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Adrenocortical Stem and Progenitor Cells—Implications for Adrenocortical Carcinoma

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Abstract

The continuous centripetal repopulation of the adrenal cortex is consistent with a population of cells endowed with the stem/progenitor cell properties of self-renewal and pluripotency. The adrenocortical capsule and underlying undifferentiated cortical cells are emerging as critical components of the stem/progenitor cell niche. Recent genetic analysis has identified various signaling pathways including Sonic Hedgehog (Shh) and Wnt as crucial mediators of adrenocortical lineage and organ homeostasis. Shh expression is restricted to the peripheral cortical cells that express a paucity of steroidogenic genes but give rise to the underlying differentiated cells of the cortex. Wnt/ β -catenin signaling maintains the undifferentiated state and adrenal fate of adrenocortical stem/progenitor cells, in part through induction of its target genes Dax1 and inhibin- α , respectively. The pathogenesis of ACC, a rare yet highly aggressive cancer with an extremely poor prognosis, is slowly emerging from studies of the stem/progenitor cells of the adrenal cortex coupled with the genetics of familial syndromes in which ACC occurs. The frequent observation of constitutive activation of Wnt signaling due to loss-of-function mutations in the tumor suppressor gene APC or gain-of-function mutation in β -catenin in both adenomas and carcinomas, suggests perhaps that the Wnt pathway serves an early or initiating insult in the oncogenic process. Loss of p53 might be predicted to cooperate with additional genetic insults such as IGF2 as both are the most common genetic abnormalities in malignant versus benign adrenocortical neoplasms. It is unclear whether other factors such as Pod1 and Pref1, which are implicated in stem/progenitor cell biology in the adrenal and/or other organs, are also implicated in the etiology of adrenocortical carcinoma. The rarity and heterogeneous presentation of ACC makes it difficult to identify the cellular origin and the molecular progression to cancer. A more complete understanding of adrenocortical stem/progenitor cell biology will invariably aid in characterization of the molecular details of ACC tumorigenesis and may offer new options for therapeutic intervention.

Keywords

stem cell; progenitor cell; adrenal; adrenocortical carcinoma; Sf1; Hedgehog signaling; Wnt Signaling; Gli1; Shh; β -catenin; IGF2; Dax1; inhibin- α ; p53; Pod1; Pref1

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Introduction

A cancer cell represents a genetically altered cell that has acquired pro-growth, anti-apoptotic properties, avoidance of immune detection, and/or a variety of other properties that delineates it from its tissue of origin (for a review of the hallmarks of cancer see (Hanahan and Weinberg, 2011)). While signaling and transcription pathways are shared between cell types, it is a unique combinatorial code of gene expression that defines both a normal and malignant cell as tissue-specific (i.e. an adrenal chromaffin cell and a pheochromocytoma cell derived from an adrenal medullary cell) (Harari and Inabnet, 2011). Therefore, an understanding of the normal development and homeostasis of an organ is essential for the molecular characterization of most cancer types.

Adrenocortical tumors (ACT) are common with benign adrenocortical adenomas (ACA) present in about 3–7% of the population (Fassnacht et al., 2011; Giordano, 2010). Malignant adrenocortical carcinomas (ACC) are rare, accounting for only 0.2% of cancer cases reported annually (Fassnacht et al., 2011; Giordano, 2010). The adrenal gland contains stem/progenitor cells that are responsible for the centripetal repopulation of the adult cortex (Kim et al., 2009). Many of the factors that are proving to play a role in the homeostatic growth of the adrenal gland also appear to be significant in adrenocortical tumorigenesis (See Table 1). Specifically, adrenocortical stem/progenitor biology is proving to be at the crossroads of normal gland maintenance and oncogenic transformation.

Development of the Adrenal Gland

The adrenal gland is two distinct endocrine organs that have separate embryological origins and physiologic functions; the mesoderm-derived cortex secretes steroid hormones while the neural crest-derived medulla secretes catecholamines (Else and Hammer, 2005). Formation of the adrenal gland occurs in several distinct developmental events (Else and Hammer, 2005; Kim and Hammer, 2007). During the 4th week of gestation in humans (E9.0 in mice), proliferation of mesoderm-derived cells of the coelomic epithelia and underlying mesonephros results in coalescence of the adrenogonadal primordium (AGP), defined by expression of the nuclear receptor NR5a1 (Steroidogenic factor 1, Sf1) (Hatano et al., 1996; Luo et al., 1994). At the 8th week of gestation in humans (E10.5 in mice), the bipotential AGP separates into discrete adrenal primordia (fetal adrenal zone) and gonadal primordia (Hatano et al., 1996; Kim and Hammer, 2007). The segregation of a discrete adrenal primordia from the AGP involves a Wilm's tumor 1 (Wt1) and Cited2-mediated upregulation of Sf1 expression (Val et al., 2007). Once separated from the AGP, the adrenal primordia activates Sf1 expression through an entirely different mechanism – the recruitment of the homeobox protein PKNOX1 (Prep1), homeobox gene 9b (Hox) and pre B-cell leukemia transcription factor 1 (Pbx1) to a fetal adrenal-specific Sf1 enhancer (FAdE) (Zubair et al., 2006). Sf1 itself maintains FAdE-dependent expression of Sf1 in the adrenal primordia over time through autoregulation of Sf1 expression. Proliferation of fetal adrenocortical cells is believed to be under control of fetal pituitary-derived adrenocorticotrophic hormone (ACTH) (Mesiano and Jaffe, 1997). However, insulin-like growth factor 2 (IGF2) is expressed throughout the fetal adrenal cortex and several studies have suggested ACTH mediates some of its effects on proliferation through IGF2 action (Coulter, 2005; Ilvesmaki et al., 1993; Mesiano et al., 1993).

Concurrent with activation of FAdE-driven Sf1 expression at embryonic day E11.5–12.5 in mice (equivalent to 8–9th week of gestation in humans), neural-crest-derived chromaffin progenitor cells migrate into the central fetal gland. These cells form the adrenal medulla followed by the coalescence of the mesenchymal capsule around the fetal adrenal gland (Else and Hammer, 2005). Before encapsulation is complete, the development of the

definitive cortex (definitive zone or adult cortex) is initiated between the capsule and the fetal zone. While the fetal cortex ultimately regresses in all species, the timing of regression is species-specific; in humans the fetal zone regression occurs at birth while in mice the zone persists until puberty in males or the first pregnancy in females (Kim et al., 2009). In humans, functional zonation of the adult cortex into unique concentric steroidogenic regions initiates at birth concurrent with the coalescence of the adrenal medulla (Keegan and Hammer, 2002).

Establishment of the Stem Cell Niche

The adrenal capsule has recently been proposed to serve as a stem cell niche/residence for adult adrenocortical stem/progenitor cells that reside within and/or underneath the capsule (Kim et al., 2009; Wood and Hammer, 2011). As such, the formation of the multicellular capsule and its role in the transition from a fetal to adult cortex and ultimately in the homeostatic maintenance of the adult gland has become a critical area of current research. While most histologic studies describe the adrenal capsule as forming from migrating mesonephric mesenchymal cells that subsequently encapsulate the fetal adrenal cortex, for decades, the capsule has been considered a simple scaffold for structural integrity of the gland. Current work indicates that the capsule is indeed more than a static support structure but may contain and/or nurture a dynamic population of adrenocortical stem/progenitor cells that serve as precursor cells for the cells of the definitive cortex (Wood and Hammer, 2011).

The FAdE enhancer defines Sf1 expression in the adrenal primordia (fetal zone). Moreover, the utilization of the FAdE is restricted to the fetal zone and does not regulate Sf1 expression in the definitive cortex (Zubair et al., 2006). Remarkably, lineage tracing studies employing an inducible Cre transgene under control of the FAdE enhancer, reveal that all Sf1(+) cells of the definitive cortex are derived from cells of the fetal cortex in which Sf1 is expressed under control of the FAdE (Zubair et al., 2008). These studies imply that the transition of a fetal adrenal cell to a definitive adrenal cell requires a novel mechanism of activation of Sf1 transcription dependent upon a not yet identified definitive adrenal enhancer.

In apparent contrast, other recent studies detail Sonic hedgehog (Shh) expression in the outer periphery (subcapsule) of the adrenal gland and its downstream effector, Gli1, expressed in the capsule (Ching and Vilain, 2009; Huang et al., 2010; King et al., 2009). During fetal development, Gli1(+), Sf1(−) cells of adrenal capsule, have been shown to differentiate into Sf1(+) cells of the underlying cortex [Fig 2; (King et al., 2009)]. These two different experimental models raise a number of questions including whether these scenarios are mutually exclusive models or whether the Gli1(+), Sf1(−) cells of the capsule are indeed derived from prior Sf1(+) FAdE-utilizing fetal zone cells? Moreover, do the Gli1(+), Sf1(−) capsular cells function as *bona fide* stem/progenitor cells to populate the definitive cortex with new Sf1(+) cells throughout life?

Homeostatic Maintenance

Replenishment of damaged or dying cells is essential for organ homeostasis, implying the existence of adult tissue stem/progenitor cells, which have since been implicated in most tissues and/or organs including bone marrow, skin, liver, small intestine and many others. Historically, the adrenal gland has been shown to also possess regenerative properties in a variety of model systems including growth of rat adrenal explants, enucleation of rat adrenals and subsequent regrowth of a functional gland *in vivo*, and compensatory growth of the remaining adrenal following adrenalectomy (Beuschlein et al., 2002; Perrone et al., 1986; Schaberg, 1955). A continual centripetal displacement of cells from the outer periphery of the cortex to the cortical-medullary boundary indicates the peripheral

generation of new cortical cells throughout life (Kim et al., 2009). Immunohistochemical staining of proliferating cell nuclear antigen (PCNA), a marker of mitotic cells, is only detected in peripheral cells of the cortex (and not the capsule) (Mitani et al., 1999; Schulte et al., 2007). Moreover, in pulse chase experiments, BrdU (Bromodeoxyuridine) and thymidine- H^3 , while initially incorporated preferentially in the outer cortex, exhibit gradual dilution of signal and increasingly centripetal localization over time. This is consistent with proliferation occurring only in cells in the outermost periphery that are centripetally displaced until they undergo apoptosis at the cortical-medullary boundary (Ford and Young, 1963; Mitani et al., 1999). Furthermore, studies on the Shh pathway provide genetic data supporting the existence of stem/progenitor cells within and/or underneath the capsule (Table 1). Huang et al. using an inducible Gli1-Cre transgene crossed with a lacZ reporter showed a centripetal detection of lacZ activity in the adrenal cortex after longer periods of Gli1-Cre activation, suggesting that Gli(+) cells originating in the capsule are centripetally displaced through the cortex (Huang et al., 2010). Using lineage tracing studies, King et al. have shown Gli(+) cells, which do not express Sf1 or markers for differentiated adrenocortical cells, become Sf1(+), Cyp11b2 (+) cells of the Zona Glomerulosa and Sf1(+), Cyb11b1(+) cells of the Zona Fasciculata, providing the first definitive genetic evidence that differentiated cells of the adrenal cortex can arise from the capsule [Fig 2; (King et al., 2009)].

The activation of the Wnt/ β -catenin pathway in peripheral cells of the definitive cortex has been implicated as a crucial factor in the homeostatic maintenance of the gland (Table 1). Using a Wnt-reporter mouse in which a concatamerized Wnt-responsive element fused to an expression vector for LacZ, Kim et al., revealed selective activation of the Wnt signaling pathway in circumferential clusters of peripheral definitive cortical cells under the adrenal capsule [Fig 1A; (Kim et al., 2008)]. Employing a tissue specific knockdown strategy utilizing the Cre-Lox system in which the Cre transgene was under control of the complete Sf1 transcriptional regulatory region, β -catenin was found to be essential for formation and maintenance of the definitive adrenal cortex. Two Sf1 Cre transgenes with differential expression were employed: Sf1-Cre^{Hi} is active in every Sf1(+) cell and hence loss of β -catenin in every Sf1(+) cell while Sf1-Cre^{Low} exhibits a mosaic expression pattern with only a subset of Sf1(+) cells capable of undergoing β -catenin excision (Kim et al., 2008). Complete ablation of β -Catenin in Sf1(+) cells in the Sf1-Cre^{Hi} model results in adrenal aplasia following formation of the adrenal capsule, indicating β -catenin is essential for adrenal development. A loss of BrdU-incorporation in newly forming definitive zone cells of the fetal gland is observed in Sf1-Cre^{Hi} β -Catenin knockout animals indicating that the Wnt pathway (β -catenin) regulates proliferation of this cell population. Furthermore, the adrenal forms normally in the Sf1-Cre^{Low} model but exhibits a progressive thinning of the cortex over time. Subcapsular β -catenin expression is maintained, implying that new Sf1(+), β -catenin(+) cells are continuously being generated, presumably to replace ablated cells lost following the Cre-mediated deletion of β -catenin. The progressive cortical thinning is predicted to be the result of a gradual depletion of the Sf1(+), β -catenin(+) stem/progenitor pool. Collectively, these studies support the role of β -catenin in maintenance of subcapsular stem/progenitor cells in addition to a model in which Sf1(+), β -catenin(+) cells are being continuously generated throughout adult life (Fig 1A,B; Fig 2).

Two unique β -catenin target genes, Dax-1 and inhibin, that serve to regulate adrenocortical stem/progenitor cell pluripotency and adrenocortical versus gonadal fate respectively, highlight the importance of the Wnt signaling pathway in adrenocortical homeostatic maintenance (Fig 1B–E). Dax1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita critical region on the X chromosome, gene 1), the gene mutated in X-linked cytomegalic adrenal hypoplasia congenita, has been molecularly defined by its role as an inhibitor of Sf1-mediated gene transcription, particularly of steroidogenic enzymes [Table 1; (Crawford et

al., 1998; Ito et al., 1997; Yu et al., 1998)]. Dax1 expression is enriched in the subcapsular region, coincident with β -catenin. Moreover, β -catenin and Sf1 bind to the Dax1 promoter and upregulate its expression, suggesting that Dax1 may mediate the effects of β -catenin to maintain the undifferentiated state of subcapsular stem/progenitor cells [Fig 1B,C; (Gummow et al., 2006; Jordan et al., 2001; Kim et al., 2008; Mizusaki et al., 2003; Scheys et al., 2011). Interestingly, Dax1 exhibits a sexually dimorphic expression pattern. Specifically, a broader expression throughout the adrenal cortex of female versus male mice predicts additional Wnt-independent roles of Dax1 in adrenocortical cell biology (Mukai et al., 2002). Young Dax1 knockout mice exhibit an initial enhancement of steroidogenic activity (Scheys et al., 2011) consistent with a loss of Dax1 mediated inhibition of Sf1-dependent differentiation (transcription of steroidogenic enzymes). As the mice age, however, they exhibit a profound loss of subcapsular proliferation, concomitant adrenal dysplasia and attenuation of steroidogenic capacity supporting a model stem/progenitor cell depletion (Scheys et al., 2011; Yu et al., 1998). Recent data confirms that a number of patients with Dax1 mutations exhibit hyperfunctional adrenals prior to development of adrenal failure – consistent with a role of Wnt-mediated Dax1 expression in the regulation of undifferentiated stem/progenitor cells (El-Khairi et al., 2011; Lin et al., 2006).

In addition to a role of Wnt-mediated Dax1 expression in the maintenance of pluripotency of the adrenocortical stem/progenitor cell, the Wnt pathway regulates the adrenocortical (versus gonadal) fate of these cells through regulation of the inhibin- α gene. The expression of inhibin- α , a member of the Transforming Growth Factor- β (TGF β) superfamily of growth and differentiation ligands, is induced by a convergence of Sf1 and β -Catenin on the inhibin- α promoter [Fig 1B,E; (Gummow et al., 2003)]. The adrenal glands of inhibin knockout mice develop differentiated ovarian tissue that secretes estradiol—an effect dependent on elevated levels of luteinizing hormone (LH) following gonadectomy (Looyenga and Hammer, 2006). This binary change from adrenal to gonadal fate is a consequence of a switch from GATA6 expression to GATA4 in the undifferentiated adrenal subcapsular progenitor cells; GATA4 defines gonadal lineages while GATA6 is specific to the adrenal gland (Looyenga and Hammer, 2006). In the normal adrenal, LH induces the subcapsular expression of GATA4 that has the potential to induce a fate switch of the multipotent subcapsular progenitor towards a gonadal fate (Looyenga and Hammer, 2006; Looyenga et al., 2010). However, it is only in the absence of inhibin that TGF β initiates expansion of the GATA4 cells into both theca and granulosa lineages arrayed in *bona fide* functional hormonal follicular structures in the adrenal gland (Fig 1D). In the normal adrenal cortex, inhibin serves to bind to and internalize the TGF β 2 coreceptor β -glycan to inhibit TGF β -dependent signaling and subsequent gonadal differentiation of adrenocortical progenitors [Fig 1D; (Looyenga et al., 2010)].

Steroidogenic Factor 1 in Normal and Neoplastic Adrenocortical Growth

The expression of the orphan nuclear receptor Sf1 defines the adrenogonadal lineages during development as evidenced by gonadal and adrenal aplasia in Sf1 knockout mice and patients with loss-of-function mutations in the Sf1 gene. While emerging data indicate that Sf1(-), Gli(+) capsular cells become Sf1(+) cells of the underlying cortex during development (Fig 2), the role of Sf1 in homeostatic proliferation of the adult gland has been delineated in other studies. The compensatory growth of the adrenal gland following unilateral adrenalectomy of the contralateral gland is dependent on Sf1 as evidenced by the lack of compensatory growth in an adrenalectomized Sf1 heterozygote mice (Beuschlein et al., 2002). The enhanced proliferation of peripheral subcapsular adrenocortical cells in Sf1 overexpressing mice highlights the role of Sf1 in adrenocortical growth homeostasis (Doghman et al., 2007). These data, together with the plethora of data detailing the role of Sf1 as the obligate activator of most steroidogenic enzymes in the adrenal cortex, supports the essential role of

Sf1 in both proliferation and differentiation (steroidogenesis) of the adult gland and predict unique mechanisms of Sf1 activation that preferentially engage transcription of genes that regulate proliferation versus differentiation. The implication of Sf1 in proliferation of adrenocortical cells predicts a potential dysregulation of Sf1 expression in the etiology of ACC (Table 1). Indeed, Sf1 is highly upregulated in ACC and mice with overexpression of Sf1 develop adrenal tumors derived from proliferating subcapsular cells (Almeida et al., 2010; Doghman et al., 2007; Pianovski et al., 2006). Furthermore, Sf1 expression is prognostic for ACC with a higher level of Sf1 expression correlating with shortened overall 5-year survival (Sbiera et al., 2010).

Signaling Pathways in Development and Homeostasis: Implications for Cancer

IGF Signaling Pathway

The insulin-like growth factor signaling pathway has been implicated in the growth of numerous tissue types and its dysregulation is a frequent occurrence in cancer (Ryan and Goss, 2008). IGF2 is normally expressed at high levels in the developing fetal adrenal where it is considered the primary mitogen for early growth of the developing gland (Ilvesmaki et al., 1993; Mesiano et al., 1993). IGF2 expression drops dramatically at birth coincident with fetal zone regression and appears to be expressed postnatally only in the adrenal capsule and the periphery of the cortex (Baquedano et al., 2005; Mesiano et al., 1993). On the contrary, IGF1 expression increases at birth throughout the cortex (Mesiano et al., 1997; Shigematsu et al., 1989). While it is unclear what different roles IGF2 and IGF1 may play in adrenocortical development and homeostasis, the expression pattern of IGF2 is consistent with regulation of proliferation of a more undifferentiated adrenocortical cell type of the developing gland and proliferating stem/progenitor cells of the subcapsular cortex [Fig 1F; (Coulter, 2005; Mesiano et al., 1993)]. Indeed, four separate mouse models in which IGF2 expression is upregulated in the adrenal cortex exhibit larger adrenals with fetal adrenal cytomegaly, fetal adrenal cysts and/or definitive zone hyperplasia (Caspary et al., 1999; Susaki et al., 2009; Weber et al., 1999; Wolf et al., 1994).

Together with the characterization of the genetic mutations in family cancer syndromes in which ACC occurs, the expression analyses of sporadic ACC have been extremely informative in identifying common genetic changes within ACC (Ragazzon et al., 2011). Beckwith-Wiedemann syndrome is a genetic disease that increases susceptibility to a wide range of childhood tumors including ACC, albeit infrequently (Sotelo-Avila et al., 1980; Weksberg et al., 2010). Beckwith-Wiedemann syndrome has been mapped to numerous genetic alterations of the 11p15 locus, which encodes IGF2 [Table 1; (Caspary et al., 1999; DeBaun et al., 2002; Weksberg et al., 2010)]. IGF2 is maternally imprinted and thus expression is limited to the paternal allele. Loss of imprinting of the IGF2 locus and resultant upregulation of IGF2 is frequently seen in BWS with similar epigenetic changes observed in sporadic ACC, suggesting a common mechanism responsible for IGF2 upregulation (DeBaun et al., 2002; Gicquel et al., 2001; Gicquel et al., 1994). Moreover, IGF2 has been consistently identified as the most upregulated gene in both pediatric and sporadic adult ACC [Table 1; (Almeida et al., 2008; Giordano et al., 2009)]. Its cognate receptor, Insulin-like Growth Factor receptor 1 (IGFR1), is also frequently upregulated whereas IGF1 is not (Faical et al., 1998; Kamio et al., 1991). Moreover, ZAC1 (PLAGL1), an additional epigenetically regulated gene involved in regulation of an IGF2 network of imprinted genes that participate in stem cell maintenance, is one of the most downregulated genes in pediatric ACC (Varrault et al., 2006; West et al., 2007). Together these data support a role of the IGF2 signaling pathway in both the normal development and homeostasis of the adrenal cortex together with adrenocortical carcinoma initiation and/or maintenance.

Gli and Shh Hedgehog Signaling

The hedgehog pathway has been implicated in development and maintenance of numerous organ systems and has recently been shown to play a prominent role in establishment of stem/progenitor cells in and/or beneath the adrenal capsule (see Laufer et al in this issue of MCE). In the mouse, Shh is expressed in the several cell layers below the capsule, with the possibility of a few cells expressed within the capsule itself [Fig 1G, Fig 2; (Ching and Vilain, 2009; Huang et al., 2010; King et al., 2009). Patched1 (Ptch1), the receptor for hedgehog ligands, and Gli1 are expressed exclusively within the adrenal capsule, but only within a subpopulation of cells (Guasti et al., 2011; Huang et al., 2010; King et al., 2009). While the restricted spatial expression of Shh and Gli1 suggest that Shh produced within cells in the periphery of the cortex activates signaling in the capsular Gli1 cell, it is unclear what role Shh signaling in the capsule plays in development and/or homeostasis of the definitive cortex. However, Shh has been shown to be required for proper adrenal gland formation. Loss of Shh in development results in adrenal aplasia and concomitant loss of Gli expression within the capsule (Ching and Vilain, 2009; Huang et al., 2010). Furthermore, patients with Pallister-Hall Syndrome (PHS), a rare disease caused by mutations in the Shh pathway transcription factor Gli3, can present with adrenal hypoplasia and aplasia [Table 1; (Hall et al., 1980; Kang et al., 1997)]. Constitutive activation of hedgehog signaling has been implicated in many cancers, most notably medulloblastoma (Ng and Curran, 2011). However, in published cDNA profiling studies of ACCs, Gli and Shh do not appear to be upregulated when compared to ACA and normal adrenals (Giordano et al., 2009). Whether a Gli1 or Shh expressing cell is a cell of origin of ACC or a signature of a rare cancer stem cell in ACC is unknown.

Wnt/ β -Catenin Pathway

The Wnt/ β -Catenin pathway has emerged in recent years to be a major regulator of both adrenocortical homeostasis and tumorigenesis. Wnt signaling is initiated through binding of Wnt ligands to cognate Frizzled (Fzd) receptors which results in inactivation of GSK-3 β /APC/Axin degradation complex and subsequent stabilization of the active signaling molecule, β -Catenin, which translocates to the nucleus, and binds to TCF/LEF transcription factors to ultimately initiate transcription of Wnt-responsive target genes [Fig 1A,B; (Polakis, 2007)]. β -catenin is expressed exclusively in the peripheral adrenal cortex of the developing and adult adrenal gland raising the question as to the origin of adrenal-specific Wnt ligands (Kim et al., 2008). While a number of Wnt signaling components such as the inhibitor Dkk3 and the ligands Wnt 2b, Wnt 4 and Wnt11 are expressed in the adrenal cortex, the expression of Wnt2b in the periphery of the gland suggests potential signaling from capsule or cortical periphery to underlying cortex (opposed to cortical to capsular signaling of Shh) (Lako et al., 1998; Lin et al., 2001; Suwa et al., 2003). The slow disappearance of the adrenal cortex in adrenal-specific β -catenin null mice, together with the restricted subcapsular activation of β -catenin suggests a role of Wnt ligands in maintaining adrenocortical homeostasis through regulation of peripheral stem/progenitor cells [Fig 2; (Kim et al., 2008)].

Dysregulation of Wnt/ β -catenin signaling has also been implicated as an initiating event in adrenocortical tumorigenesis. Adenomatous polyposis coli (APC), a critical component of the β -catenin destruction complex is the causative mutation in familial adenomatous polyposis, a colon cancer syndrome that frequently manifests with ACTs [Table 1, (Marchesa et al., 1997; Smith et al., 2000)]. Because loss of APC results in stabilization and constitutive activation of β -catenin, it might be predicted to be a possible mechanism for the nuclear accumulation of β -catenin seen in sporadic ACA and ACC [Fig 1A,B; (Gaujoux et al., 2010)]. However, APC mutations are rare in sporadic ACC, perhaps because APC acts

as a tumor suppressor and requires inactivation of both alleles (Gaujoux et al., 2011; Gaujoux et al., 2010).

Indeed, it is not APC but β -catenin that is commonly dysregulated in sporadic ACTs, both benign ACAs and malignant ACCs. 15–25% of ACA and ACC have been shown to exhibit stabilized nuclear β -catenin (El Wakil and Lalli, 2011). While a majority of these ACTs with nuclear β -catenin have been found to harbor activating mutations on β -catenin, a subset do not, suggesting perturbation of additional upstream mechanisms of Wnt activation (beyond APC and β -catenin mutations) are involved in the constitutive stabilization of β -catenin (Bonnet et al., 2011; Gaujoux et al., 2008; Tissier et al., 2005). For example, in the highly inbred mouse strain DBA2/J, in which gonadectomy has been shown to induce subcapsular hyperplasia followed by *bona fide* adrenocortical tumorigenesis, a loss of the Wnt antagonist and putative tumor suppressor SFRP1 (Secreted Frizzled-Related Protein 1) is observed in the post-gonadectomy ACTs suggesting a dysregulation of Wnt signaling [Fig 1A; (Bernichtein et al., 2008)]. Moreover, in a recent screen of a large cohort of human cancers from a variety tissue types including ACTs, a downregulation of SFRP1 was observed [Table 1; (Dahl et al., 2007)]. Unfortunately, a retrospective review of previous ACC cDNA microarray studies does not support SFRP1 as a greatly downregulated gene in sporadic ACC with the caveat that SFRP1 expression has not been specifically analyzed in the cohort of ACC exhibiting abnormal β -catenin nuclear accumulation (Giordano et al., 2009).

The lack of β -catenin mutations in the majority of ACAs (75–85%) suggests that there are indeed other mechanisms of ACT initiation and progression. Moreover, the presence of β -catenin mutations in both benign ACA and malignant ACC suggests that Wnt/ β -catenin dysregulation is not sufficient for ACC formation. Indeed, the forced constitutive expression of activated β -catenin in the mouse adrenal cortex results in adrenal hyperplasia and benign tumors in young mice and even local invasion in aged mice, a feature typical of malignant ACTs (Berthon et al., 2010). Together these data support the hypothesis that β -catenin might serve as an early genetic abnormality that initiates hyperplastic growth of adrenocortical cells but is not sufficient to drive development of malignant neoplasm.

It is interesting that in a recent mouse model of adrenal-specific constitutive β -catenin stabilization, most β -catenin(+) cells do not colocalize with markers of differentiated adrenocortical cells further supporting β -catenin's role in conferring an undifferentiated, stem/progenitor-like phenotype to adrenocortical cells (Berthon et al., 2010). However, whether such a sporadic disease-causing mutation in the human adrenal normally arises in an undifferentiated subcapsular progenitor cell that normally responds to Wnt ligands and/or in a differentiated cell that normally does not exhibit activated Wnt signaling is unknown. Dysregulation of Wnt signaling by multiple mechanisms is predicted to be sufficient to define a progenitor-like fate capable of proliferating and accumulating additional harmful genetic perturbations, such as upregulation of IGF2, that participate in malignant transformation.

Other Factors in Homeostatic Maintenance and Cancer

P53

The *TP53* gene, which encodes the versatile p53 protein, is the most frequently mutated gene in all cancers with a frequency of ~50% thus aptly earning its nickname “the guardian of the genome” (Vousden and Prives, 2009). p53 modulates the expression of an impressive array of genes (~100 have been identified as direct p53 targets) to ultimately engage in a variety of functions (cell cycle inhibition, DNA repair, apoptosis, and senescence) aimed at protecting against the propagation of harmful mutations (Riley et al., 2008; Vousden and Prives, 2009). ACC is a defining cancer of the hereditary autosomal dominant Li-Fraumeni

syndrome, which results from germline mutations in p53 [Table 1; (Malkin et al., 1990)]. p53 acts as a tumor suppressor and, in most cases, both *TP53* alleles must acquire loss of function mutations in order for the gene to be silenced. Li-Fraumeni syndrome patients inherit one nonfunctioning allele thus increasing the probability of a second mutation occurring *de novo* and potentially resulting in ACC (Latronico et al., 2001). Interestingly, a unique germ-line mutation in p53 (R337H) in Southern Brazil results in a markedly increased incidence of adrenocortical cancer yet a paucity of other Li-Fraumeni cancers (Ribeiro et al., 2001; Varley et al., 1999).

Approximately 25% of sporadic ACC exhibit mutations in p53 while benign ACAs do not exhibit p53 mutations, suggesting that p53 may be a late event in the potential transformation of a benign to malignant adrenocortical neoplasm [Table 1; (Ohgaki et al., 1993; Ragazzon et al., 2010; Reincke et al., 1994)]. The relatively low proliferative rate of the adrenal cortex and hence propagation of initiating carcinogenic events targeting both alleles may explain why the frequency of p53 mutations in sporadic ACC is lower than other types of cancers (similar to the low incidence of SFRP1 and APC mutations). p53 is likely not sufficient for such transformation either, as p53 knockout mice fail to develop ACC without additional genetic events such as telomere dysfunction (despite 100% penetrance of tumor development in a wide range of tissues in p53 null mice) (Donehower et al., 1992; Else et al., 2009; Jacks et al., 1994). However, since p53 loss is not observed in ACA, mutations in p53 may be important for malignant transformation in some ACC patients.

Pod1

Pod1 (Tcf21, Capsulin, Epicardin) is a bHLH transcription factor that may play a significant role in adrenal homeostasis (Lu et al., 1998; Robb et al., 1998). Pod1 is expressed in the outer periphery of the adrenal cortex during development as shown by *in situ* hybridization (Quaggin et al., 1998; Tamura et al., 2001). In the adult mouse, Pod1 is expressed exclusively in the capsule as shown by using a mouse with a lacZ reporter under control of the Pod1 regulatory region (Kim et al., 2009; Lu et al., 1998). Pod1 has been shown to suppress the transcriptional ability of Sf1 and it has been suggested it may directly regulate Sf1 expression within the adrenal capsule (Cui et al., 2004; Tamura et al., 2001). Indeed, in Pod1 knockout mice, ectopic Sf1 expression is seen in the capsule and a more intense Sf1 staining pattern is observed throughout the cortex (Kim et al., 2009). In the gonad, enhanced expression of Sf1 in Leydig cells in Pod1 knockout mice is provocative and suggests a similar mechanism may be occurring the adrenal capsule [Fig 2; (Cui et al., 2004; Tamura et al., 2001)].

Pod1 is downregulated in ACC in addition to several other cancers such as melanoma, lung, and HNSCC (Arab et al., 2011; Giordano et al., 2009; Smith et al., 2006). The fact that 1) Sf1 is overexpressed in ACC, 2) Sf1 overexpression can lead to increased proliferation of adrenocortical cells, and 3) Pod1 is downregulated in ACC, suggests that a Pod1(+), Sf1(−) cell may develop a mutation leading to increased generation of Sf1(+) progenitors (Doghman et al., 2007; Giordano et al., 2009). However, Pod1 downregulation may simply be secondary to an expansion of Sf1(+) cortical cells. Interestingly, it has been shown that Pod1 is epigenetically silenced in lung and HNSCC (Smith et al., 2006). In melanoma, it has recently been shown that epigenetic silencing of Pod1 results in a loss of transcriptional inhibition of a gene involved in metastasis (Arab et al., 2011). Currently there is no information on the epigenetic status of Pod1 in normal or pathologic adrenal or the contribution of Pod1 to the development of ACC.

Pref1

Pre-adipocyte factor 1 (Pref1/Dlk1/FA1/PG2/ZOG), a transmembrane protein related to Notch receptors and ligands, was defined as a critical mediator of the maintenance of undifferentiated adipocyte progenitors (preadipocytes) (Smas and Sul, 1993). Pref1 expression in fetal liver and a subpopulation of adult hepatic oval cells (putative liver progenitor cells) in a liver regeneration model in the rat suggests a role in tissue homeostasis in multiple organ systems (Tanimizu et al., 2004; Yanai et al., 2010). Indeed, Pref1 was also identified as a markedly upregulated transcript in the subcapsule of the rat adrenal cortex during regeneration following enucleation (Halder et al., 1998; Okamoto et al., 1997). Other studies have confirmed Pref1 expression in the peripheral cortex of the adult adrenal gland (Floridon et al., 2000; Okamoto et al., 1997). These data suggest Pref1 expression may be an important factor to the maintenance of quiescence of many types of stem/progenitors including those within the adrenal cortex.

While it is unknown whether Pref1 expression participates in adrenocortical tumorigenesis by conferring a progenitor-like phenotype upon adrenocortical tumor cells, Turanyi et al. detected Pref1 protein in 100% of the 32 ACA and 5 ACC samples. However, no differences in Pref1 expression, staining pattern, or correlation with clinical data could differentiate ACA from ACC (Turanyi et al., 2009). The observed expression of Pref1 and nuclear β -catenin in the periphery of the adrenal cortex together with the role of both proteins in progenitor cell biology in multiple systems supports the early acquisition of adrenocortical stem/progenitor-like properties as a component of adrenocortical tumorigenesis. Furthermore, both proteins show a lack of stratification of expression in ACA versus ACC.

Cellular Origin of ACC

In addition to the acquisition of mutations that instill upon tumors the properties of stem/progenitor cells, it is equally plausible that such properties are the result of the cell of origin of these tumors being an adrenocortical stem/progenitor cell. For example, the presence of nuclear β -catenin found in ACA and ACC might reflect a subcapsular progenitor-like cell of origin while elevated IGF2 expression in pediatric ACC might be an indication of a fetal adrenal-like cell of origin in those cancers.

The relaxed genomic state of a rapidly dividing DNA-duplicating adrenocortical stem/progenitor cells may increase the probability of accumulating harmful genetic changes, since these alterations would be promoted to successive generations of cells that might result in the eventual manifestation of a malignant phenotype. The cancer stem cell model posits that only a small minority of cells within a tumor is capable of providing all the various types of cells within a heterogeneous tumor and has the ability to induce tumor recurrence in cancers that appear to be grossly eliminated (Korkaya and Wicha, 2010). However, a cancer stem cell does not necessarily have to be derived from a normal adult stem cell. No studies have identified cancer stem cells within ACC. Regardless of whether a rare population of cancer stem cells are present in a subset of ACC, an understanding of normal adrenocortical stem/progenitor cells may give insight into the various aspects of adrenocortical tumorigenesis. The lineage of a Sf1(+) cortical cell from a Gli(+), Sf1(-) capsular cell implies that any mutation acquired in a capsular cell could be propagated to all subsequent generations of differentiated adrenocortical cells. Indeed, clonal analysis of ACT suggests a monoclonal origin; mutations arise within a single cell while additional genetic hits occur in subsequent cellular generations ultimately resulting in polyclonal cancers (Beuschlein et al., 1994; Gicquel et al., 1994). Furthermore, β -catenin stabilization may confer a transiently amplifying progenitor-like cell fate with a characteristic undifferentiated molecular signature upon adrenocortical cells that will allow them to accumulate additional genetic mutations. Whether the steroidogenic activity of the large percentage of ACC that present with

hormone excess such as Cushing's syndrome (hypercortisolism) reflects a more differentiated cellular origin of the tumor (i.e. Zona Fasciculata cell), remains unknown (Stratakis and Boikos, 2007). Indeed, the increased expression of Sf1 observed in many ACCs might be predicted to induce steroidogenic enzyme expression regardless of basal expression of these enzymes in the cell of origin.

As such, it is currently unknown where or how adrenocortical carcinogenesis is initiated. Numerous zonal-specific factors have been identified with prominent roles in normal and dysregulated adrenocortical cell growth. Given the knowledge of the structure and function of the adrenal gland, three potential origins can be postulated: 1) cell autonomous mutations of stem/progenitors cells of the capsule or non-cell autonomous effects of such a mutation, 2) mutations in transiently amplifying stem/progenitors of the subcapsule 3) mutations in differentiated adrenocortical cells of the Zona Glomerulosa, Zona Fasciculata or Zona Reticularis. Although not mutually exclusive, these scenarios require rigorous preclinical testing and model validation before concluding singular or multiple cells of origin of adrenal tumorigenesis.

Concluding Remarks

The current body of literature supports the hypothesis that stem/progenitor cells reside within the capsule/subcapsule of the adrenal. A great deal of work remains to fully characterize these cells, their lineage relationships and the coincident signaling and transcription pathways involved in organ homeostasis. However, since the turnover rate of the adrenal gland is relatively slow, defining adrenocortical lineages *in vivo* is relatively time consuming. Similarly, due to the rarity of ACC and its clandestine progression *in vivo*, molecular characterization of adrenocortical tumorigenesis remains superficial. Despite these drawbacks, a remarkable understanding of ACC has emerged in recent years. β -catenin stabilization and its constitutive transcriptional activity appear to be necessary for initial adrenal tumor formation but IGF2 overexpression may be required for driving ACT cells towards a malignant phenotype. Wnt/ β -catenin dysregulation is proposed to be an early event in the development of ACT since the frequency of nuclear accumulation of β -catenin is comparable in both ACA and ACC though it is insufficient to drive formation of ACC. IGF2 upregulation is unique to ACC versus ACA consistent with a later role in oncogenic transformation. In pediatric ACTs, however, IGF2 expression alone is unable to distinguish benign from malignant neoplasm, suggesting that in children, IGF2 dysregulation may be a much earlier genetic event in the transition from benign to malignant adrenocortical tumors (Rosati et al., 2008; West et al., 2007). Interestingly, in mouse models of colorectal carcinoma, APC mutations and subsequent constitutive β -Catenin activation have been shown to induce tumorigenesis yet progression to carcinoma is dependent on IGF2 expression (Harper et al., 2006). Data supporting this interaction in ACC is currently lacking. However, it is unlikely that only β -catenin and IGF2 are sufficient to drive ACC generation. The heterogeneous classification of ACC suggests panoply of dysregulated adrenal biology and most likely a host of less prominent factors. An increased understanding of adrenocortical stem/progenitor cell biology is predicted to advance insights into the pathology of ACC by helping to characterizing the shared molecular footprint of normal and dysregulated growth of adrenocortical cells.

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Abbreviations

ACC	adrenocortical carcinoma
ACA	adrenocortical adenoma
ACT	adrenocortical tumor
FAdE	fetal adrenal enhancer

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Highlights

- A population of stem/progenitor cells centripetally repopulates the adrenal cortex.
- Recent data suggest a dual fetal and capsular origin of the adult adrenal cortex.
- Stem/progenitor cell fate is regulated in part by Wnt-activated Dax1 and inhibin- α .
- Activated β -catenin and elevated IGF2 are implicated in adrenal tumorigenesis.
- Stem/progenitor cell-like and/or fetal adrenal-like alterations contribute to ACC.

Figure 1.

Signaling Pathways in Adrenocortical Subcapsular Stem/progenitor Cell. **A.** Wnt ligands secreted from the periphery of the adrenal gland (adrenal capsule and peripheral cortex) acts on subcapsular stem/progenitor cells to activate canonical Wnt signaling (secreted inhibitors such as Wifs and SFRPs are predicted to attenuate Wnt activation) resulting in an inhibition of the degradation complex composed of APC, Gsk-3 β , Axin, and DVL and subsequent stabilization of β -catenin. **B.** Nuclear accumulation of stabilized β -catenin results in formation of β -catenin/Sf1 transcriptional complexes that induce expression of Dax-1 and Inhibin. **C.** Dax-1 inhibits the ability of Sf1 to transactivate steroidogenic enzymes such as Cyp11 β 1, Cyp11 β 2, and 3 β -hsd and prevents differentiation into subsequent differentiated adrenocortical lineages of the Zona Glomerulosa Zona Fasciculata and Zona Reticularis respectively. **D.** Inhibin binds to and internalizes β -glycan thus blocking activation of the TGF β 2/TGF β -R2 complex and hence **E.** preventing the expansion of LH-dependent Gata4 expressing cells poised for gonadal differentiation. **F.** IGF2 secreted from the capsule binds to the Igf-1R to activate Akt and downstream signaling involved in cell proliferation and survival. **G.** Shh is secreted from the subcapsular cell to activate Hedgehog signaling in the capsule.

Figure 2.

Hypothesized Stem/progenitor cell Populations in the Adrenal Capsule and Subcapsular Cortex. Gli(+), Sf1(−) stem/progenitor cells residing in the capsule have been shown to differentiate into cells of the cortex (King et al., 2009). Pod1(+), Sf1(−) are hypothesized to be an additional population of stem/progenitor cells. However, It is unclear if Gli1 and Pod1 are expressed in the same cell or comprise mutually distinct populations. Shh(+), Sf1(+) stem/progenitor cells residing within the outer periphery of the adrenal cortex and have been shown to give rise to differentiated adrenocortical cells of the Zona Glomerulosa [Cyp11β2(+)] and the Zona Fasciculata [Cyp11β1(+)] (King et al., 2009). It is unclear if Shh(+) cells are derived from Gli1(+) capsular cells or are of fetal adrenal origin. β-Catenin(+), Sf1(+) cells are essential for maintenance of subcapsular stem/progenitor cells but it is unknown if Shh and β-Catenin are expressed within the same population of cells.

Table 1

Molecules involved in adrenocortical development, homeostasis, and disease.

Molecule	Expression	Adrenal Disease	Function	Refs
Shh	Peripheral Subcapsular Cortex	None	Required for adrenal development and differentiation of Shh(+), Sf1(+) cells into all other Sf1(+) adrenocortical cell types	(Huang et al., 2010; King et al., 2009)
Gli	Capsule	None	Gli(+), Sf1(-) cells differentiate into all other Sf1(+) adrenocortical lineages during fetal development	(King et al., 2009)
Gli3	Capsule	Mutated in Pallister-Hall Syndrome; Adrenal hypoplasia and aplasia	Unknown, proposed similar function as Gli1	(Kang et al., 1997)
β -catenin	Peripheral Subcapsular Cortex	Nuclear accumulation and mutations in ACA and ACC	Role in maintenance of stem/progenitor cells, initiation of adrenocortical tumorigenesis	(Berthon et al., 2010; Kim et al., 2008; Tissier et al., 2005)
APC	Cortex	Mutated in Familial Adenomatous Polyposis, loss of expression in sporadic ACC	Attenuation of Wnt/ β -catenin signaling in adrenocortical cells	(Gaujoux et al., 2010)
SFRP1	Cortex	Loss of expression in sporadic ACA and ACC	Attenuation of Wnt/ β -catenin signaling in adrenocortical cells	(Dahl et al., 2007)
Sf1	Cortex	Mutated in Adrenal Hypoplasia, upregulated in childhood and adult ACC	Master regulator of steroidogenesis; regulation of adrenocortical cell proliferation	(Doghman et al., 2007)
IGF2	Capsule/Peripheral Subcapsular Cortex	Upregulated in majority of ACC; Mutated in Beckwith-Wiedemann Syndrome	Regulation of fetal adrenal cell proliferation	(Almeida et al., 2008; Giordano et al., 2009; Mesiano et al., 1993)
Dax1	Peripheral Subcapsular Cortex	X-linked Cytomegalic Adrenal Hypoplasia Congenita	Repression of Sf1 transcriptional ability; Maintenance of stem/progenitor cells	(El-Khairi et al., 2011; Scheys et al., 2011)
p53	Cortex	Mutated in Li-Fraumeni syndrome and sporadic ACC	Protection of genomic integrity, prevention of the accumulation of mutations	(Malkin et al., 1990; Ribeiro et al., 2001)