-high phenotype clearly being associated to worse prognosis (99).

A study integrating transcriptome, miRNome, copy number alterations, methylome, and whole exome sequencing data in adult ACC was recently published (66), showing that major pathways involved by mutation or homozygous deletion include beta-catenin (*CTNNB1* and *ZNRF3*), p53/Rb signaling (*TP53*, *CDKN2A*, and *RB1*), and chromatin remodeling (*MEN1*, *DAXX*, *ATRX*, *MED12*, and *TERT*) (**Table 1**). In addition, recurrent homozygous deletions were found in 4q34, similarly to children ACT. This study also showed that a substantial overlap exists among the different omics classifications of ACC: the previously identified gene expression profile clusters (C1A, C1B, and C2; see section on *Distinct Patterns of Coding Genes Expression in Children vs. Adult ACT*) (54) are strongly correlated with subgroups based on DNA methylation and miRNA expression, mutation rate, and alteration of key molecular pathways.

PERSPECTIVES

Children ACT represent a distinct pathological entity compared to tumors in adults concerning their origin, clinical manifestations, molecular alterations, and prognostic evolution. Important fields of investigation in the future will be the search for genetic and environmental factors that modulate penetrance of ACT in carriers of germline *TP53* mutations in order to orient screening procedures to detect disease at an early stage, the identification of robust biomarkers of malignancy, which are still lacking, and the clinical testing of targeted therapies against the major molecular pathways that are altered in this disease (100).

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microRNAs as Potential Biomarkers in Adrenocortical Cancer: Progress and Challenges

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Adrenocortical carcinoma (ACC) is a rare malignancy with poor prognosis and limited therapeutic options. Over the last decade, pan-genomic analyses of genetic and epigenetic alterations and genome-wide expression profile studies allowed major advances in the understanding of the molecular genetics of ACC. Besides the well-known dysfunctional molecular pathways in adrenocortical tumors, such as the IGF2 pathway, the Wnt pathway, and TP53, high-throughput technologies enabled a more comprehensive genomic characterization of adrenocortical cancer. Integration of expression profile data with exome sequencing, SNP array analysis, methylation, and microRNA (miRNA) profiling led to the identification of subgroups of malignant tumors with distinct molecular alterations and clinical outcomes. miRNAs post-transcriptionally silence their target gene expression either by degrading mRNA or by inhibiting translation. Although our knowledge of the contribution of deregulated miRNAs to the pathogenesis of ACC is still in its infancy, recent studies support their relevance in gene expression alterations in these tumors. Some miRNAs have been shown to carry potential diagnostic and prognostic values, while others may be good candidates for therapeutic interventions. With the emergence of disease-specific blood-borne miRNAs signatures, analyses of small cohorts of patients with ACC suggest that circulating miRNAs represent promising non-invasive biomarkers of malignancy or recurrence. However, some technical challenges still remain, and most of the miRNAs reported in the literature have not yet been validated in sufficiently powered and longitudinal studies. In this review, we discuss the current knowledge regarding the deregulation of tumor-associated and circulating miRNAs in ACC patients, while emphasizing their potential significance in pathogenic pathways in light of recent insights into the role of miRNAs in shaping the tumor microenvironment.

Keywords: adrenocortical carcinoma, circulating miRNA, biomarker, diagnosis, prognosis, therapeutic targets

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INTRODUCTION

Adrenocortical cancer is a rare and aggressive malignancy (with an incidence of 0.7–2.0 cases per million per year). Patients with adrenocortical carcinoma (ACC) generally have a poor prognosis, with a 5-year survival rate ranging from 15 to 30% in most series (1). Most patients present with advanced disease or develop local recurrence and distant metastasis post-operatively. In addition,

despite the development of systematic classification algorithms (2), it is sometimes challenging to discriminate malignant tumors from their benign counterparts. Currently, the only curative approach to localized ACC is complete tumor resection. Adjuvant mitotane therapy has been shown to improve recurrence-free survival following complete surgical resection (3).Nevertheless, this adrenolytic drug causes significant toxicity and adverse effects (4). Adjuvant radiation therapy showed no advantage in terms of overall survival (5–7). In metastatic disease, mitotane has produced very limited objective response (8) and remains the only drug approved by the U.S Food and Drug Administration (FDA) and the European Medicine Executive Agency (EMEA). The First International Randomized trial in locally advanced and Metastatic Adrenocortical Carcinoma Treatment (FIRM-ACT) reported that the combination of mitotane with the chemotherapeutic agents etoposide–cisplatin–doxorubicin was associated with a better progression-free survival than the association of mitotane with streptozotocin (9). However, the overall survival did not differ between both arms. In light of these therapeutic failures, molecular targeted therapies have been tested in patients with metastatic ACC. These approaches include epidermal growth factor receptor (EGFR) inhibitors (10), anti-vascular growth factor antibodies (bevacizumab) (11), and tyrosine kinase inhibitors (Sorafenib, Sunitinib) (12, 13). More recently, drugs targeting the IGF2/IGF-1R signaling pathway have been evaluated (14–16). All these targeted therapies yielded disappointing results in terms of progression-free and overall survival. In this context, there is a critical need for additional tools to improve diagnosis and prognosis and to explore new targeted therapies. Over the last decade, gene expression profiling using DNA microarray analysis has emerged as a useful technique for tumor classification (17–25). Increased *IGF2* expression was identified in most studies as one of the most dominant transcriptional change specifically present in ACC relative to benign tumors (adenomas, ACA) and normal adrenal (NA). More recently, an integrated genomic characterization of ACC, combining exome sequencing, SNP arrays, DNA methylation analysis, mRNA expression arrays, and microRNAs (miRNAs) sequencing provided a comprehensive overview of known drivers genes (*CTNNB1, TP53, CDKN2A, RB1*, and *MEN1*) and newly identified altered pathways (*ZNRF3, DAXX, TERT*, and *MED12*) in ACC (26). It appeared that aggressive and non-aggressive ACC are two distinct diseases with specific gene signatures and alterations.

In mammals, miRNAs were discovered a decade ago as an abundant class of small non-coding RNA (18–24 nt in length) that silence their target genes at the post-transcriptional level, either by degrading mRNA or by inhibiting translation (27). Comparative sequence analyses combined to computational methods predict that miRNAs could regulate the expression of more than 50–60% of human coding genes (28). The latest version of miRBase (Release 21, June 2014) has annotated over 2000 miRNA sequences in the human genome and novel ones are reported at a constant rate as more tissues are sequenced to greater depth. The biogenesis of miRNAs consists of multiple steps (29) (**Figure 1**). The primary miRNA (pri-miRNA) transcript is transcribed by RNA polymerase II, then cleaved by the complex Drosha to release a hairpin-structured miRNA precursor (pre-miRNA) in the nucleus. Pre-miRNA is transported from the nucleus to the cytoplasm by exportin-5-Ran-GTP-dependent double-stranded (ds) RNA-binding protein then processed into a short ds miRNA duplex by the ribonuclease III Dicer. Following unwinding of the duplex, the resultant guide strand mature miRNA is preferentially assembled into the RNA-induced silencing complex (RISC) composed of Dicer, Argonaute 2 (Ago2), and the dsRNA-binding protein TRBP. The association of the miRNA–RISC complex to complementary sequences in the 3′-untranslated region (3′-UTR) of target mRNA leads to inhibition of protein translation or degradation of the mRNA. More recently, it has been shown that miRNAs may target protein coding as well as 5′-UTR regions (30). An additional layer of complexity has been added since miRNAs were demonstrated to modulate gene expression at transcriptional level through their interaction with the transcription machinery or promoter sequences (31). Many miRNAs exhibit tissue-specific pattern of expression, suggesting that they play critical role in tissue and organ development, function, and maintenance. Each miRNA can control hundreds of genes and a single transcript harbors binding sites for several miRNAs. Due to their potential multi-target actions, it is not surprising that miRNAs regulate a plethora of basic biological mechanisms, such as cell cycle control, apoptosis, cell proliferation, differentiation, migration, and invasion, that impact systems biology in cancer.

A link between miRNAs and cancer was brought by the seminal observation of Croce's group who reported that miR-15 and miR-16, two miRNAs located in chromosome 13 (13q14) are frequently deleted in chronic lymphocytic leukemia (CLL) and function as tumor suppressors (32, 33). Since then, miRNAs have been studied most intensively in the field of cancer research and growing evidence suggests that altered miRNA expression is involved in the pathogenesis of cancers. The causes of the deregulation of miRNA expression in cancer cells are only partially elucidated. So far, at least three different mechanisms that could function independently or together have been described. The first one is that half of the known miRNAs are located in regions of chromosomal instability associated with cancer, including regions of loss of heterozygosity (LOH), regions of amplification, and fragile sites (34). The second mechanism involves epigenetic regulation of miRNA expression. DNA hypomethylation, CpG island hypermethylation and histone-modification losses have been shown to also affect miRNA expression (35). For example, histone deacetylase inhibition in breast cancer cells was followed by the extensive alteration of miRNA levels (36). The third mechanism is abnormalities in miRNA-processing genes and proteins (35). As the machinery involved in the biogenesis and maturation of miRNAs involve multiple protein complexes, one can anticipate that alterations of these proteins should have dramatic effects on miRNA expression. An analysis of gene expression in a wide range of primary tumors revealed that the downregulation of miRNAs observed in cancer was due to a failure at the Drosha processing step although the mechanisms underlying these dysregulations were not elucidated in this study (37). Interestingly, it was subsequently reported that p53 promotes the Drosha-mediated processing of certain miRNAs

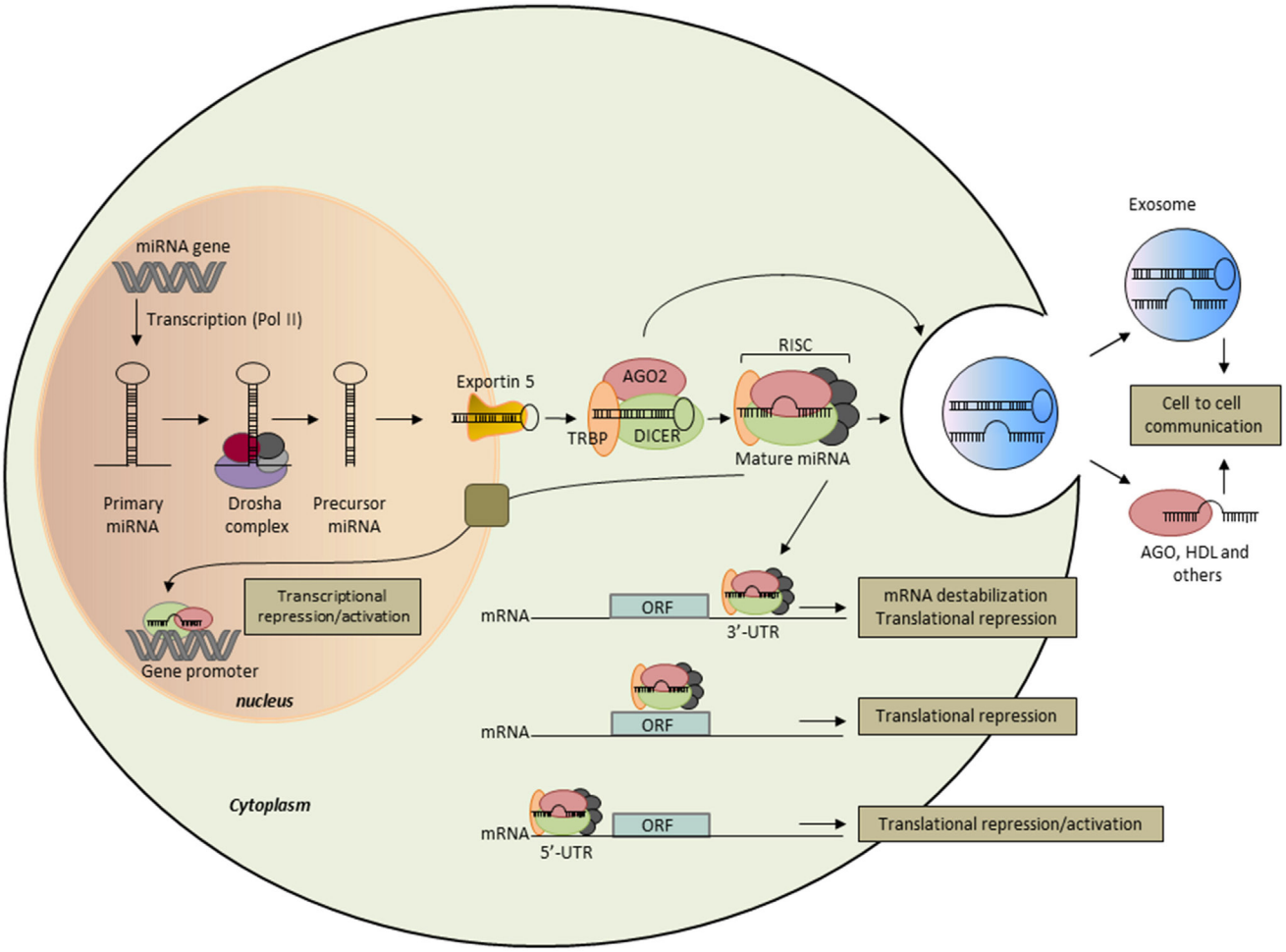

FIGURE 1 | Biogenesis and function of microRNAs. miRNA genes are transcribed as primary miRNAs (Pri-miRNA) by polymerase II (Pol II) in the nucleus. Pri-miRNAs are cleaved by the RNAse III endonuclease DROSHA and its proteins partners (DROSHA complex) to produce the 60- to 70-nt stem–loop precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported to the cytoplasm by exportin 5 and further processed by DICER1, a type III RNAse that produces the 22-nt mature miRNAs. One strand of the mature miRNA is selectively loaded into the miRNA-induced silencing complex (RISC), which contains DICER1, Argonaute (AGO) proteins, and the transactivation-responsive RNA-binding protein (TRBP). In the cytoplasm, mature miRNAs bind essentially to the 3′-UTR of the target mRNA and repress its expression through both translational repression and mRNA destabilization. Some miRNAs have been shown to bind to the open reading frame (ORF) and the 5′-UTR of the target mRNA, and to activate or repress its translation efficiency. In the nucleus, miRNAs were shown to bind to gene promoter to regulate gene expression. miRNAs are also released into the extracellular space and are possibly involved in intercellular communication when transferred to target cells. Extracellular miRNAs are encapsulated within microvesicles, such as exosomes, or bound to RNA-binding proteins, such as Ago2, or lipoproteins, such as HDL.

with growth-suppressive function (38). Consequently, p53 gene mutations may lead to decreased processing of pri-miRNAs by Drosha and decreased levels of mature miRNAs in cancer cells.

The abnormal levels of miRNAs in tumors have important pathogenic consequences: miRNAs that are overexpressed in tumors contribute to oncogenesis by downregulating tumor suppressor genes, whereas underexpressed miRNAs contribute to oncogene expression. However, certain miRNAs may function as tumor suppressors or oncogenes depending on the cell-type-specific microenvironment, which may provide a different repertoire of available target genes. Identification of specific miRNA expression patterns for different tumor histological types is a useful complement for the classification of tumors that otherwise cannot be accurately diagnosed by classical morphology-based methods. Interestingly, Lu et al. showed that the expression levels of 217 miRNAs classified poorly differentiated tumors better than information obtained from microarray analysis of about 16,000 mRNAs (39). The diagnostic power of these miRNA profiles strongly support the key role of miRNAs in developing and maintaining cellular fates. On the other hand, the potential role of miRNAs as prognostic and predictive biomarkers in cancer patient management has been suggested by numerous studies. One of the most recent exciting findings is that cell-derived miRNAs exist with remarkable stability in various types of body fluids, including blood. Circulating miRNAs are encapsulated in microparticles (microvesicles, exosomes, and apoptotic bodies)

or associated with RNA-binding proteins (Ago2), or lipoprotein complexes (high-density lipoproteins) (40). Although previously considered to be cellular waste products, recent studies have demonstrated that exosomes are "bioactive vesicles" that promote intercellular communication by shuttling molecules between cells (41). Importantly, specific circulating miRNA concentrations correlate with the development and progression of cancer (42). Circulating miRNAs fulfill several properties of non-invasive and good biomarkers, such as availability in various body fluids, sequence conservation between human and various preclinical models, and available sensitive technologies for their quantification. The technologies used to measure miRNA levels include microarray, next-generation sequencing (NGS), and reverse-transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). Both microarray and NGS-based platforms are suitable for screening and discovery purposes, but qPCR remains the top choice for validation and clinical tests. Regarding miRNA target identification, it is worth mentioning that this task is largely limited due to the imperfect complementarity of miRNAs and target transcripts. Canonical miRNA targeting is characterized by the perfect pairing of the miRNA's seed sequence, usually comprising 2–7 nt at the 5′-end of the miRNA, which is accompanied by base pairing at the miRNA's 3′-end (43). However, non-canonical targeting that lacks continuous seed pairing, but relies on increased complementarity toward the miRNA's center and/or 3′-end has been reported (44). Notably, only canonical targeting of miRNAs can be predicted by available *in silico* tools. Although these algorithms provide useful insights in some cases, these approaches remain challenging. Integrated transcriptome, proteome, and miRnome analyses to identify functional mRNA targets of miRNAs with altered expression may add valuable information on changes in gene regulation. On the other hand, because miRNAs affect the expression of multiple genes and thereby tune multiple steps in oncogenic pathways, they represent interesting therapeutic targets. The potential for using miRNAs in cancer therapy is now being explored thanks to the new advances in delivery of miRNA inhibitors or miRNA mimics (45). An overview of miRNA research tools for isolation, detection, target determination, regulation, and clinical applications is presented in **Figure 2**. In the present review, we summarize the findings related to miRNA deregulation in ACC with a focus on specific miRNA members and discuss their intrinsic merits and challenges for their use as diagnostic and prognostic biomarkers as well as potential therapeutic targets in ACC.

GLOBAL CHANGES IN miRNA EXPRESSION AND IN miRNA BIOGENIC MACHINERY IN ADRENOCORTICAL CANCER

Due to the rarity of adrenocortical cancer, our understanding of the relevance of miRNAs in the pathogenesis of this disease is still in its infancy. While the role of miRNAs in the development and progression of a most common cancer, such as lung cancer, has reached several hundred publications in Medline, about 30 studies have been published on miRNAs in ACC. The number of validated target genes for deregulated miRNAs in ACC remains very limited (46–50). Thus, in-depth analyses of the mechanisms underlying miRNA deregulations in ACC and their role in aberrant gene expression remain to be conducted. Network algorithms could be effective in testing for potential associations between miRNA clusters and gene expression alterations. For example, integration of certain dysregulated miRNAs into gene networks established from ACC omics datasets revealed their potential role in specific signaling pathways in adrenocortical cancer (51). A new intricate dimension has been added to miRNA regulation since it was discovered that miRNAs are themselves targeted by regulatory RNA species (52). Recent studies identified competing endogenous RNAs (ceRNAs) or natural miRNA sponges that titrate miRNA availability (53). Such miRNA sponges bind miRNAs and competitively sequester them from their physiologically relevant targets. This class of sponges includes endogenously transcribed pseudogenes, long non-coding RNAs (lncRNA), and recently discovered circular RNAs. They may act in large complex networks in conjunction with miRNAs to regulate protein levels. Interestingly, lncRNA dysregulation has been recently reported in adrenocortical tumors (54). The impact of lncRNAs on miRNA expression and function in ACC awaits further investigations.

Besides chromosomal alterations, major dysfunctional pathways in ACC, such as somatic mutations of the tumor suppressor gene TP53, overexpression of IGF2, and activation of the Wnt/β catenin signaling pathway, are likely to impact miRNA expression. Global alterations in the expression of miRNAs in ACC compared to ACA or to normal adrenocortical tissue (NA) have been reported in nine retrospective studies conducted in adult patients (**Table 1**). Only one study has assessed the expression of a set of miRNAs in childhood adrenocortical tumors (46). Microarrays and qPCR have been the main strategies applied to investigate the link between miRNAs and adrenocortical tumors (**Table 1**). More recently, NGS brought a new informative landscape on miRNA expression in adrenocortical cancer (26) (discussed below). Despite this rapid progress, many challenges related to miRNA biomarker development for ACC include variations in sample collection and processing, in quantification methods and normalization controls as well as in data analysis. Independent studies using small cohorts and different miRNA detection platforms have often reported poorly overlapping results. So far, none of the miRNAs identified as potential biomarkers for ACC have been validated in appropriately powered clinical studies. International collaborative studies using large cohorts, standardized procedures, and consensual rules for statistical analyses would enable to establish robust miRNA signatures. In this context, the use of RNA sequencing would enable to address different questions that remain unanswered by RT-qPCR or microarray approaches, such as the detection of single nucleotide variants and copy number as well as the discovery of novel miRNAs. In addition, RNA sequencing is not biased by thermodynamics, a drawback of qPCR and microarrays platforms. However, potential limitations of NGS include the high cost and the computational infrastructure needed for data analysis and interpretation. A detailed description of miRNAs that were found deregulated in ACC has been provided in previous reviews (55, 56). Here, we will focus on miRNAs that were consistently reported as differentially

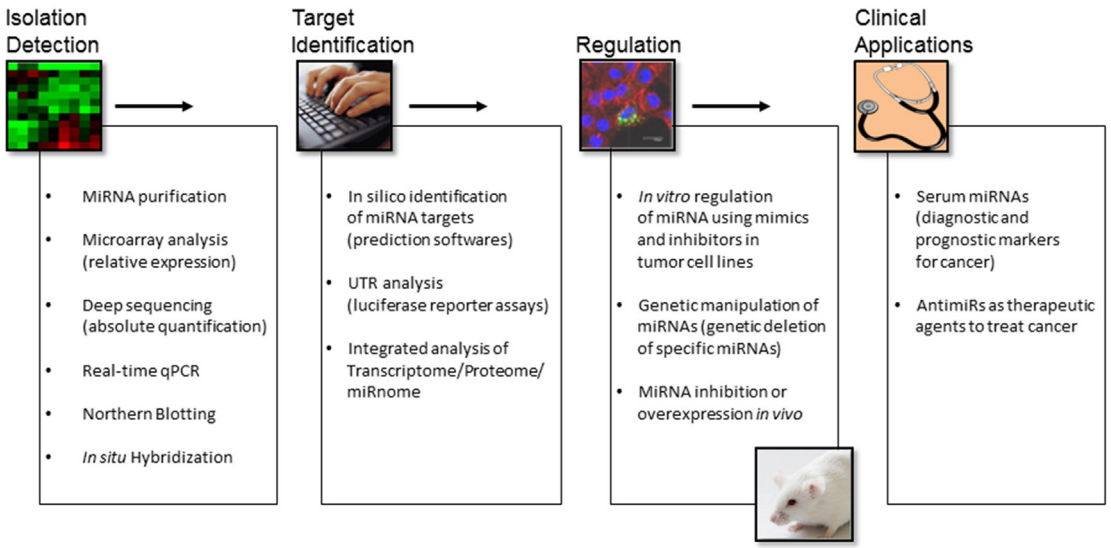

FIGURE 2 | An overview of miRNA research tools for isolation, detection, target determination, regulation, and clinical applications.

expressed in tumor tissue and serum or plasma among different studies.

miR-483-5p and miR-483-3p

miR-483-5p overexpression in ACC was consistently found in seven studies out of nine, whereas miR-483-3p overexpression was reported in five studies only (**Table 1**). Increased expression of miR-483-5p also identified a subgroup of patients that had significantly poorer prognosis (57). Upregulation of miR-483-5p was also observed in malignant pheochromocytomas as compared to benign tumors and associated with a poorer disease-free survival (64). miR-483 gene, which encodes both strands 5p and 3p, is located at 11p15.5 within the second intron of *IGF2* gene. The high expression of miR-483-5p observed in ACC was found to be correlated with the high expression of *IGF2* (58). However, the potential contribution of others mechanisms to miR-483-5p overexpression remains to be evaluated. Indeed, a functional β-catenin-dependent and *IGFI2*-independent-transcription start site located upstream of miR-483 locus has been reported in hepatocarcinoma and colon cancer cells (65). Oncogenic features of miR-483-5p and miR-483-3p have been suggested in Wilms' tumors as well as in liver, breast, and colon cancers (66, 67). Veronese et al. further demonstrated that the oncogenic mechanism of miR-483-3p could be partially attributed to its ability to modulate the pro-apoptotic protein BBC3/PUMA, thereby protecting cells from apoptosis (66). Similar observations were subsequently made in ACC by Ozata et al. in the NCI-H295R ACC cell line (47). In the same study, downregulation of both miR-483-5p and miR-483-3p resulted in decreased proliferation. Using *in situ* hybridization, Wang et al. observed that miR-483-3p was overexpressed in 68% (17 of 25) of ACCs and in 12% (3 of 25) of ACAs (68). A combination of miR-483-3p and Smad4 expression improved the diagnostic accuracy provided by the Weiss score system. Interestingly, miR-483-3p but not miR-483-5p was found to be upregulated in childhood adrenocortical tumors (46). It is worth mentioning that overexpression of miR-483-5p or miR-483-3p in several human neoplasms suggests a wider involvement of this miRNA in human tumorigenesis. However, the dramatic increase of IGF2 and miR-483 expression in ACC (up to several hundreds of times as compared to ACA or NA) when compared to that of other cancers suggest a critical role for IGF2 locus in adrenocortical cancer development and progression. miR-483-5p but not miR-483-3p was recently shown to induce epithelial to mesenchymal transition (EMT) and to promote lung adenocarcinoma cell migration *in vitro* by targeting Rho GDP dissociation inhibitor alpha (RhoGDI1) and activated leukocyte cell adhesion molecule (ALCAM) (69). *In vivo*, miR-483-5p promotes lung adenocarcinoma metastases (69). Interestingly, IGF2 overexpression was not sufficient for tumor formation in transgenic mouse models (70–72). Along the same line, Veronese et al. showed that miR-483-3p inhibition could suppress tumorigenicity of HepG2 cells while no antitumor effect was elicited by inhibition of IGF2 (66). These results clearly indicate crucial oncogenic functions of miR-483 within *IGF2* gene and might explain why transgenic animals for IGF2 overexpression did not develop tumors as IGF2 transgenes were lacking miR-483 locus.

miR-503

miR-503 was found significantly overexpressed in ACCs as compared to their normal and benign counterparts (26, 47, 61, 62) and in childhood adrenocortical tumors (46). Survival analysis indicated that high miR-503 was significantly associated with poor survival of ACC patients. In the study by Chabre et al., overexpression of miR-503 in ACC was observed in the discovery cohort but did not reach significance (59). miR-503 overexpression has been reported in retinoblastoma (73) as well as in parathyroid carcinoma (74). The role of miR-503 in ACC pathogenesis deserves further investigation. Indeed, miR-503

TABLE 1 | Significantly deregulated microRNAs in adrenocortical cancer.

Validated miRNA in ACC compared to ACA or NA	Sample type and method	Cohort composition	Signature	Reference
miR-483-5p	Microarray/RT-qPCR	22 ACC, 27 ACA, 6 NA	↑[c]	Soon et al. (24, 57)
	Microarray/RT-qPCR	10 ACC, 26 ACA, 21 NA	↑	Patterson et al. (58)
	Microarray/RT-qPCR	25 ACC, 43 ACA, 10 NA	↑	Ozata et al. (47)
	RT-qPCR	18 ACC[a], 10 ACA, 3 NA	↑	Chabre et al. (59)
	RT-qPCR	51 ACC	↑	Duregon et al. (60)
	NGS	45 ACC[a], 3 NA	↑	Assie et al. (26)
	Microarray/RT-qPCR	17 ACC, 29 ACA	↑	Feinmesser et al. (61)
miR-483-3p	Microarray/RT-qPCR	10 ACC, 26 ACA, 21 NA	↑	Patterson et al. (58)
	Microarray/RT-qPCR	25 ACC, 43 ACA, 10 NA	↑	Ozata et al. (47)
	RT-qPCR	51 ACC	↑	Duregon et al. (60)
	NGS	45 ACC[a], 3 NA	↑	Assie et al. (26)
	Microarray/RT-qPCR	17 ACC, 29 ACA	↑	Feinmesser et al. (61)
miR-210	TLDA	7ACC, 19 ACA, 10 NA	↑	Tombol et al. (25, 62)
	Microarray/RT-qPCR	25 ACC, 43 ACA, 10 NA	↑	Ozata et al. (47)
	RT-qPCR	51 ACC	↑[b,c]	Duregon et al. (60)
	NGS	45 ACC[a], 3 NA	↑[b]	Assie et al. (26)
	Microarray/RT-qPCR	17 ACC, 29 ACA	↑	Feinmesser et al. (61)
miR-503	TLDA	7ACC, 19 ACA, 10 NA	↑	Tombol et al. (25, 62)
	Microarray	25 ACC, 43 ACA, 10 NA	↑[c]	Ozata et al. (47)
	NGS	45 ACC[a], 3 NA	↑[b]	Assie et al. (26)
	Microarray/RT-qPCR	17 ACC, 29 ACA	↑	Feinmesser et al. (61)
miR-184	TLDA	7ACC, 19 ACA, 10 NA	↑	Tombol et al. (25, 62)
	NGS	45 ACC[a], 3 NA	↑[b]	Assie et al. (26)
miR-21	Microarray/RT-qPCR	25 ACC, 43 ACA, 10 NA	↑	Ozata et al. (47)
miR-1202	*id.*	*id.*	↑[c]	*id.*
miR-1275	*id.*	*id.*	↑[c]	*id.*
miR-139-5p	Microarray/RT-qPCR	12 ACC[a], 6 ACA + validation cohort (18 ACC[a], 10 ACA, 3 NA)	↑[b]	Chabre et al. (59)
	NGS	45 ACC[a], 3 NA	↑[b]	Assie et al. (26)
miR-376a	Microarray/RT-qPCR	12 ACC[a], 6 ACA + validation cohort (18 ACC[a], 10 ACA, 3 NA)	↑[b]	Chabre et al. (59)
	NGS	45 ACC[a], 3 NA	↑[b]	Assie et al. (26)
miR-195	Microarray/RT-qPCR	22 ACC, 27 ACA, 6 NA	↓[c]	Soon et al. (24, 57)
	Microarray/RT-qPCR	10 ACC, 26 ACA, 21 NA	↓	Patterson et al. (58)
	Microarray/RT-qPCR	25 ACC, 43 ACA, 10 NA	↓	Ozata et al. (47)
	Microarray/RT-qPCR	12 ACC[a], 6 ACA + validation cohort (18 ACC[a], 10 ACA, 3 NA)	↓	Chabre et al. (59)
	NGS	45 ACC[a], 3 NA	↓	Assie et al. (26)
	Microarray/RT-qPCR	17 ACC, 29 ACA	↓	Feinmesser et al. (61)
miR-335	Microarray/RT-qPCR	22 ACC, 27 ACA, 6 NA	↓	Soon et al. (24, 57)
	TLDA	4 ACC, 9 ACA, 4 NA + validation cohort (n = 15)	↓	Schmitz et al. (63)
	Microarray/RT-qPCR	12 ACC[a], 6 ACA + validation cohort (18 ACC[a], 10 ACA, 3 NA)	↓	Chabre et al. (59)
	NGS	45 ACC[a], 3 NA	↓	Assie et al. (26)
	Microarray/RT-qPCR	17 ACC, 29 ACA	↓	Feinmesser et al. (61)
miR-214	TLDA	7 ACC, 19 ACA, 10 NA	↓	Tombol et al. (25, 62)
	NGS	45 ACC[a], 3 NA	↓	Assie et al. (26)
	Microarray/RT-qPCR	17 ACC, 29 ACA	↓	Feinmesser et al. (61)
miR-375	TLDA	7 ACC, 19 ACA, 10 NA	↓	Tombol et al. (25, 62)
miR-511	TLDA	*id.*	↓	*id.*
miR-100	Microarray/RT-qPCR	10 ACC, 26 ACA, 21 NA	↓	Patterson et al. (58)
miR-125b	*id.*	*id.*	↓	*id.*
	Microarray/RT-qPCR	17 ACC, 29 ACA	↓	Feinmesser et al. (61)
miR-1974	Microarray/RT-qPCR	25 ACC, 43 ACA, 10 NA	↓	Ozata et al. (47)
miR-497	Microarray/RT-qPCR	*id.*	↓	*id.*
	NGS	45 ACC[a], 3 NA	↓	Assie et al. (26)
	Microarray/RT-qPCR	17 ACC, 29 ACA	↓	Feinmesser et al. (61)
miR-139-3p	TLDA	4 ACC, 9 ACA, 4NA + validation cohort (n = 15)	↓	Schmitz et al. (63)
miR-675	TLDA	*id.*	↓	*id.*

microRNAs highlighted in bold have been validated in several studies.

ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma; NA, normal adrenal cortex.

↑ Upregulated, ↓ downregulated in ACC as compared to adenoma or normal adrenal cortices.

[a]*The ACC group was composed of aggressive (poor prognosis) and non-aggressive (good prognosis) ACC.*

[b]*Overexpressed in aggressive versus non-aggressive ACC.*

[c]*Associated with shorter survival.*

id, idem refers to the line above.

was reported as a tumor suppressor in several other cancers. miR-503 was found underexpressed in hepatocellular carcinoma (HCC) and was shown to inhibit angiogenesis *in vitro* and *in vivo* by downregulating expression of both fibroblast growth factor 2 (FGF2) and vascular growth factor A (VEGFA) (75). Low expression levels of miR-503 were associated with worse overall survival of HCC patients (76). Functional studies showed that miR-503 suppressed the proliferation of HCC cells by induction of G1 phase arrest through Rb-E2F signaling pathways (76). Furthermore, tumor suppressive effect of miR-503 was suggested in glioblastoma multiform (77). In this study, miR-503 was shown to exert its effect not only through suppression of cell proliferation by inducing G0/G1 cell cycle arrest and apoptosis but also through inhibition of cancer cell migration and tumor invasion. In addition, insulin-like growth factor-1 (IGF–1R) receptor mRNA was identified as a target of miR-503.

miR-210

Overexpression of miR-210 in ACC has been observed in five studies out of eight (**Table 1**). High miR-210 was found associated with ACC aggressiveness and poor prognosis (60). miR-210 is a master miRNA in the cellular response to hypoxia (78). As hypoxia is a major hallmark of solid tumors, it is therefore not surprising that miR-210 is overexpressed in many tumors types. The expression of miR-210 is elevated in head and neck carcinoma (79), lung adenocarcinoma (80), late-stage small cell lung cancer (81), glioma (82), malignant melanoma (83), pancreatic ductal adenocarcinomas (84), ovarian cancer (85), and renal cancer (86). The stem–loop of miR-210 is located in an intron of a non-coding RNA on chromosome 11p15.5 (87). miR-210 is regulated by both HIF1α and HIF2α transcription factors (88, 89). However, Akt activation induces hypoxia-associated accumulation of miR-210 in a HIF-independent manner, suggesting that several signaling pathways can upregulate miR-210 in response to hypoxic stress (90).

miR-195

miR-195 is also a major miRNA deregulated in ACCs (**Table 1**). miR-195 is significantly downregulated in ACCs compared to ACAs and its low expression in ACCs is significantly associated with poor overall survival (57). miR-195 levels are also significantly downregulated in childhood adrenocortical tumors (46). miR-195 gene is located on the chromosome 17p13.1 and is a member of the miR-15/16/195/424/497 family of miRNAs. Numerous studies have suggested that miR-195 promotes apoptosis while inhibiting cell proliferation. Restoration of miR-195 expression in the ACC cell line NCI-H295R impaired their proliferation *in vitro* (47). miR-195 is aberrantly expressed in multiple types of cancers, including human breast cancer (91), glioblastoma multiforme (92), gastric cancer (93), human HCC (94), and bladder cancer (95). Cyclin D1, CDK6, and E2F3 were identified as direct targets, suggesting that miR-195 plays a role in regulating G1/S transition. In colorectal cancer, miR-195 was shown to target Bcl-2 and thereby to inhibit tumorigenicity through apoptosis (96). In breast cancer, the methylation state of CpG islands upstream of the miR-195/497 gene was found to be responsible for the downregulation of both miRNAs (91). A forced expression of miR-195 or miR-497 suppressed breast cancer cell proliferation and invasion. In this study, Raf-1 and Cyclin D1 were identified as direct targets of miR-195. In addition, miR-195 expression in breast cancer was found to be inversely correlated with malignancy.

miR-335

miR-335 was highly significantly downregulated in ACCs as compared to ACAs and normal adrenocortical tissue in several studies (**Table 1**). miR-335 has been shown to act as tumor suppressor or oncogene depending on cancer types. These findings suggest a tissue-specific role for miR-335. miR-335 is located at 7q32.2. It is downregulated in breast cancer (97, 98), while it is upregulated in colon cancer (99) and pediatric acute leukemia (100). The genetic deletion and epigenetic promoter hypermethylation occurring at miR-335 locus has been correlated with breast cancer metastases and ovarian cancer recurrence (98). In breast cancer, miR-335 suppresses metastasis and migration through targeting of the progenitor cell transcription factor SOX4 and extracellular matrix component tenascin C (101). More recently, miR-335 was shown to act as a tumor suppressor to regulate clear cell renal cell carcinoma cell proliferation and invasion through downregulation of BCL-W expression (102). Moreover, miR-335 suppresses breast cancer cell migration by negatively regulating the HGF/c-Met pathway (103).

microRNAs Differentiating between Aggressive and Non-Aggressive ACC: The miR-506-514 and DLK1-MEG3 Clusters

Recent genomic studies led to the identification of two distinct molecular subgroups of ACC with different outcomes: the C1A group, associated with poor prognosis, and the C1B group, associated with better prognosis (21, 26). Using Illumina sequencing to determine miRNA expression in 45 ACC, Assie et al. identified three ACC clusters characterized by three distinct miRNA profiles Mi1, Mi2, and Mi3. Mi1 and Mi2 clusters belong to the C1B group, while the Mi3 cluster characterizes the C1A group. Strikingly, the Mi1 cluster displayed the largest differences in miRNA expression relative to NA samples. This group was characterized by upregulation of 11 miRNAs belonging to the miR-506-514 cluster located at Xq27.3. Interestingly, these observations were in agreement with those reported by Chabre et al. in a small cohort of ACC [discovery cohort: 6 aggressive ACC (aACC, poor prognosis), 6 non-aggressive ACC (naACC, good prognosis) and 6 ACA; validation cohort: 9 aACC, 9 naACC, and 10 ACA] (59). In this study, miR-508-3p, miR-509-3p, miR-513-3p, and miR-514, which belong to the miR-506-514 cluster, were also found upregulated in naACC as compared to aACC. An oncogenic role for the miR-506-514 cluster was reported in melanoma where these miRNAs promote not only melanoma progression but also melanocyte transformation (104). The mechanisms underlying the upregulation of this oncogenic miRNA cluster in non-aggressive ACCs (C1B group) then its downregulation in aggressive ACC (C1A group) remain to be determined. Along the same line, comparing the miRNAs related to melanoma early progression to those involved in metastasis, Mueller et al. identified miR-506

and miR-507 as upregulated during early progression and subsequently downregulated in metastatic colonization (105). One can speculate that the functions of sub-clusters of the miR-506-514 cluster versus the full miR-506-514 cluster support shifting roles for various members depending on the stage of ACC progression. Identifying downstream targets of the miR-506-514 cluster may reveal important pathways contributing to ACC pathogenesis.

In humans, the DLK1-DIO3 genomic region, located on human chromosome 14 (14q32) contains the paternally expressed imprinted genes DLK1, RTL1, and DIO3 and the maternally expressed imprinted genes MEG3 and MEG8, and antisense RTL1. This region hosts, in addition to the two long intergenic RNAs MEG3 and MEG8, one of the largest miRNA clusters in the genome, with 53 miRNAs in the forward strand and one in the reverse strand (106). Assie et al. found downregulation of 38 miRNAs belonging to the imprinted DLK1-MEG3 cluster located at 14q32.2 in the good prognosis group of ACC (Mi1 tumors) (26). LOH of chromosome arm 14q was detected in all Mi1 tumors, associated with full methylation of MEG3 promoter. In line with these data, Chabre et al. reported that several miRNAs belonging to the DLK1-MEG3 (miR-370, miR-376a, miR-376b, miR-376c, miR-377, miR-379, miR-382, miR-411, miR-487a, miR-494, and miR495) were downregulated in non-aggressive ACC as compared to aggressive ACC (59). Quantitative PCR analysis further confirmed that miR-376a, miR-376b, and miR-376c were significantly underexpressed in naACC. Using microarray expression and qRT-PCR assays, Teferedegne et al. found that increases in the expression of miR-376a correlated with the acquisition of tumorigenic phenotypes in cell lines of non-human primates (107). miR-376a overexpression was associated with nodal metastasis in the progression of gastric cancer (108). Interestingly, overexpression of the DLK1-MEG3 was positively correlated with HCC stem cell markers and associated with poor survival rate in HCC patients (109). In another study, overexpression of miR-376c in ovarian cancer cells was found to block cisplatin-induced cell death (110). The investigators suggested that miR-376c enhances proliferation, survival, and chemoresistance by targeting activin receptor-like kinase 7 (ALK7). The role of the DLK1-MEG3 cluster in ACC aggressiveness awaits further investigations.

Deregulation of the miRNA-Processing Machinery in Adrenocortical Cancer

In addition to genomic or transcriptional alterations, deregulated miRNA expression can arise from failure in miRNA biogenesis. Several studies have shown that miRNA expression is globally suppressed in cancer cells compared with normal tissue, suggesting that miRNA biogenesis might be defective in cancer (39). A decreased expression of Dicer1 and Drosha has been reported in lung and ovarian cancers (111, 112). In addition, low Drosha or Dicer1 expression levels were associated with advanced tumor stage and poor clinical outcome in patients with ovarian cancer (112). On the contrary, Dicer1 overexpression was reported in melanomas (113) and was associated poor survival in colorectal cancer (114). Higher expression of Drosha was found in cervical squamous cell carcinomas (115) and epithelial skin cancers (116). Its overexpression was associated with poor prognosis in esophageal cancer (117). These variations of Dicer1 and Drosha expression levels among different tumor types suggest that miRNA-processing complexes act as tumor suppressors or oncogenes depending on cellular context. In adrenocortical cancer, two studies analyzed Tarbp2, Dicer1, and Drosha expression in ACA and ACC. Using RT-qPCR, Caramuta et al. reported a significant overexpression of Tarbp2, Dicer1, and Drosha transcripts in carcinomas compared with adenomas or NA cortices (43 ACA, 30 ACCs, and 9 NA cortices) (48). In addition, mRNA expression of Tarbp2, but not Dicer1 and Drosha could discriminate between ACAs and ACCs. Copy number gain of the *Tarbp2* gene was observed in 57% of the ACCs analyzed in this study. Inhibition of Tarbp2 expression in NCI-H295R cells resulted in a decreased cell proliferation and induction of apoptosis. Tarbp2 and Dicer1 were demonstrated as targets of miR-195 and miR-497, two miRNAs downregulated in ACC, suggesting that miRNAs might contribute to deregulation of their own biogenesis. de Sousa et al. analyzed Tarbp2 and Dicer1 expression in a cohort of 75 ACAs and 79 ACCs (118). Immunohistochemical analysis revealed that Dicer1 protein overexpression was found in 49% of ACCs and 32% of ACAs, while its mRNA was overexpressed in 60% of ACCs and 23% of ACAs. Nevertheless, the authors reported that metastatic ACC were characterized by a weak Dicer1 expression as compared to their non-metastatic counterparts. Furthermore, a weak Dicer1 expression was associated with reduced disease-free and overall survival. In contrast to Caramuta et al. study, no significant differences were found between ACCs and ACAs in terms of Tarbp2 protein or mRNA levels. The reasons for these discrepancies between the two studies remain unclear. They might be due to the size and the heterogeneity of the cohorts. Another regulator of miRNA biogenesis, LIN28, has been studied in adrenocortical tumors (119). LIN28 is an RNA-binding protein that binds to let-7 miRNA precursors (pri- and pre-let-7) and blocks their processing by Drosha in the nucleus and by Dicer in the cytoplasm (120). LIN28 was found underexpressed in aggressive ACC as compared to their non-aggressive counterparts (119). In the same study, Faria et al. reported that both weak expression of LIN28 and overexpression of miR-9, a negative regulator of LIN28, were associated with poor outcome of ACC patients. Nevertheless, a direct functional interaction between LIN28 and miR-9 was not investigated. When analyzing the global expression profile of miRNAs in the ACC cohort studied by Assie et al., it seems that miRNAs are rather overexpressed in the poor prognosis group as well as in a subpopulation of good prognosis group (26). Indeed, among the significantly deregulated miRNAs in ACC with poor outcome (C1A group), 86% were found upregulated and 14% were downregulated (Mi3 cluster). In the C1B group with good prognosis, 85% of the miRNAs were upregulated and 15% were downregulated in the Mi2 cluster, while only 45% were upregulated and 55% were downregulated in the Mi1 cluster. Chabre et al. also observed that all the discriminatory miRNAs between aggressive and non-aggressive ACC were upregulated in aggressive ACCs (59). Putting all these data together, it seems that the contribution of the miRNA-processing machinery disruption to the global deregulation of miRNA expression in ACC needs further clarifications.

CIRCULATING microRNAs AS POTENTIAL NON-INVASIVE DIAGNOSTIC AND PROGNOSTIC BIOMARKERS IN ACC

Since the discovery of cell-free circulating miRNAs, numerous studies have reported that specific miRNA levels in body fluids reflect various disease states (42). Although the precise mechanism of miRNA release into the extracellular environment is not completely elucidated, some miRNAs are probably released as a result of normal or pathology-associated cell death (41, 121). Other cellular miRNAs were shown to be released into body fluids through active secretion. Notably, a ceramide-dependent secretory pathway that involves sphingomyelinase 2 has been described (122). Circulating miRNAs are either encapsulated in small vesicles that are referred to as microvesicles or exosomes depending on their size, or complexed to HDL and RNA-binding proteins. Nevertheless, Turchinovich et al. reported that most extracellular miRNAs in blood plasma and cell culture conditioned media are not associated with exosomes or microvesicles but are bound to Ago2, a component of the RISC complex (123). The role of HDL-mediated miRNA transport in the context of adrenocortical tumorigenesis deserves further investigations as HDL may also function as a source of ApoA1-dependent selective uptake of cholesterol in steroidogenic cells through the scavenger receptor SR-B1 (124). A potential connection between cholesterol uptake and miRNA internalization in adrenocortical cells remains an open and fascinating question.

The field of circulating miRNA research in ACC is emerging and we are still far from having a clear picture. The transfer to the clinic of circulating miRNA-based test requires the establishment and implementation of standardized operating procedures. Unspecific fluctuations of circulating miRNAs may arise upon different serum/plasma preparation methods, different storage conditions of samples, and the presence of hemolysis (125–127). Another major concern is the potential interference of the therapy with circulating miRNA levels that may confound the interpretation of the results. Prospective studies in which blood samples will be timely collected before and after treatment of ACC patients are needed. Three studies analyzed circulating miRNA levels in ACC patients (**Table 2**). All three studies reported an increase in miR-483-5p in ACC patients, which seems to accompany the previously identified increase of miR-483-5p in tumor tissue (59, 128, 129). However, there are substantial differences in the findings of these studies, which may be in part due to the different blood material used, i.e., serum or plasma and the normalization strategies. Chabre et al. spiked-in *C. elegans* cel-miR-39 not only to monitor the efficiency of RNA extraction but also to use it as a normalization miRNA. Based on the identification of deregulated levels of miR-195, miR-335, miR-139-5p, miR-376a, and miR-483-5p (**Table 2**), they assessed their potential diagnostic value. The most informative miRNA for the discrimination of ACA from ACC patients was miR-195 [area under curve (AUC) = 0.948, 95% CI: 0.819–0.994, $p < 0.0001$]. miR-195 could detect individuals with adrenocortical cancer with 90.9% sensitivity and 100% specificity. miR-335 and miR-376a were also good markers of malignancy with an AUC of 0.837, and 0.811, respectively. Although miR-139-5p displayed high sensitivity for the discrimination of ACA from ACC patients (87.5%), its specificity was moderate (65%, AUC = 0.714, $p = 0.023$). Importantly, miR-483-5p could distinguish non-aggressive ACC from aggressive ACC patients with 85.7% sensitivity and 100% specificity (AUC = 0.929, 95% CI: 0.741–0.994, $p < 0.0001$). Moreover, low levels of miR-195 and high levels of miR-483-5p were predictive of recurrence risk in ACC patients. Using plasma samples and endogenous miR-16 as a reference, Szabo et al. identified hsa-miR-100, hsa-miR-181b, hsa-miR-184, hsa-miR-210, and hsa-miR-483-5p miRNAs as significantly differentially expressed between ACA and ACC patient plasma samples (129). By combining endogenous hsa-miR-16 and spiked-in cel-miR-39, they found hsa-miR-181b and hsa-miR-483-5p as significantly differentially expressed. The $dCT_{hsa\text{-}miR\text{-}210}$–$dCT_{hsa\text{-}miR\text{-}181b}$ and the $dCT_{hsa\text{-}miR\text{-}100}$/$dCT_{hsa\text{-}miR\text{-}181b}$ pairs yielded the highest AUC values (0.87 and 0.85, respectively). In Patel's study, it was found that the levels of miR-34a were increased in the serum of patients with ACC, while miR-34a was reported to be decreased in ACC tumors (128). Along the same line, Chabre et al. observed that miR-376a was significantly upregulated in ACC tumors, while it was significantly downregulated in the serum of patients with ACC. Opposite differential expression profiles of miRNAs in the circulation compared to parental cells are increasingly reported (130). These observations raise the question of an active

TABLE 2 | Deregulated circulating microRNAs in patients with adrenocortical cancer.

Validated miRNA in ACC compared to ACA or NA	Sample type and cohort composition	Signature	Reference
miR-483-5p	Serum, 23 ACC, 14 ACA, 9 NA	↑[a,b,c]	Chabre et al. (59)
	Plasma, 13 ACC, 12 ACA	↑	Szabo et al. (51, 129)
	Serum, 17 ACC, 22 ACA	↑	Patel et al. (128)
miR-100 miR-181b miR-184 miR-210	Plasma, 13 ACC, 12 ACA	↑	Szabo et al. (51, 129)
miR-34a	Serum, 17 ACC, 22 ACA	↑	Patel et al. (128)
miR-195	Serum, 23 ACC, 14 ACA, 9 NA	↓[a,c]	Chabre et al. (59)
miR-335	Serum, 23 ACC, 14 ACA, 9 NA	↓[a]	Chabre et al. (59)

ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma; NA, normal adrenal cortex.

↑ Upregulated, ↓ downregulated in the serum or plasma of patients with ACC as compared to patients with adenoma or healthy subjects.

[a]The ACC group was composed of aggressive (poor prognosis) and non-aggressive (good prognosis) ACC.

[b]Overexpressed in the serum from patients with aggressive versus patients with non-aggressive ACC.

[c]Associated with shorter survival and recurrence risk.

Circulating microRNA levels were determined by RT-qPCR in the three cited studies.

mechanism by which selected miRNAs are promoted toward the extracellular space. Given the small cohorts used these studies, validation of circulating miRNAs as biomarkers for adrenocortical cancer requires an in-depth analysis in larger cohort of samples. Combinatorial use of multiple miRNAs should improve the sensitivity and specificity of biomarkers panels.

ADRENOCORTICAL CANCER CELL-DERIVED EXOSOMES: PLAYERS IN THE COMMUNICATION WITH THE TUMOR MICROENVIRONMENT?

Although the release of apoptotic bodies during apoptosis has long been recognized (131), the fact that healthy cells also shed vesicles from their plasma membrane has only recently become appreciated. Numerous studies are beginning to decipher the molecular mechanisms of exosomes sorting and release. Notably, the content of cancer cell-derived exosome differs from exosomes derived from normal healthy cells and cancer cells have an increased rate of exosome release (132). The concept that exosomes are signaling entities in the cross-talk between various cell types is expanding (133). One can anticipate that exchange of exosomes between adrenocortical cancer cells and their neighboring components in the tumor microenvironment (TME), such as vascular endothelial cells, immune cells, and fibroblasts, might occur (**Figure 3**). The cellular origin of the multiple significantly deregulated miRNA in ACC tumor tissue as well as in the serum of the patients has not been deciphered so far. The expression profiles of miR-335, miR-195, miR-376a, miR-376b, miR-376c, miR-139-5p, and miR-483-5p in the NCI-H295R cell line were similar to their expression in the patients ACC samples, suggesting that their deregulation occurs in cancer cells (59). Increased circulating levels of miR-483-5p paralleled its marked upregulation in ACC. Nevertheless, defining the cellular localization of the other deregulated miRNA in ACC by performing *in situ* hybridization may help to unravel the potential interaction between ACC cancer cells and their surroundings and also the relationship between intratumoral and circulating miRNAs. Luga et al. reported a key role for cancer-associated fibroblast-derived exosomes in mobilizing autocrine Wnt-planar cell polarity (PCP) signaling in breast cancer cells to stimulate invasive behavior and metastasis in animal models (134). Transfer of exosomal miRNAs to endothelial cells has been shown to disrupt the vascular endothelial barrier by targeting the tight junction protein ZO-1 during early breast premetastatic niche formation (135). A seminal study performed by the group of Liberman demonstrated that exosomes released by metastatic cancer cells can transfer metastatic capabilities to non-metastatic cells. This transformation is directed by the miR-200 family that is known to mediate the mesenchymal-to-epithelial transition (136). The exchange of exosomal miR-21 and miR-155

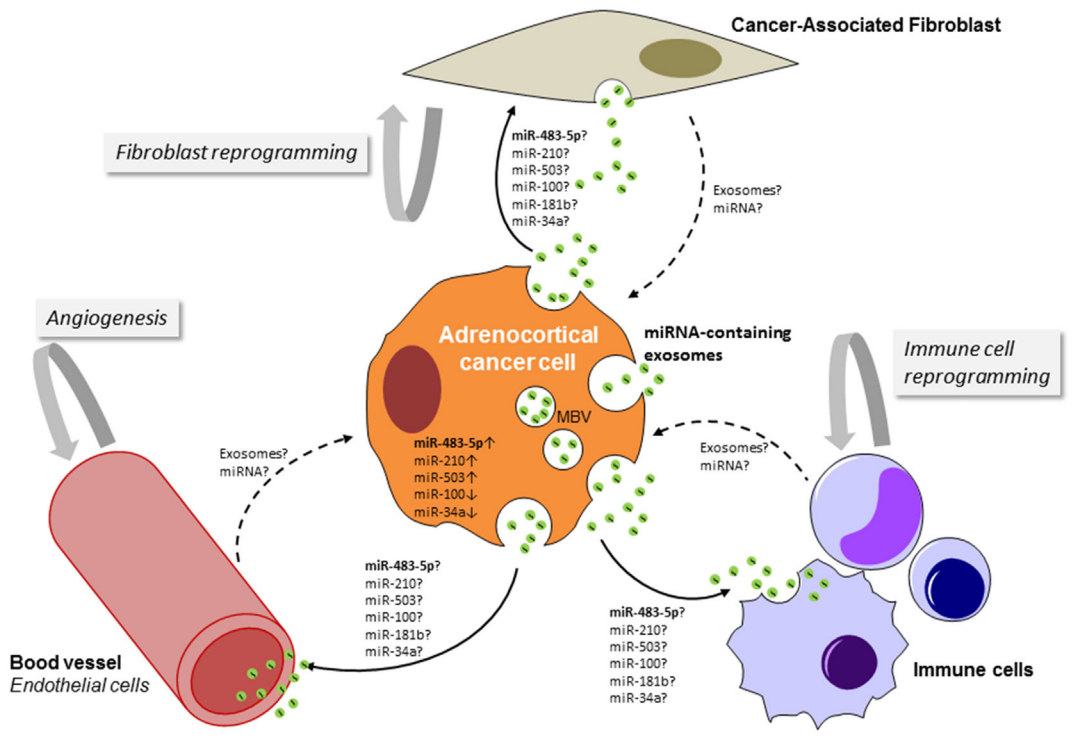

FIGURE 3 | Schematic representation of the potential cross-talk between adrenocortical cancer cells and cells of the tumor microenvironment (TME). By targeting cells of the TME, adrenocortical cancer cell-derived exosomes could favor stimulation of angiogenesis, production of pro-inflammatory cytokines, alterations of the extracellular matrix, and generation of a pre-metastatic niche. The TME-derived exosomes (dashed lines) could enhance growth and survival of cancer cells, promote invasion and induction of epithelial to mesenchymal transition, and also drug resistance. MBV, multivesicular body.

between neuroblastoma cells and human monocytes has been implicated in the development of resistance to chemotherapy (137). All these observations open new perspectives in the field of exosome-mediated cell-to-cell communication within the TME in ACC.

SILENCING AND RECOVERY OF ALTERED microRNAs: A FUTURE THERAPEUTIC APPROACH IN ADRENOCORTICAL CANCER

microRNAs are at the center of a complex combinatorial code regulating gene expression. Thus, identifying the relationships between miRNA signatures and adrenocortical cancer could help to understand the mechanisms behind the pathological processes and to develop therapeutic strategies. The biosynthesis, maturation, and activity of miRNAs can be manipulated by specific oligonucleotides that are complementary to mature miRNAs (138). Overexpression of miRNAs can be triggered by using synthetic miRNA mimics. Conversely, overexpressed miRNAs can be silenced by antagomiRs or miRNA sponges to restore miRNA balance in cancer networks (139). For example, inhibition of miR-21 and miR-17-92 was associated with reduced tumor growth, invasion, angiogenesis, and metastasis (140, 141). The therapeutic potential of miR-122 antagonist, miravirsen, in the treatment of Hepatitis C was evident from a multi-centric phase II trial (142). Although such findings are exciting, targeted miRNA therapeutics remain in the early stages of development and are essentially limited to *in vitro* and murine models of cancer. The development of relevant animal models of ACC is essential to the preclinical testing of miRNA-based therapies. On the other hand, though miRNAs possess tremendous therapeutic potential for cancer, a major concern remains their delivery system that may induce off-target effects. Lipid-based vehicles, viral systems, and cationic polymers are the main delivery tools for miRNA-based therapeutics (143). Each of these strategies has its own challenges and still needs improvements to address problems, such as cytotoxicity, immunogenicity, and low efficiency. Due to their natural role in miRNA secretion and shuttling between different cells, exosomes are of great interest in miRNA therapeutics. Their non-synthetic nature potentiates them for more efficient and non-immunogenic delivery of cargo while they maintain the cargo integrity and stability. Moreover, exosomal membranes contain proteins that have specific receptors on the surface of recipient cells. Therefore, they can selectively target cell types of interest and modifying their miRNA contents. Two delivery systems using liposome formulated miRNAs or miRNAs packaged in EnGeneIc Delivery Vehicles (EDVs) (144, 145) have reached the clinic and are currently under evaluation in cancer clinical trials. Recently, Glover et al. first reported that systemic administration of miR-7-containing EDV reduces ACC xenograft growth through the targeting of Raf-1 proto-oncogene and mechanistic target of rapamycin (mTOR) (49). This work is the first study investigating the therapeutic potential of miRNAs in ACC and many others should be expected.

CONCLUSION

The discovery of miRNAs has considerably changed our understanding of gene regulation and new findings over the last decade have established that miRNA are key players in cancer molecular biology. Deregulations of miRNAs expression and activity are important steps in the development of many cancers, including adrenocortical cancer. On the basis of expression profiling of miRNA in ACC, several groups have identified miRNAs enabling diagnosis and prognosis of ACC. These findings need to be validated in larger cohorts and in prospective studies. Another important question for the management of ACC is the possibility of predicting patient response to therapy. Identification of specific miRNAs as significant indicators for response to mitotane or chemotherapy may guide the clinicians and provide an opportunity for personalized medicine. To improve our knowledge as to the role of miRNAs in ACC pathogenic pathways, functional effects of specific miRNAs need more comprehensive and thorough studies. The occurrence of miRNAs in the serum and plasma of ACC patients lays the groundwork for their development as minimally invasive biomarkers. The fact that miRNAs can function as cellular master regulators, show broad activity across multiple cancer types, and appear to specifically inhibit metastasis suggests that they could be used as therapeutic agents in cancers for which there are no or few treatment options, such as ACC. Nevertheless, a number of scientific and technical considerations must be addressed before we could reach these promising prospects.

AUTHOR CONTRIBUTIONS

NC conceived and wrote the manuscript.

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