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Development of Adrenal Cortex Zonation

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1. FETAL AND EARLY ADULT DEVELOPMENT OF THE ADRENAL CORTEX

1.1 Formation of the adrenal cortex

1.1.1 Origin of the adrenogonadal primordium (AGP)—The adrenal glands develop from two separate embryological tissues: the medulla is derived from neural crest cells originating in proximity to the dorsal aorta, while the cortex develops from the intermediate mesoderm [1]. The appearance of the adrenal gland in the form of the adrenogonadal primordium (AGP) at 28–30 days post conception (dpc) in humans (embryonic day (E) 9.0 in mice) is marked by the expression of steroidogenic factor 1 (SF1, NR5A1), a nuclear receptor essential for adrenal development and steroidogenesis [2, 3]. The bilateral AGP first appears as a thickening of the coelomic epithelium between the urogenital ridge and the dorsal mesentery. Each AGP contains a mixed population of adrenocortical and somatic gonadal progenitor cells. SF1 positive AGP cells then delaminate from the epithelium and invade the underlying mesenchyme of the intermediate mesoderm [4].

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1.1.2 Separation of AGP (formation of the adrenal gland)—Following delamination, the majority of AGP cells migrate dorsolaterally to form the gonadal anlagen (gonadal primordial; GP). A subset of AGP cells that express higher levels of SF1 migrate dorsomedially to form the adrenal anlagen (adrenal primordial; AP or adrenal fetal zone; FZ), ultimately settling ventrolateral to the dorsal aorta [3]. At about 48 dpc in humans (E11.5-E13.5 in mice), neural crest cells migrate from the dorsal midline just lateral to the neural tube to the area where the AP is developing [5]. These cells persist as discrete islands scattered throughout the embryonic adrenal until birth and ultimately coalesce and differentiate into the catecholamine-producing chromaffin cells of the adrenal medulla [6, 7]. Meanwhile, the adrenal gland starts to separate from surrounding mesenchyme and becomes encapsulated with the formation of a fibrous layer overlying the developing cortical cells, a process largely complete by 52 dpc in humans (E14.5 in mice) [8].

Adrenocortical and chromaffin cells have an intimate relationship during embryonic development and postnatal homeostasis. Adrenal glucocorticoids play an essential role in chromaffin cell hormone production by regulating the expression of phenylethanolamine N-methyltransferase (PNMT) that results in epinephrine (as opposed to nor-epinephrine) being the dominant catecholamine produced in the post-natal adrenal medulla[9, 10]. However, mutant mice lacking the glucocorticoid receptor exhibit normal embryonic neural crest cell migration to the adrenal and normal early fetal chromaffin cell development [11]. Similarly, even in the setting of a hypoplastic (*Sf1* heterozygous ($Sf1^{+/-}$) mice) or aplastic (*Sf1* null ($Sf1^{-/-}$) mice) adrenal cortex, a rudimentary adrenal medulla develops[12], albeit in an ectopic location in the hypoplastic gland[1213]. Further studies are needed to yield insights into the molecular mechanisms that dictate the interplay between the steroidogenic adrenocortical cells and the catecholamine-producing chromaffin cells of the adrenal gland [14, 15].

1.2 Fetal development of the adrenal cortex

1.2.1 Fetal zone formation and function—After encapsulation, the embryonic adrenal cortex expands rapidly. In humans, the enlargement of the fetal cortex (fetal zone; FZ) accounts for the majority of the prenatal growth, especially during the last 6 weeks of gestation. Indeed, the human fetal adrenal is one of the largest organs at term (0.2 % of total body weight and nearly the size of the kidney), with 80% of the gland composed of FZ cells[16]. These large steroidogenic cells (20–50mm) exhibit a high cytoplasmic/nuclear ratio and robustly express cytochrome P450 17 alpha (CYP17), a bifunctional enzyme with both 17 hydroxylase and 17,20 lyase activities that converts pregnenolone to dehydroepiandrosterone (DHEA). Due to the high activity of CYP17 at this stage, the human fetal adrenal cortex produces large amounts of DHEA and DHEA-S, which is then converted by the placenta to oestrogens for the maintenance of normal pregnancy. While large amounts of other sulphated Δ5 steroids, including pregnenolone sulphate and 17α-hydroxypregnenolone sulphate are also produced by FZ cells, it is unclear if such steroids play a functional role in human biology.

1.2.2 Emergence of the definitive zone—By the 8th week of gestation, new adrenocortical cells emerge between the capsule and FZ, forming the definitive zone (DZ) that later develops into the adult cortex. The DZ is composed of SF1-positive, densely

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packed basophilic cells arranged in a narrow band of cellular clusters[17, 18]. Small in size (10–20 mm), DZ cells are lipid-poor during midgestation and exhibit structural characteristics typical of cells in a proliferative state. However as gestation advances, inner cells of the DZ form arched cords with finger-like columns of cells reaching the outer rim of the FZ. During the third trimester, the cells of these cords continue to expand and begin producing cortisol under the regulation of adrenocorticotropin hormone (ACTH) defining the emergence of the ZF of the adult adrenal cortex.

1.2.3 Factors involved in fetal adrenal development (regulatory mechanism)

SF1: The nuclear receptor SF1 (also known as NR5A1) has emerged as a pivotal factor for the initiation and fetal maturation of the adrenal cortex. In the absence of SF1 expression, the adrenal gland does not form (adrenal aplasia in *Sf1*-knockout mice[19] and most human patients[20, 21]). Moreover, Sf1 haploinsufficiency ($Sf1^{+/-}$) results in delayed and incomplete development of the adrenal gland in mice[22], while overexpression of Sf1 results in aberrant proliferation, gonadal differentiation and ultimate neoplasia in the mouse adrenal[23].

The regulation of *Sf1* gene expression is complex, with different enhancer elements controlling the spatial and temporal expression in the developing adrenal. Current data indicate that while Wilms tumor 1 (WT1) regulates *Sf1* expression in the AGP, Cbp/P300-Interacting transactivator, with Glu/Asp-Rich carboxy-terminal domain, 2 (CITED2) expression in the AGP is necessary for proper differentiation of the adrenal primordial (AP or fetal zone- FZ) [24, 25]. Zubair, et al identified the mouse fetal adrenal-specific enhancer (FAdE) in the *Sf1* gene as the critical mediator of *Sf1* gene expression in the AP[26]. The transcription complex containing the homeobox protein PKNOX1 (Prep1), homeobox gene 9b (Hox9b) and pre B-cell leukemia transcription factor 1 (Pbx1), initiates FAdE-mediated *Sf1* expression in the AGP[26]. Sf1 subsequently regulates itself by maintaining FAdE-mediated *Sf1* expression in the AP. After E14.5 in mice, FAdE is no longer utilized[27]. In the emerging DZ, *Sf1* regulation is shifted to a different definitive enhancer that has not yet been characterized. In humans, however, no similar FADE or DZ enhancers have yet been confirmed[26].

Dax1: A Sf1 target gene, *Dax1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1, Nr0b1), is an orphan nuclear receptor. *DAX1* was first cloned as the gene responsible for X-linked Cytomegalic Adrenal Hypoplasia Congenita [28, 29]. In the adrenal, Dax1 primarily serves as a co-repressor of Sf1-mediated transcription of steroidogenic genes [4, 20, 30]. Consistent with this role and the enriched expression of Dax1 in the outer cortex, knockdown of *Dax1* results in premature differentiation of mouse adrenocortical progenitor cells. However, this occurs at the expense of depleting this essential cell population, ultimately resulting in adrenal failure[31]. As such, Dax1 plays an essential role in the maintenance of stem/progenitor cell pluropotency. Moreover, Dax1 may serve as the repressor for FAdE activity in the mouse adrenal gland during the FZ to DZ transition [27]. (The relationship between Dax1, Sf1, ACTH and Wnts are summarized in Figure 1)

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Accordingly, regulation of *Dax1* expression is predicted to be a dynamic process, balancing progenitor renewal and adrenocortical differentiation/steroidogenesis. Sf1 activates *Dax1* transcription in cooperation with paracrine Wnt signaling and glucocorticoids that are synthesized in the differentiated adult cortex[32]. Conversely, ACTH, the well-established glucocorticoid stimulator, has been shown to effect release of Sf1 complexes from the *Dax1* promoter, thus leading to effective inhibition of *Dax1* transcription. This is predicted to promote the response of Sf1-positive progenitor cells to ACTH and subsequently initiate steroidogenesis. In an analogous fashion, Dax1 plays a similar role in mouse embryonic stem (ES) cells. It is transcriptionally activated by the other member of the NR5A family, Liver Receptor Homolog 1 (LRH1), and the homeobox transcription factor Nanog [33]. Moreover, knockdown of *Dax1* leads to premature spontaneous differentiation of ES cells into cells of all three germ layers, establishing Dax1 as a critical mediator of stem/progenitor cell pluripotency.[34]

ACTH and other hormonal factors: ACTH is a 39 amino acid peptide secreted from the anterior pituitary gland under control of corticotropin-releasing hormone (CRH) [35]. As a major component in the hypothalamic- pituitary-adrenal axis, ACTH binds to the transmembrane receptor melanocortin receptor 2 (MC2R) specifically in adrenocortical cells and exerts its effect through downstream cAMP and Ras/MEK/ERK signaling pathways[36]. While adrenocortical growth and differentiation are independent of ACTH during the first trimester of human pregnancy, ACTH begins to play an essential role in the morphological and functional development of the adrenal gland after 15 weeks of gestation[37]. The growth promoting effects of ACTH are mediated in part through the stimulation of locally produced growth factors such as insulin-like growth factor 2 (IGF2) and fibroblast growth factor beta (FGFβ) [38, 39]. During development, as the outer DZ emerges, ACTH participates in the regulation of steroidogenesis, cell differentiation and cell growth[40–42].

As mentioned above, CRH, a 41-amino acid peptide, is commonly considered to regulate adrenal function indirectly through the central nervous system (CNS) via the hypothalamic-pituitary-adrenal (HPA) axis[43]. Secreted by the parvocellular neurons of the paraventricular nucleus (PVN) in the hypothalamus in response to central and peripheral stimuli, CRH stimulates corticotroph cells in the anterior pituitary to produce POMC, which is subsequently cleaved to ACTH[44].

Interestingly CRH, CRH-homologous peptides (Urocortin 1–3, UCN1-3) and their receptors (CRF1 and CRF2 receptor) are all found locally in the both adult and fetal adrenal gland with different distribution patterns[46, 47]. In adults, while CRH is found to be expressed throughout the gland, UCN1 mainly presents in chromaffin cells in the medulla[44, 45]. On the contrary, UCN2 is observed in all three zones of human cortex but is only weakly expressed in adrenal medulla, and UCN3 also appears to be expressed in human adrenals[45, 46]. The expression patterns of CRF and UCNs in fetal adrenals parallel the developmental changes in human, where UCN1, UCN3 and the CRF1 and CRF2 receptors all appear to be expressed in both FZ and DZ of the adrenal cortex[47, 48].

The presence of all CRH peptides and their receptors in the adrenal suggests that the CRH system can function locally within human and rodent adrenals. Another important source of

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CRH is the human placenta, which releases CRH into the fetal circulation. As gestation advances CRH concentration increases: the remarkable increase in placental CRH production at the end of gestation has been suggested to contribute to the parturition process by forming a feed-forward loop that leads to increased productions of cortisol and DHEA/DHEA-S in human fetal adrenals. Studies have shown that CRH stimulates cortisol production in primary cultures of fetal adrenocortical cells by elevating the mRNA levels of steroidogenic acute regulatory protein and other steroidogenic enzymes, including *3β-HSD2*, *CYP21* and *CYP11B1*[49]. In addition, CRH enhances the adrenal response to ACTH, further driving the production of cortisol and DHEA/DHEA-S[49, 50]. A further study has shown that the stimulatory effect of CRH on cortisol production may require the presence of chromaffin cells[51], consistent with the potential paracrine relationship between cortex and medulla.

Insulin-like growth factor 2 (IGF2): Both IGF1 and IGF2 are mitogens expressed in the adrenal gland. IGFs bind dimeric/heterodimeric cell surface receptors (insulin receptor (IR) or IGF receptor 1 (IGF1R)) and induce autophophorylation of the intracellular portion of the receptor, leading to activation of Ras/MEK/ERK and PI3K/AKT[52, 53]. Although both IGF1 and IGF2 are present during human adrenal development, IGF2 is more highly expressed in early fetal development, where it likely plays a major role [54–56]. In contrast, IGF1 is dominant in the adult adrenal gland, functioning as a regulator of postnatal growth maintenance. Although at lower levels compared to fetal adrenal cortex, IGF2 is expressed in the adult adrenal gland with expression restricted to the peripheral cortex and capsule of the adult adrenal, coinciding with the stem/progenitor cell niche [53]. In the mouse however, the expression pattern is a little different: in the fertilized mouse embryo, mRNA transcripts encoding *Igf2* are present in low amounts almost as soon as the embryonic genome is activated, at the 2- to 4-cell stage, while the *Igf1* mRNA appears later, around the time of implantation at 8 to 9 d of gestation. Besides their mitogenic effects, IGFs and FGF have been shown to be essential factors for maintenance of the stem cell niche in multiple other organ systems [56, 57], supporting a potential role for IGF2 in adrenal stem/progenitor cell maintenance. A more recent study found the IGF pathway essential in adrenogonadal development and primary sex determination, since knocking down the receptors causes adrenal agenesis, possibly by decreased expression of Sf1 in AGP and subsequent failure of adrenal primordia specification. Growth retardation, male-to-female sex reversal and delayed ovarian development are additionally observed in these mice[58].

1.2.4 Transition of FZ to DZ/ adult cortex—Studies by Zubair, et al provided significant insights into the relationship between the FZ and the DZ/adult cortex [26, 27, 59, 60]. Using the FAdE-Cre mouse model, these investigators observed proliferating cells in a scattered pattern throughout the adrenal gland until E13.5 [13, 60]. At later time points, proliferating cells assembled in the periphery of the adrenal gland [61]. Similar to the developing human adrenal cortex, the mouse FZ is first encapsulated by surrounding cells of the intermediate mesoderm. Following encapsulation a second group of cells emerge as the densely packed DZ or definitive (adult) cortex. As previously noted, *Sf1* is activated by FAdE only in the FZ before E14.5 but this enhancer does not activate *Sf1* expression in the DZ. By breeding a Rosa26 mouse with a transgenic mouse harboring the Cre-recombinase gene driven by a basal *Sf1* promoter and the FAdE enhancer, Zubair et al traced the fate of

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FZ cells during development. Their results demonstrated that all DZ cells are derived from FAdE-expressing cells of the fetal adrenal [60]. To complement these experiments, Zubair et al also utilized a tamoxifen-inducible FAdE-cre mouse model. DZ staining was only observed when tamoxifen was administrated early in embryogenesis (E11.5-E12.5). No LacZ-positive cells were found when tamoxifen was administered after E14.5. These results are consistent with the absence of FAdE activity during later developmental stages and support the conclusion that the FZ gives rise to the DZ.

In contrast, studies focused on a downstream activator of the hedgehog pathway, Glioma-Associated Oncogene Homolog 1 (Zinc Finger Protein) (Gli1) provide evidence that the adrenal capsular cells also give rise to the DZ [62, 63]. Gli1-expressing cells are specifically located in the adrenal capsule and do not express Sf1. As demonstrated by King et al, this subpopulation of cells is capable of giving rise to Sf1-expressing, differentiated adrenocortical cells during embryonic development[63]. However, these data are in conflict with the above-mentioned lineage tracing experiments using FAdE-LacZ reporter mouse that revealed that all DZ cells arise from FZ cells. While these apparently conflicting observations may reflect two temporally distinct lineages of the definitive cortex, in which both the Sf1-positive FZ and the Sf1-negative capsule cells can give rise to Sf1-positive DZ adrenocortical cells, recent studies support a single developmental and homeostatic mechanism of adrenal growth. Wood et al demonstrated that FZ cells that once expressed *Sf1* under control of the FAdE enhancer gave rise to a subset of capsular cells[64]. These FZ cell descendants within the adrenal capsule express Gli1, suggesting some FZ cells can transition into Sf1-negative capsular cells which in turn can give rise to the underlying DZ / adult cortex (Figure 2).

1.3 Postnatal adrenal development

The human adrenal gland continues to undergo significant remodeling during neonatal and pubertal periods. Immediately after birth, cells within the FZ undergo apoptosis, resulting in involution of the remaining FZ [65]. On the other hand, under the influence of the trophic hormones angiotensin II (AngII) and ACTH, the adult zona glomerulosa (zG) and zona fasciculata (zF) mature. Later, at age 6–8 years old in females and 7–9 years old in males, the adrenal zona reticularis (zR) begins to form between the zF and the medulla. This process, known as adrenarche [66], is characterized by increased proliferation and production of adrenal androgens. Due to zR hormonal influence, secondary sexual characteristics appear, particularly development of pubic hair and altered sweat composition, which produces adult body odor. Additionally, increased oiliness of the skin and hair and mild acne may occur [67]. In most males, these changes are indistinguishable from early testicular testosterone effects occurring at the beginning of gonadal puberty. In females, adrenal androgens from adrenarche drive most of the early androgenic changes of puberty. Adrenarche can occur prematurely in many children who are overweight and/or born small for gestational age, suggesting links between placental hormonal, intrauterine growth retardation, metabolic disease and zG function [68–71]. However, the exact mechanisms for zR growth and the control factors for adrenarche remain elusive.

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In rodents, the remaining remnants of the FZ (classically called X-Zone in mouse) ultimately regress at the onset of puberty in male and during the first pregnancy in females [72, 73]. However, because FAdE activity is lost by E14.5, a definitive enhancer likely begins functioning before birth. Current efforts aim to identify and characterize a definitive/adult *Sf1* enhancer in rodents. (Figure 3 summarizes the major milestones of human and mouse adrenal gland development)

2. STRUCTURE AND FUNCTION OF THE ADULT ADRENAL CORTEX

2.1 Adrenal cortical zonation

2.1.1 Anatomy—In conjunction with FZ regression, the DZ forms discrete functional compartments within the adrenal cortex, termed the zG and the zF, respectively. The zG, composed of ovoid shaped cells, is the most outer layer . Although in kids with low salt diets, zG is normally presented as a full zone, due to high-salt consumption in western diet, zG cells in adults are actually scattered in clusters with a width of 200–1300 μm and a depth of 100–500 μm beneath the capsule[74–76]. These so-called aldosterone-producing cell clusters (APCCs) express CYP11B2 enzyme and is responsible for aldosterone production[74]. The zF constitutes the majority of the adrenal cortex, sitting directly under the zG. Importantly, zF cells are bigger cells organized into bundles, which led to its name, “fascicles”. Lastly, zR constitutes the innermost layer of the adrenal cortex, between the zF and the adrenal medulla. Here, ZR cells are arranged as cords that project in different directions, resulting in the net-like appearance that gives the zone its name (reticulum= net).

Extracellular matrix (ECM): ECM, which is composed of glycoproteins and associated integrin receptors, has been shown to play an important role in both the fetal and adult adrenal[77]. Using second-trimester human fetal adrenal glands, in situ studies have demonstrated that collagen type IV is the major ECM glycoprotein present in the fetal adrenal and that it is evenly distributed throughout the gland. In contrast, other ECM glycoproteins, including laminin and fibronection, display more restricted expression patterns. Specifically, laminin is mainly expressed in the early expanding DZ while fibronectin is found with a gradient highest in the FZ and decreasing in the outer layers. Interestingly, the receptors for ECM components--integrin subunits also distribute in different zones of the human fetal adrenal. Integrin α1 chain is found primarily in the DZ, while α2-subunits are found abundantly at the interface of the FZ/DZ. The α3 chain (a low-affinity ligand for fibronectin), however, is observed in the DZ/FZ and FZ. Consistent with this pattern, in vitro studies using primary human fetal adrenal cells cultured on matrix-coated dishes found that the ECM not only contributes to maintenance of cell morphology of primary cultures, but also influences cellular behavior. For example, increased proliferation rates are observed on laminin- or collagen-coated dishes, while fibronectin supports higher levels of apoptosis[78]. Additionally, collagen IV strongly enhances ACTH-stimulated DHEA/DHEA-S as well as cortisol secretion [78, 79]; fibronectin increases DHEA and DHEA/DHEA-S secretion through increased P450C17 gene expression[79]. These studies demonstrate that the unique ECM environment within each region of the fetal adrenal gland supports zone-specific cellular behavior.

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Similar to its effects in the fetal gland, the ECM is also thought to influence behavior and function within the adult adrenal gland. Studies in adult rat adrenal glands have shown varying expression gradients for all four major glycoproteins. In adult rat adrenal glands, collagen I is the major glycoprotein in the outer capsule, with moderate levels of collagen IV, fibronectin, and laminin also present. Collagen IV, fibronectin, and laminin are found surrounding each glomerulosa cell in the zG. In the zG, collagen IV is present as thick fibrils while fibronectin and laminin transition to discontinuous and short fibrils, respectively. Interestingly, the expression of each of these glycoproteins progressively decreased in the inner cortex regions. In the zR, only collagen IV is observed. In addition to varying expression patterns, ECM components also have distinct effects on adult adrenal function in vitro[80]. Specifically, fibronectin, collagen I, and collagen IV increase 3β-HSD expression and subsequent aldosterone production in zG cells as well as corticosterone secretion in zF cells. In contrast, laminin decreased protein synthesis, 3β-HSD expression, and corticosteroid release[80]. Morphological changes are also detected on cells growing on different ECM, indicating potential changes in function as well. Similarly, induction of 11β-hydroxylase and 21-hydroxylase enzyme expression is observed in bovine adrenal cells cultured with ECMs[81]. These results suggest that complex interactions between different matrix gradients likely contribute to the precise control of cellular behaviors within each zone.

2.1.2 Adrenal blood supply and innervation—Three critical arteries supply each adrenal gland: the superior suprarenal artery provided by the inferior phrenic artery, the middle suprarenal artery provided by the abdominal aorta and the inferior suprarenal artery provided by the renal artery. Perhaps due to the energy intensive nature of steroidogenesis, the adrenal glands have one of the greatest blood supplies per gram of tissue of any organ, with up to 60 arterioles entering each adrenal gland. This may account for the high prevalence of cancer metastases found in the adrenal gland [82–84]. Arterial blood first reaches the outer surface of adrenal cortex where the cortical arteries branch to form the subcapsular capillary plexus[85]. Most of the subcapsular plexus become fenestrated capillaries that follow the cord like structures of the ZF down into the ZR before dumping into the medullary veins, which then form the suprarenal veins. Some of the arterioles continue through the cortex and provide blood directly to parts of the medulla. The right suprarenal vein drains into the inferior vena cava and the left suprarenal vein drains into the left renal vein or the left inferior phrenic vein [86–89]. This unique anatomy of blood supply in adrenal cortex provide an interesting spatial gradient of adrenal steroids in different zones and may be a contributing regulatory mechanism for the distinct characters of cells in each zone.

The adrenal gland is also richly innervated, with the majority of nerve plexuses residing in the capsular region. Their fibres mainly originate from the greater splanchnic nerve and associated abdominal plexuses of the sympathetic autonomic nervous system, together with some parasympathetic contributions from the phrenic and vagal nerves. The nerve bundles penetrate the cortex, mostly in association with blood vessels[90]. Similar to the vasculature structure, the majority of these postganglionic nerves terminate in the medulla. While controversial, it has been hypothesized that the innervation of the superficial blood vessels regulates blood flow in the cortical capillary bed. Moreover, in vitro studies have reported

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the regulation of cortical blood flow by neuropeptides released in response to splanchnic nerve stimulation. Specifically, vasoactive intestinal polypeptide and Met-enkephalin increase, while neuropeptide Y decreases, cortical flow rate[91]. Lastly, while of unclear pathophysiologic relevance, these same neuropeptides are present at lower levels in associated nerve fibers in hyperplastic cortical tissue[92].

2.2 Function of adrenal cortical zones

The main function of the adrenal cortex is to produce a variety of steroids hormones. Although each zone uses cholesterol as the precursor molecule, distinct steroids result from differential enzyme expression.

Mineralocorticoids—Angiotensinogen (synthesized in liver) is converted by renin (from the juxtaglomerular cells of the kidney) to Angiotensin I that is later converted to Angiotensin II by angiotensin converting enzyme (ACE) in the lung. Aldosterone is the primary mineralocorticoid produced by zG cells under the control of angiotensin II and extracellular potassium[93–96]. Aldosterone functions as the ligand for the nuclear receptor MR (mineralocorticoid receptor) in target tissues that include the colon, salivary gland and the renal distal convoluted tubule and collecting ducts, where it causes increased reabsorption of sodium and increased excretion of both potassium (by principal cells) and hydrogen ions (by intercalated cells of the collecting duct).

Glucocorticoids—Glucocorticoids are produced by zF cells at a basal level, but can be stimulated by ACTH secreted from the anterior pituitary under stress [97–99]. In humans, cortisol is the main glucocorticoid produced by the adrenal cortex under normal conditions and its actions include mobilization of fats, proteins, and carbohydrates [100–102]. In mice and rats, due to the lack of *Cyp17* gene expression, corticosterone is the major glucocorticoid produced and utilized [103–105]. Once produced and released into the bloodstream, glucocorticoids facilitate the release of energy stores for utilization during stress. Integral to the feedback control of the activated HPA axis, glucocorticoids inhibits production and secretion of ACTH from the HPA axis [106, 107].

Adrenal androgens—In primates, cells in the zona reticularis produce precursor androgens including DHEA and androstenedione[108–110]. DHEA is further converted to DHEA-sulfate by the action of dehydroepiandrosterone sulfotransferase (SULT2A1). These precursors are weak androgens and get released into the bloodstream and are taken up in peripheral tissues that include hair follicles, genital skin, and prostate for production of more potent androgens including testosterone[111–113]. Recent work using human adrenal vein sampling has detected testosterone and other downstream androgen metabolites from the adrenal cortex, suggesting the presence of a wider variety of adrenal androgens than previously appreciated [114]. The regulatory mechanism of adrenal androgen production has not been fully elucidated. However, LH/hCG receptors have been shown to be present in zR[115] and hCG can stimulate DHEA-sulfate synthesis in cultured human fetal adrenals[116, 117]. This suggested that LH/hCG might regulate androgen synthesis in an ACTH-independent manner. Moreover, the illicit action of LH has been implicated in excess cortisol production in primary adrenal macronodular hyperplasia (PMAH) patient[118].

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2.3 Homeostasis of adult cortex

2.3.1 Homeostatic Maintenance of the DZ/adult cortex—After the functional adult adrenocortical zones are established, they are maintained by stem or progenitor cells. Beginning in the 1930s, numerous studies have suggested that cells from the outer region of the adrenal cortex proliferate and migrate to repopulate the adrenal cortex[119–121] Several studies show that most proliferating cells are located in the outer layer of the mature adrenal gland, while most cell death occurs near the boundary of cortex and medulla. These historic data are consistent with the presence of a stem/progenitor cell population in or directly under the capsule of the cortex[122–125].

Shh pathway: In 2009, three laboratories independently utilized lineage-tracing to provide evidence that the Shh signaling pathway was essential for mouse adrenal gland development and maintenance [63, 126, 127]. In these studies, Shh was found in the adrenal gland at E11.5, primarily in the subcapsular zG where stem/progenitor cells reside. In adult adrenal glands, Shh colocalizes with Sf1 in non-steroidogenic cortical cells of the zG region but not in differentiated zG or zF cells, which express both Sf1 and markers of fully differentiated steroidogenic cells (Cyp11b2 and Cyp11b1, respectively). However, descendants of Shh expressing cells express adrenocortical differentiation markers, suggesting Shh-positive cells serve as progenitor cells for the adrenal cortex[63]. Mice in which *Shh* is ablated specifically in *Sf1*-expressing cells reveal marked adrenal hypoplasia, decreased proliferation, and a thin capsule[126]. However, despite a decrease in size, the adrenal glands in these mice maintain proper zonation, suggesting Shh does not have a role in the initiation of differentiation. Together, these data implicate the Shh pathway in actively promoting maintenance of the adrenal cortex.

Wnt pathway: The Wnt/β-catenin signaling pathway is also critical for adrenocortical homeostasis. Studies performed in the mouse adrenal revealed that β-catenin expression and activity is present early in the fetal cortex. By E18.5, with the emergence of the DZ, β-catenin is restricted to the subcapsular zG[128]. Employing a cre-lox technology to ablate β-catenin specifically in Sf1-expressing cells of the adrenal cortex[129], Kim et al observed adrenal aplasia at birth in mice expressing a highly penetrant *Sf1*-cre transgene. Careful examination of these mice showed normal adrenal development until E12.5, after which adrenal failure became evident precisely as DZ cells emerged between the coalescing capsule and the FZ. Intriguingly, in mice that bear a weakly penetrant *Sf1*-cre transgene (express β -catenin in about half of the adrenocortical cells), adrenal development progresses normally. However, as the mice age (i.e. 30 weeks) a progressive cortical thinning and a decreased steroidogenic capacity is observed. This progressive failure of the cortex is hypothesized to be a result of the loss of adrenocortical progenitor cells.

Additional support for a role of Wnt/β-catenin signaling pathway in the maintenance of adrenocortical stem/progenitor cells comes from studies in which the pathway has been genetically “over-activated” in the mouse adrenal cortex. In these studies, both an expansion of undifferentiated progenitor-like cells and ultimate tumor formation are observed. In one model of elevated Wnt signaling, hyperaldosteronism due to zG-specfic expansion is characterized[129, 130]. Moreover, activation of the Wnt pathway is frequently observed in

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adrenocortical neoplasms[131–133]. Although the exact function of Wnt/ β-catenin signaling in the adrenal cortex remains unknown, the fact that β-catenin and Sf1 can directly activate *Dax1* suggests that Dax1 may be a critical mediator of Wnt action in the adrenal cortex[134]. Recent data indicates that Wnt/ β -catenin signaling may additionally regulate adrenal homeostasis by inhibiting fasciculata differentiation, promoting the undifferentiated state of progenitor cells and priming the progenitor cell for zG fate[135, 136].

2.3.2 Homeostasis of cortical zones—Homeostasis of the adrenal cortex also entails the maintenance of zonal structure and function in the context of continual centripetal cellular repopulation of the gland. Because stem/progenitor cells reside primarily in the outer/subcapsular zG, investigators have asked whether differentiated cells first adopt a zG fate and later transition to zF and zR, or alternatively whether the stem/progenitor cells differentiate directly into each of the three cell types?

As previously mentioned, lineage-tracing studies using an inducible GFP marker to track the fate of Shh-descendants revealed that GFP expression was more prevalent in zG cells initially following induction of Shh-GFP. At later times however, an increasing number of GFP-expressing zF cells were observed[63]. These results suggest that the majority of progenitor cells become zG cells first and then transition to zF cells.

More direct evidence for this model comes from recently published work using a zG-specific *Cyp11b2*-promoter-driven GFP knock-in mouse, wherein zG cell descendants are labeled by GFP [137]. In this model, GFP-positive cells are rare in the adrenal gland of newborn mice. However, within three months, the entire cortex becomes GFP-positive, suggesting all cells within the zF and x-zone originated from the zG cells. Furthermore, treatment with dexamethasone, a cortisol analog used to repress ACTH, can prevent zG cells from transitioning to zF cells, manifesting in atrophy of the zF. These results implicate ACTH as an important mediator of zF homeostasis. Surprisingly, when Sf1is ablated in zG cells, the mice are able to maintain a normal functional zF zone, despite the complete absence of zG-derived GPF cells within the zF, suggesting perhaps that progenitor cells can (although they usually do not) directly adopt a zF phenotype without first becoming a *Cyp11b2*-expressing zG cell. (Figure 2)

3. DEFECTS IN CORTICAL ZONAL DEVELOPMENT

3.1 Adrenal hypoplasia

The term “adrenal hypoplasia” refers to distinct clinical conditions characterized by the underdevelopment or hypotrophism of the adrenal cortex. Patients with complete adrenal hypoplasia or aplasia characteristically develop adrenal insufficiency soon after birth, or less commonly, in a more insidious manner throughout childhood or even in young adulthood. Adrenal insufficiency is potentially life-threatening if not promptly recognized and treated.

Based on its physiopathology, adrenal hypoplasia can be divided into two broad categories: primary and secondary. Primary adrenal hypoplasia is characterized by a hypotrophic (and hypofunctional) adrenal cortex in the presence of normal hypothalamic/pituitary function. The causes of primary adrenal hypoplasia can be further categorized into 1) defects of

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adrenal cortex formation or differentiation – a condition known as congenital adrenal hypoplasia (AHC) and 2) "ACTH resistance" and related conditions (also known as familial glucocorticoid deficiency). AHC is usually associated with a severe phenotype, which might also include disorders of sexual development caused by gonadal failure, severe adrenal insufficiency associated with salt-wasting and other developmental disorders[138–141]. The most common form of AHC is the X-linked congenital adrenal hypoplasia due to disruption of the nuclear receptor *DAX1* (OMIM 300200). Mutations of *SF1* are one contributing cause of the autosomal form of AHC[139–141]. Rare autosomal forms of AHC include the IMAGE syndrome (OMIM 600856) and the SERKAL syndrome (OMIM 603490), caused by mutations of *CDKN1C* and *WNT4*, respectively[138, 141]. Familial isolated glucocorticoid deficiency syndromes (broadly known as "ACTH resistance" syndromes) are autosomal recessive diseases caused by mutations in genes that participate in the ACTH signaling. These include mutations in *MC2R* and *MRAP (a gene that codes for* an accessory protein of the ACTH receptor, essential for its targeting and function)[142, 143]. More recently, mutations in the *NNT* and *MCM4* genes were also associated with familial glucocorticoid deficiency [143–145]. Patients with ACTH resistance usually do not present with the salt-wasting phenotype because aldosterone-producing cells are not affected by these mutations.

"Secondary adrenal hypoplasia" is caused by a pituitary or hypothalamic dysfunction that results in the disruption of ACTH synthesis, processing and/or release by the pituitary gland. Thus the adrenal zF becomes hypotrophic and hypofunctioning due to the lack of adequate stimulus. Causes of secondary adrenal hypoplasia include 1) developmental abnormalities of the CNS such as anencephaly, septo-optic dysplasia (OMIM 182230) and holoprosencephaly/Pallister-Hall syndrome 2 spectrum (OMIM 615849), the last two caused by mutations in *HESX1* and *GLI2*; 2) diseases of pituitary development such as pituitary combined hormone deficiency syndromes (caused by mutations in *PROP1, POU1F1, OTX2, LHX3 and LHX4*); 3) isolated ACTH deficiency (OMIM 201400) (caused by *TBX19* mutations)); 4) defects in *POMC* and neuroendocrine convertase 1 (*PCSK1*), which leads to deficiencies in all POMC-related peptides[146, 147]; and 5) miscellaneous non-genetic causes that lead to hypothalamic or pituitary dysfunction such as idiopathic, intracranial tumors, infections or birth-related traumatic injuries[143, 148–151].

Considerable progress has been made in the understanding of the genetic basis of many of these forms of adrenal hypoplasia in the past 20 years. These breakthroughs have been very important to allow for counseling families and screening of other family members at risk of adrenal failure, as well to provide insight into the molecular mechanisms of adrenal development in humans. However, for a significant percentage of cases of primary adrenal hypoplasia, the underlying cause remains unknown.

3.2 Disorders of excessive growth

3.2.1 Adrenal hyperplasia—Disorders of the adrenal cortex characterized by excessive growth include hyperplasias and tumors. Hyperplasias are invariably polyclonal disorders defined by bilateral enlargement of the adrenal cortex. Adrenal hyperplasia can be secondary to ACTH overstimulation (also referred as ACTH-dependent hyperplasia), such as in Cushing's disease or congenital adrenal hyperplasia. (CAH). The latter is a common

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denomination of a heterogeneous group of autosomal recessive disorders that affects the function of key enzymes of the steroidogenic cascade, resulting in deficient cortisol production. As a consequence, the pituitary produces higher amounts of ACTH, which leads to 1.accumulation of steroid precursors before the enzymatic defect and 2.increase in adrenal size, ultimately leading to nodules formation. The symptoms will depend on the level of the enzymatic blockage (which in turn will determine which type of steroid precursors will accumulate) and the severity of the defect (how much does it impair cortisol production). The most common form of CAH is the 21-hydroxylase deficiency (*CYP21A2*), accounting for more than 95% of the cases[152]. More than 100 mutations have been described in this disease and a strong genotype-phenotype correlation is observed[153]. The classical form is characterized by the presence of virilization at birth with genital ambiguity on the female fetuses (due do androgen precursor accumulation) and severe adrenal insufficiency, that might be aggravated by mineralocorticoid deficiency in the salt-wasting forms[154]. The non-classical form is usually characterized by mild signs of hyperandrogenism that frequently goes unnoticed until adulthood[154]. Other types of CAH (each of these with distinct clinical manifestations) may result from mutations in other genes that code for enzymes of the steroidogenesis cascade, including *STAR, CYP11A1, HSD3B2, CYP17A1, CYP11B1* and *POR*[155–157]. Primary causes of adrenal hyperplasias are by definition ACTH-independent. However, intra-adrenal ACTH production has recently been described in some cases of primary adrenal hyperplasia, suggesting that some of the growth might be mediated by paracrine/autocrine ACTH stimulation from the hyperplastic tissue itself[158]. Primary adrenal hyperplasias are often associated with a genetic syndrome. Some of these syndromes, such as Multiple Endocrine Neoplasia type 1 (MEN1), Gardner syndrome, McCune-Albright syndrome and Carney complex, are characterized by multiple organ involvement. In other syndromes, such as primary adrenal macronodular hyperplasia (PMAH) and isolated primary pigmented micronodular adrenal hyperplasia (PPNAD), the adrenals are the only organs affected[159, 160]. Both PMAH and PPNAD have been described in familial and isolated forms. Recently, germline mutations in *ARMC5* have been described in about half of the PMAH cases, being the most frequent genetic alteration associated with this disease. Remarkably, the prevalence of germline *ARMC5* mutations is high even among patients with apparent sporadic disease, suggesting that familial forms are actually much more prevalent than previously appreciated. Regarding the endocrine manifestations, the adrenal hyperplasia can either functioning or non-functioning. The most common endocrine manifestation is Cushing syndrome, but rare cases of mineralocorticoid and androgen production have been reported[161, 162]. Abnormal activation of the protein kinase A (PKA) pathway by different molecular mechanisms, such as ectopic expression of G-protein-coupled receptors by adrenal cells, activating somatic mutations in *GNAS*, inactivating mutations of *PRKAR1A* (the gene that codes for one of the regulatory subunits of the PKA complex), inactivating mutations of *PDE11A* and *PDE8B* and gene amplification of a catalytic subunit of the PKA complex (*PRKACA*), is usually present in the hormonally-active group[163, 164]. (Table 1)

3.2.2 Adrenal tumors—Adrenocortical tumors (ACTs) are common neoplasms especially in older adults, in whom the prevalence can reach up to 6% of the population[165]. Unlike the hyperplasias, ACTs are usually unilateral, although bilateral tumors are not an infrequent

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finding[165, 166]. The majority of them are benign and non-functioning adenomas (ACA) and incidentally found during abdominal imaging for unrelated reasons (being also designated "incidentaloma" in this setting). ACAs can also be hormonally active, being able to secrete cortisol (Cushing syndrome), aldosterone (Conn syndrome) or androgens[165, 166]. Recently, the molecular mechanisms that lead to the formation of both aldosterone-producing adenomas (APA) and cortisol-producing adenomas (CPA) have been elucidated by next-generation sequencing approaches. APAs are the second most common cause of idiopathic (non-familial) primary aldosteronism, accounting for ∼30% of the cases (hyperplasias are the leading cause, accounting for 70%) [167154]. Molecularly, APAs are characterized by somatic mutations in several genes involved in the regulation of the intracellular calcium concentration, leading to abnormal activation of the calcium-calmodulin-dependent protein kinase 1/II (*CAMK1/2*), the main regulators of angiotensin-II and potassium-stimulated *CYP11B2* transcription[154, 155]. Somatic mutations in *KCNJ5* have been identified in approximately 40% of the APAs[168]. Interestingly, heterozygous germline *KCNJ5* mutations have also been described as the cause of familial primary aldosteronism type III[169]. This gene encodes an inward-rectifying potassium channel (GIRK4) localized at the plasma membrane, which is responsible for the maintenance of the resting membrane potential, by regulating the potassium efflux. *KCNJ5* is highly expressed in normal adrenals, specifically at ZG and outer ZF with its activity stimulated by AngII. Mutations in *KCNJ5* increase its permeability to potassium, favoring membrane depolarization (which in turn lead to the opening of membrane voltage-gated calcium channels, increasing the intracellular calcium concentrations resulting in activation of the *CAMK1/2*)[170, 171]. Subsequently, mutations in other genes that regulate intracellular calcium concentrations have been described in APAs, including mutations of the sodium/potassium-transporting ATPase subunit alpha-1 (*ATP1A1*), the plasma membrane calcium-transporting ATPase 3 (*ATP2B3*) and the voltage-dependent L-type calcium channel subunit alpha-1D (*CACNA1D*). Taken together, somatic mutations in these genes are present in ∼15% of APAs. Importantly, mutations in these genes are mutually exclusive, reinforcing their causative role in intracellular calcium homeostasis[172]. Cortisol-producing adenomas are characterized by abnormally high levels of PKA activation[163]. Recently, three different groups have described the recurrent somatic activating mutations of a catalytic subunit of the PKA (*PRKACA*) in a large subset of cortisol-producing adenomas[173–175]. This mutation leads to a defective protein-protein interaction with the regulatory subunit (encoded by the gene *PRKAR1A*), causing its constitutive activation.

The malignant counterparts of ACAs, adrenocortical carcinoma (ACC), however, are rare tumors with an incidence of one case per 0.5–2 million per year[176, 177]. A bimodal age distribution has been described, with a first peak occurring early in childhood and a second one around the fourth of fifth decades. Clinically, around 60% of ACCs are hormonally active and unlike ACAs, they can secrete more than one class of steroids (e.g., cortisol + androgens)[178, 179]. ACC is a highly malignant tumor with few therapeutic options once surgical cure cannot be achieved. At the time of diagnosis, about 50% of the patients will present with a stage III (locally advanced) and IV (metastatic) disease. Even after a complete surgical resection in early stage disease, the recurrence rates are remarkably high[178–180]. In comparison to other types of tumors, little is known about the molecular pathogenesis of

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ACTs. Much of the current knowledge comes from the understanding of the molecular basis of rare cancer syndromes of which ACC is one manifestation, although the great majority of ACCs are sporadic[163].

3.2.3 Molecular pathways involved in adrenal tumorigenesis—The few studies focusing on the clonality of human ACTs have concluded that, while adenomas are either monoclonal or polyclonal, carcinomas are primarily monoclonal, indicating that they are derived from a single cell that undergoes transformation and clonal expansion after a set of mutational events[181, 182]. Although there are no clear data to identify the origin of this initiating cell, whether it is a stem, progenitor or even differentiated cell, it is reasonable to expect that mutations in genes involved in maintenance of stem cell properties are critical for the above transformation process. As mentioned in previous sections, the IGF system and the Wnt pathway are of key importance for the maintenance and homeostasis of adrenal stem cell and progenitor cell populations. In fact, abnormalities of the IGF system and of the Wnt pathway are present in a large subset of ACCs, as will be discussed below.

Wnt/β-Catenin: Adrenal hyperplasia and tumors are manifestations of the Gardner syndrome[183, 184]. This syndrome is caused by germline mutations of the *APC* gene, which is part of a multiprotein complex (destruction complex) that ubiquitinates cytoplasmatic β-catenin, targeting it to proteosomal degradation[185]. Without the proper function of the destruction complex, β-catenin accumulates in the cytoplasm and is translocated to the nucleus where it activates Wnt pathway target genes. Thus, nuclear accumulation of β-catenin, as observed by immunohistochemistry, is an indicator of pathway activation. Studies have shown that about a third of both ACAs and ACCs exhibit nuclear β-catenin immunostaining[186]. Accordingly, somatic activating *CTNNB1* mutations are also observed in both ACAs and ACCs, suggesting that some ACAs may be precursor lesions of ACCs [186, 187]. Animal models further corroborate these observations. A study using a transgenic mouse model with constitutively active β-catenin specifically expressed in adrenocortical cells shows elevated Axin2 levels and β-catenin scattered throughout the cortex and in clusters of cells at the cortical/medullary boundary[130]. Although differing in degree, these mutant mice all exhibit adrenal hyperplasia and dysplasia, and disruption of normal adrenal zonation, with zG cells expanding into the zF. By 10 months of age, Sf1 expression can be found in the medulla region, suggesting that these abnormal cells are resistant to apoptosis. More importantly, all the mice between 5–10 months of age show increased Vegf expression, and by 17 months, some females develop large masses with malignant characteristics, such as high proliferation rate and decreased *Sf1* expression[130].

These results suggest that abnormal activation of canonical Wnt signaling pathway can lead to an expansion of the stem/progenitor cell compartment. Alternatively, autonomous Wnt signaling in a differentiated cell results in the acquisition of stem/progenitor cell characteristics and coincident cellular expansion. Ultimately, imbalances in proliferation, differentiation and apoptosis of these cells favor tumor formation. Further extending the importance of the Wnt pathway in adrenocortical tumorigenesis, biallelic inactivation of *ZNRF3* (an ubiquitin ligase that is a negative regulator of the Frizzled receptor), have been described as the most common changes in a large cohort of adrenocortical carcinomas[188].

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Interestingly, these mutations are mutually exclusive with those of the *CTNNB1* gene, further suggesting that either mutation resulting in elevated Wnt signaling can have a causative role in adrenocortical tumorigenesis.

IGF2: As discussed before, the IGF system has an important role in adrenal organogenesis and homeostasis. The link between abnormal activation of the IGF system and adrenocortical tumorigenesis came from observations of patients with the Beckwith-Wiedemann syndrome (BWS). BWS is an overgrowth disorder characterized by organomegalia, congenital malformations and a predisposition to embryonic tumors and adrenocortical cancer [189]. The cause of this disorder is an imprinting defect at the 11p15 region, which leads to high postnatal levels of *IGF2*[189]. Interestingly, very high expression levels of IGF2 are observed in ∼90% of adult ACC[190–192]. Somatic parental isodissomy of the 11p15 locus have been described in these tumors[193]. Mutant mice overexpressing Igf2 exhibit a BWS-like phenotype, including adrenal hyperplasia and cytomegaly. However, these mice do not develop ACTs[194]. In a recent study, Igf2 overexpressing mice are crossed with mutant mice that have stabilized β-catenin (*Apc* mutant). The offsprings of these mice that exhibit both abnormalities develop bigger tumors than those mice with stabilized β-catenin alone. Additionally, malignant transformation occurred at an earlier age in mice harboring both elevated Igf2 and stabilized β-catenin than in the single mutant mice[195]. Taken together, the data support the role of *IGF2* in tumorigenesis and provide a rationale for molecular targeted therapy. Unfortunately, although preclinical studies indicate that blocking the IGF1R, (one of the most important mediators of the IGF2 mitogenic effects) had anti-growth effects *in-vitro* and *in-vivo*, phase I and phase II clinical studies combining different agents displayed low therapeutic efficacy[196–200]. However, long-term anti-tumor activity was indeed observed in a small cohort of patients. Molecular markers that can predict which patients would respond to IGF1R blockage would be extremely useful for personalized medicine[36, 198, 199].

TP53: The *TP53* tumor suppressor gene is a critical component of the DNA repair and the senescence pathways. The clinical association between *TP53* and ACC was identified in patients with the Li-Fraumeni syndrome (LFS). This syndrome is caused by germline inactivating mutations of the *TP53* and is characterized by various types of early-onset malignant tumors, such as breast cancer, brain tumors and sarcomas[201]. ACC is also a manifestation of the syndrome, occurring in ∼10% of the patients[202]. In sporadic ACC, somatic inactivating mutations of *TP53* are present in a significant proportion of cases (∼25%-30%), usually associated with a more aggressive phenotype[203, 204]. Among sporadic ACC patients, germline *TP53* mutations are estimated to account for ∼5%[205, 206]. However, unlike in adults, the prevalence of germline *TP53* mutations in the pediatric population is high, ranging from 50 to 90%[207–209].

Telomere maintenance machinery: One of the fundamental properties of the stem/progenitor cells is self-renewal, the ability to give rise to two daughter cells, at least one of which is an exact copy of the stem/progenitor cell itself. With each cell division and coincident DNA replication, the telomeres at the end of each chromosome shorten. Unabated, the chromosomes become too short to engage in further DNA replication.

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Moreover, the protective cap on the end of the chromosome is disassembled, leaving the chromosome short and unprotected. Such DNA is recognized as damaged DNA and can initiate the DNA damage response with the cell ultimately undergoing apoptosis or senescence. Therefore to perpetually engage in cell division /self renewal, stem/progenitor cells must maintain telomere length and protect the naked telomere end[210]. This task is accomplished by increased telomerase activity and by maintaining the integrity of the shelterin complex. The shelterin complex is a set of proteins that caps and protects the ends of the chromosomes and regulates the activity of the repair mechanisms and telomerase[211]. Telomere dysfunction is a universal feature in cancer. In order to expand its replicative potential, many cancers overexpress telomerase[212]. A recent study has demonstrated that *TERT*, a gene that codes for the catalytic subunit of the telomerase enzyme, is frequently amplified in ACC[188]. The importance of the telomere maintenance mechanisms for both normal stem cell function and cancer can be exemplified by a mouse model. The adrenocortical dysplasia mouse (*acd*) bears mutations in the *ACD/TPP1* gene, which codes for one component of the shelterin complex[213]. Since the shelterin complex is critical for protecting the ends of chromosomes and ensuing complete replication of coding sequences, loss of function promotes non-proliferative states and may explain some of the phenotypes seen in the *acd* mouse. These mice display early onset adrenal failure, with dysplasia and cytomegaly of the cortex. Interestingly, crossing the *acd* mutant mice with *Tp*53 null mice results in rescue of adrenal senescence and overall dysplasia, highlighting the role of the DNA repair mechanisms in removing cells with dysfunctional telomeres from the pool[214]. Both the *acd/acd* mice and the *Tp*53 −/− mice develop a spectrum of cancers during their lifespan. Interestingly, the cross between the two strains results in offsprings with earlier onset of a larger spectrum of tumors including adrenocortical carcinoma, suggesting that mutations that affect genes involved in telomere maintenance together with cell cycle checkpoint genes may act by synergistic mechanisms[214].

3.2.4 Development and stem cell implications for adrenal cancers—Genetic profiling indicates that most cancers are clonal (derived from a single cell) but cells within a given cancer are heterogeneous in terms of both differentiation state and proliferation potential. The significant heterogeneity within a given cancer may be a result of secondary mutations in daughter cells. Two models have been proposed to explain cancer cell proliferation potentials. The “stochastic model” predicts that all cells in a tumor are biologically equivalent and any heterogeneity is caused by extrinsic/environmental factors; every cancer cell has the ability to proliferate and metastasize[215]. In contrast, the “cancer stem cell” model posits that only a distinct, generally rare subpopulation of tumor cells possess the so-called stem cell potential and the ability to differentiate into multiple cell types in a cancer and originate new metastatic lesions[215]. The properties of such cells theoretically have a profound impact on therapeutics, since they are able to repopulate the entire tumor if they are not targeted by a given treatment. Indeed, subsets of cancer cells with these unique properties have been identified in a variety of cancers, including hematologic malignancies, breast, brain, colon and pancreatic cancers[215]. However, the discovery of cancer stem cell does not rule out the existence of the stochastic model, which may be more appropriate for some tumor types. Alternatively, both models may also coexist in the same tumor, depending on the context (i.e., tumor cells may undergo a phenomenon

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called stem cell plasticity, in which they may fluctuate in a range of different degrees of differentiation and “stemness” when challenged)[216, 217].

Studies have been directed at determining whether ACC expand through the stochastic or cancer stem cell model. Lichtenauer et.al took advantage of the multidrug resistant properties of progenitor cells to study the “side population” of H295R human adrenocortical carcinoma cell line[218]. Cells that are able to efflux the Hoescht 33342 vital dye by increased activity of members of the ATP-binding cassette family have been demonstrated to exhibit stem cell properties in a wide variety of tissues and tumor samples. Based on these properties, these cells can be sorted by FACS after being treated with Hoescht 33342. These cells, which constitute a small fraction of the total cell pool, are consequently called side populations. Side populations derived from the H295R cells exhibited a less differentiated phenotype in terms of steroidogenic enzyme expression. However, in contrast to other cancer stem cells, these cells do not show a difference in growth potential or resistance to cytotoxic drugs compared to the predominant population[218]. Furthermore, after the side population cells are maintained for some time in culture, they give rise to a population of cells similar to the original H295R culture in terms of differentiation and the proportion of cells with properties of side populations. Although these observations argue against the existence of cancer stem cells in the H295R cell line, it does not completely rule out this possibility. Additionally, since the study used a cell line rather than cells directly isolated from ACC patients, it remains unclear if the side population with multidrug resistance properties is a major contributor to ACC initiation and/or maintenance.

As detailed in this review, the most common mutated genes in both adrenocortical hypoplasia and adrenocortical tumors are also stem/ progenitor cell factors critical for proper adrenal development and homeostasis. It is reasonable to hypothesize that defects in the adrenocortical stem cells serve as drivers for a spectrum of adrenal disorders of growth and differentiation. Future studies utilizing novel genomic approaches in tumor samples and model systems should shed insight into the genetic and cellular defects underlying these diseases of the adrenal cortex.

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KEY POINTS

- The human adult adrenal cortex is composed of three different zones: zona glomerulosa (zG), zona fasciculata (zF) and zona reticularis (zR), responsible for production of mineralocorticoids, glucocorticoids, and adrenal androgens, respectively.
- The establishment of the adrenal zG and zF occurs late in fetal development with a transition from the fetal to adult cortex; however, the final completion of cortical zonation in humans does not occur until puberty with the establishment of the zR and its production of adrenal androgens, a process called adrenarche.
- The maintenance of the adrenal cortex involves the centripetal displacement and differentiation of peripheral Sonic hedgehog (Shh) positive progenitors cells into zG that later transition to zF cells and subsequently zR cells.

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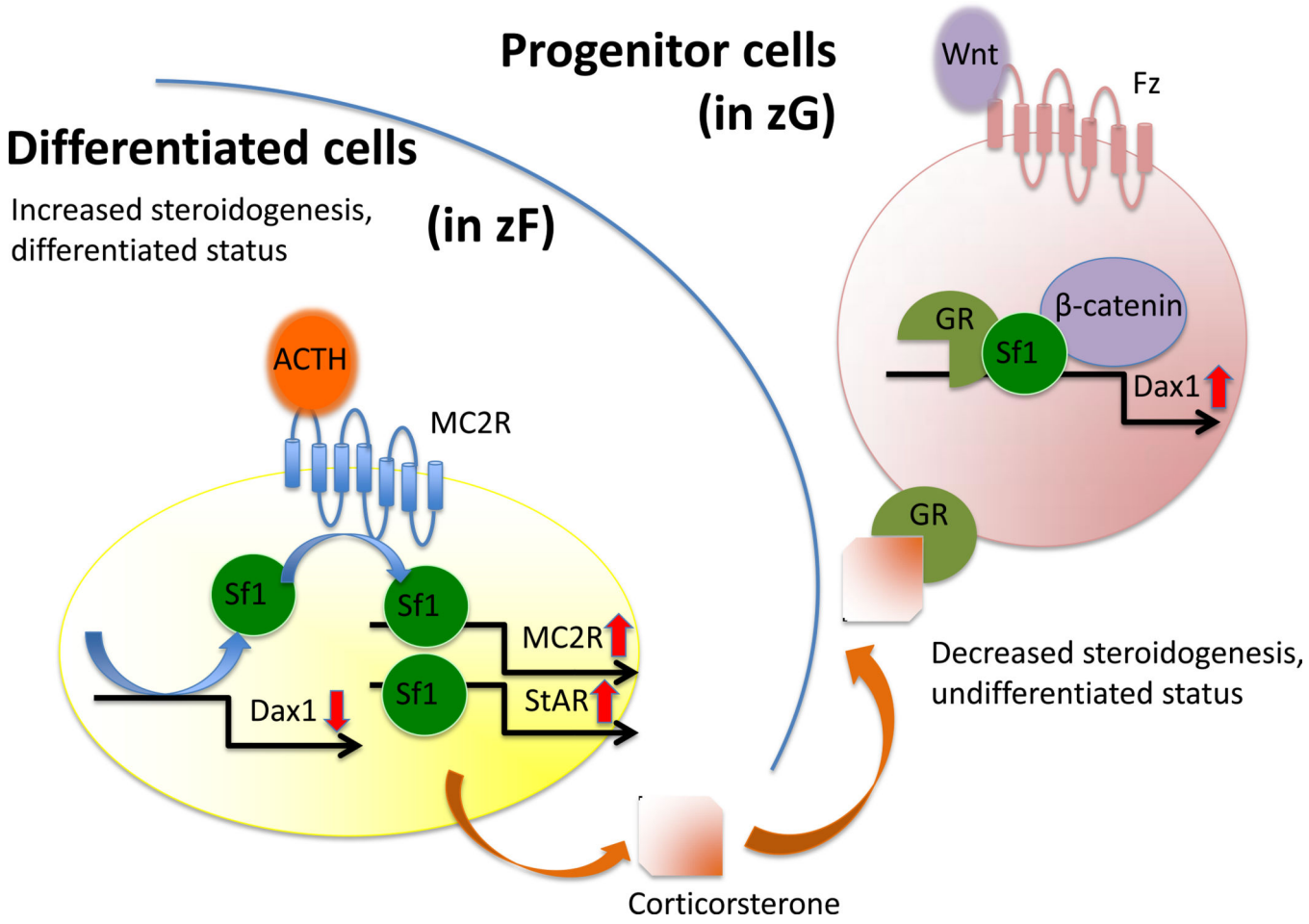

Figure 1.
Interaction of Sf1, GR, β-catenin on Dax1 regulation

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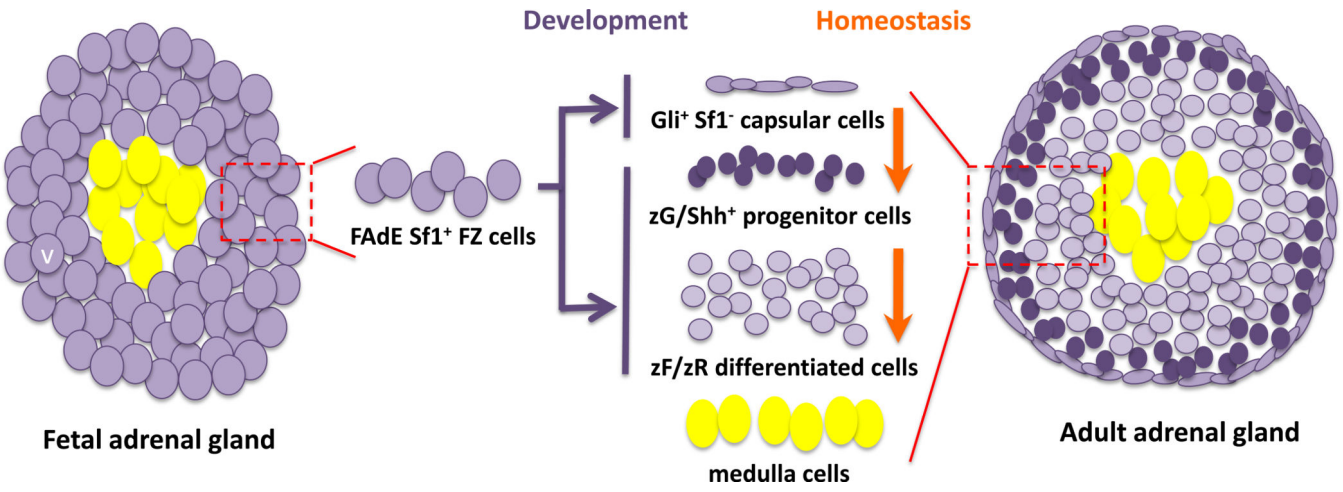

Figure 2.
Stem cell theory of adrenal development and homeostasis

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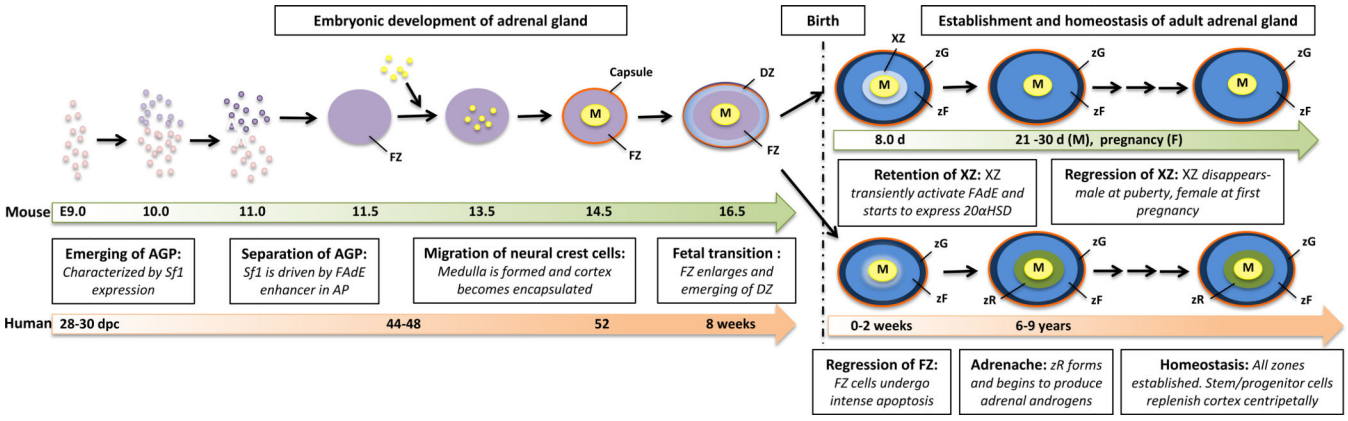

Figure 3.

Development and zonation of the adrenal gland.

FZ: fetal zone. DZ: definitive zone. XZ: remnant of fetal zone (x-zone). zG: zona Glomerulosa. zF: zona Fasciculata. zR: zona Reticularis.

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TABLE 1

Genetic Syndromes Associated with Adrenal Hyperplasia/Neoplasia

Syndrome	Heritage	Locus	Gene	Clinical features	Adrenal manifestations	Comments
Multiple endocrine neoplasia type 1	Autosomal dominant	11q13	MEN1	Primary hyperparathyroidism, gastric, pancreatic, and duodenal neuroendocrine tumors, pituitary adenomas, thymic carcinoid tumors	Non-functioning macronodular hyperplasia in up to 40% of patients. ACCs rarely described	Somatic MEN1 mutations have been frequently described in sporadic ACC; 11q LOH is a frequent finding
Carney's complex	Autosomal dominant	17q22–24	PRKAR1A	Cutaneous lentigens, pituitary adenomas, cardiac myxomas, pancreatic, and cutaneous tumors	Micronodular pigmented adrenal hyperplasia	Somatic PRKAR1A have been described in functioning ACAs; ACCs have been described in patients previouly diagnosed with CC; 17q LOH frequently described in ACTs
McCune–Albright syndrome	Sporadic (post-zygotic somatic mosaicism)	20q13.3	GNAS1	Polyostotic bone dysplasia, gonadotropin-independent precocious puberty, café-au-lait spots, pituitary adenomas	Cortisol-producing bilateral nodular hyperplasia	Activating GNAS1 mutations have been described in cortisol-producing ACAs and PMAH
Gardner's syndrome	Autosomal dominant	5q21–q22	APC	Familial adenomatosis polyposis, increased risk for colon cancer, thyroid tumors, osteomas of the skull	Bilateral adrenocortical hyperplasia in 7–13%	Somatic APC mutations have not been described in sporadic ACTs. Abnormal nuclear β-catenin staining has been described in one-third of ACCs and ACAs
Primary adrenalmacronodular hyperplasia (PMAH)	Sporadic/autosomal dominant	16p11.2	ARMC5/	Intracranial meningiomas have been described in some patients, suggesting that these tumors are also a manifestation of the disease.	Bilateral macronodular enlargement of adrenal glands associated with Cushing's syndrome	Overexpression of GPCRs are virtually omnipresent; intraadrenal ACTH production with paracrine/autocrine stimulation of cortisol production have been described in some cases
Li–Fraumeni syndrome	Autosomal dominant	17p13	TP53	Increased risk for sarcomas, hematologic malignancies, lung tumors, breast tumors	ACCs in 5%	Germline inactivating TP53 mutations are very frequent in pediatric ACCs but rarely seen in adults. Somatic inactivating TP53 mutations are present in 30% of samples
Beckwith–Wiedemann syndrome	Autosomal dominant/sporadic	11p15	IGF2	Organomegalia, omphalocele, microcephalia, mental retardation, fetal neoplasms (Wilm's	ACT in 1.5%	IGF2 overexpression and structural abnormalities of 11p15 are present in up to 90% of sporadic ACCs.

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Syndrome	Heritage	Locus	Gene	Clinical features	Adrenal manifestations	Comments
				tumor, hepatoblastoma, ACC)		