ROLE OF β-CATENIN SIGNALING IN ADRENOCORTICAL DEVELOPMENT AND TUMORIGENESIS

by

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TO DAD, MOM, AND YOUNGER BROTHER CHRIS
FOR THEIR LOVE, SUPPORT, AND SACRIFICES.

AND TO MAIKE FOR LOVE AND SUPPORT.

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I owe great and sincere gratitude to my wonderful mentor, Gary Hammer. I am in debt to you for giving me the chance to become your student. The positive environment as well as academic and emotional support that you provided since the first day allowed me to constantly challenge myself as a scientist. Thank you so much for providing me with such wonderful guidance, motivation, encouragement, and friendship. This thesis exists because of you.

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My group of best friends, David, Thai, Neethan, Nick B., Nick C., and Geewah, thank you guys for constant encouragements and unconditional support.

PREFACE

The work described in this thesis represents both peer-reviewed and manuscript-in preparation, first author data that I have generated as a graduate student at the time of my thesis defense – April 21, 2009. For Chapter 2, Anne Reuter (University of Texas – Southwestern Medical Center, Dallas, TX) performed experiments for the embryonic studies involving the Sf1-Cre^{high} transgenic mice and the southern blotting. I performed all other experiments and generated data represented. For Chapter 3, I performed all of the experiments and generated data represented. For Chapter 4, I performed all the experiments and generated data represented.

Chapter 1 represents hypotheses that were tested during my graduate studies. This chapter was published in *Mol Cell Endocrinol. 2007 Feb; 265-266:10-6.* and in *Endocrine Reviews, May 2009 Special Stem Cells Issue ER080039*. I was the first author of both reviews. Chapter 2 investigates the effect of loss of Wnt/β-catenin signaling on adrenal gland development and tissue maintenance. This chapter was published in *Development. 2008 Aug;135(15):2593-602.* I was a first co-author along with Anne Reuter. Chapter 3 examines the role of constitutive activation of Wnt/β-catenin signaling in adrenal gland cell fate determination and tumorigenesis. This chapter constitutes a manuscript in preparation for submission, and I will be the first author. Chapter 4 investigates the role of constitutive Wnt/β-catenin activation on gonadal development, primarily male development, and tumorigenesis. This chapter is also in preparation for

submission, and I will be the first author. All the work was performed under the guidance of Gary D. Hammer.

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ROLE OF β-CATENIN SIGNALING IN ADRENOCORTICAL DEVELOPMENT AND TUMORIGENESIS

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Wnt/β-catenin signaling plays many important roles during mammalian development. In the adrenal cortex, perturbations in the signaling pathway are associated with abnormal development and adrenocortical tumorigenesis. Loss- and gain-of-function mutations in *Wnt4* result in profound defects in embryonic specification of the mouse adrenal cortex, metanephric kidney, and gonad. Moreover, stabilization of β-catenin has been reported in sporadic adrenocortical carcinoma (ACC) as well as ACC associated with Gardner Syndrome. In order to gain further insights into the role of β-catenin in the adrenal gland development and tumorigenesis, we utilized a conditional knockout (KO) approach. Mice harboring homozygous floxed-β-catenin or floxed-APC alleles were crossed to transgenic mice containing adrenal specific Cre drivers (Sf1-Cre^{low} and Sf1-Cre^{high}). Inactivation of β-catenin mediated by *Sf1-Cre^{high}*, a transgene expressed at high levels, caused adrenal aplasia in newborn mice. Analysis of fetal adrenal development

with *Sf1-Cre*^{high}-mediated β-catenin inactivation showed decreased proliferation in presumptive adrenocortical precursor cells. In contrast, the *Sf1-Cre*^{low} transgene effected a lesser degree of β-catenin inactivation that did not affect all adrenocortical cells, permitting adrenal survival to reveal age-dependent degeneration of the cortex. Conversely, to examine the role of constitutive activation of β-catenin, we performed conditional knockout of *Apc* alleles (Apc^{high} KO and Apc^{low} KO) using an adrenal-specific Cre-expressing transgenic mice, *Sf1-Cre*^{high} and *Sf1-Cre*^{low}. In Apc^{high} KO, we observe an abnormally developed adrenal that resembles an embryonic adrenal gland. These adrenals are small in size and never fully maturity. On the other hand, Apc^{low} KO reveal an initial expansion of less differentiated aberrant cells. These cells exhibit high cytoplasmic and nuclear concentration of β-catenin, coincident with an increased proliferation. At >30 weeks of age, these mice presented with adrenocortical adenomas and later presented with adrenocortical carcinomas. These results define critical roles for β-catenin in both embryonic development of the adrenal cortex, in the adult organ maintenance, and tumorigenesis.

CHAPTER 1

INTRODUCTION

ADRENAL CORTEX: DEVELOPMENT AND CANCER

In 1883, Gottschau described the processes of adrenal gland replenishment from the cells of an outer germinal layer and adrenal cellular breakdown in the "zona consumptive" at the interface of the adrenal cortex and medulla (1). In 1909, Bongomolez confirmed these findings and observed that proliferation in the adrenal cortex was restricted to the subcapsular gland, and that cells from this region "migrated" centripetally to populate the inner cortex. In the late 1930's, as Edward Kendall was making great contributions to the field of clinical endocrinology by defining the syndrome of primary autoimmune adrenal failure and purifying the major adrenocortical hormones, respectively, basic researchers were uncovering the regenerative potential of the adrenal capsule/subcapsular unit through a series of innovative enucleation and lineage tracing studies. While these studies provided seminal observations in support of stem and/or progenitor-like cells in the adrenal cortex, work in this area was soon eclipsed by the emergence of powerful cell and molecular techniques that were applied to the growing field of steroidogenesis. Only recently as gene-targeting technology has emerged to apply molecular approaches to whole organ studies have scientists begun to readdress the questions raised by our scientific predecessors of the early 1900s. What are

the mechanisms of adrenocortical cellular replenishment and maintenance? What are the mechanical and chemical stimuli that induce subcapsular proliferation? What is the relationship of the adrenal capsule to the proliferating subcapsular cells? In addition to the contribution of these cells to the development and the maintenance of the adrenal cortex, do these cells play a role in pathogenic states of the organ, namely hypoplasia and cancer? Such questions are at the heart of the burgeoning field of adrenal biology. In this thesis, I will address the concept of adrenocortical somatic progenitor cells through an examination of potential roles of such cells in development and homeostatic maintenance of the organ as well as their contributions to developmental pathogenesis and tumorigenesis. Specifically, I will detail the contribution of the WNT/β-catenin signaling pathway in adrenocortical development and carcinogenesis as well as its role in gonadal development and carcinogenesis.

ADRENAL ANATOMY

The adrenal gland, a component of the hypothalamic-pituitary-adrenal (HPA) axis, is a major hormone-secreting organ. The gland is composed of two functionally distinct organs (Figure 1.1). The medulla, derived from neural crest cells of neuroectoderm lineage, synthesizes catecholamines that facilitate the acute mammalian stress or "fight-or-flight" response. The cortex, derived from the cells of the intermediate mesoderm, synthesizes steroid hormones that mediate body homeostasis and chronic stress responses. The cortex is organized into three concentric zones, zona glomerulosa (zG), zona fasciculata (zF), and zona reticularis (zR), each responsible for the production of different steroid hormones. In 1866, Arnold first described the zonal organization of

the cortex with nomenclature that is still in use today (2, 3). The cells of the zG are organized in rounded clusters around capillary coils or "glomeruli" and synthesize mineralocorticoids. The cells of the zF synthesize glucocorticoids and are arranged in radial rows separated by trabeculae and blood vessels. The cells of the zR are arranged in a uniform reticular net of connective tissue and blood vessels and synthesize the neurohormone dehydroepiandosterone (DHEAS) and a subset of sex steroid precursors. Although histological and functional differences exist between the adrenal cortices of various mammalian species (mainly the absence of zR and the presence of the fetal/X-zone in some rodents), common developmental principles appear to mediate the formation and homeostatic maintenance of the gland (2-4).

ADRENAL GLAND DEVELOPMENT

Adrenal gland development in the mammal is defined by discrete histological events (Figure 1.2) (4-6). The first milestone is marked by the proliferation of the mesoderm-derived, Nr5a1-expressing (Nuclear Receptor Subfamily 5, Group A, Member 1: Sf1 or Ad4BP) coelomic epithelia and underlying mesonephric mesenchymal cells, forming the embryonic adrenogonadal primordia (AGP) which resides between the primitive urogenital ridge and the dorsal mesentery (5, 7, 8). Although *Sf1* expression has proven essential for the formation of the adrenogonadal primordial, a variety of loss-of-function studies indicate that additional transcription factors including pre-B-cell leukemia homeobox 1(Pbx1), odd-skipped related 1 (Odd1, Osr1), and polycomb group protein M33 also participate in specification and/or expansion of the AGP (9-11). The bilateral AGP then divides into the adrenal primordia (adrenal blastema, often referred to

as the fetal adrenal or fetal zone) and the gonadal primordia. The molecular specification of the adrenal primordia is initiated through the upregulation of *Sf1* expression by Wilm's tumor 1 (Wt1) and Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (Cited2) (12). Upon separation of the adrenal primordia from the AGP, a different transcription complex containing the homeobox protein PKNOX1 (Pknox1, Prep1), homeobox gene 9b (Hox9b), and Pbx1 is recruited to maintain fetal zone expression of *Sf1*. Subsequently, Sf1 itself provides feed forward activation of its own expression in this compartment (13). Coincident with this transcriptional cascade is the coalescence of the mesenchymal capsule around the fetal cortex (5, 8). Once encapsulation is complete, the development of the definitive cortex (definitive zone or adult cortex) becomes evident between the capsule and fetal cortex (Figure 1.3). In a previous review, we provided two distinct possibilities for the origin of the adult cortex (8). First, the adult cortex arises from the precursors from the mesenchymal capsule (8). Second, the fetal cortex contains precursors, which gives rise to the adult cortex (8). From our recent studies on Wnt signaling detailed in later chapters of this thesis (14) and additional data from the Morohashi's group (8), it is probable that both mechanisms participate in organ development and maintenance of the adrenal cortex. The migration of the neural crest cells to establish the adrenal medulla follows. Upon birth, significant remodeling of the adrenal cortex occurs with regression of the fetal cortex and maturation of the zG and the zF (8). Following maturation of the gland, the adult organ maintains organ homeostasis through constant replenishment of the adrenocortical cells. We hypothesize that cells in the periphery of the gland are adrenocortical somatic progenitor cells that remain mostly quiescent and relatively undifferentiated with the potential to

self-renew or differentiate as dictated by the homeostatic need of the adrenal cortex. Mitogenic stimuli induce these cells to proliferate while differentiation signals (angiotensin II and ACTH) induce differentiation.

ADRENOCORTICAL REGENERATION

The persistent proliferative capacity of the definitive cortex and the observation of centripetal cellular turnover within the cortex support a centripetal-repopulation model of adrenocortical cytogenesis and suggest the possible existence of stem-like cells in the outer compartment of the gland (Figure 1.4) (15-17). Following adrenal enucleation, (removal of the inner content of the adrenal gland leaving the only capsule and underlying subcapsular cells intact), the cortex undergoes a dynamic process regeneration (Figure 1.5) (18-21). By the 8^{th} day following enucleation, foci of newly formed cells spread out beneath the capsule and extend towards the center of the gland. Cells continually proliferate from the capsular/subcapsular region to repopulate the newly forming cortex. The regeneration takes approximately 30 days until the gland assumes a normal histological appearance with proper cortical zonation. This regenerated gland recapitulates the normal steroidogenic functions of the adrenal cortex. Transplantation of primary adrenocortical cells results in the formation of similarly functional adrenocortical tissue within the host animal (Figure 1.6) (22-25). The host animal, often adrenalectomized, survives with physiologic replacement of adrenal function by the transplanted tissue (22-25). Moreover, the tissue resulting from these transplantation studies assumes normal adrenocortical architecture (22-25). Hence, both enucleation and transplantation models support a model whereby a pool of cells within the adrenal cortex

have the ability to regenerate and replenish the adrenal cortex continually throughout the life of the organism. From these studies, it can be presumed that the outer cortical cells are responsible for this replenishment.

CLONAL ORIGIN OF ADRENOCORTICAL CELLS

While enucleation studies indicate that the capsular/subcapsular unit has the capacity to proliferate and differentiate, the experiments have not been designed to establish the lineage relationships between inner and outer cells of the intact cortex. The histological analyses of the adrenal glands of chimeric and transgenic mice (utilizing β-galactosidase reporters under the control of either the cytomegalovirus (CMV) or steroidogenic gene promoter) reveal variegated expression of chimeric or reporter genes in cord-like radial stripes extending from the periphery to the cortico-medullary boundary, consistent with a clonal origin of cells within each radial stripe (Figure 1.7). The data support the hypothesis that the adrenal cortex is maintained through proliferation and clonal replenishment of peripheral cells that undergo centripetal displacement and differentiation in response to endocrine stimulation (26-29).

ADRENOCORTICAL CELL FATE DETERMINATION: WNT/β-CATENIN SIGNALING PATHWAY

As described in the historical data presented above, it is hypothesized that the differentiated adrenocortical cells, with the capacity to synthesize steroid hormones, are derived clonally from pool of presumably less differentiated cells located in the outer periphery of the gland, specifically within the subcapsular region (5, 8). Moreover, loss-

of-function studies involving transcription factors, such as Sf1, Dax1, Pbx1, and Cited2, allowed for identification of important regulators of adrenal gland development. However, tissue-specific assessment of molecular mechanism governing the adrenocortical subcapsular cell fate determination was lacking. With the advent of conditional (tissue-specific) knockout technology, we began to investigate for such mechanisms within the adrenal cortex, specifically the Wnt/β-catenin signaling pathway.

WNT/β-CATENIN SIGNALING PATHWAY

Wnt signaling is subcategorized into distinct intracellular pathways with different molecular mediators and cellular consequences: Wnt/β-catenin signaling, Wnt/planar-cell polarity, and Wnt/Ca2+ pathways (30-33). In this doctoral dissertation, we focus on the Wnt/β-catenin signaling pathway and its role in cell fate determination in the adrenal cortex.

Wnt ligands represent a large family of highly conserved morphogens that are characterized by repetitive cysteine residues (30, 34, 35). Activation of the Wnt/β-catenin pathway occurs through binding of a Wnt ligand to its respective Frizzled (Fzd) receptor, a seven-pass transmembrane receptors with an extracellular N-terminal cysteine-rich domain (CRD), and co-receptor low-density lipoprotein receptor-related protein (LRP) (30, 34, 35). The complex of Fzd and LRP5/6 interact with Dishevelled (Dsh), a cytosolic phosphoprotein, and Axin, a scaffold protein, to stabilize cytoplasmic concentration of β-catenin, the main effector of the canonical Wnt signaling pathway. In the absence of Wnt/Fzd/LRP5/6 complex, a low cytoplasmic β-catenin concentration is maintained by the destruction complex consisting of Axin/adenomatous polyposis

coli/glycogen synthase kinase 3β/casein kinase 1 (Axin/Apc/Gsk3β/CK1). The binding of β-catenin to the destruction complex allows for Gsk3β and CK1 to phosphorylate serine/threonine residues at the N-terminus of β-catenin. The phosphorylated β-catenin is then recognized by the F box/WD repeat protein β-TrCP, an E3 ubiquitin ligase component. As a result, the β-catenin is ubiquitinated and targeted for proteosomal degradation.

Upon Wnt ligand binding, disruption of the degradation complex occurs, presumably through sequestering of Axin to LRP5/6. As a result, increase in nonphosphorylated cytoplasmic β-catenin concentration is observed and, ultimately, nuclear accumulation of β-catenin. Inside the nucleus, β-catenin interacts with members of the lymphoid enhancer-binding factor/T-cell factor (Lef/Tcf) family of transcription factors to activate expression of target genes. Through transcription of target genes, the Wnt/ β-catenin pathway regulates processes such as proliferation, specification of cell fate, stem cell maintenance, and differentiation.

WNT/ B-CATENIN PATHWAY: ADRENOCORTICAL DEVELOPMENT AND MAINTANENCE

Wnt/ β-catenin signaling pathway plays an important role in many developmental processes as well as orchestrating the homeostatic tissue self-renewal for the lifetime of an organism. In organ systems such as gut, skin, and blood, studies revealed the importance of Wnt/ β-catenin signaling in cell-fate determination, specifically the stem/progenitors, during tissue renewal (30, 33). As such, recent studies have suggested that Wnt signaling pathway play a role in adrenocortical development and maintenance.

For example, mice harboring mutation in the *Wnt4* gene displayed aberrant presence of adrenocortical cells in the gonads as well as abnormal differentiation of the adult/definitive cortex (36-38). With focus on the Wnt/β-catenin pathway, we initially examined the temporal and spatial expression of the β-catenin protein as well as the localization of the active β-catenin signaling using the transgenic Wnt reporter mouse lines that expresses β-galactosidase only in cells with active β-catenin signaling. Through the study, we observed specific localization of the active β-catenin signaling to the subcapsular region of the developing and adult adrenal cortex (14). To further examine the role of β-catenin in the adrenal cortex, we utilized the Cre-loxP conditional knockout strategy to inactivate β-catenin alleles in the adrenal cortex, which is detailed in Chapter 1 of this dissertation. The study revealed the essential role of β-catenin in adrenal gland development as well as tissue renewal maintenance in the adult organ (14).

ADRENOCORTICAL TUMORS

Benign adrenal tumors are relatively common with occurrences of 3-7% of the population. Malignant adrenal tumors or adrenocortical carcinomas (ACC) are relatively rare with the incidence rate of ~2 cases per million people per year and representing only 0.2% of cancer deaths in the United States. Although rare, this form of cancer is highly malignant and presents with extremely poor prognosis as a consequence of metastasis or local invasion at the time of diagnosis (39-41).

WNT/ B-CATENIN PATHWAY AND ADRENOCORTICAL TUMORS

The importance of Wnt/β-catenin signaling in normal tissue renewal is what connects this pathway to disease development, such as cancer. For example in the gut, Wnt/β-catenin serves as one of the main regulators of crypt progenitor cell fate. As such, the germline APC mutations, resulting in a truncated and/or non-functional APC protein, are the cause of the hereditary cancer syndrome, familial adenomatous polyposis (FAP), in which patients typically present with innumerable colonic polyps and ultimate colonic cancer. These patients also have increased risks of other cancers in the extracolonic organs including the pancreas, thyroid, and adrenal glands (42-45).

The molecular mechanism of tumorigenesis resulting from APC gene mutations has been widely characterized (42, 46-48). The product of the APC gene functions as a tumor suppressor mainly through regulation of β-catenin protein stability and hence β-catenin-mediated transcription. Inactivating mutations of APC result in upregulation of the β-catenin target genes, with subsequent abnormal growth and tumor formation. Although recent evidence suggests additional functions of APC in cell adhesion, cell polarity and migration, chromosome segregation, and mitochondria-mediated apoptosis, this review focuses on the role of APC as a member of the β-catenin degradation complex (46). Predicated on the known role of APC in the regulation of the canonical Wnt signaling pathway in tissue development and homeostasis, we and others began analyzing the status of β-catenin in sporadic adrenocortical tumors. In earlier work by Tissier et al, an increase in the nuclear/cytoplasmic ratio of β-catenin in sporadic ACC was observed (49). Initially, we began screening a panel of ACC samples for stabilization of β-catenin and observed a subset of carcinoma samples with strong nuclear accumulation of β-catenin indicative of active signaling. Utilizing expression array

profiling, we compared carcinomas with strong nuclear β-catenin staining to carcinomas with only membranous β-catenin staining. In the analysis, we observed marked upregulation of classical β-catenin-mediated transcription targets, but only in ACC samples did we observe increased nuclear β-catenin. Such results support a potential role of active β-catenin signaling in a subset of patients with adrenocortical carcinoma.

To further test a hypothesis that active β-catenin signaling may initiate adrenocortical carcinogenesis through aberrant activation in adrenocortical subcapsular cells, we again utilized a conditional knockout mouse approach. The analysis of the *Apc* knockout mice, detailed in Chapter 2, revealed the presence of aberrant clusters of undifferentiated adrenocortical cells at the cortical-medullary apoptotic boundary and even within the adrenal medulla. The gene expression profile of these adrenals at 9 weeks of age revealed an expansion of cells with active β-catenin signaling but with diminished steroidogenic capacity. With increase in age however, the cells begin to undergo differentiation as evidenced by a substantially upregulated steroidogenic gene profile. Lastly, at 45 weeks of age, these mice present with tumors that are histologically comparable to ACC. As such, we hypothesize that canonical Wnt signaling regulates the proliferation and undifferentiated state of adrenocortical stem/progenitor cells. With constitutive activation of the canonical Wnt pathway, expansion of this population is followed by ultimate cancer formation.

Figure 1.1 Anatomy of Mouse Adrenal Gland. The adrenal gland is composed of mesoderm-derived cortex and of neuroectoderm-derived medulla. The mesenchymal-like capsule (C) surrounds and provides support for the organ. The cortex is further organized into concentric zones, zona glomerulosa (zG) responsible for output of aldosterone and zona fasciculate (zF) responsible for output of corticosterone. In mice, there exists an X/fetal zone (X), which regresses in males following puberty and in females following first pregnancy. The cortex and the medulla are held separate by the cortico-medullary boundary (CM). The cells in the periphery of the cortex, capsular and/or subcapsular, proliferate and then differentiate to continually renew the tissue. The differentiated cells travel towards the CM via centripetal displacement and undergo apoptosis when reaching CM.

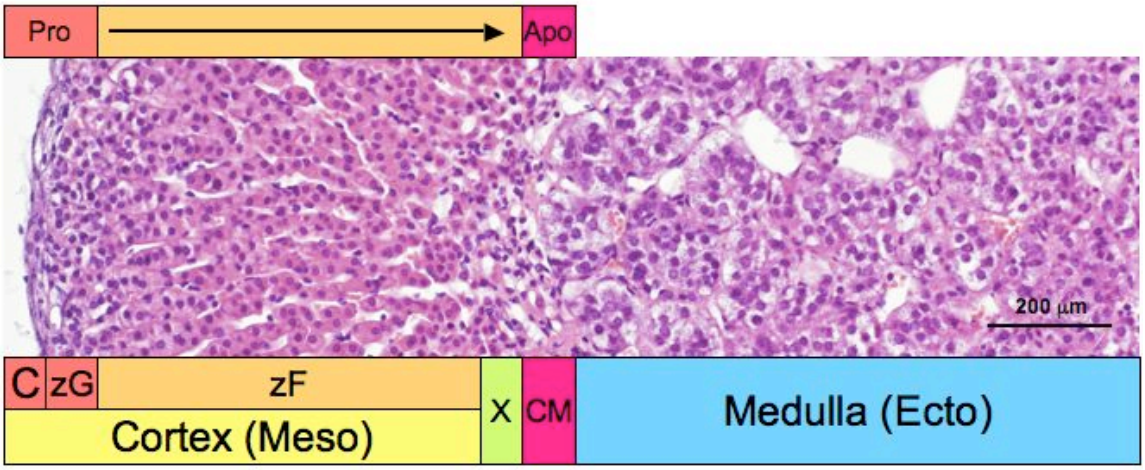

Pro
Apo
200 µm
C
zG
zF
Cortex (Meso)
X
CM
Medulla (Ecto)

Figure 1.2 Progression of Adrenal Gland Development. The condensation of cells from the coelomic epithelium and the intermediate mesoderm form the urogenital ridge, a precursor structure that provides cells for the adrenal, gonadal, and kidney development. Separation of the adrenogonadal primordium from the mesonephros and metanephros is mediated by the expression of Sf1, Dax1, Odd1, and M33. Further separation of the adrenogonadal primordium into the adrenal primordium is initiated by additional expression of *Wnt4*, *Wt1*, and *Cited2*. In embryonic stages, the adrenal primoridum differentiation is initiated by added expression of *Pbx1*, *Prep1*, and *Hox9b*. Immediately, prior to birth and following birth, Pomc-derived peptides drive development of the adult adrenal gland.

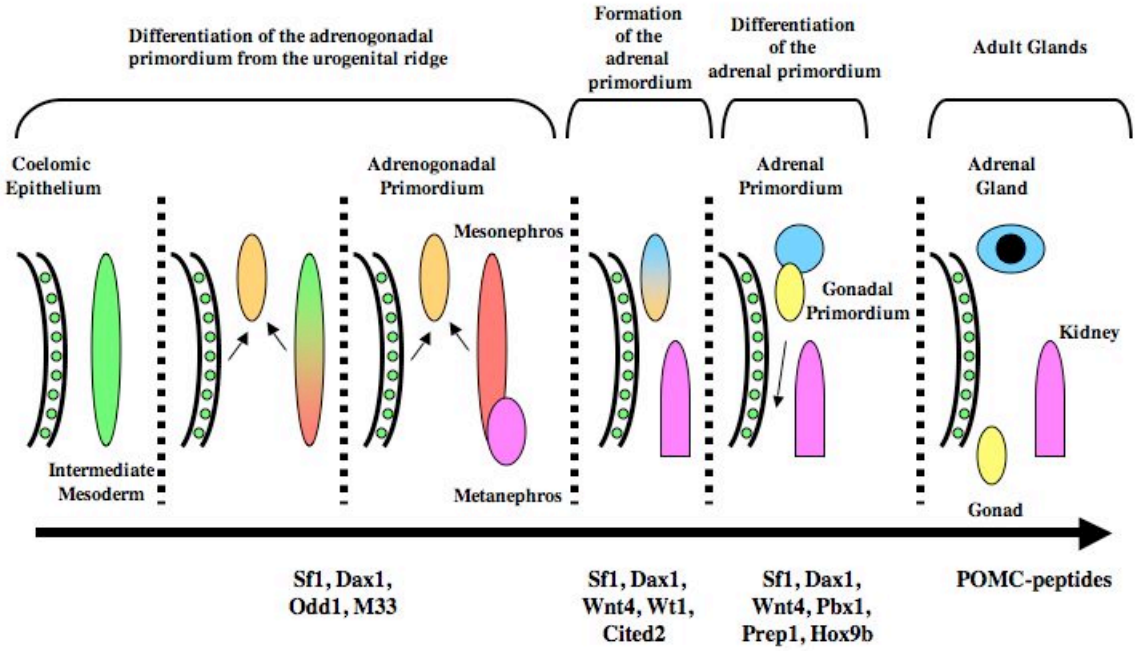

Differentiation of the adrenogonadal primordium from the urogenital ridge
Formation of the adrenal primordium
Differentiation of the adrenal primordium
Adult Glands
Coelomic Epithelium
Adrenogonadal Primordium
Adrenal Primordium
Adrenal Gland
Mesonephros
Gonadal Primordium
Kidney
Intermediate Mesoderm
Metanephros
Gonad
Sf1, Dax1, Odd1, M33
Sf1, Dax1, Wnt4, Wt1, Cited2
Sf1, Dax1, Wnt4, Pbx1, Prep1, Hox9b
POMC-peptides

Figure 1.3 Focused View of Adrenal Gland Development. Model of mammalian adrenal organogenesis detailing the sequential formation of the fetal zone, mesenchymal capsule and definitive zone followed by migration and differentiation of neural crest cells into chromaffin cells of the adrenal medulla and postnatal regression of the fetal zone.

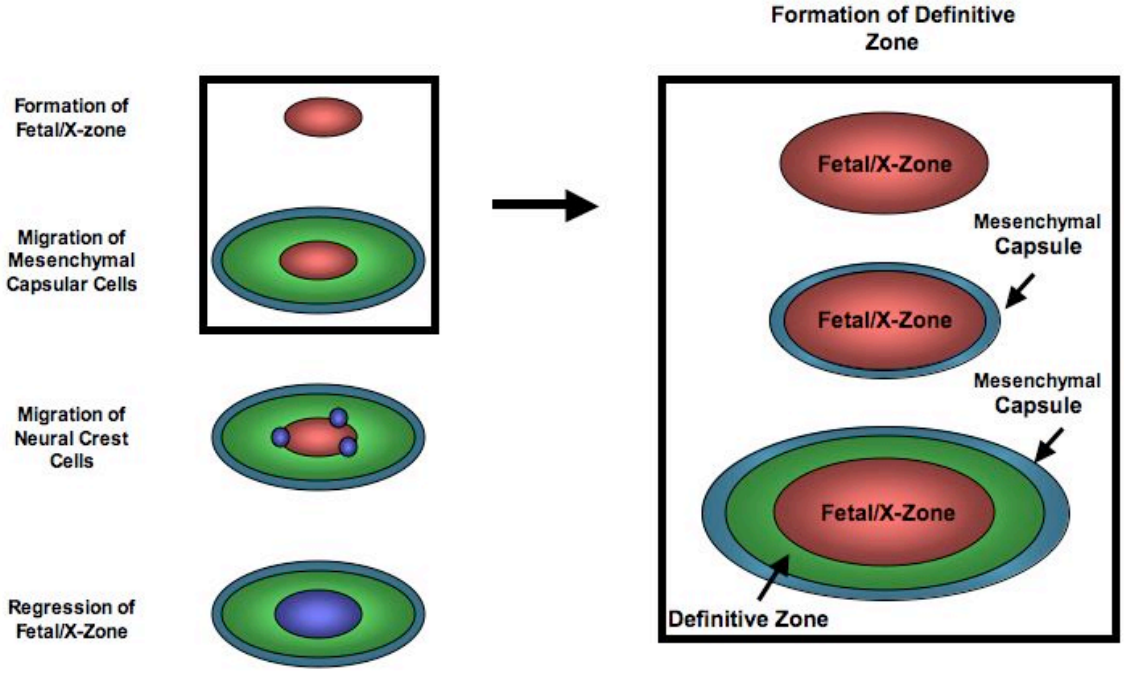

Formation of Definitive Zone
Formation of Fetal/X-zone
Migration of Mesenchymal Capsular Cells
Migration of Neural Crest Cells
Regression of Fetal/X-Zone
Fetal/X-Zone
Mesenchymal Capsule
Fetal/X-Zone
Mesenchymal Capsule
Fetal/X-Zone
Definitive Zone

Figure 1.4 Assessment of Proliferation and Centripetal Displacement of Adrenocortical Cells. Initial pulse with thymidine-H3 and bromodeoxyuridine (BrdU) reveal peripheral adrenocortical cells, capsular and/or subcapsular, undergoing S-phase of the cell cycle. Exposure or staining of the tissue, following initial pulse called "chase", at various timepoints reveal centripetal movement of adrenocortical cells. Adapted from Ford, JK. et al. (thymidine-H3) (16) and Mitani, F. et al. (BrdU) (17).

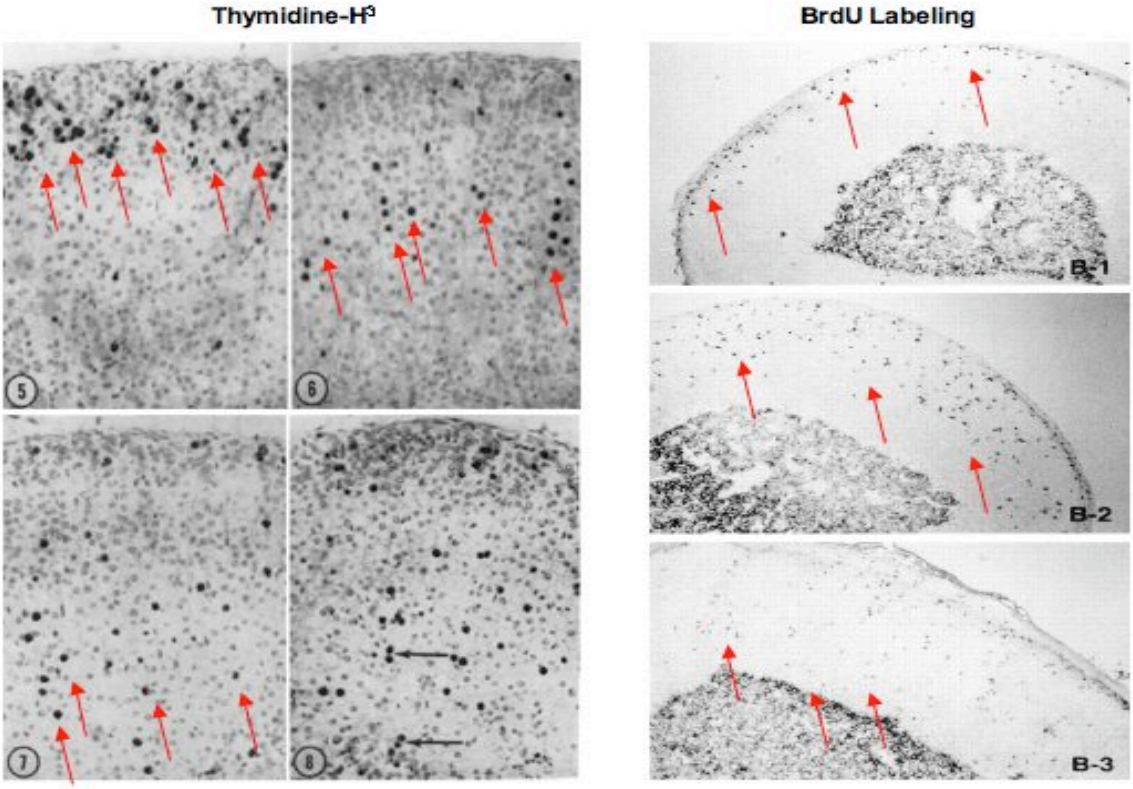
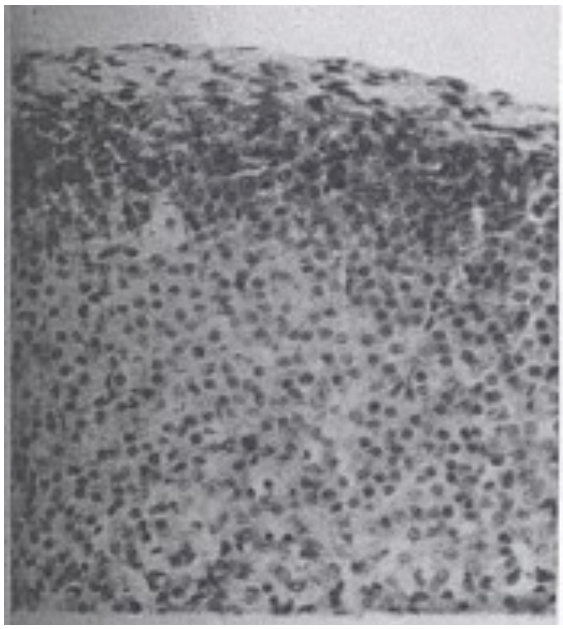
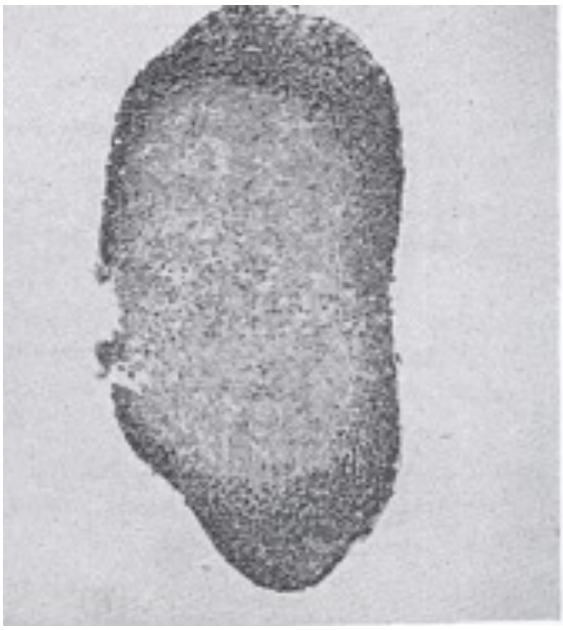
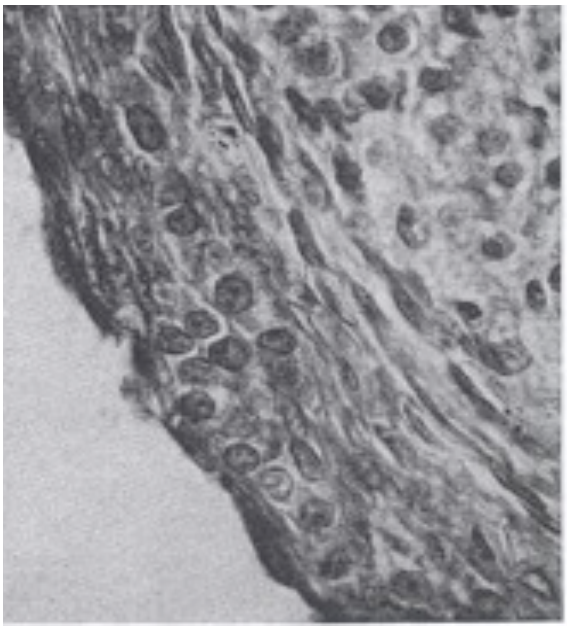

Thymidine-H³
BrdU Labeling
5
6
7
8
B-1
B-2
B-3

Figure 1.5 Regeneration of Functional Adrenal Gland Post-Enucleation.
Enucleation experiment involves a gross surgical removal of inner content of the adrenal gland leaving the capsule and some subcapsular cells intact. Following the enucleation, a functional adrenal gland is reformed within 30 days in vivo post-procedure. Upper left and lower left panels reveals the increased density of peripheral cells in two different enucleated adrenals during regeneration. Upper right and lower right panels exhibit histology cross-section of the regenerated adrenal glands at 30 days post enucleation. Adapted from Schaberg, A., et al. (20).







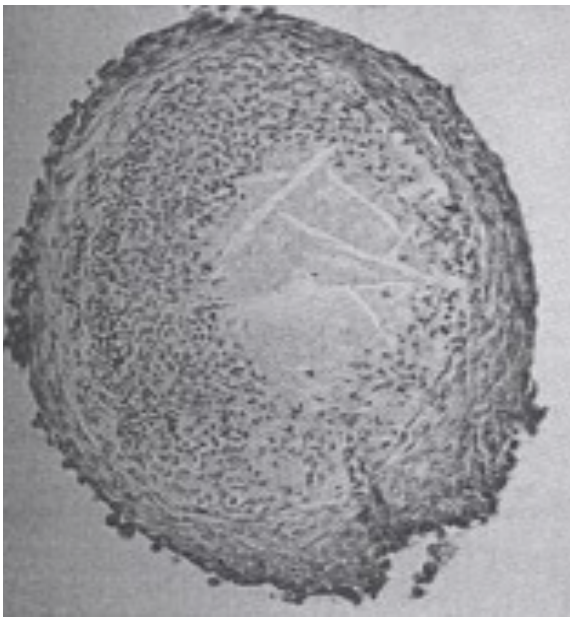


Figure 1.6 Transplantation of Primary Adrenocortical Cells and Formation of Functional Tissue. Primary adrenal cells, when transplanted sub-kidney capsule, regenerate a functional adrenocortical tissue to support life of a host mouse that underwent bilateral adrenalectomy. (A) Cross-section of the kidney containing tissue formed from transplanted adrenocortical cells. (B, C) Haematoxylin and eosin staining reveal the newly formed adrenocortical tissue from subcapsular kidney transplantation (B: lower magnification, C: higher magnification). Adapted from Thomas, M. et al. (23).

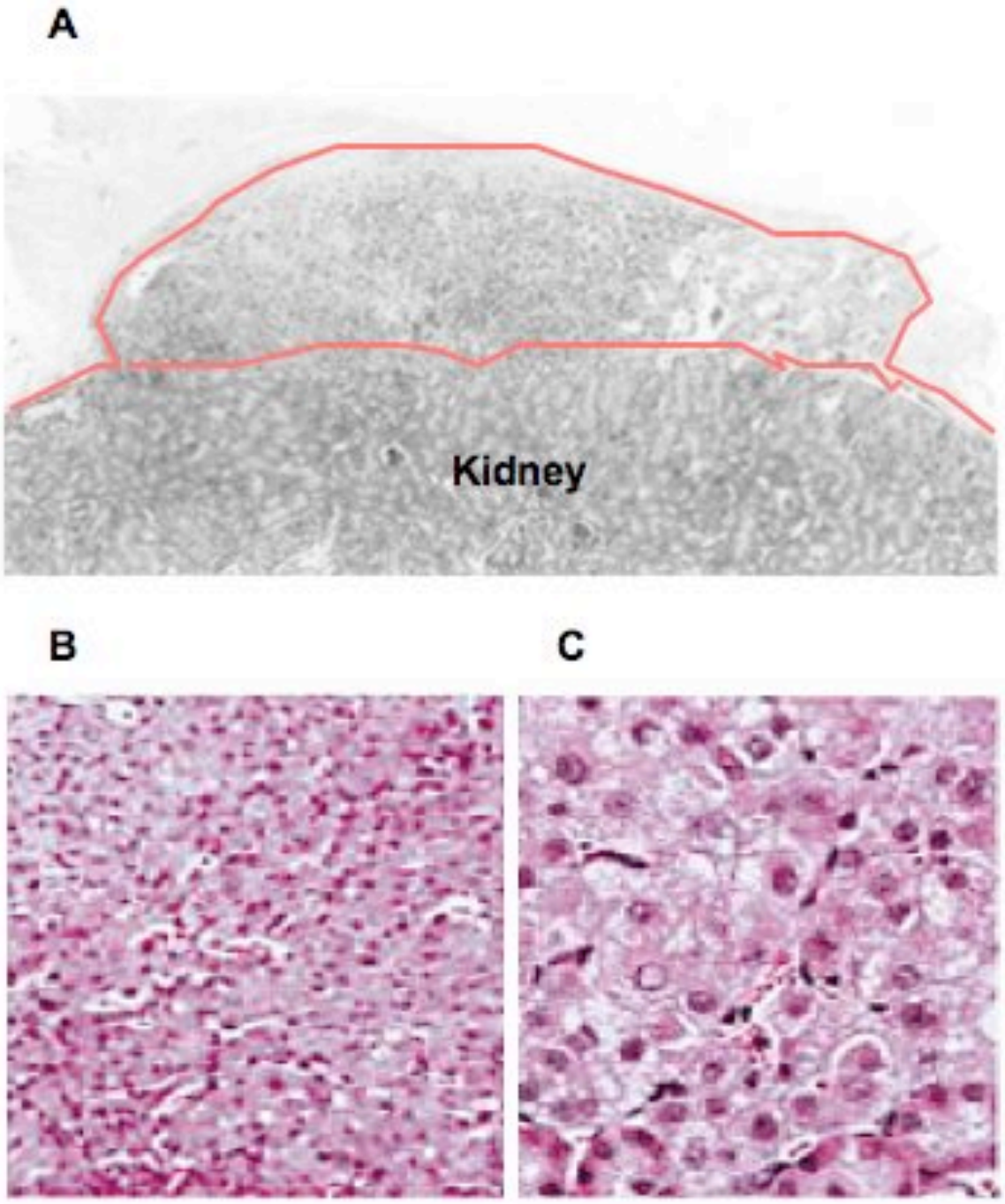
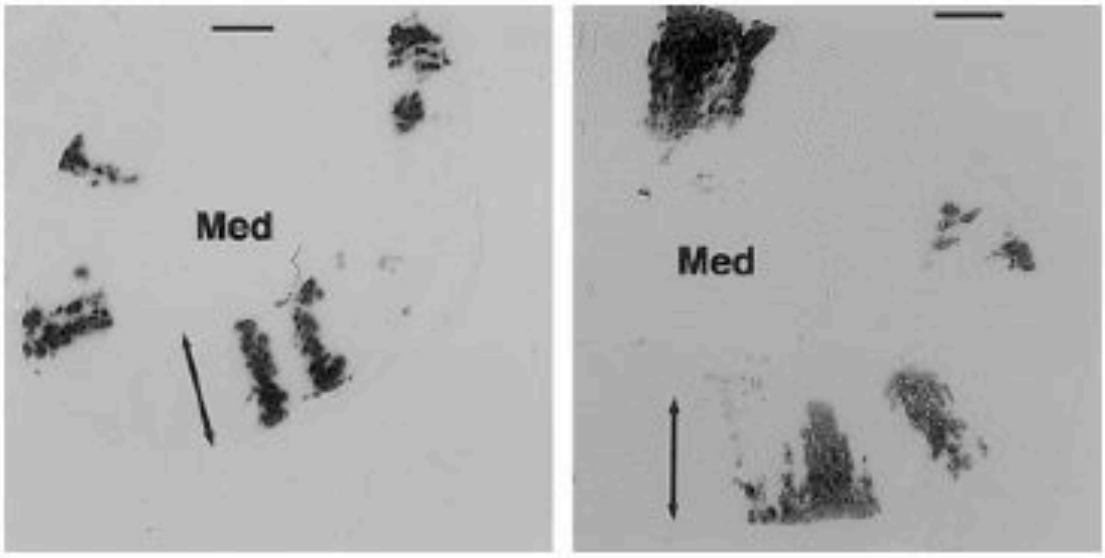
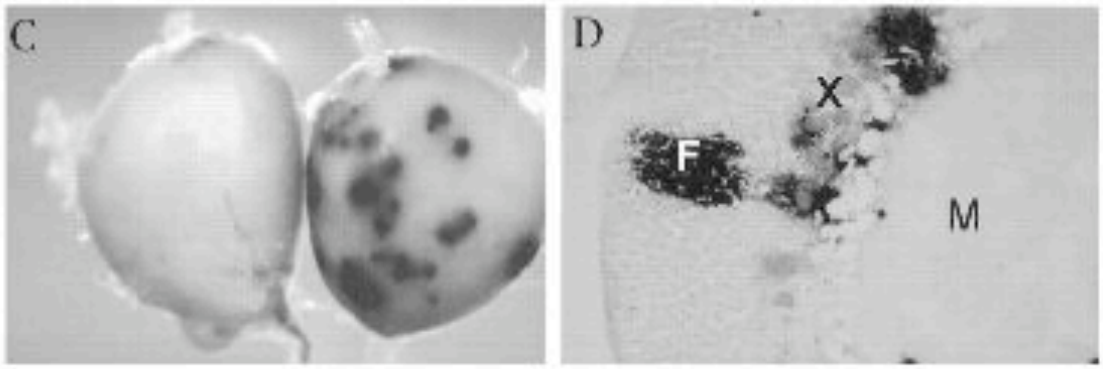

A
Kidney
B
C

Figure 1.7 Clonality of Adrenocortical Cells. The adrenals were from transgenic knock-in mice containing β-galactosidase (LacZ) gene within Cyp21b (21-OH) or Cyp11a1 (p450scc). Adrenocortical cells, as they renewal the tissue through centripetal displacement, arise from a common precursor, as evidenced by variegated staining in 21-OH-LacZ and p450scc-LacZ adrenals. Adapted from Hu, MC. et al. (21-hydoxylase-LacZ) (26) and Morley, SD. et al. (p450scc-LacZ) (29).

21-Hydroxylase-LacZ



p450scc-LacZ



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CHAPTER 2

TARGETED DISRUPTION OF β-CATENIN IN SF1-EXPRESSING CELLS IMPAIRS DEVELOPMENT AND MAINTENANCE OF THE ADRENAL CORTEX

ABSTRACT

The nuclear receptor steroidogenic factor 1 (Sf1, Nr5a1) is essential for adrenal development and regulates genes that specify differentiated adrenocortical function. The transcriptional coactivator β-catenin reportedly synergizes with Sf1 to regulate a subset of these target genes; moreover, Wnt family members, signaling *via* β-catenin, also are implicated in adrenocortical development. To investigate the role of β-catenin in the adrenal cortex, we used two Sf1-Cre transgenes to inactivate conditional β-catenin alleles. Inactivation of β-catenin mediated by *Sf1-Cre*^{high} (β-cat^{high} KO), a transgene expressed at high levels, caused adrenal aplasia in newborn mice. Analysis of fetal adrenal development in β-cat^{high} KO revealed decreased proliferation in presumptive adrenocortical precursor cells. In contrast, the *Sf1-Cre*^{low} transgene effected a lesser degree of β-catenin inactivation (β-cat^{low} KO) that did not affect all adrenocortical cells, permitting adrenal survival to reveal age-dependent degeneration of the cortex. These results define critical roles for β-catenin—presumably as part of the Wnt canonical signaling pathway—in both embryonic development of the adrenal cortex and in the adult organ maintenance.

INTRODUCTION

The adrenal cortex is part of the hypothalamic-pituitary-adrenal (HPA) axis that mediates the response to stress through synthesis and release of corticosteroid hormones. Although there are differences among species, the adrenal cortex in both mice and humans is initially derived from the proliferation and migration of coelomic epithelial cells and intermediate mesoderm of the urogenital ridge to form the adrenogonadal primordium (1). In mice, a distinct adrenal primordium is first detected around the 8th week of gestation in humans and embryonic day 12 (E12.0) in mice (2). After formation of the adrenal primordium, the adrenal cortex undergoes further maturation and development to form a transient fetal zone (x-zone), which is particularly well developed in the human adrenal gland, and the definitive (adult) cortex. After birth, the fetal zone regresses, while presumptive "stem/progenitor" cells adjacent to the capsule proliferate and renew the definitive cortex through centripetal cellular repopulation (1, 3).

Analyses of humans with congenital adrenal hypoplasia and knockout mice have identified various factors required for the initial specification and subsequent development of the adrenal cortex, including the nuclear receptors Sf1 and Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1 (Dax1, Nr0b1), the transcriptional coactivator CREB-binding protein/p300-interacting transactivator, with ED-rich tail, 2 (Cited2), and the Pre-B-cell leukemia homeobox1 (Pbx1) (4-9). In addition to transcriptional regulators, paracrine and morphogenic factors play key roles in the development of the adrenal cortex. Targeted disruption of *Wnt4*, a member of the "wingless-like MMTV integration site" family of morphogens, was associated with abnormal differentiation of the definitive zone of the adrenal cortex and

ectopic expression of "adrenal-like" cells in the gonads that was attributed to abnormal migration of adrenocortical progenitor cells (10-12). Analysis of kindred with a complex phenotype that includes renal and adrenal hypoplasia and lung abnormalities similarly implicated *WNT4* in human adrenal development (13).

Wnt members participate in various developmental processes during embryogenesis; in adult tissues such as the skin, mammary gland, and hematopoietic and central nervous systems, Wnts function in proliferation, specification of cell fate, stem cell maintenance, and differentiation (14, 15). In this manuscript, we have focused on the role of the β-catenin signaling. In the absence of Wnt ligands, the pool of β-catenin is sequestered to the cellular membrane/cell adherence junctions and the cytoplasmic concentration is maintained at low levels by ubiquitin-mediated proteolysis through a degradation complex consisting of Axin/adenomatous polyposis coli/glycogen synthase kinase 3 beta (Axin/Apc/Gsk3β). Upon binding of Wnt ligands to their respective Frizzled receptors, the degradation complex is disrupted, which permits cytoplasmic and nuclear accumulation of β-catenin. Inside the nucleus, β-catenin interacts with members of the lymphoid enhancer-binding factor/T-cell factor (Lef/Tcf) family of transcription factors to activate expression of target genes. β-catenin has also been shown to interact functionally with Sf1 to synergistically activate target genes, including *Nr0b1* (Dax1), *Inha* (inhibin-α), *Star* (steroidogenic acute regulatory protein), *Hsd3b1* (3β-hydroxysteroid dehydrogenase), *Cyp19a1* (aromatase), and *Lhb* (β-subunit of luteinizing hormone) (16-20). These studies raised the possibility that β-catenin also plays important roles in adrenocortical development and function. Knockout (KO) mice that are globally

deficient in β-catenin undergo embryonic lethality during gastrulation and lack mesoderm, precluding analysis of these potential defects in adrenal development (21).

To define the role of β-catenin in the adrenal cortex, we used the Cre-loxP transgenic strategy to conditionally inactivate β-catenin alleles in the adrenal cortex. Depending on the extent of β-catenin inactivation, these studies revealed either complete adrenal aplasia during development or defects in maintenance of the adult cortex resulting in depletion of adrenocortical cells. Thus, β-catenin plays a critical role in development and maintenance of the adrenal cortex.

MATERIALS AND METHODS

Mice

All experiments involving mice were performed in accordance with institutionally approved and current animal care guidelines from the respective universities. The *Sf1-Cre*^{high} transgene targets high levels of expression of Cre recombinase to the urogenital ridge by E10.0 and activates a Cre-dependent reporter gene throughout the adrenal cortex (22); other sites of expression include pituitary gonadotropes, the ventromedial hypothalamic nucleus, somatic cells of the gonads, and the spleen. The *Sf1-Cre*^{low} transgene is a single-copy transgene that is expressed at lower levels in the same sites. Mice carrying the floxed β-catenin allele (*Ctnnb1*^{tm2kem}) were purchased from The Jackson Laboratory (Bar Harbor, ME); this conditional allele contains *loxP* sites flanking exons 2-6, resulting in complete inactivation upon Cre-mediated recombination (23). The Cre-dependent reporter Z/AP [*Tg(ACTB-Bgeo/ALPP)1Lbe*] was purchased from The Jackson Laboratory (24). The LEF/Tcf-LacZ (Wnt-Gal) reporter transgenic mice were generously provided by Daniel Dufort (25).

Following timed matings, embryos were staged by designating noon of the day on which the copulatory plug was detected as E0.5. Correct staging was verified by appropriate morphological criteria as described (26). Genotyping for the *Sf1-Cre* transgenes and the β-catenin loxP alleles was performed on the amnion of each embryo and in adult mice, as previously described (Bingham et al., 2006; Brault et al., 2001; Truett et al., 2000). Sexes were determined by PCR analysis with primers specific for the Y-chromosome gene *Zfy* (27).

Analysis of Adrenal Histology, Immunohistochemistry, and In Situ Hybridization Analysis

Adrenal glands were collected at the indicated ages and fixed for 2–3 h in 4% paraformaldehyde/phosphate buffered saline (PBS). Tissues were dehydrated in graded ethanol solutions and embedded in paraffin before sectioning. Sections were cut at 6 μm thickness and processed using standard procedures.

For immunohistochemical analyses, adrenal glands were processed as above and washed in Tris-buffered saline/0.1% Tween-20 (TBST, pH 7.5). Antigen retrieval was performed by boiling rehydrated sections in 10 mM sodium citrate (pH 6.0) for 20 min, followed by one wash in deionized water and two washes in TBST at room temperature. Antibody stainings were conducted using VECTASTAIN ABC kits and Vector Mouse on Mouse (M.O.M.) kits according to manufacturer's protocol (Vector Laboratories, Burlingame, CA). Tissue sections were blocked in antibody diluent solution for 1 h, and then incubated overnight at 4 C with anti-β-catenin (H-102) (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-tyrosine hydroxylase (1:500, Pel-Freez Biologicals, Rogers, AR), or either of two antibodies against Sf1: A (1:1000 dilution and generously provided by Dr. Ken Morohashi) or B [1:1500 dilution of a rabbit antiserum raised against recombinantly expressed, full-length SF1 protein that was affinity purified as a GST fusion protein and then liberated by thrombin cleavage using standard methods (Invitrogen, Carlsbad, CA)]. The next day, sections were washed, exposed to secondary antibodies, and processed for signal detection according to the manufacturer's protocol.

For X-gal staining, tissues were collected at indicated ages. The tissues were prefixed in a LacZ fixation solution (2.7% Formaldehyde, 0.20% Glutaraldehyde, 2mM

$MgCl_2$, 5mM EGTA, 0.02% NP-40, PBS) for 10 min, followed by three washes in PBS. The X-gal staining was performed using the β-Gal Staining Set (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol. The tissues were stained for 24 hours and then post-fixed in 4% formaldehyde/PBS solution for 1 hour. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for DNA fragmentation was performed using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) following manufacturer's protocol.

For in situ hybridization, embryos were collected at the indicated stages, fixed for 4 h in 4% paraformaldehyde (PFA), and cryoprotected in 20% sucrose overnight. Embryos were embedded in OCT compound (Tissue Tek Sakura, Torrance, CA), and transverse sections were cut at 12 μm. Nonradioactive in situ hybridization analysis using digoxigenin-labeled probes was performed according to standard procedure; a specific protocol is available from the authors upon request. Probes used in this study were 3β-hydroxysteroid dehydrogenase (3β-HSD, Acc. NM_008293, 512-1523), side-chain cleavage enzyme (Cyp11a1, Acc. NM_019779, 132-691), and 21-hydroxylase (Cyp21, Acc. NM_009995, 469-1554).

To examine cell proliferation in E12.5 and E13.5 embryos, the BrdU Labeling and Detection Kit II (Roche, Indianapolis, IN) was used. Pregnant mothers were injected at the appropriate stages with BrdU (B-500, Sigma-Aldrich, St. Louis, MO; 50 mg/kg body weight) and embryos were harvested 1 hr later and then processed as described above. Slides containing 5 μm sagittal sections were treated according to the manufacturer's protocol with alkaline phosphatase (AP) as the detection agent, followed by NBT/BCIP visualization. The slides were then stained with anti-Sf1 antiserum B to

identify adrenocortical cells. Singly- and doubly-stained nuclei were counted in serial (at least three) sections of each genotype (WT and KO) at each age. Statistical significance was calculated using Student's t-test.

Southern Blotting

The *Sf1-Cre*^{high} and *Sf1-Cre*^{low} transgenic mouse lines were prepared and analyzed as previously described (22), except that copy number was determined by ImageJ (NIH, Bethesda, MD). Briefly, the probe contains sequences from the 1st intron of Sf1; following digestion of genomic DNA with restriction endonucleases (EcoRI, EcoRV, NcoI), the probe hybridizes to DNA fragments of 4 kb (endogenous gene) and 2.6 kb (*Sf1-Cre* transgene), respectively. The copy number is determined by relative intensities of the signal for the endogenous gene (2 copies) and that produced by the transgene (5 copies for *Sf1-Cre*^{high} and 1 copy for *Sf1-Cre*^{low}).

Real-Time PCR

Adrenal glands were removed, cleaned, and snap frozen. Frozen tissues were lysed in Trizol reagent using an electric tissue homogenizer, and total RNA was prepared according to the manufacturer's protocol. Total RNA was treated with DNase (Ambion, Austin, TX) to remove residual genomic DNA and quantitated by UV spectrometry. 1 μg of total RNA was used to synthesize cDNA using the iScript kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The final cDNA products were purified and eluted in 50 μl of Tris-EDTA buffer using PCR purification columns (QIAGEN, Hilden, Germany) or directly diluted to final volume. Primer sequences for each gene

are: human placental alkaline phosphatase (*hAP*): Fwd-5' ctgctgccctccagacat, Rev-5' cgggttctcctcctcaact; *Axin2*: Fwd-5' gcaggagcctcacccttc, Rev-5' tgccagtttctttggctctt; Tyrosine Hydroxylase (*Th*): Fwd-5' cccaagggcttcagaagag, Rev-5' gggcatcctcgatgagact, *Sf1*: Fwd-5' acaagcattacacgtgcacc, Rev-5' tgactagcaaccaccttgcc; Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*): Fwd-5' aatgtgtccgtcgtggatct, Rev-5' cccagctctccccatcacta. *Hsd3b1* (3β-Hsd): Fwd-5' cagtttgtgtcttgggcttaaca, Rev-5' gcagatcacagtgggagtga. *Cyp11b2*: Fwd-5' gcaccaggtggagagtatgc, Rev-5' gccattctggcccatttag. *Cyp21a1*: Fwd-5' gacccaggagttctgtgagc, Rev-5' tccaaaagtgaggcaggaga, *Star*: Fwd-5' aaggctggaagaaggaaagc, Rev-5' ccacatctggcaccatctta, *Actb1* (β-actin): Fwd-5' ctaaggccaaccgtgaaaadg, Rev-5' accagaggcatacagggaca.

For quantitative, real-time PCR (qRT-PCR) analyses of mRNA abundance, reactions were performed with a 2x SYBR Green PCR mastermix (Applied Biosystems, Foster City, CA) and gene-specific primers in the ABI 7300 thermocycler (Applied Biosystems, Foster City, CA). Each quantitative measurement was normalized to Rox dye as an internal standard and performed in triplicate. Transcript abundance was normalized in each sample to the average Ct value for mouse Gapdh and β-actin (Livak et al., 2001). For mRNA quantitation, a minimum of three samples from differing genotypes was analyzed. Statistical significance was calculated using Student's t-test.

Semi-quantitative determination of LacZ Expression

To quantitate LacZ positive cells in *Wnt-Gal/β-catenin*$^{loxP/loxP}$ (Wnt-Gal) and *Sf1-Cre*^{low}*/Wnt-Gal/β-catenin*$^{loxP/loxP}$ (Wnt-Gal/β-cat^{low} KO) adrenals, we used at least three representative sections from adrenal glands stained for LacZ activity and counterstained

with eosin. Images were captured using an Optiphot-2 microscope (Nikon, Melville, NY) with an Olympus DP-70 camera and software system (Olympus, Hauppauge, NY). Images were further analyzed using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA) and ImageJ (NIH, Bethesda, MD). Statistical significance was calculated using Student's t-test.

ACTH Measurements

All mice were individually housed for 24 hours preceding all procedures in a low stress environment. Baseline blood samples were obtained at 0900 hours by decapitation and collection of core-trunk blood within 30 sec of initial mouse handling to minimize stress-induced ACTH secretion. Blood plasma was collected using the Microvette CB 300 blood collection tube (Sarstedt, Germany) and stored at -80°C prior to analysis. The ACTH analysis was conducted through Vanderbilt Hormone Assay & Analytical Services Core (Vanderbilt University, Nashville, TN). Statistical significance was calculated using one-way analysis of variance (ANOVA) and Tukey post-hoc test.

RESULTS

Canonical Wnt Signaling Increasingly Becomes Restricted to Cells Adjacent to the Developing Adrenal Capsule

To define the temporal and spatial organization of canonical Wnt signaling in the developing adrenal cortex, we examined the embryonic and postnatal adrenal expression of β-catenin, as well as the expression of LacZ driven by the canonical Wnt/β-catenin reporter gene, *Wnt-Gal* (Figure 2.1). At E12.5, β-catenin and Sf1 staining overlapped throughout cells of the adrenal primoridum. In contrast, a few Sf1-negative cells in the adrenal primordium expressed tyrosine hydroxylase (*Th*), a marker for chromaffin cell precursors that will form the adrenal medulla. This close correlation between sites of Sf1 and β-catenin expression was not maintained at later stages. In adrenals at E18.5 and postnatal day 0 (P0), Sf1 expression was seen in nuclei throughout the adrenal cortex, while β-catenin expression was preferentially localized to the subcapsular region. This apparent restriction of β-catenin expression in only a subset of Sf1 positive cells persisted at 3 weeks after birth.

At E12.5, LacZ staining (indicative of canonical Wnt signaling) was seen in a few cells in the outer region of the gland, immediately adjacent to the emerging adrenal capsule. The establishment of the capsule and the restricted subcapsular localization of LacZ were more evident at E18.5. In the newborn adrenal gland (Figure 2.1, P0), active canonical Wnt signaling, as visualized by LacZ expression, was seen in discrete clusters of cells at the periphery of the adrenal cortex, again immediately beneath the capsule. By three weeks of age, LacZ expression in the immediate subcapsular region was more uniform and corresponded more closely to the expression pattern for β-catenin.

Moreover, the expression and the active β-catenin signaling in the fetal/X-zone are not observed. These observations were confirmed using a different Wnt-reporter strain (*BAT-Gal*, data not shown). Importantly, we observed active β-catenin signaling—as revealed by LacZ expression—in only a subset of subcapsular cells expressing β-catenin protein (Figure 2.1). This observation is consistent with the premise that only a subset of adrenocortical cells maintains active canonical β-catenin signaling through Lef/Tcf transcription factors at any given time. Together, these data define the establishment of canonical Wnt signaling, which activates β-catenin dependent transcription in the presumptive adrenocortical stem/progenitor cells of the definitive cortex as it organizes under the developing capsule.

Differential Effects of the Sf1/Cre Transgenes on Adrenocortical Expression of Cre Recombinase

The two Sf1-Cre transgenes differ in copy number (5 copies for *Sf1-Cre*^{high} versus one copy for *Sf1-Cre*^{low} as revealed by quantitative Southern blotting data in Figure 2.2A) but have similar sites of expression (22). To visually and quantitatively determine the efficiency of the Cre-mediated recombination in the adrenal cortices of the two Sf1-Cre transgenic lines, we generated mice carrying the Cre transgenes and the Cre-dependent reporter, *Z/AP* (24). These reporter mice initially express the *LacZ* reporter gene; following Cre-mediated recombination, they silence *LacZ* and express human placental alkaline phosphatase (*hAP*). As revealed by expression of LacZ (Figure 2.2B), the recombination efficiencies of the two Sf1-Cre transgenes differed in adrenal glands at 6 weeks of age. The *Sf1-Cre*^{low} transgene mediated only partial recombination of the

reporter gene, as evidenced by persistent LacZ staining in some cortical cells. In contrast, the *Sf1-Cre*^{high} transgene completely abolished LacZ expression in the adrenal cortex, suggesting that it mediates Cre-dependent recombination in the adrenal cortex in a highly efficient manner. Moreover, quantitative PCR analyses of adrenal expression of hAP confirmed the different efficiencies of Cre-mediated recombination for the two *Sf1-Cre* transgenes (Figure 2.2C), with approximately 3-fold higher expression of hAP in Z/AP mice carrying the *Sf1/Cre*^{high} transgene than in those with the *Sf1-Cre*^{low} transgene. The results indicate that the Cre protein in *Sf1-Cre*^{low} adrenals is expressed in a lower number of cells of the cortex than that in *Sf1-Cre*^{high} mice.

β-catenin KO Mediated by the *Sf1-Cre*^{high} Transgene Causes Adrenal Aplasia

Having characterized the relative efficiencies of the two *Sf1-Cre* transgenes in driving Cre-mediated recombination, we next examined their functional effect on the conditional β-catenin allele (*Ctnnb1*^{tm2kem}), crossing mice with either the *Sf1-Cre*^{low} or the *Sf1-Cre*^{high} transgene and one copy of the floxed β-catenin allele with mice that were homozygous for the floxed β-catenin allele.

Direct effects of *Sf1-Cre*^{high}-mediated β-catenin KO (β-cat^{high} KO) on β-catenin expression were examined utilizing immunohistochemical assays with an anti-β-catenin antibody. As shown in Figure 2.3A (and similar to results in Figure 2.1), β-catenin at E12.5 was expressed in the WT adrenal primordium, as well as in other regions of the embryo (top panels). Thereafter (E14.5 and E16.5), the cells that expressed β-catenin again localized as a thin layer of cells near the subcapsular zone at the periphery of the adrenal cortex. In the β-cat^{high} KO mice, in contrast, adrenal immunoreactivity for β-

catenin was not detected in sections at any of these stages (Figure 2.3C, bottom panels), indicating that the *Sf1-Cre*^{high} transgene caused complete ablation of β-catenin expression. These studies document that Cre recombinase driven by the *Sf1-Cre*^{high} transgene abrogates expression of β-catenin at very early stages of adrenal development.

Based on the striking effect on β-catenin expression, we next examined adrenal development at different stages, focusing both on histology (Figure 2.3B) and on expression of Sf1 (Figure 2.3C). At E12.5, the developing testis was visible as a group of cells under the coelomic epithelium, some of which expressed Sf1. Immediately adjacent to this gonadal precursor are cells that comprise the adrenal primordium, which also expressed Sf1 (Figure 2.3B). At this early developmental stage, we observed relatively subtle histological differences between WT and β-cat^{high} KO mice (Figure 2.3B), although the apparent decrease in the number of Sf1-positive cells (Figure 2.3C) suggests that the adrenal primordium is already affected by the conditional β-cat^{high} KO.

By E14.5, the β-cat^{high} KO adrenal glands were smaller than their WT counterparts (Figure 2.3B) and contained considerably fewer Sf1-positive cells (Figure 2.3C); very similar findings were observed in sections from E16.5 embryos. In fact, many of the cells remaining in the region where the adrenal gland normally resides at E16.5 expressed *Th*, identifying them as chromaffin cell precursors derived from the neural crest. Finally, by E18.5, all remnants of an adrenal gland, including the presumptive chromaffin cells (Figure 2.3B), had disappeared.

Based on the striking effect of β-catenin inactivation on adrenal structure, we also used in situ hybridization analyses to examine the effect on expression of several steroidogenic enzymes. As shown in Figure 2.4, the cholesterol side-chain cleavage

enzyme (Cyp11a1), 3β-hydroxysteroid dehydrogenase (3β-HSD), and 21-hydroxylase (Cyp21) normally are expressed in a subset of cells within the adrenal primordium at E12.5, with higher expression seen at E13.5. In marked contrast, expressions of Cyp11a1 and 3β-HSD were decreased relative to WT levels at E12.5; even greater apparent differences in expression of all three steroidogenic enzymes in β-catenin KO mice was apparent at E13.5. These studies suggest that β-catenin is directly or indirectly required for the expression of multiple components of the steroidogenic pathway in the adrenal gland from very early stages of differentiation.

Adrenal and gonadal regression in Sf1 KO mice is associated with increased apoptosis (4), whereas studies in mice with Leydig cell-specific disruption of Sf1 showed that gonadal proliferation is markedly decreased (27). We therefore used BrdU labeling to assess proliferation in β-cat^{high} KO. As shown in Figure 2.5, BrdU incorporation in the region of the adrenal primordium in the β-cat^{high} KO mice did not differ from that seen in WT mice at E12.5 but was decreased considerably at E13.5. Consistent with our previous observation that the absence of β-catenin affects adrenal development from very early stages (Figure 2.3B), the total number of Sf1-expressing cells in the KO mice was significantly decreased at both time points, The decreased number of cells labeled with BrdU supports an important role for β-catenin in regulating cell proliferation in the embryonic adrenal gland. In contrast, apoptosis—as revealed by TUNEL staining for DNA fragmentation—did not differ significantly in the same region of the developing adrenal gland (data not shown). Thus, decreased proliferation apparently is the predominant factor in the adrenocortical regression seen in β-cat^{high} KO mice.

Effect of β-catenin inactivation mediated by the Sf1-Cre^{low} transgene on adrenal structure and function

We also examined the effect of β-catenin disruption mediated by the *Sf1-Cre^{low}*(β-cat^{low} KO) transgene on adrenal structure (Figure 2.6A). Both in analysis of the intact urogenital region (top panels) and in histological sections (bottom panels), the WT adrenal glands immediately after birth were located rostral to the kidneys. Very similar findings were seen in β-cat^{low} KO mice. Consistent with the developmental studies described above, β-cat^{high} KO mice exhibited complete absence of the adrenal (Figure 2.6A; top and bottom panels). These data demonstrate that either the timing or the extent of β-catenin ablation mediated by the two Sf1-Cre transgenes differs in functionally important ways.

To explore the β-cat^{low} KO postnatal adrenocortical function, we examined the extent of inhibition of subcapsular canonical Wnt signaling mediated by the *Sf1-Cre^{low}* transgene. Crossing the *Sf1-Cre^{low}* transgene into the Lef/Tcf-LacZ (*Wnt-Gal*) reporter revealed that the field of subcapsular cells still expressing *LacZ* was diminished by approximately 50% in these mice (Figure 2.6B), coincident with a roughly comparable decrease in the expression of the canonical Wnt/β-catenin target gene, Axin2 (Figure 2.6C). Therefore the *Sf1-Cre^{low}* transgene inactivates the β-catenin gene (and hence canonical Wnt signaling) in only a subset of adrenocortical cells. This finding was supported by direct analysis of Cre-mediated recombination of β-catenin in genomic DNA samples from adrenal glands of mice with the differing genotypes (Figure 2.6D). Although the presence of adrenal medullary cells can potentially influence these analyses, the cortical-specific expression of the *Sf1-Cre* transgene and lack of active Wnt signaling

in the medulla makes these concerns negligible. Therefore, these data are consistent with the model that partial but not complete recombination of β-catenin occurs in the *Sf1-Cre^{low}* mice. Similarly, the presence of LacZ positive cortical cells in *Sf1-Cre^{low}/Z/AP* mice (Figure 2.2B) indicates that some cells in the *Sf1-Cre^{low}* adrenal did not undergo Cre-mediated recombination, which presumably permits survival of cells that have the potential to engage β-catenin signaling.

In order to further determine the effects of β-catenin deficiency mediated by the *Sf1-Cre^{low}* transgene, we analyzed adrenocortical structure and steroidogenic capacity in these mice at different time-points. At 15 weeks of age (Figure 2.7), the adrenal glands of WT and β-cat^{low} KO mice had comparable histology and Sf1 expression. In addition, the adrenal cortex at this stage maintained some degree of subcapsular β-catenin staining, presumably in cells that escaped Cre-mediated recombination of β-catenin (Figure 2.7). At 30 weeks of age, we began to observe histological thinning and disorganization of the adrenal cortex in a subset of the KO mice. As shown in Figure 2.6, the width of the adrenal cortex in mid-adrenal sections was markedly reduced in 50% of β-cat^{low} KO mice (n=6) relative to the WT glands (n=6). By 45 weeks of age, all β-cat^{low} KO mice (n=4) exhibited histologic disorganization and thinning of the adrenal cortex (Figure 2.7). The adrenal cortex in these mice also appeared to have a decreased number of Sf1-positive cells, revealing the importance of β-catenin in adult adrenocortical organ maintenance. Thus, although β-catenin staining was still seen in some cells, the partial deficiency of β-catenin mediated by the *Sf1-Cre^{low}* transgene eventually led to striking changes in the adrenal cortex, presumably secondary to depletion of at least some of the population of adrenocortical stem/progenitor cells. These findings argue that there is a cumulative

effect of the *Sf1-Cre*^{low}-mediated depletion of β-catenin activity over time that ultimately affects adrenocortical maintenance in all mice of this line.

To explore the mechanism of this postnatal adrenocortical depletion, we performed TUNEL staining to assess DNA fragmentation, indicative of apoptosis (Figure 2.7). Although the adrenal glands in the β-cat^{low} KO mice at 15 weeks were relatively intact histologically, an increase in TUNEL staining in the adrenal cortex was consistent with increased cell death *via* apoptosis occurring at this time. The marked increase in TUNEL staining in the adrenals of β-cat^{low} KO mice at 30 weeks (Figure 2.7) indicates that the loss of β-catenin in the adrenal cortex progressively contributes to loss of adrenocortical tissue *via* apoptosis. We also observed increased TUNEL staining in the adrenal medulla of 30-week old β-cat^{low} KO mice (Figure 2.7), consistent with the known roles of the cortex in maintaining medullary function.

Following the observation of cortical thinning and disorganization in 50% of β-cat^{low} KO mice at 30 week, we stratified these mice into two groups (histological failure versus no histological failure) and analyzed these mice in more detail in regards to adrenal size, ACTH levels, and steroidogenic enzyme expression. We predicted that mice with histological failure would have smaller adrenal glands with a compensatory elevation in ACTH levels with or without a decrease in steroidogenic enzyme expression. As shown in Table 2.1, the KO mice with clear histological failure (n=3) have a significant reduction in adrenal mass compared to both WT and the KO mice without evidence of histological failure, consistent with the observed stochastic rate of cortical depletion. In addition, these mice have significantly elevated basal ACTH levels compared to WT (n=6) and KO mice without histological failure (n=3). Lastly, the

expression of a panel of steroidogenic genes in individual KO mice with histological failure was routinely decreased compared to mean values in WT mice (Figure 8). These qPCR studies also supported the apparent decrease in immunohistochemical detection of Sf1 described above. The reciprocal elevation in ACTH and reduction in cortical size with concordant decrease in steroidogenic enzyme expression is consistent with developing adrenal failure.

DISCUSSION

The complete absence of adrenal glands in mice with efficient disruption of β-catenin driven by the *Sf1-Cre*^{high} transgene unequivocally establishes the essential role of β-catenin in mouse adrenocortical development. This effect is qualitatively different than that seen in *Wnt4* KO mice (10), perhaps due to potential roles of Wnt4 in additional noncanonical Wnt signaling (28-30), the activation of the canonical pathway in the adrenal gland by additional Wnt ligands, or Wnt independent actions of β-catenin (31, 32).

Regardless, β-catenin can be included in the small group of transcriptional regulators, including Sf1, Dax1, Wt1 Pbx1, and Cited2, whose deficiency causes complete adrenal absence. Although further studies will be needed to define the molecular mechanisms of β-catenin signaling in adrenocortical cells, the reported synergy between β-catenin and Sf1 suggests that these two genes may interact to regulate the expression of critical target gene(s) whose expression is essential to stimulate adrenocortical proliferation and/or inhibit apoptosis. Of interest, proposed target genes of both β-catenin and Sf1 include a number of genes that regulate proliferation, providing plausible candidates for this co-regulation (16-20, 33). Moreover, mutations and/or amplification of both Sf1 and β-catenin have been linked to adrenocortical tumorigenesis in humans, again suggesting that these two genes play key roles in adrenocortical cell proliferation *in vivo* (34, 35). Sf1 exhibits marked dose-dependent effects on growth as revealed by the impaired adrenal development seen in mice with Sf1 haploinsufficiency (36-38). In contrast, haploinsufficiency for β-catenin apparently is compatible with normal adrenocortical function, as we observed no obvious adrenal phenotype in mice

carrying the *Sf1-Cre*^{high} transgene and one conditional β-catenin allele. Given our model that these transcriptional co-regulators cooperate in adrenocortical organogenesis, the basis for their differing dose dependence is an important area for further investigation.

Our direct analyses of immunoreactive β-catenin and indirect analyses of β-catenin-dependent transcription using the Wnt-Gal reporter revealed that some adrenocortical cells retaining immunoreactive β-catenin in the β-cat^{low} KO mice did not activate β-catenin-dependent gene expression. Thus, β-catenin activates downstream events of its canonical signaling pathway in only a subset of adrenocortical cells where it is expressed. The precise mechanisms that convey competence for β-catenin-mediated transcription to a subset of cells expressing the gene remain to be determined, as does the relative distribution of β-catenin protein between the cytoplasmic and nuclear compartments. It is tempting to speculate that other transcription factors/co-regulators also are permissive for β-catenin-dependent transcription and that the expression of these factors "marks" a specific population of cells within the adrenal cortex. Further studies will be needed to identify these putative co-regulators and to define just how they may interact with β-catenin to affect the pool of adrenocortical progenitors.

The presence of cells that express *Th* in the region of the adrenal gland argues strongly that the common sympathoadrenal precursors can differentiate into chromaffin cells despite the marked depletion of Sf1-expressing adrenocortical cells. Although cell culture studies suggested an obligatory role for steroid hormones in the differentiation of these precursors into chromaffin cells (39), studies with Sf1 KO mice demonstrated that the complete loss of steroidogenic adrenocortical cells was compatible with the differentiation of sympathoadrenal precursors into cells that exhibited several

characteristics of chromaffin cells (40). Although we have not explored the function of these cells in detail, they apparently disappear from the region of the adrenal gland by E18.5, arguing that the adrenal cortex plays important roles in supporting their continued survival. The enhanced TUNEL staining in the postnatal adrenal medulla of β-cat^{low} KO mice (Figure 2.6) is consistent with this model.

The Sf1-Cre transgenes are expressed in the anterior pituitary gland (22), and defects in pituitary expression of corticotropin are associated with impaired development of the adrenal cortex. However, the Sf1-Cre transgenes are not expressed in pituitary corticotropes, and even complete absence of corticotropin does not cause agenesis/aplasia of the adrenal gland. Moreover, ACTH levels in the β-cat^{low} KO mice with histologic failure were higher than those in WT mice and KO mice without clear histologic failure, suggesting a primary defect in adrenal function. Although defining the effects of Sf1-Cre-mediated disruption of β-catenin in other sites such as the anterior pituitary, ventromedial hypothalamic nucleus, and gonads is an important area for future studies, the finding that surviving adrenal cells are those that have not inactivated the floxed LacZ Cre reporter (Figure 2.2) argues that these are cell-autonomous adrenal effects rather than the result of external perturbations. Thus, it is extremely unlikely that the phenotype observed here reflects secondary effects on the adrenal cortex due to disruption in other sites.

The available data from other tissues suggest the importance of canonical Wnt signaling in the development and maintenance of organ systems (41-45). For example, Wnt signaling in hair follicles is localized to the stem cell population. Within the adrenal cortex, active canonical signaling is seen in the developing adrenal primordium from E12.5. As the definitive cortex subsequently emerges, Wnt signaling increasingly

becomes restricted to the subcapsular area of the cortex, coincident with the organization of the surrounding capsule. Although definitive studies identifying the capsular and subcapsular cells as the *bona fide* adrenocortical niche/stem-progenitor unit are lacking (1), the data presented here indicate that canonical Wnt signaling in these cells is critical for the development of the definitive cortex and maintenance of the adult gland.

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Author Contributions: Alex C. Kim and Anne L. Reuter (University of Texas – Southwestern, Dallas, TX) were first co-authors on the manuscript represented in this chapter. Alec C. Kim performed experiments and analysis depicted on Figure 2.1, 2.2B, 2.2C, 2.6, 2.7, 2.8, and Table 2.1 Anne L. Reuter performed experiments and analysis depicted on Figures 2.2 A, 2.3, 2.4, and 2.5. This work was published in *Development. 2008 Aug;135(15):2593-602.*

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Figure 2.1 Developmental Profile of β-catenin and Canonical Wnt Signaling in the Embryonic and Adult Adrenal Glands. Immunohistochemical analyses of Sf1 (using antiserum A), β-catenin, and tyrosine hydroxylase (Th) were performed as described in Materials and Methods. Scale Bars: 100 μm. Colorometric determinations of LacZ activity, as a surrogate for active canonical Wnt signaling, in adrenal glands from Wnt-Gal mice at E12.5, E18.5, P0, and P21 were performed as described in Materials and Methods. The black arrows highlight LacZ staining in the E12.5 section. Scale Bar: 100 μm. The inset in the E12.5 section shows a 40x magnification of the area stained for LacZ. The scale bar= 20μm. A: Adrenal Cortex; X: Adrenal Fetal/X-zone; M: Adrenal Medulla.

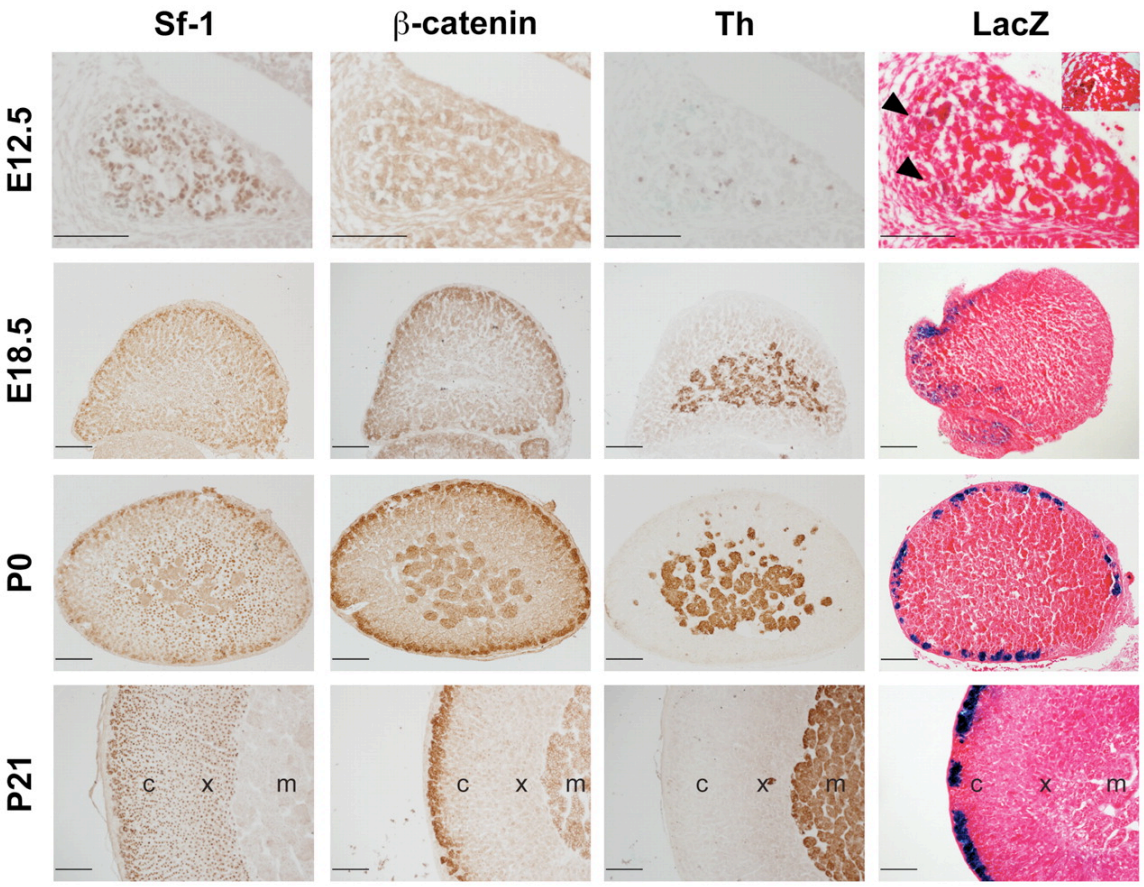
Sf-1
β-catenin
Th
LacZ
E12.5
E18.5
P0
P21
c
x
m

Figure 2.2 Cre Expression in the Adult Adrenal Gland. (A) Southern blot analysis of isolated genomic DNA from WT, Sf1-Cre^{low}, and Sf1-Cre^{high} mice. (B) LacZ activity staining of adrenal glands from 6-week old male Z/AP, Sf1-Cre^{low} x Z/AP, and Sf1-Cre^{high} x Z/AP, as described in Materials and Methods. Scale Bars: 100 μm. (C) Quantitative-PCR analysis of hAP expression in adrenal glands from 6-week old male WT, Z/AP, Sf1-Cre^{low} x Z/AP, and Sf1-Cre^{high} x Z/AP mice.

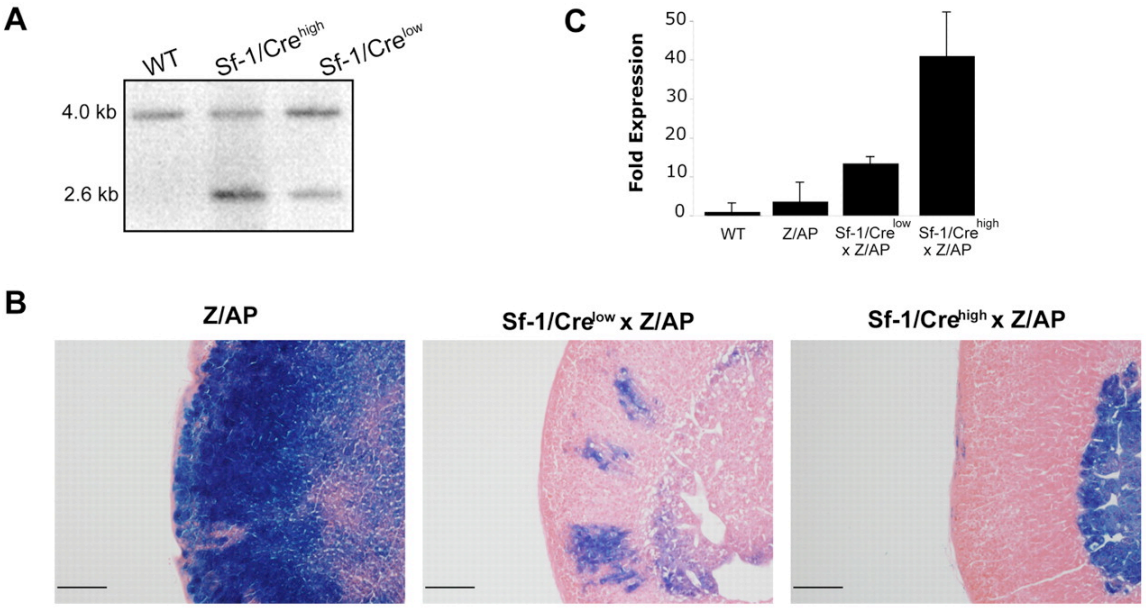
A
WT
Sf-1/Cre^high
Sf-1/Cre^low
4.0 kb
2.6 kb
C
Fold Expression
50
40
30
20
10
0
WT
Z/AP
Sf-1/Cre^low x Z/AP
Sf-1/Cre^high x Z/AP
B
Z/AP
Sf-1/Cre^low x Z/AP
Sf-1/Cre^high x Z/AP

Figure 2.3 Comparison of WT and Sf1-Cre^{high}-Mediated β-catenin KO Embryos at Different Developmental Stages. Embryos were harvested from timed-pregnant dams at the indicated stages and sagittal sections were processed and analyzed as described in Materials and Methods. (A) Immunohistochemical analysis of β-catenin expression. (B) Hematoxylin & eosin (H&E) staining of sections. (C) Immunohistochemical analysis of SF1 expression using antiserum B against Sf1 to identify adrenocortical progenitors. The offset shows a section from an E16.5 embryo stained with an antiserum against tyrosine hydroxylase, which detects chromaffin cell precursors. DA: dorsal aorta; G: gonad.

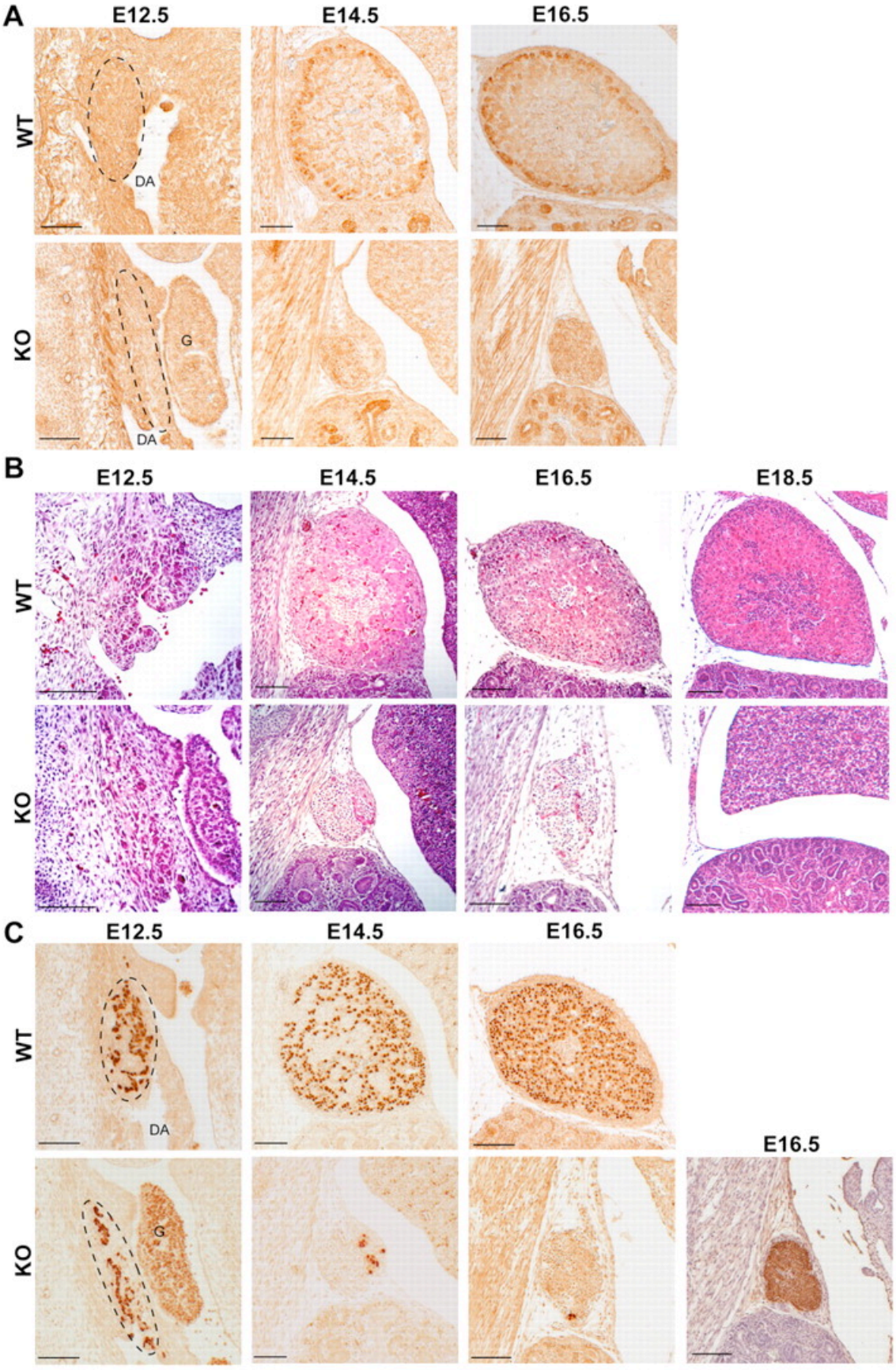
A
E12.5
E14.5
E16.5
WT
KO
DA
G
DA
B
E12.5
E14.5
E16.5
E18.5
WT
KO
C
E12.5
E14.5
E16.5
WT
DA
E16.5
KO
G

Figure 2.4 Effect of Sf1-Cre^{high}-Mediated β-catenin KO on Adrenal Expression of Steroidogenic Enzymes. Embryos from mice of the indicated genotypes were harvested at E12.5 or E13.5, processed as described in Materials and Methods, and used for in situ hybridization analyses. Probes included cholesterol side-chain cleavage enzyme (Cyp11a1), 3β-hydroxysteroid dehydrogenase (3β-HSD), and 21-hydroxylase (Cyp21).

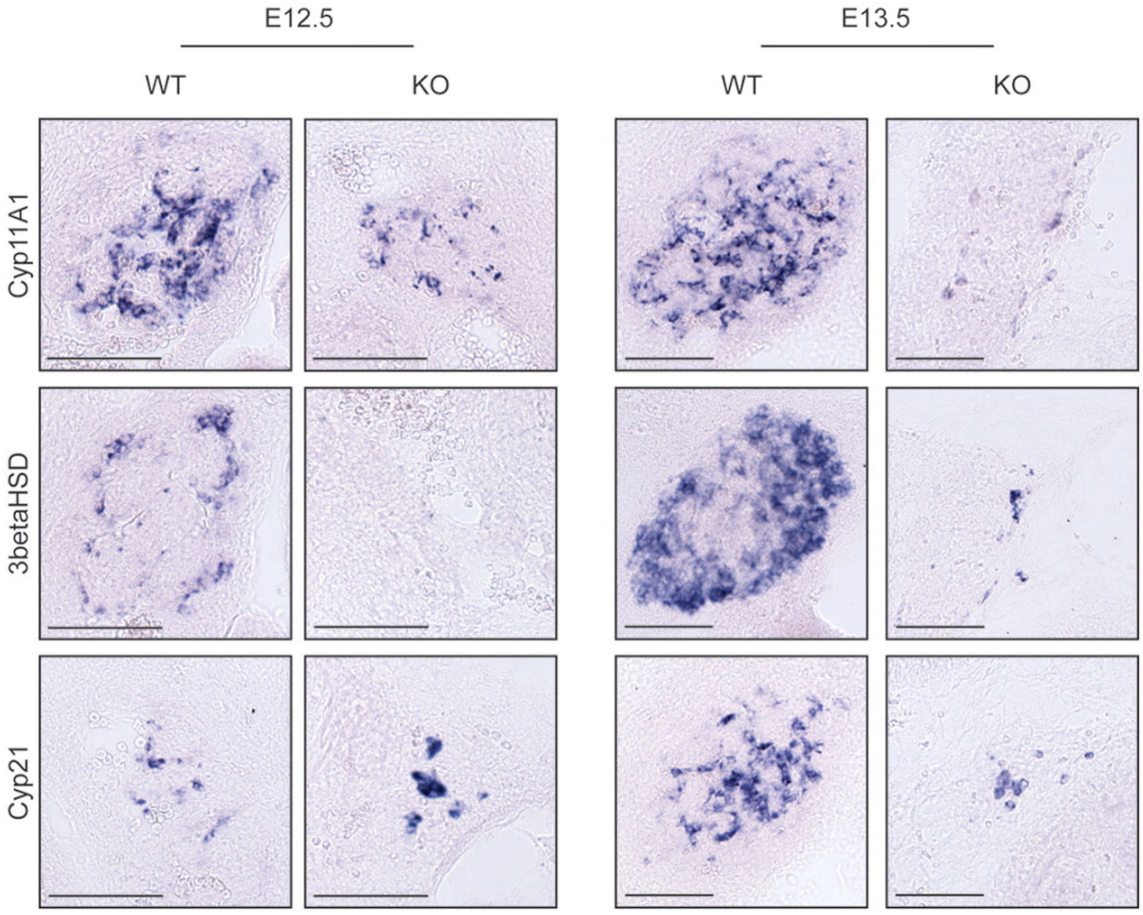
E12.5
E13.5
WT
KO
WT
KO
Cyp11A1
3betaHSD
Cyp21

Figure 2.5 Loss of β-catenin Mediated by the Sf1-Cre^{high} Transgene Results in a Decreased Number of Adrenocortical Cells at Least Partly Due to Decreased Proliferation. BrdU incorporation into WT and Sf1-Cre^{high}-mediated KO mice was determined at the indicated stages as described in Materials and Methods. Mice with Sf1-Cre^{high}-mediated β-catenin KO appeared to have decreased BrdU staining in the region of the adrenal primordium. The offset shows quantitation of Sf1 positive cells (top) and Sf1 positive cells that were also positive for BrdU (bottom) in E12.5 and E13.5 embryos. The number of Sf1 positive cells was decreased significantly at both E12.5 and E13.5, whereas the number of doubly labeled cells was only decreased significantly at E13.5 ($p<0.01$ versus WT).

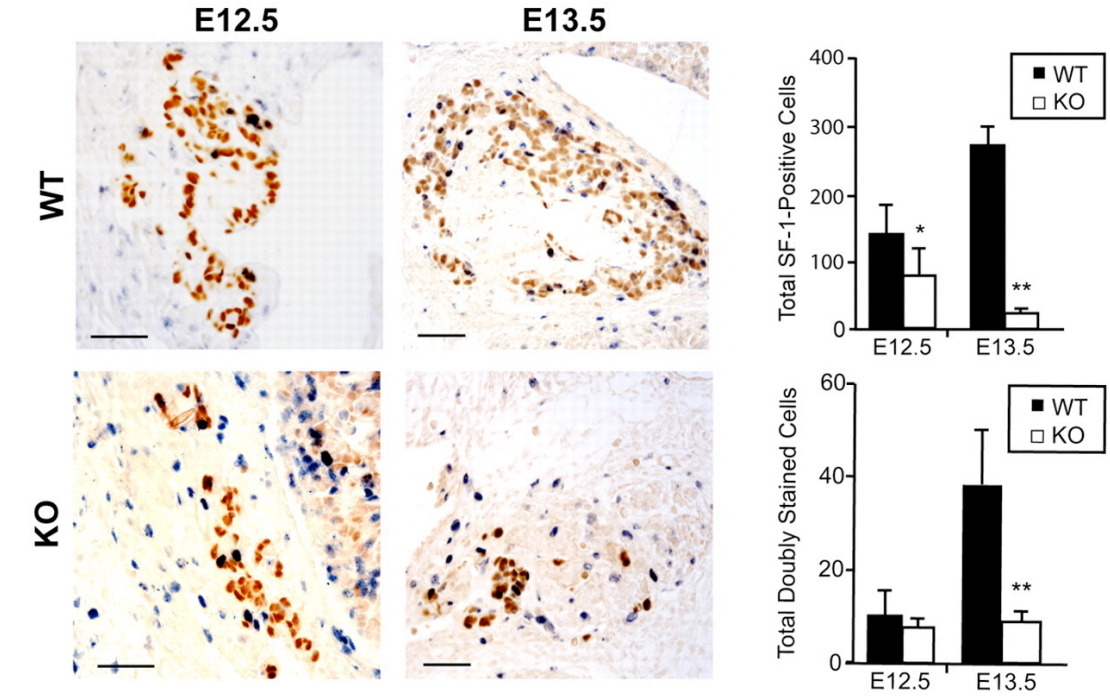
E12.5
E13.5
WT
KO
Total SF-1-Positive Cells
400
300
200
100
0
WT
KO
*
**
E12.5
E13.5
Total Doubly Stained Cells
60
40
20
0
WT
KO
**
E12.5
E13.5

Figure 2.6 Sf1-Cre^{low}-Driven Loss of β-catenin Permits Adrenal Survival Due to Incomplete Inactivation of β-catenin. (A) Photograph of the urogenital region of newborn (PO) WT, Sf1-Cre^{low}-, and Sf1-Cre^{high}- β-catenin KO mice. H&E staining of sagittal sections in the region of the adrenal gland from PO WT mice or from mice with β-catenin KO mediated by the indicated Sf1-Cre transgenes. (B). Demonstration of recombination of the β-catenin gene in DNA samples from WT mice and those with β-catenin inactivation mediated by the Sf1-Cre^{low} transgene. (C) LacZ stained sections of Wnt-Gal and Wnt-Gal/β-catenin KO^{low}, as described in Materials and Methods. Scale Bar: 200 μm. (D) Quantitation of active canonical Wnt signaling by comparing the area of LacZ staining versus the total adrenal area ($p<0.05$ versus Cre-negative littermates). Quantitative-PCR analysis of Axin2 expression in adrenal glands from WT and Sf1-Cre^{low}-β-catenin KO mice ($p<0.05$ versus Cre-negative littermates). Ad, adrenal gland; K, kidney; l, liver.

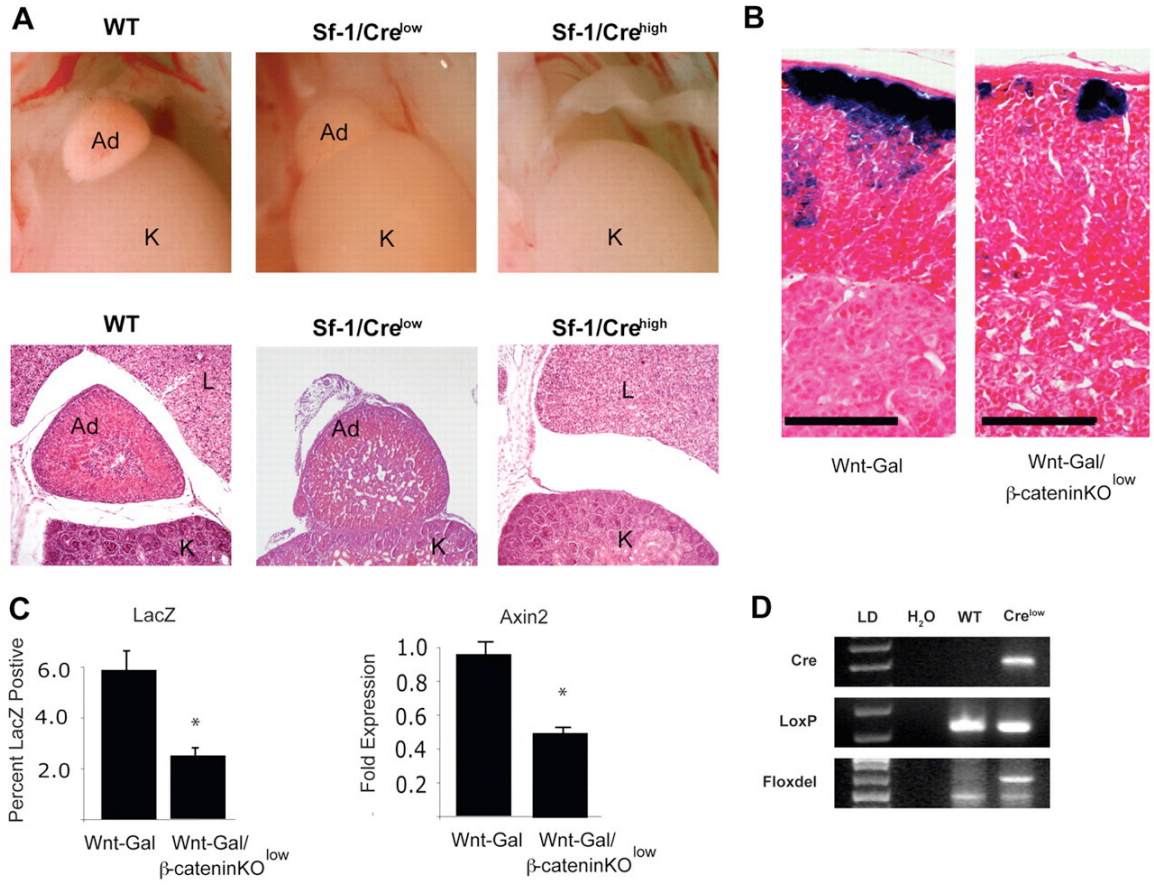
A
WT
Sf-1/Cre^low
Sf-1/Cre^high
Ad
K
L
B
Wnt-Gal
Wnt-Gal/
β-cateninKO^low
C
LacZ
Percent LacZ Postive
6.0
4.0
2.0
*
Wnt-Gal
Wnt-Gal/
β-cateninKO^low
Axin2
Fold Expression
1.0
0.8
0.6
0.4
0.2
D
LD
H2O
WT
Cre^low
Cre
LoxP
Floxdel

Figure 2.7 Progressive Decline of Adrenocortical Volume in Sf1-Cre^{low}–Mediated β-catenin KO Mice. (A) Histological analysis of the adrenal glands from WT and Sf1-Cre^{low}–mediated β-catenin KO mice at different ages. Adrenal glands were isolated from mice of the indicated genotypes as described in Materials and Methods and processed for immunohistochemical detection of Sf1 (antiserum A), β-catenin, and tyrosine hydroxylase (Th). TUNEL assay was conducted on the section as described in Materials and Methods. (B) H&E staining and Sf1 immunohistochemistry (antiserum A) of adrenal from WT and Sf1-Cre^{low}–mediated β-catenin KO mice at 45 weeks of age. The black bar highlights the adrenal cortex. Scale Bar: 100 μm. C: Adrenal Cortex; M: Adrenal Medulla.

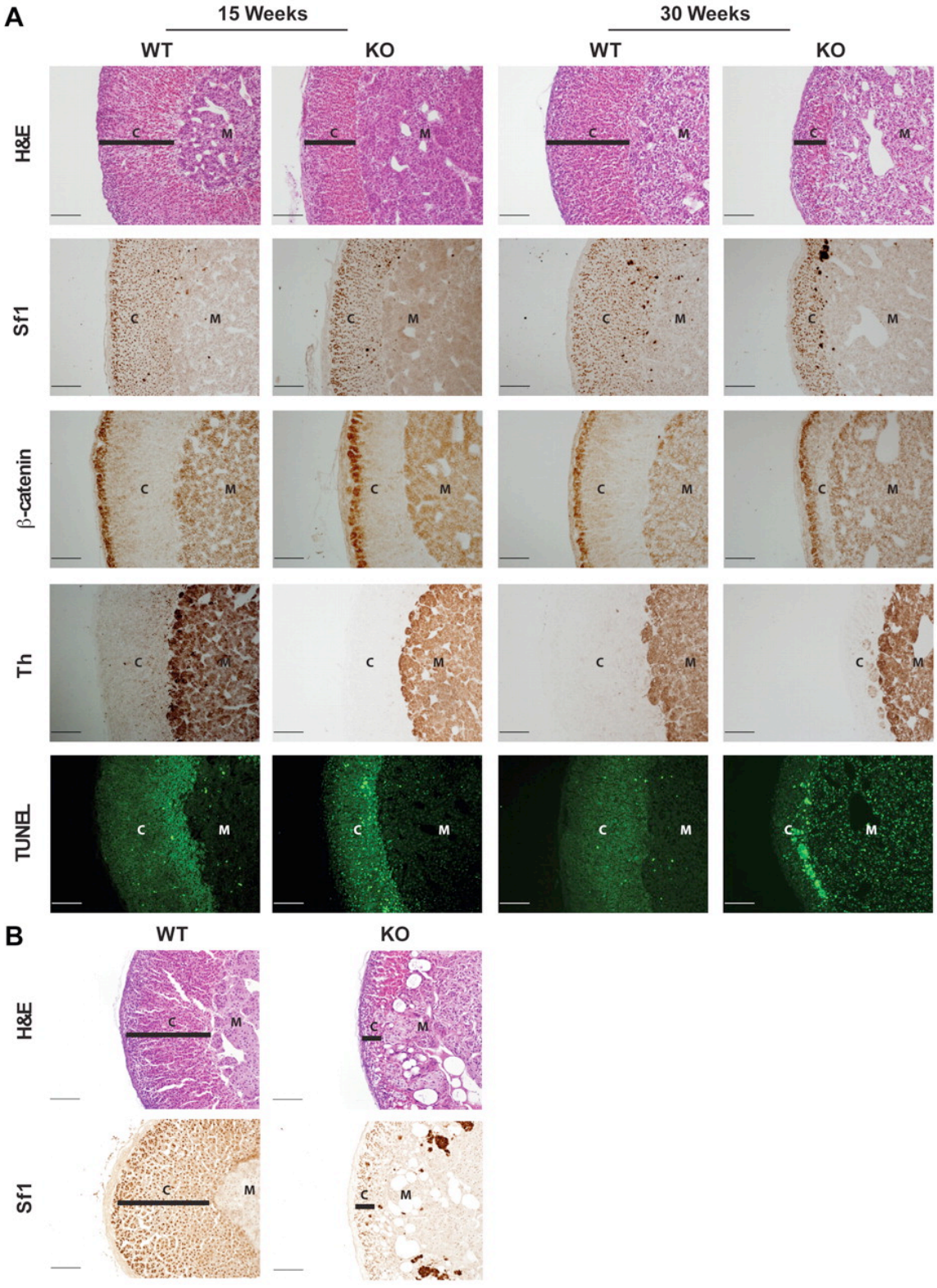
A
15 Weeks
30 Weeks
WT
KO
WT
KO
H&E
Sf1
β-catenin
Th
TUNEL
C
M
B
WT
KO
H&E
Sf1

Figure 2.8 Analysis of Differentiated Adrenocortical Markers. The quantitative PCR was performed on adrenal cDNA from the 30 weeks old WT and Sf1-Cre^{low}-β-catenin KO-histological failure mice as described in Materials and Methods.

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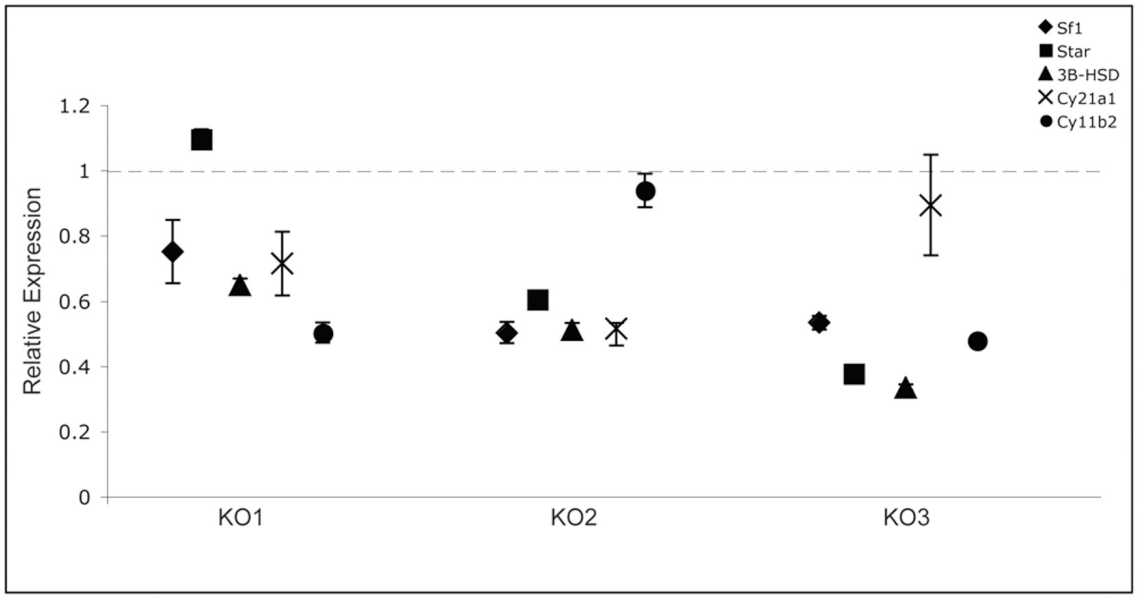

	Gene	Relative Expression w/ S.E.M.
KO1	Sf1	0.75 ± 0.10
	Star	1.09 ± 0.03
	3β-HSD	0.65 ± 0.02
	Cyp21a1	0.71 ± 0.10
	Cyp11b2	0.50 ± 0.03
KO2	Sf1	0.50 ± 0.03
	Star	0.60 ± 0.00
	3β-HSD	0.51 ± 0.02
	Cyp21a1	0.51 ± 0.02
	Cyp11b2	0.94 ± 0.05
KO3	Sf1	0.53 ± 0.02
	Star	0.38 ± 0.01
	3β-HSD	0.33 ± 0.01
	Cyp21a1	0.89 ± 0.15
	Cyp11b2	0.48 ± 0.01

Table 2.1 Analysis of Adrenal Weight and Basal Level ACTH Measurement of 30 Weeks Old Sf1-Cre^{low}-β-catenin KO Mice. The mice were separated into WT, KO-histological failure, and KO-non-histological failure groups. The mass of adrenal glands and the whole mouse were obtained at time of euthanasia. The adrenal mass was normalized to the total body weight ($p<0.05$ versus WT and KO-non-histological failure). The basal level ACTH were measured as described in the Materials and Methods ($p<0.05$ versus WT and KO-non-histological failure).

Table 1

	Adrenal Mass (Normalized)	Basal ACTH (pg/ml)
WT	1.31E-04 ± 1.83E-05	64.29 ± 31.16
KO-Cortical Decline	9.88E-05 ± 5.73E-06*	168.77 ± 80.90*
KO-Non-Cortical Decline	1.35E-04 ± 1.99E-05	59.58 ± 38.07
		*p<0.05 (Analysis of Variance)

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CHAPTER 3

THE ROLE OF CONSTITUTIVE β-CATENIN STABILIZATION ON ADRENAL DEVELOPMENT AND TUMORIGENESIS

ABSTRACT

Wnt signaling plays many important roles in the determination of cell polarity, migration, and differentiation during mammalian development. In the adrenal cortex, perturbations in the Wnt signaling pathway are associated with abnormal development and adrenocortical carcinogenesis. Stabilization of β-catenin has been reported in sporadic human adrenal tumors while loss of function mutation in APC (familial adenomatous polyposis) has been observed in adrenocortical tumors associated with Gardner Syndrome. To examine the role of constitutive activation of β-catenin, we performed conditional knockout of *Apc* using adrenal-specific Cre-expressing transgenic mice, *Sf1-Cre*^{high} and *Sf1-Cre*^{low}. In *Sf1-Cre*^{high}*/Apc* KO mice, in which the *Apc* gene is excised in most adrenocortical cells, we observe an abnormally developed adrenal gland. These adrenals are small in size and never fully maturity. In contrast, *Sf1-Cre*^{low}*/Apc* KO mice, in which the *Apc* gene is excised in fifty percent of adrenocortical cells, exhibit an expansion of less differentiated adrenocortical cells. These cells display a high cytoplasmic and nuclear concentration of β-catenin and undergo a high level of

proliferation. At >30 weeks of age, *Sf1-Cre*^{low}/*Apc* KO mice begin to present with adrenocortical adenomas and at later ages present with adrenocortical carcinomas. Additional studies with transgenic mice reveal the phenotype to be β-catenin dependent.

INTRODUCTION

Adrenal tumors are relatively common, affecting three to seven percent of the population. Most cases are benign adrenocortical adenomas (ACA), which carry an excellent prognosis. Infrequently, the tumors present as adrenocortical carcinoma (ACC), a highly malignant tumor with annual incidence of ~2 cases per million population (1-7). ACC has a bimodal age distribution with increased incidences in the first and fifth decade, with higher rates observed among females (1-7). Sixty percent of ACC patients exhibit hormone excess (aldosterone, cortisol, and/or sex steroids) resulting in Cushing's syndrome or virilization (1, 2, 7). Thirty to forty percent of patients, at the time of diagnosis, present with advanced disease and evidence of distant metastases (1-7). Consequently, these patients carry a dismal mean 5-year survival rate of less than five percent (1-7). Currently, 1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) ethane (mitotane) is the only FDA approved drug for ACC, specifically for advanced stages of disease (1-3, 8). However, the limited efficacy of mitotane and other treatment options highlight the requirement for a better understanding of the molecular etiology of ACC.

Activating mutations of the Wnt/β-catenin pathways have been implicated in adrenocortical tumors (9-11). Patients affected by familial adenomatous polyposis (FAP) have an autosomal-dominant disorder with a mutation in the adenomatous polyposis coli (*APC*) gene, a tumor suppressor gene located on the chromosome 5q21-22. The protein product of the *APC* gene functions as an inhibitory regulator of β-catenin. Most mutations in APC result in inappropriate accumulation of β-catenin, causing cellular transformations via constitutive activation of downstream β-catenin targets. Consequently, FAP patients present with colonic polyps at a young age (12). These

polyps, if left untreated, progress to colorectal carcinomas by the fourth decade (9). In addition, case studies have reported FAP patients exhibiting extracolonic tumors, such as adrenocortical adenomas and carcinomas (9). Other studies have reported the prevalence of adrenal tumors in thirteen percent of FAP population (9, 13).

In addition to *APC* mutations, mutations in the phosphorylation sites of the β-catenin gene, allow for stabilization of β-catenin via evasion of the destruction complex (14). Such β-catenin stabilizing mutations are found in multiple tumors including colorectal, desmoid, endometrial, hepatocellular, thyroid, ovarian, and adrenocortical (14). A study conducted by Tissier et al revealed that twenty-seven percent of adrenocortical adenomas and thirty-one percent of ACC have phosphorylation site mutations of the β-catenin (10). In addition, thirty-eight percent of adrenocortical adenomas and eighty-five percent of ACC have abnormal cytoplasmic and nuclear accumulation of β-catenin as assessed by immunohistochemistry (10). Together, these data demonstrate that alterations in cellular β-catenin levels may contribute to adrenocortical tumorigenesis.

While Wnt/β-catenin research identified roles of this pathway in many different tissue systems, only few studies have examined the importance of Wnt/β-catenin signaling in adrenal gland biology. Predicated on a) our earlier work indicating the role of the Wnt/β-catenin signaling pathway in both development and maintenance of adrenal tissue and b) the clinical evidence showing elevated β-catenin in adrenocortical tumorigenesis, we hypothesized that adrenal specific ablation of *Apc* in mice will constitutively stabilize β-catenin in the adrenal cortex and result in adrenocortical tumorigenesis.

In this study, we utilized the two Sf1-Cre transgenic mice (*Sf1-Cre*^{high} and *Sf1-Cre*^{low}), described in our prior study, to conditionally ablate the *Apc* gene in the adrenal cortex (15).

MATERIALS AND METHODS

Mice

All experiments involving mice were performed in accordance with institutionally approved and current animal care guidelines from the respective universities. The *Sf1-Cre*^{high} transgene targets high levels of expression of Cre recombinase to the urogenital ridge by E10.0 and activates a Cre-dependent reporter gene throughout the adrenal cortex (16); other sites of expression include pituitary gonadotropes, the ventromedial hypothalamic nucleus, somatic cells of the gonads, and the spleen. The *Sf1-Cre*^{low} transgene is a single-copy transgene that is expressed at lower levels in the same sites. Mice carrying the floxed Apc allele (*Apc*$^{loxP/loxP}$) were purchased from Bart Williams Van Andel Instititue (Grand Rapids, MI); this conditional allele contains *loxP* sites flanking exons 14, resulting in frameshift mutation generating a null product (17). Mice carrying the floxed β-catenin allele (*Ctnnb1*^{tm2kem}) were purchased from The Jackson Laboratory (Bar Harbor, ME); this conditional allele contains *loxP* sites flanking exons 2-6, resulting in complete inactivation upon Cre-mediated recombination (18).

Following timed matings, embryos were staged by designating noon of the day on which the copulatory plug was detected as E0.5. Correct staging was verified by appropriate morphological criteria as described (19). Genotyping for the *Sf1-Cre* transgene and the loxP alleles of *Apc* and β-catenin were performed on the amnion of each embryo and in adult mice, as previously described (16, 17).

Western Blotting

Adrenals were removed, dissected from surrounding adipose and connective tissue, and immediately snap frozen in liquid nitrogen. Protein lysates were collected by brief sonication of frozen tissues in lysis buffer (40 mM HEPES, 120 mM sodium chloride, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1 mM EDTA, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1% Triton X-100) containing protease inhibitor cocktail (Sigma), followed by 1-h rotation at 4 C to solubilize proteins. Soluble protein was collected from centrifuged total lysates and quantified by Bradford assay. SDS-PAGE was performed on 9–10% polyacrylamide Tris-glycine gels loaded with 10–40 μg of protein per sample, and separated proteins were transferred to nitrocellulose membranes using a semidry transfer unit. After transfer, membranes were blocked 1 h in 4% nonfat dry milk in TBST, and then incubated overnight at 4 C with primary mouse antibodies against unphosphorylated β-catenin (active β-catenin) (1:1000; Abcam, Cambridge, MA), primary mouse antibodies against proliferating cellular nuclear antigen (PCNA) (1:1000, Santa Cruz, Santa Cruz, CA), primary mouse antibodies against ß-actin (1:5000; Sigma, St. Louis, MO). The next day, membranes were washed three times for 5–10 min in TBST, and then incubated with horseradish peroxidase-labeled goat antirabbit or antimouse secondary antibody for 1 h at room temperature. After three more washes in TBST, membranes were incubated for 2–5 min in West Dura ECL reagent (Pierce, Rockford, IL), and then exposed to film for detection.

Analysis of Adrenal Histology and Immunohistochemistry

Adrenal glands were collected at the indicated ages and fixed for 2–3 h in 4% paraformaldehyde/phosphate buffered saline (PBS). Tissues were dehydrated in graded ethanol solutions and embedded in paraffin before sectioning. Sections were cut at 6 μm thickness and processed using standard procedures.

For immunohistochemical analyses, adrenal glands were processed as above and washed in Tris-buffered saline/0.1% Tween-20 (TBST, pH 7.5). Antigen retrieval was performed by boiling rehydrated sections in 10 mM sodium citrate (pH 6.0) for 20 min, followed by one wash in deionized water and two washes in TBST at room temperature. Antibody stainings were conducted using VECTASTAIN ABC kits and Vector Mouse on Mouse (M.O.M.) kits according to manufacturer's protocol (Vector Laboratories, Burlingame, CA). Tissue sections were blocked in antibody diluent solution for 1 h, and then incubated overnight at 4 C with anti-Dax1 (1:500, generously provided by Dr. Ken Morohashi), anti-20α-Hsd (1:10,000, generously provided by Dr. Yacob Weinstein), anti-β-catenin (610154) (1:1000, BD Biosciences, San Jose, CA), anti-β-catenin (H-102) (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-tyrosine hydroxylase (1:500, Pel-Freez Biologicals, Rogers, AR), and anti-Sf1 (1:1000 dilution and generously provided by Dr. Ken Morohashi). The next day, sections were washed, exposed to secondary antibodies, and processed for signal detection according to the manufacturer's protocol.

Real-Time PCR

Adrenal glands were removed, cleaned, and snap frozen. Frozen tissues were lysed in Trizol reagent using an electric tissue homogenizer, and total RNA was prepared

according to the manufacturer's protocol. Total RNA was treated with DNase (Ambion, Austin, TX) to remove residual genomic DNA and quantitated by UV spectrometry. 1 μg of total RNA was used to synthesize cDNA using the iScript kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The final cDNA products were purified and eluted in 50 μl of Tris-EDTA buffer using PCR purification columns (QIAGEN, Hilden, Germany) or directly diluted to final volume. Primer sequences for each gene are: *Axin2*: Fwd-5' gcaggagcctcacccttc, Rev-5' tgccagtttctttggctctt; *Lef1*: fwd-5' ctgaaatccccaccttctacc, Rev-5' tgggataaacaggctgacct; Tyrosine Hydroxylase (*Th*): Fwd-5' cccaagggcttcagaagag, Rev-5' gggcatcctcgatgagac;, *Cyp11a1* (p450scc): Fwd-5' aagtatggccccatttacagg, Rev-5' tggggtccacgatgtaaact; *Cyp11b2*: Fwd-5' gcaccaggtggagagtatgc, Rev-5' gccattctggcccatttag; *Cyp21a1*: Fwd-5' gacccaggagttctgtgagc, Rev-5' tccaaaagtgaggcaggaga; *Star*: Fwd-5' aaggctggaagaaggaaagc, Rev-5' ccacatctggcaccatctta; *Actb1* (β-actin): Fwd-5' ctaaggccaaccgtgaaaadg, Rev-5' accagaggcatacagggaca.

For quantitative, real-time PCR (qRT-PCR) analyses of mRNA abundance, reactions were performed with a 2x SYBR Green PCR mastermix (Applied Biosystems, Foster City, CA) and gene-specific primers in the ABI 7300 thermocycler (Applied Biosystems, Foster City, CA). Each quantitative measurement was normalized to Rox dye as an internal standard and performed in triplicate. Transcript abundance was normalized in each sample to the average Ct value for mouse β-actin (Livak et al., 2001). For mRNA quantitation, a minimum of three samples from differing genotypes was analyzed. Statistical significance was calculated using Student's t-test.

ACTH Stimulation Test

Dexamethasone (Sigma-Aldrich Corp., St. Louis, MO) was reconstituted in saline and injected IP at a dose of 5 μg/g mouse body weight at 1800 h the night before and at 0800 h the morning of the procedure. ACTH (Peninsula Laboratories, Inc., Belmont, CA) was reconstituted in saline and injected IP at a dose of 1 μg/g body weight at 1000 h the morning of the procedure. Tail blood was collected at 0-, 15-, 30-, and 60-min intervals for determination of serum corticosterone levels using a commercially available assay (MP Biomedicals, Solon, OH). Statistical significance was calculated using Student's t-test.

RESULTS

Conditional ablation of *Apc* causes aberrant adrenocortical development and cell expansion

To assess the effect of constitutive activation of β-catenin pathway in the adrenal cortex, we performed a conditional ablation of the *Apc* gene using the *Sf1-Cre*^{high} or *Sf1-Cre*^{low} transgenic mice. We crossed the *Sf1-Cre*^{high} or *Sf1-Cre*^{low} transgenic mice to the conditional *Apc* mutant mice carrying the floxed *Apc* gene (*Apc*$^{loxP/loxP}$) to produce progeny with *Sf1-Cre*^{high} (*Sf1-Cre*^{low})*/Apc*$^{loxP/loxP}$ genotype, which will be referred to as Apc^{high} KO or Apc^{low} KO, respectively (15-17). Conditional *Apc* mutant mice were originally developed by Shibata, H. et al to induce loss of function mutation of *Apc* caused by a resultant frameshift mutation from Cre-recombination of exon 14 (Figure 3.1A) (17).

Mice of both genotypes, Apc^{high} KO and Apc^{low} KO, were born at expected Mendelian ratios (Data not shown). PCR analysis of the genomic adrenal DNA confirmed the Cre-mediated excision of the *Apc* gene from both conditional knockout strains (Figure 3.1B). Initial analysis of Apc^{low} KO or Apc^{high} KO adrenals at 9 weeks revealed contrasting phenotypes. Adrenals from the Apc^{high} KO were considerably smaller than adrenals from wildtype (WT) littermates. By histological examination, Apc^{high} KO adrenals displayed a disorganized adrenal cortex (Figure 3.1C). In addition, there was a complete absence of recognizable X/fetal zone or adrenal medulla in the Apc^{high} KO adrenals (Figure 3.1C). This phenotype was one hundred percent penetrant in both males (n=6) and females (n=6).

In contrast, Apc^{low} KO adrenals were consistently larger, compared to WT littermates (Data not shown). Although histological examination of Apc^{low} KO adrenals revealed preservation of both the cortex and the medulla, we also observed abnormal cells intermingled within the adrenal medulla (Figure 3.1C). The observed phenotype in the Apc^{low} KO adrenals was one hundred percent penetrant in both males (n=6) and females (n=6).

Despite our initial hypothesis that conditional ablation of Apc and subsequent constitutive stabilization of β-catenin would result in tumorigenesis, no adrenal tumors were observed at 9 weeks from either the Apc^{high} KO (n=12) or the Apc^{low} KO (n=12) adrenals. In addition, there were no discernable differences between WT adrenals and Apc heterozygote, *Sf1-Cre^{high}/Apc$^{loxP/+}$* and *Sf1-Cre^{low}/Apc$^{loxP/+}$* , adrenals from both crosses. However, the phenotypes described above indicate that the constitutive activation of β-catenin in the adrenal cortex may play a role in both development and in adult tissue maintenance.

Presence of disorganized cortex and absence of adrenal medulla in APC^{high} KO

To further investigate the Apc^{high} KO phenotype of disorganized adrenal cortex and absence adrenal medulla (Figure 3.1C), marker analysis was performed in adrenals from 9 week old mice utilizing immunohistochemistry (IHC) with α-Sf1, a cortical marker, and α-Th, a gene product specifically expressed in the adrenal medullary cells. Confirming the histological observations, nearly all cells in the adrenal gland were positive for Sf1 expression while there was complete absence of Th expression (Figure 3.2A). In these Apc^{high} KO mice, where Cre-recombinase is expressed in nearly all cells

of the adrenal cortex, we expected a majority of adrenal gland cells of to express high cytoplasmic and nuclear β-catenin. However, the analysis identified only a few cells, in clusters, that were positive for β-catenin expression, while the majority of cells were devoid of β-catenin expression (Figure 3.2A). To further evaluate β-catenin expression, we carried out western blot analysis using an antibody that detects only stabilized, active (unphosphoryled) β-catenin protein. As seen in Figure 3.2B, unphosphorylated β-catenin (active β-catenin) was not elevated in Apc^{high} KO adrenals, but was only comparable to WT levels. This result is consistent with the IHC results. Additionally, because β-catenin is reported to promote proliferation, we examined the proliferation status of these Apc^{high} KO adrenals. As assayed by western blot for proliferating cell nuclear antigen (PCNA), the Apc^{high} KO adrenals revealed reduced proliferation (Figure 3.2B). From these results, we concluded that excision of Apc in most cells resulted in disturbed adrenal gland development resulting in absence of adrenal medulla and disorganization of the adrenal cortex, presumably due to loss of proliferation.

Aberrant cells are adrenocortical in origin

In WT mice, the adrenal gland is clearly compartmentalized into the cortex and the medulla, as both tissues are derived from distinct embryonic origins (cortex: mesoderm, medulla: neuroectoderm). Moreover, adrenocortical cells arising from the peripheral region of the gland are centripetally displaced inward to repopulate the gland. Upon reaching the corticomedullary boundary, these cells undergo apoptosis, by a yet to be identified stimulus (20-22). However, in Apc^{low} KO adrenals, the histological analysis revealed the presence of abnormal cells intermingled within the adrenal medulla (Figure

3.1C). To identify the origin of these cells, we again utilized the IHC marker study, described above. In the Apc^{low} KO adrenals, the aberrant, intermingled cells stained positive for Sf1 while lacking expression of Th (Figure 3.2A). This identified the aberrant cells to be of adrenocortical in origin.

Next, we set out to determine whether the aberrant cells were a direct result of conditional ablation of *Apc* gene and subsequent stabilization of β-catenin. Normally, in WT adrenals, the subcapsular region of the adrenal cortex is the only site of β-catenin expression and active signaling. As expected, the aberrant cells in the Apc^{low} KO stained positive for both cytoplasmic and nuclear β-catenin (Figure 3.2A). In addition, we observed cells in the cortex, stained positive for cytoplasmic and nuclear β-catenin, indicated by the arrows (Figure 3.2A). The model of adrenocortical repopulation of the cortex is hypothesized to occur by proliferation of progenitor cells in the periphery of the gland, the subcapsular zone (SZ), followed by centripetal displacement of differentiated cells. Consistent with this model, we hypothesize that cytoplasmic/nuclear β-catenin positive cells originate from the SZ, also a region of active β-catenin signaling in WT mice.

When we examined the adrenal lysates of the Apc^{low} KO adrenals by western blot, we observed higher expression of stabilized β-catenin, compared to the WT (Figure 3.2B). Moreover, the KO adrenals had increased levels of PCNA suggesting that there were more cells undergoing S-phase (Figure 3.2B). These data collectively suggested that in Apc^{low} KO adrenals the aberrant cells were a result of stabilized β-catenin, high level of proliferation, and share characteristics similar to the cells of the SZ of the adrenal cortex.

Expansion of subcapsular adrenocortical cells in the Apc^{low} KO but not in Apc^{high} KO

In our previous study, depletion of β-catenin, which is expressed only in the subcapsular region, resulted in an age dependent depletion of adrenocortical cells and eventual failure to maintain the adult cortex (15). We hypothesize that these subcapsular cells are tissue specific precursor cells that differentiate into the functional steroidogenic cells of the adrenal cortex. Therefore, constitutive stabilization of β-catenin in these subcapsular/precursor cells would result in an expansion of less differentiated adrenocortical cells.

To test this hypothesis, we performed IHC for an orphan nuclear receptor superfamily 0 group B1 (Nr0b1, Dax1), which is a marker for the subcapsular region and a target of Sf1/β-catenin transcriptional complex (23). Although the gene expression of *Dax1* is regulated by the Sf1/β-catenin mediated transcription, Dax1 functions to inhibit Sf1 transactivation and, recently been shown, to play a role in maintenance of undifferentiated state of embryonic stem cells (24, 25). In sections from Apc^{high} KO adrenals, a population of Dax1 expressing cells was present (Figure 3.3A), but Dax1 expression was not observed in the clusters of β-catenin expressing cells. It is possible that the absence of Dax1 expression in these cells is due to activation of β-catenin transcription independent of Sf1. Moreover, due to the abnormal development and absence of clear zonation in the Apc^{high} KO adrenals, we hypothesized that Sf1-positive cells of these adrenals may be X/fetal zone cells. Therefore, we performed additional IHC utilizing 20α-Hsd, a marker of mature X/fetal zone (26). Interestingly, we did not

detect expression of 20α-Hsd in Apc^{high} KO adrenals (Figure 3.3A), suggesting that a) X/fetal zone never formed or b) these X/fetal zone underwent atrophy. Also it does not rule out the possibility that these adrenals maintain the embryonic state, as embryonic adrenals are negative for 20α-Hsd (26).

In the Apc^{low} KO adrenals, we observed expansion of Sf1-positive/β-catenin-positive cells within the medulla and few of these cells in the cortex. Therefore, to further identify the origin of these aberrant cells, we stained the Apc^{low} KO adrenal sections with Dax1. In contrast to the results in adrenals from Apc^{high} KO mice, adrenals from Apc^{low} KO mice exhibit Dax1 staining in the SZ as well as in the aberrant cell (Figure 3.3A). The result suggested the origin of these aberrant cortical cells to be the subcapsular zone. However, due to the location of these cells, within the area of the X/fetal zone, we tested whether these aberrant cells were of X/fetal zone origin by staining for 20α-Hsd. Although expression of 20α-Hsd was observed in the X/fetal zone at the corticomedullary boundary, the aberrant cells were devoid of 20α -Hsd expressions. Therefore a relation of the aberrant cells to the X/fetal zone was unlikely.

If the aberrant cells of Apc^{low} KO are indeed an expansion of less differentiated cells of the adrenal cortex, it would be predicted that these cells would exhibit a decrease in steroidogenesis, the defining characteristic of a differentiated adrenocortical cells. To test this hypothesis, we extracted RNA from the Apc^{low} KO adrenals and their WT littermates and generated cDNA to perform quantitative RT-PCR. Until 15 weeks of age, we consistently observed a downregulation of steroidogenic gene expression in the Apc^{low} KO adrenals, specifically, a 50% downregulation of expression of *Star*, *Cyp11a1* (p450scc), and *Cyp21a1* (21OH) in Apc^{low} KO compared to WT adrenals. In accordance

Cyp11b1 and *Cyp11b2* were downregulated 25% and 40%, respectively, when compared to Apc^{low} KO to WT (Figure 3.3B). As expected, *Axin2* and *Lef1* levels were upregulated in Apc^{low} KO adrenals suggesting that stabilized β-catenin was participating in active signaling (Figure 3.3B). Taken together, our results demonstrate that Apc^{low} KO adrenals exhibit an expansion of less differentiated adrenocortical cells.

Aberrant adrenocortical cells are present during embryonic stages of development

Next we explored the embryonic origin of the aberrant adrenocortical cells in both Apc^{low} KO and Apc^{high} KO. To determine the earliest time of appearance of these cells, we examined embryos from timed pregnancies. In embryos of the Apc^{high} KO, the adrenal glands are significantly smaller than those of WT (Figure 3.4). We detected a cluster of Sf1 positive cells with high cytoplasmic/nuclear β-catenin as early as e14.5 (Figure 3.4). In addition, we observed failed migration of neural crest cells into the developing gland and thus the adrenal medulla fails to form (Figure 3.4). These cells remain outside of the abnormally developing adrenal gland (Figure 3.4), suggesting that stabilization of β-catenin results in loss of an unidentified morphogen gradient resulting in an inability of neural crest cells to migrate to their usual anatomical site within the adrenal gland.

In the Apc^{low} KO adrenals, as early as e14.5, a population of adrenocortical cells displayed cytoplasmic and nuclear accumulation of β-catenin (Figure 3.4). The dual staining revealed that Sf1 positive cells began to display high nuclear expression of β-catenin (Figure 3.4). In our previous work, we defined the β-catenin expressing cells of the developing adrenal gland to be the precursors, which we hypothesized to become

progenitor cells in the SZ of the adrenal cortex. With SZ cells in the WT adrenal being the only cells expressing β-catenin, we hypothesize that in Apc^{low} KO embryos, precursor cells that underwent Cre-recombination maintain constitutive stabilization of β-catenin and are trapped in a less differentiated state.

Age dependent formation of adrenocortical tumors in Apc^{low} KO mice but not in Apc^{high} KO mice

Initially, we hypothesized that conditional ablation of *Apc* gene, with subsequent constitutive stabilization of β-catenin, would result in formation of adrenocortical tumors. However, we failed to observe any tumor formation from birth to 15 weeks in both the Apc^{low} KO and Apc^{high} KO mice (Data not shown). To test whether our mouse model parallels the human pathology, where increasing incidences of adrenocortical tumors, specifically ACC, occur with age, we analyzed later timepoints of our Apc KO mice. When we assessed the mass of the adrenals (normalized to body mass) from Apc^{low} KO and Apc^{high} KO mice, we consistently observed an age-dependent increase in adrenal mass of Apc^{low} KO while Apc^{high} KO adrenals remained small, even at timepoints >30 weeks (Figure 3.5). Also, the Apc^{low} KO adrenals at 30 weeks exhibited increased steroidogenic gene expression (Figure 3.5). Such increased steroidogenic gene expression was confirmed through an ACTH stimulation test, which quantified the plasma corticosterone levels in response to plasma ACTH concentration. Importantly, at 30 weeks of age adrenocortical tumors begin to form (Figure 3.5E, F). At >30 weeks timepoints, a spectrum of tumors from low-grade benign adrenocortical adenomas (n=10) to high-grade adrenocortical carcinoma (n=1) was detected.

Phenotypic rescue of Apc KO adrenals through a concurrent β-catenin KO

Recent studies provided evidence that Apc protein can function independent of β-catenin stabilization. To determine whether tumorigenesis in our mouse model is mediated by constitutive stabilization of β-catenin rather than by some other function of Apc, we generated mice with adrenal specific KO of Apc and β-catenin (Sf1-Cre^{low}/Apc$^{loxP/loxP}$/β-catenin$^{loxP/loxP}$). The concurrent study utilizing Sf1-Cre^{high} mice could not be conducted as Sf1-Cre^{high}/β-catenin$^{loxP/loxP}$ mice exhibited adrenal aplasia at birth (15). If the phenotype is a result of constitutive stabilization of β-catenin, we expect to see a rescue in the double KO mice. When we examined the adrenals from the 6 week old Sf1-Cre^{low}/Apc$^{loxP/loxP}$/β-catenin$^{loxP/loxP}$ mice, we observed a complete absence of the aberrant cells present in the adrenal medulla (Figure 3.6). IHC with α-Sf1 and α-Th revealed a clear delineation of the adrenal cortex and the medulla in the Sf1-Cre^{low}/Apc$^{loxP/loxP}$/β-catenin$^{loxP/loxP}$ mice (Figure 3.6). Moreover, staining of the tissue with α-β-catenin revealed an absence of adrenocortical cells with cytoplasmic/nuclear β-catenin accumulation (Figure 3.6). These results strongly suggest that tumor formation in the adrenal cortex in Apc^{low} KO is dependent on overexpression of β-catenin.

DISCUSSION

The role of the Wnt/β-catenin signaling pathway in adrenocortical development and maintenance was demonstrated through loss of function studies of *Wnt4* and β-catenin (15, 27, 28). In vitro studies confirmed the transcriptional role of β-catenin, which synergize with Sf1 leading to an activation of adrenocortical specific target genes, such as *Dax1*, *Inha, and Star* (23, 29, 30). These genes are essential for the development and establishment of the adrenal cortex. Moreover, mutations resulting in constitutive activation of β-catenin, including mutation in *APC* and β-catenin, have been identified in both benign and malignant adrenal tumors in humans (9, 10, 13). Our goal in this chapter was to model and analyze the role of constitutive activation of β-catenin in the adrenal cortex, specifically in tumorigenesis

We utilized two Sf1-Cre transgenic mouse strains that express Cre-recombinase at different levels. To stabilize β-catenin in the adrenal cortex, we conditionally ablated the *Apc* gene. We originally hypothesized that both Apc^{low} KO and Apc^{high} KO adrenals would develop tumors. However, the knockout mice revealed contrasting phenotypes. Apc^{high} KO mice displayed a developmental phenotype while Apc^{low} KO exhibited aberrant cell expansion followed by tumorigenesis. Our studies confirmed that the observed phenotype is β-catenin dependent. Importantly, we present here the first mouse model of adrenocortical carcinoma.

Regulation of β-catenin protein levels is essential for normal adrenocortical differentiation

Similar to our previous conditional β-catenin ablation study, different degrees of conditional *Apc* ablation resulted in both developmental and adult tissue maintenance phenotypes.

In the Apc^{high} KO, where nearly all cells of the developing adrenal cortex were deficient in *Apc*, we expected to observe an increased stabilization of β-catenin and resultant early tumorigenesis. However, the Apc^{high} KO presented with abnormally developed, hypoplastic adrenals that were significantly smaller in size compared to WT. In addition, there was a complete absence of the adrenal medulla, which also contributed to its smaller mass. Although we analyzed later timepoints of Apc^{high} KO mice up to >30 weeks, these abnormal adrenal glands never developed adrenocortical tumors and maintained a less differentiated state. Other investigators have reported that complete lack of *Apc* halts the ability of embryonic stem cells to differentiate *in vitro*. Homozygous loss of function mutation of *Apc* results in an embryonic lethality by a developmental failure of primitive ectoderm (31, 32). Further investigations of this phenotype identified an Apc-dosage dependent β-catenin accumulation that results in a spectrum of differentiation blockade (33, 34). Similarly, tissue specific complete *Apc* ablation, leading to high β-catenin accumulation, was described as causing a tissue "dilemma", whereby high β-catenin dosage led to improper development rather than tumorigenesis (35-38). For example, in thymus, bone, and mammary gland, consequences of complete *Apc* ablation were failure of thymocyte maturation, prevention of osteoblast differentiation, and formation of severe metaplasia, respectively. Perhaps, the most interestingly observation is the complete absence of neoplasms (35, 36, 39, 40). Although β-catenin accumulation is the initiating event in these developmental defects, it

is believed that suppression of downstream targets, direct or indirect, may be the reason for such phenotypes (35, 41). Our results are consistent with these observations: the Apc^{high} KO result in elevated β-catenin accumulation in the developing adrenocortical cells leading to the inhibition of proper developmental programming but not tumorigenesis. In contrast, Apc^{low} KO results in both developmental defects and increased tumorigenesis.

Expansion of less differentiated adrenocortical cells

In the adrenal gland, the subcapsular zone (SZ) serves as the residence of less differentiated (progenitor-like) adrenocortical cells. Historical studies involving adrenal enucleation (complete removal of inner adrenal gland with exception of capsule and few subcapsular cells), transplantation studies, and BrdU/^{1}H-thymidine pulse-chase studies suggest that subcapsular/peripheral cells give arise to the differentiated adult cortex (42-48). Moreover, our previous study revealed that expression and active β-catenin only occurs in the SZ (15). Ablation of β-catenin in the adult cortex resulted in failure to maintain self-renewal of the adrenocortical tissue (15). Given the proposed role of β-catenin in adult gland maintenance as well as in adrenocortical tumorigenesis, we decided to employ the Sf1-Cre^{low} transgenic mice to ablate *Apc* and analyze constitutive activation of β-catenin in this organ.

In contrast to Apc^{high} KO, in Apc^{low} KO adrenals approximately 50% of the adrenocortical cells undergo Cre-mediated ablation of the Apc gene. By allowing some cells to escape Cre-recombination, Apc^{low} KO adrenals matured into relatively normal adult glands with presence of normal cortex and medulla, but with aberrant cortical cells.

Therefore it was possible to study the regulation of cell fate decision by the constitutively active Wnt/β-catenin signaling pathway in adult tissue.

As described in other systems, such as lung, haematopoeitic, and intestinal, stabilization of β-catenin resulted in initial expansion of somatic progenitor cells (37, 38, 49, 50). In the Apc^{low} KO adrenals, we observed a population of aberrant adrenocortical cells that share main characteristics with SZ cells. Immunohistochemical assays revealed high cytoplasmic and nuclear β-catenin concentrations as well as expression of *Dax1*, a nuclear target of Sf1/β-catenin and marker of subcapsular cells (Figure 3.3). Moreover, gene expression profiling of Apc^{low} KO adrenals compared to WT littermates revealed a downregulation of steroidogenic enzymes. These results suggested an overall expansion of the SZ population presumably caused by stabilization of β-catenin, with a preserved subcapsular population and the formation of a SZ population within the adrenal medulla.

We were intrigued by the medullary location of these cells. As such, we would like to entertain three explanations for this observation. First, in a WT adrenal gland, the corticomedullary boundary serves as an apoptotic boundary allowing for separation cortex from the medulla. However, we hypothesize that constitutive activation of β-catenin in these cells acts as a pro-survival signal and allows these cells to safely bypass the boundary. Although there are conflicting reports of involvement of Apc in apoptosis, loss of function mutations in Apc^{low} KO may contribute to the aberrant cells bypassing the apoptosis zone to reside in the adrenal medulla. (51, 52). Second, there are reports of Apc and β-catenin acting as a "zonation keeper", a phenomenon more defined in liver (53). Through stabilization of β-catenin in the adrenal cortex, where it is normally expressed in SZ, we have disrupted the gradient allowing for expansion of SZ cells.

Third, the aberrant cells represent the fetal adrenocortical precursor cells, which did not regress while the adult organ was forming. In case of the Sf1-Cre^{low} driver, normal adrenal cortex developed from cells that escaped Cre-recombination while the cells that underwent Cre-recombination were left as immature adrenocortical cells interspersed in the medulla. On the contrary, the Apc^{high} KO adrenal cortex represents immature (fetal) adrenocortical cells. This latter model is especially intriguing as it also suggests that formation of the adult cortex is dependent on the presence of a functional fetal cortex. Indeed, the recent data indicated that adult cortex is derived from the fetal cortex (54).

Development of adrenocortical tumors in Apc^{low} KO adrenals

We did not observe any tumor formations in the Apc^{low} KO adrenal glands until 15 weeks of age. However, analogous to other systems, such as colon and skin, we began to see age-dependent tumorigenesis beginning at 30 weeks of age. At 30 weeks, adenomas appear to form from the aberrant cells in the medulla. Concurrent with tumorigenesis, the steroidogenic gene expression profile was significantly increased and mirrored by hyper-responsiveness in ACTH stimulation test.

Older mice (>45 weeks) developed adrenocortical carcinomas. These tumors had high mitotic index, contained nuclear β-catenin, and were adrenocortical in origin (Sf1 positive). Whether ACC arise from existing ACA is unknown. While there may be *in vivo* mechanisms to prevent progression from adrenocortical adenoma to carcinoma, our mouse model suggest that constitutive activation of the Wnt/β-catenin pathways can contribute or predispose ACA to transition into ACC. Gene expression profiling of adrenocortical carcinomas has identified a classic ACC signature, of which dysregulation

of the IGF2 locus is a hallmark. In both sporadic and hereditary human ACC, an upregulation of IGF2 with downregulation of $p57^{KIP2}$ is observed (55). Analysis of our Apc^{low} KO carcinoma revealed a similar expression pattern with increased Igf2 and decreased $p57^{kip2}$, suggesting that we established a mouse ACC model that closely resembles ACC in human patients.

β-catenin dependent tumorigenesis

Although other groups have reported that expansion of cells in Apc models are a consequence of β-catenin-dependent mechanism (38, 56, 57), emerging data suggest multiple roles for Apc forming addition to its classical role in β-catenin stabilization. To confirm the β-catenin-dependence of tumorigenesis in our mouse model, we conditionally ablated both Apc and β-catenin. Although Sf1-Cre^{high}/β-$catenin^{loxP/loxP}$/$Apc^{loxP/loxP}$ could not be analyzed because these mice lack an adrenal gland, we were able to analyze Sf1-Cre^{low}/β-$catenin^{loxP/loxP}$/$Apc^{loxP/loxP}$ (15). In these mice, we observed a complete phenotype rescue, a complete absence of aberrant cells. Thus the presented phenotype maybe caused by a β-catenin-dependent mechanism.

In this chapter, we report results of our investigation into the role of stabilized β-catenin in the adrenal cortex. Through controlling the dosage of Apc ablation, and consequent β-catenin stabilization, we revealed the importance of β-catenin signaling in adrenocortical development, adult tissue maintenance and tumorigenesis. We showed that stabilization of β-catenin using Sf1-Cre^{low} resulted in initial expansion and displacement of progenitor cells. Moreover, stabilization of β-catenin resulted in disruption of adrenal architecture. Lastly, we present the first bona fide model of

adrenocortical tumorigenesis favoring a model of classical adenoma to carcinoma progression in Apc deficient organisms.

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Figure 3.1 Initial Analysis of Apc KO Mice Reveal Contrasting Phenotypes. A) Diagram demonstrates the conditional inactivation of Apc. $Apc^{loxP/loxP}$ mice harbor flanking loxP site of exon 14. Cre-recombination causes a frameshift mutation resulting in a loss of function of Apc (17). B) PCR genotyping for Cre-recombinase, loxP sites, and Cre-mediated recombination. bp: base pair, std: DNA standard. C) Initial examination of males and females at 9 weeks of WT (6 males, 6 females), Apc^{low} KO (6 males, 6 females), and Apc^{high} KO (6 males, 6 females) adrenals by H&E. C: cortex, M: medulla, X: fetal/x-zone. Scale bar = 100 μm.

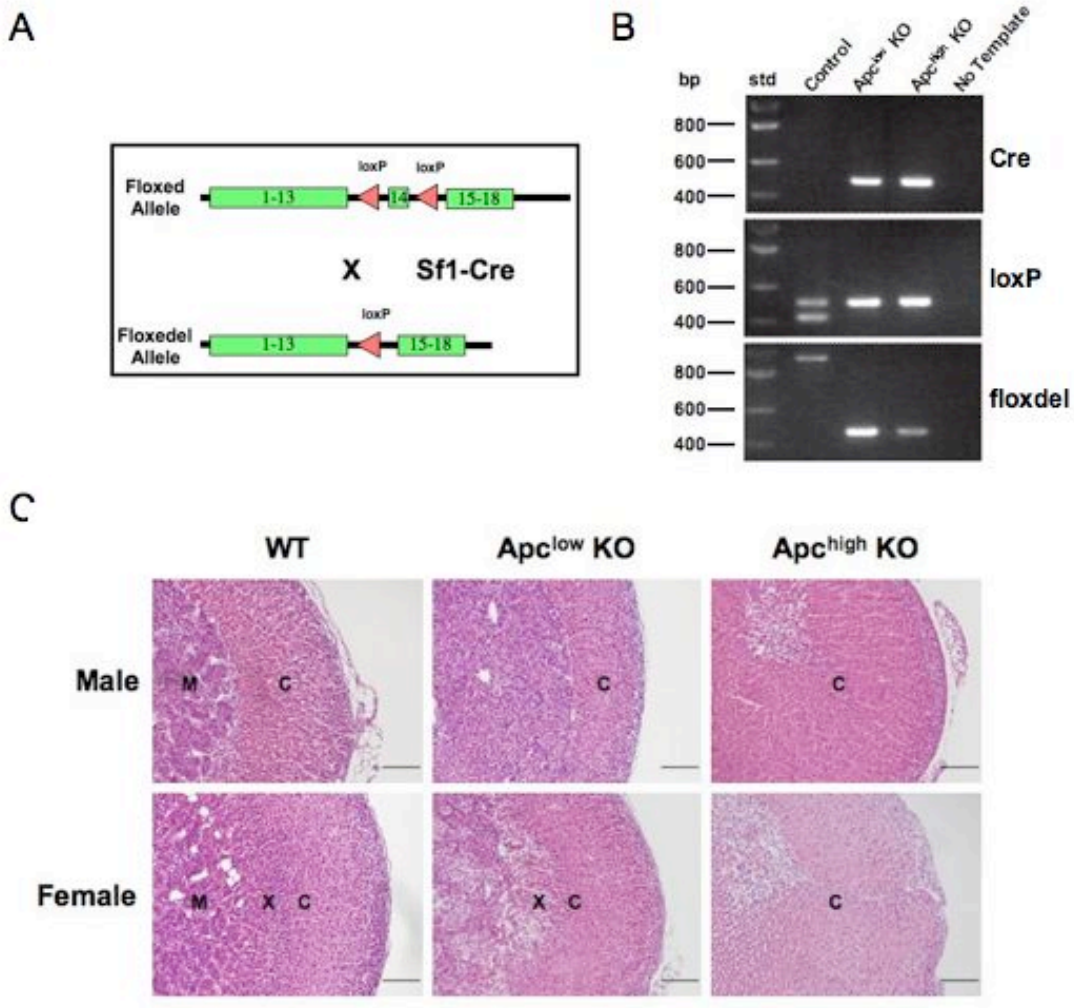

A
Floxed Allele
loxP
loxP
1-13
14
15-18
X
Sf1-Cre
Floxedel Allele
loxP
1-13
15-18
B
bp
std
Control
Apc low KO
Apc high KO
No Template
800
600
400
Cre
loxP
floxdel
C
WT
Apc low KO
Apc high KO
Male
Female
M
C
X

Figure 3.2 Assessment of 9 Weeks Apc KO Adrenals via IHC and Western Blot Analysis. A) IHC of WT, Apclow KO, and Apc^{high} KO adrenals using α-Sf1, α-Th, and α-β-catenin. Scale Bar = 100 μm. B) Western blot analysis of protein lysates from WT, Apc^{low} KO, and Apc^{high} KO adrenals. Lanes were loaded with 10 μg of protein lysates. Blots were probed with α-active-β-catenin (unphosphorylated β-catenin), PCNA, and α-β-actin.

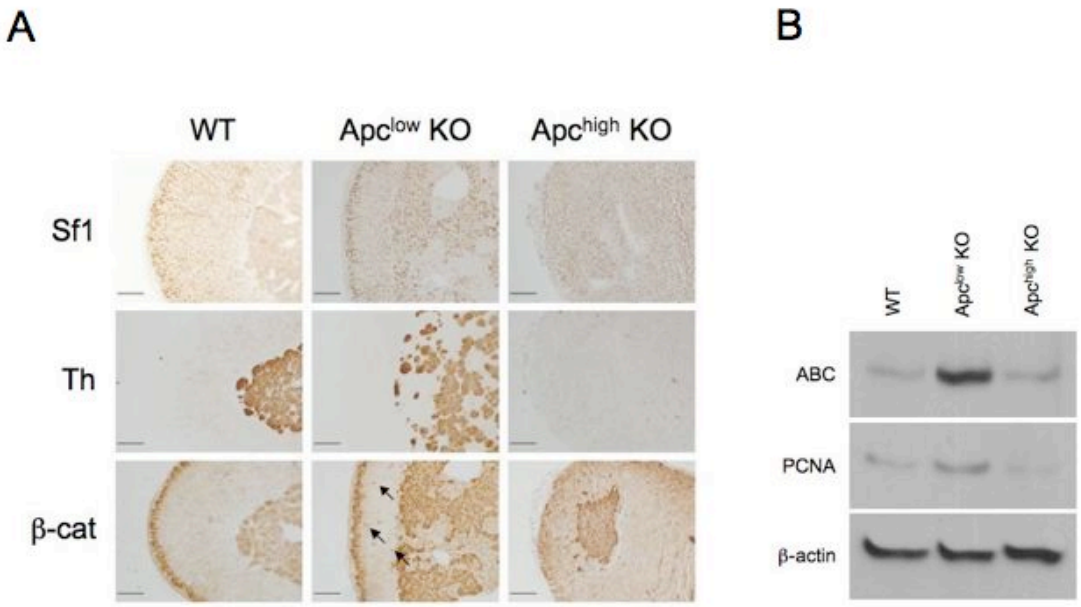
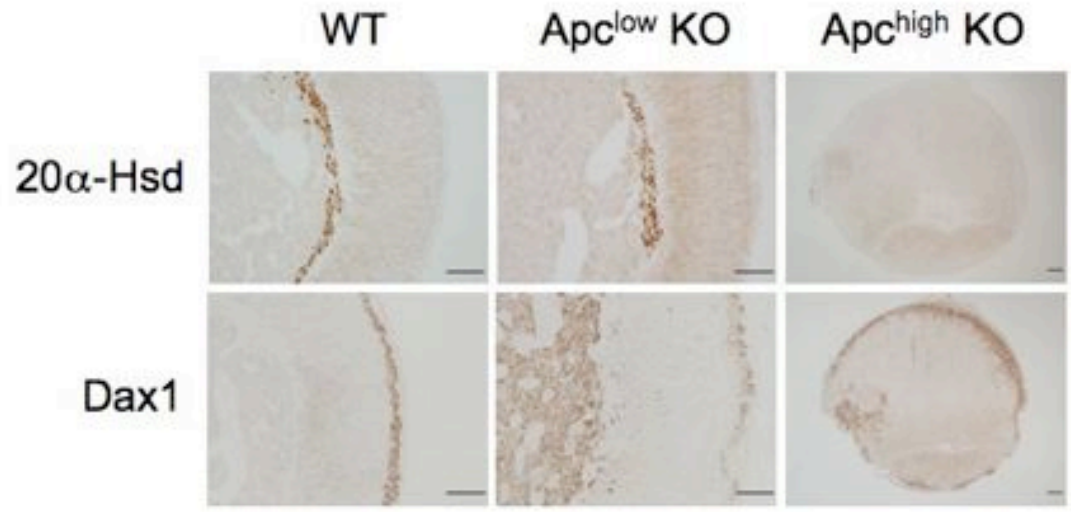

A
WT
Apc^low KO
Apc^high KO
Sf1
Th
β-cat
B
WT
Apc^low KO
Apc^high KO
ABC
PCNA
β-actin

Figure 3.3 Marker Analysis and Differential Gene Expression Profiling on Apc KO Adrenals. A) 9 weeks old adrenals of WT, Apc^{low} KO, and Apc^{high} KO adrenals using α-Dax1, a subcapsular marker, and α-20α-Hsd, a X/fetal zone marker. Scale Bar: 100 μm. B) Quantitative RT-PCR on 12 weeks adrenals from WT and Apc^{low} KO.

A


WT
Apclow KO
Apchigh KO
20α-Hsd
Dax1

B

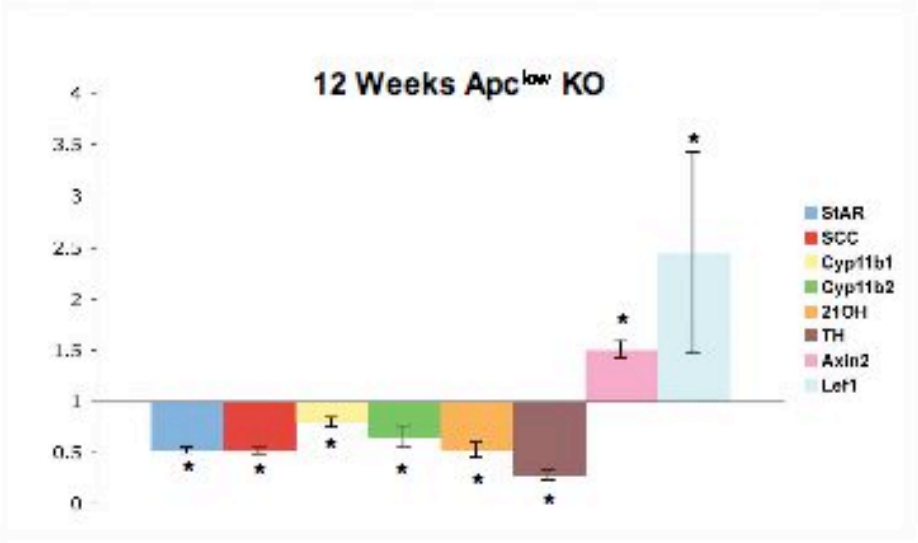

12 Weeks Apclow KO
4
3.5
3
2.5
2
1.5
1
0.5
0
StAR
SCC
Cyp11b1
Cyp11b2
21OH
TH
Axin2
Lef1

Figure 3.4 Assessment of Apc KO Embryonic Adrenal Gland in the e14.5. In the Apc^{low} KO adrenals there is an expansion of adrenocortical cells with emergence of aberrant cells expressing high cytoplasmic and nuclear β-catenin. The analysis of Apc^{high} KO reveals absence of medullary cells and defect in formation of adrenal gland. Red: α-Sf1 (Middle Panels) and α-Th (Lower Panels), Green: α-β-catenin (Middle and Lower Panels). Scale Bar: 100 μm.

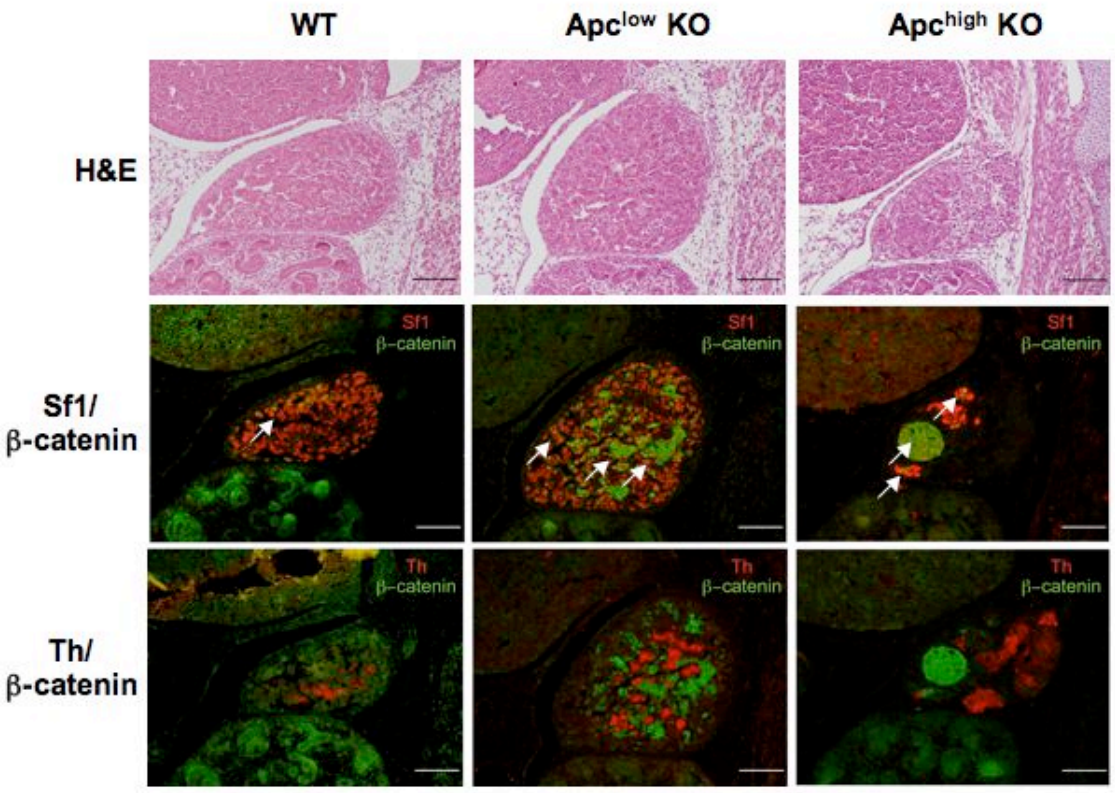
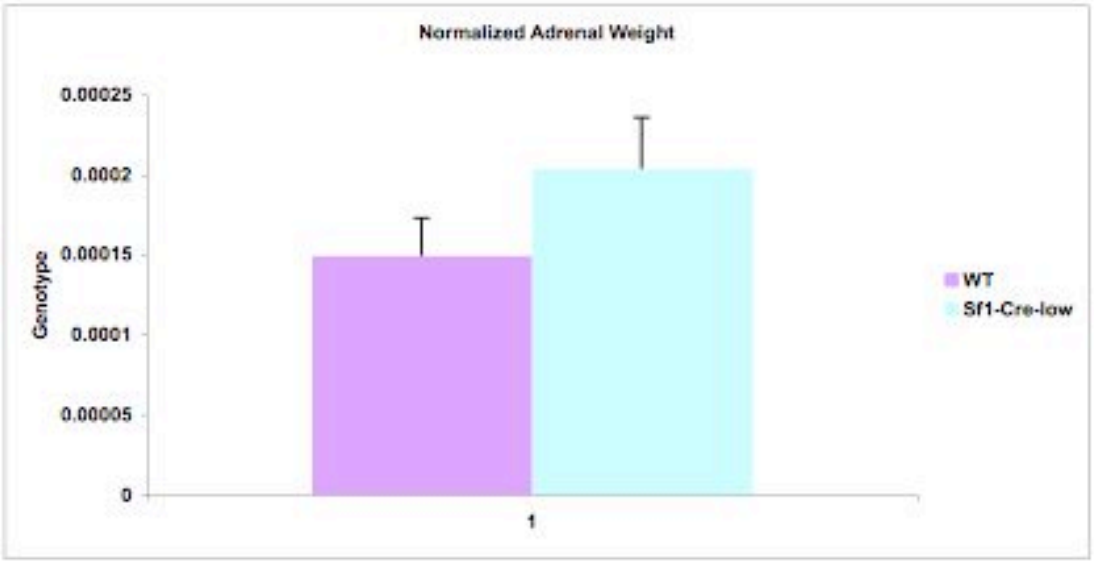
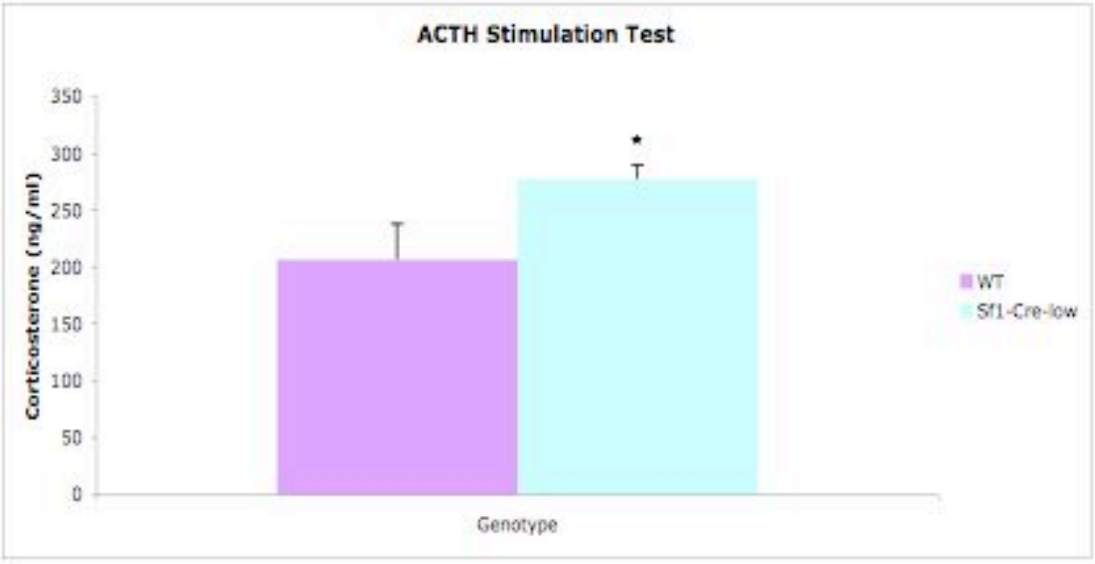

WT
Apc^low KO
Apc^high KO
H&E
Sf1/
β-catenin
Th/
β-catenin
Sf1
β-catenin
Th
β-catenin

Figure 3.5 Analysis of >30 Weeks Adrenals and Formation of Adrenal Tumors. A) Normalized adrenal mass of $Apc^{low}KO$ at 30 weeks reveal larger adrenals in the KO ($p<0.05$ by Student TTEST). B) ACTH stimulation test on 30 week adrenals from WT and $Apc^{low}KO$. The value represents the mean log-phase corticosterone level following ACTH stimulation ($p<0.05$ by Student TTEST). C) Steroidogenic gene expression profile via Q-PCR of the 30 week adrenal of WT and $Apc^{low}KO$. The values represent fold expression of individual gene normalized to WT levels ($p<0.05$). D) Q-PCR on Igf2 and $p57^{kip2}$ on 12 weeks and 30 weeks adrenals from $Apc^{low}KO$ normalized to WT. E) Quantitation of tumor incidences in 15 (n=12), 30 (n=9), 45 (n=10), and >45 week (n=6) adrenals from $Apc^{low}KO$. F) Panel of representative $Apc^{low}KO$ adrenals with increase in age. Scale Bar: 100 μm.

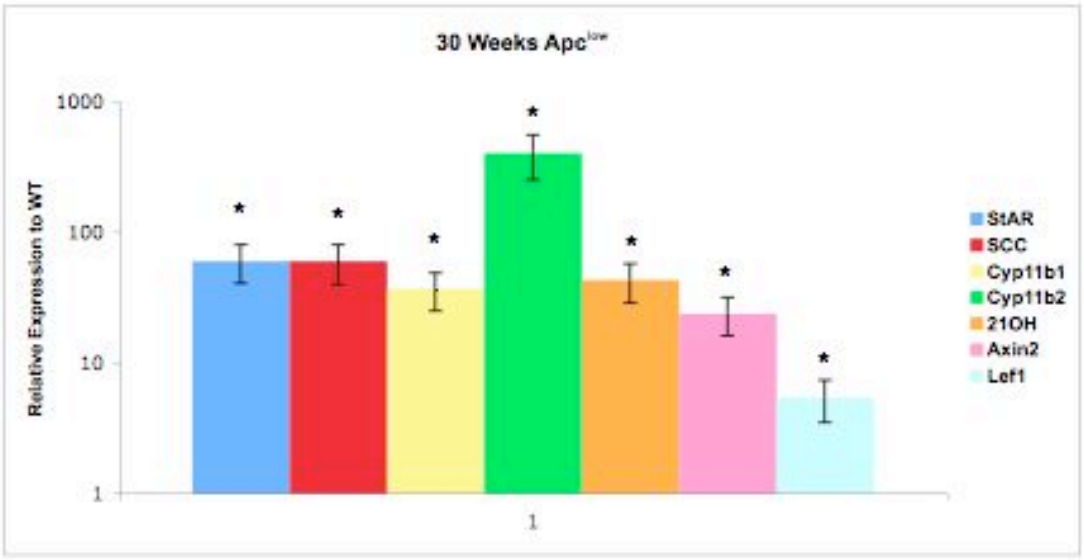
A


Normalized Adrenal Weight
0.00025
0.0002
0.00015
0.0001
0.00005
0
Genotype
1
WT
Sf1-Cre-low

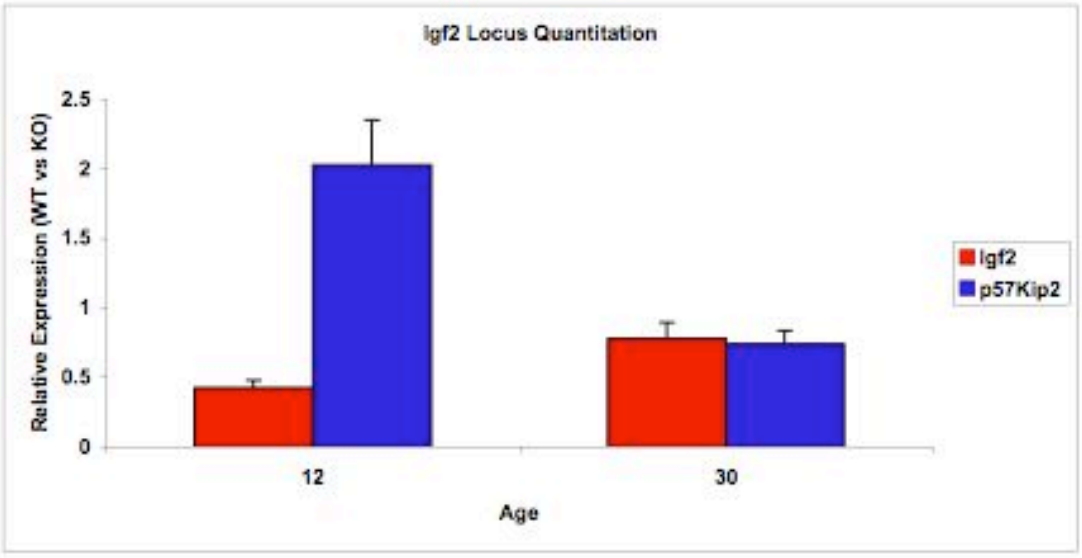
B


ACTH Stimulation Test
350
300
250
200
150
100
50
0
Corticosterone (ng/ml)
*
Genotype
WT
Sf1-Cre-low

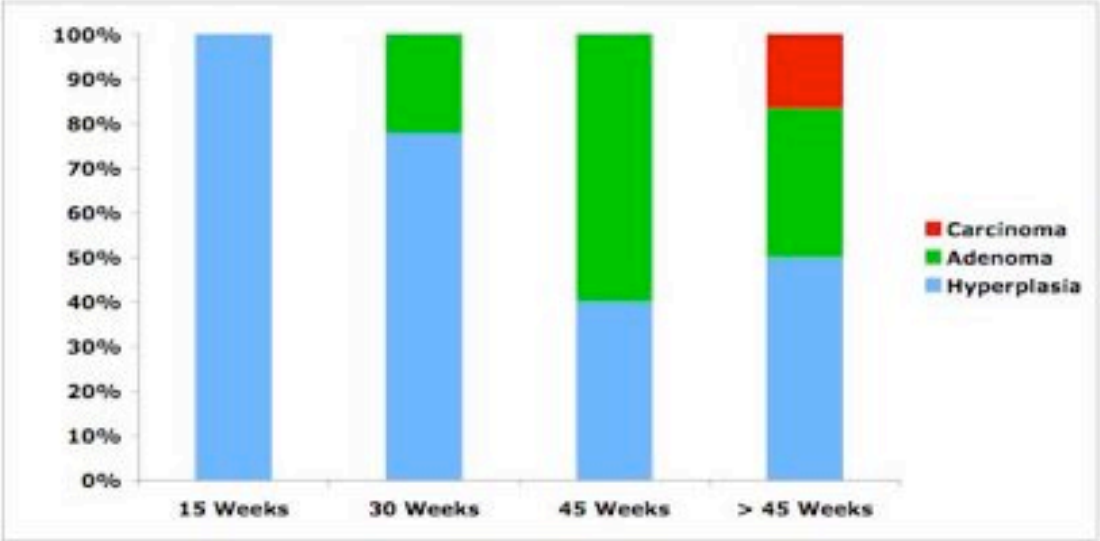
C


30 Weeks Apc^Lox
Relative Expression to WT
1000
100
10
1
1
StAR
SCC
Cyp11b1
Cyp11b2
21OH
Axin2
Lef1

D


Igf2 Locus Quantitation
Relative Expression (WT vs KO)
2.5
2
1.5
1
0.5
0
12
30
Age
Igf2
p57Kip2

E


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
15 Weeks
30 Weeks
45 Weeks
> 45 Weeks
Carcinoma
Adenoma
Hyperplasia

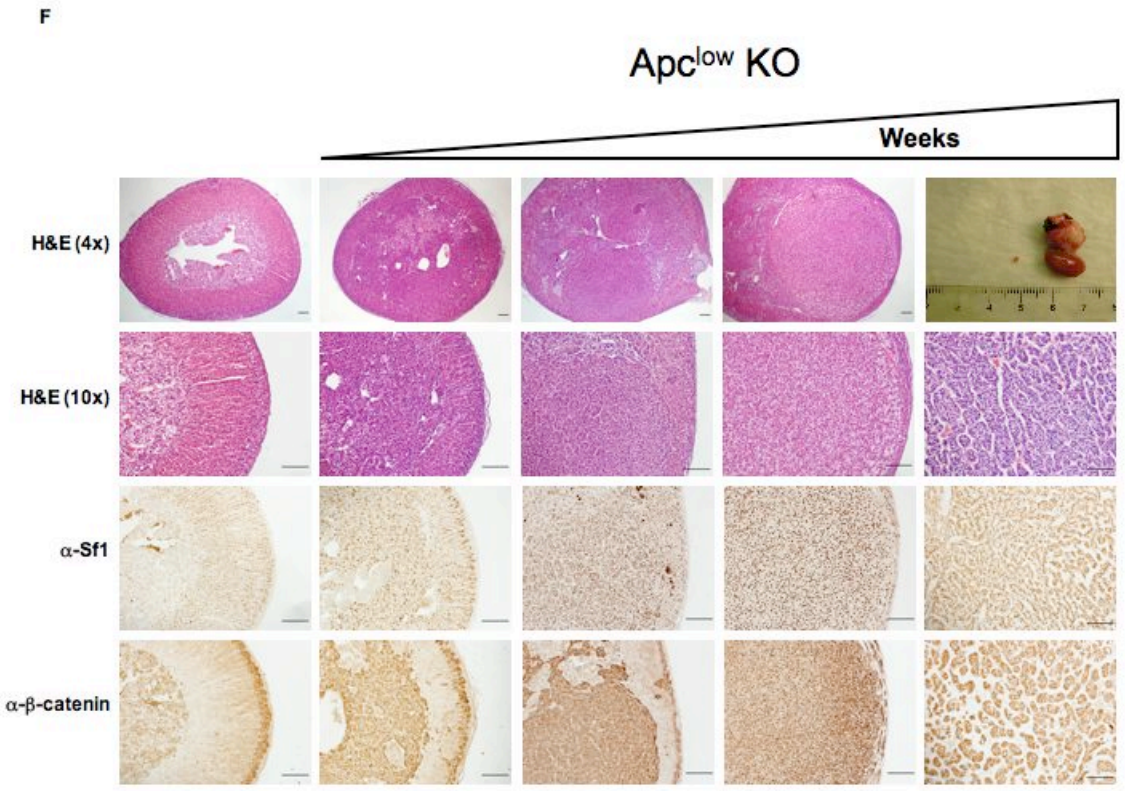
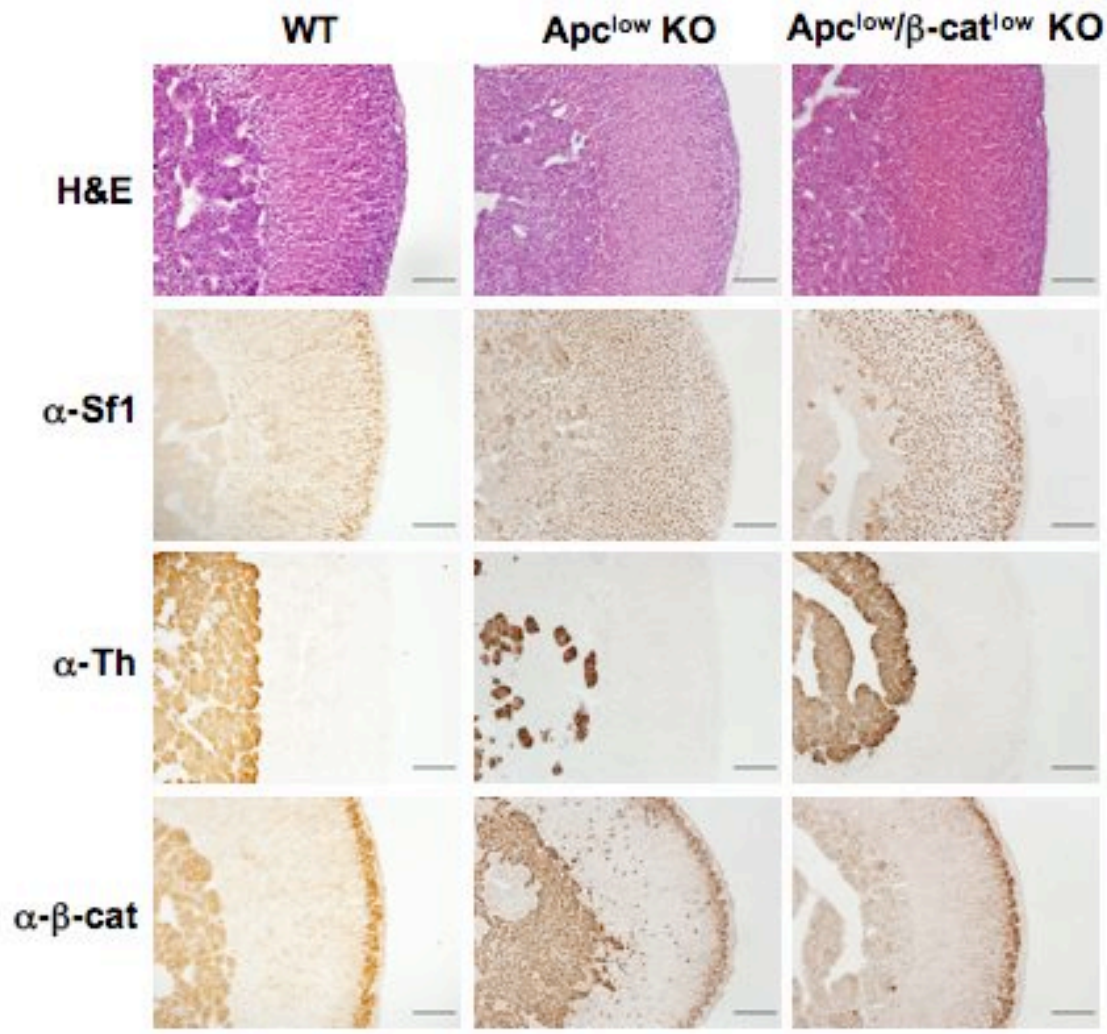

F
Apc^low KO
Weeks
H&E (4x)
H&E (10x)
α-Sf1
α-β-catenin

Figure 3.6 Depletion of Aberrant Cells in Apc^{low} KO via Ablation of β-catenin. IHC on 6 weeks old WT, Apc^{low} KO, and Apc^{low}/β-catenin^{low} KO using α-Sf1, α-Th, and α-β-catenin. In the Apc^{low}/β-catenin^{low} KO (n=3), there was absence of Sf1(+) and β-catenin(high cytoplasmic and nuclear) aberrant cells. Scale Bar: 100 μm.


WT
Apc^low KO
Apc^low/β-cat^low KO
H&E
α-Sf1
α-Th
α-β-cat

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CHAPTER 4

THE ROLE OF β-CATENIN IN SEX-DETERMINATION AND TUMORIGENESIS OF THE GONADS

ABSTRACT

Tumorigenesis, resulting from perturbation of canonical Wnt signaling, is a well-documented phenomenon in many different tissues. Recent studies demonstrated that the stabilization of β-catenin, mainly through a stabilizing β-catenin mutation is a sufficient factor leading to tumorigenesis. However, studies on the specific role of APC, an inhibitor of β-catenin, in the gonads are thus far limited to drosophila as a model organism. Utilizing the Wnt reporter mice (*Wnt-Gal*), we observed expression and active β-catenin signaling in the somatic gonadal cells, Sertoli and Leydig cells in the testis and thecal and granulose cells in the ovary. These cells support germ cell development and maturation through the secretion of paracrine factors and steroid hormones. Mutations or dysregulation of signaling pathways in these somatic can cells result in formation of sex-cord stromal tumors. Therefore, to determine whether constitutive activation of the Wnt pathway is sufficient for *in vivo* gonadal tumorigenesis, we employed a conditional knockout approach as described in Chapter 3. Mice harboring homozygous floxed-Apc alleles $Apc^{lox/lox}$ were crossed to transgenic mice containing a steroidogenic-specific Cre driver (*Sf-1-Cre*^{low}, and *Sf1-Cre*^{high}). The initial analysis at 9 weeks of the *Sf1-Cre*^{low} mediated Apc knockouts (Apc^{low} KO) revealed no discernable differences in the gonads

of both sexes in WT and KO. However, in 45 week-old mice, we observed one hundred percent penetrant bilateral gonadal tumors. Histological analysis of these tumors reveals an expansion of Sf-1-positive and β-catenin-positive cells in the testis and the ovaries. Moreover, the ovarian tumors contained both granulosa and theca cells while the testicular contained "granulosa/Leydig"-like cells. In contrast, the examination of Apc^{high} KO revealed a phenotypic XY to XX reversal, resulting from β-catenin stabilization. Genotyping confirmed that approximately fifty percent of the Apc^{high} KO mice have a Y chromosome. By Q-PCR analysis, we determined that Sox9, a male-determining gene, was significantly downregulated, potentially accounting for the development of female characteristics. Moreover, 100% of older mice developed bilateral gonadal tumors. Taken together, the data suggest that conditional KO of Apc results in subsequent constitutive activation of β-catenin, which disrupts early-male development and contributes to later gonadal tumorigenesis.

INTRODUCTION

Mammalian gonadal development occurs in a biphasic process, bipotential gonad formation and subsequent sex-determination (1-4). Initially, the bipotential gonads arise from the condensation of coelomic epithelial cells of the urogenital ridge at embryonic day 9.0 (e9.0) (3-5). The urogenital ridge consists of three segmented regions, the pronephros that regresses during development, the mesonephros that gives arise to the adrenogonadal primordium, and the metanephros that gives arise to the definitive kidney (4). The development of the urogenital ridge coincides with the expression of a transcription factor, Wilms' tumor 1 (*WT1*). Examinations of patients with Frasier syndrome (caused by point mutation in intron 9 splice donor site of *WT*; patients present with Wilms' tumor, XY sex reversal, and renal failure) and Denys-Drash syndrome (caused by various mutations in WT1; patients present with renal failure, pseudohermaphordism, and susceptibility to Wilms' tumor) revealed the importance of *WT1* in urogenital structure developments (5-7). Moreover, *Wt1* knockout mice exhibit an absence of all the structures arising from the urogenital ridge including the kidneys, adrenal glands, and gonads (8).

Between e9.0 to e11.0, the development of the adrenogonadal primordium occurs, coincidentally with the expression of *Sf1*. As described in previous chapters, *Sf1* is crucial to both adrenal and gonadal development, as evidenced by the complete absence of both adrenal glands and gonads but presence of kidneys in the mice with targeted disruption of *Sf1* (5, 9).

Ultimately, separation of the gonadal primordium from adrenal primordium occurs around e11.0 (3). The undifferentiated cells of the gonadal primordium provide

two different somatic cell populations, the supporting cells (male-specific Sertoli cells or female-specific granulosa cells) and the steroidogenic cells (male-specific Leydig cells or female-specific thecal cells) (4). In contrast, the primordial germ cells (PGC) are specified in the epiblast and develop from the primitive streak around e7.5 (3). During e10.5 to e11.5, the PGCs proliferate and migrate through the gut mesentery into the gonadal primordium (2-4).

The sex-determination phase of the gonadal development is initiated by the expression of the genes in the sex-determining region of chromosome Y (*Sry*) in Sertoli cells of the XY gonads (1-4). *Sry* (encoding a high mobility group (HMG)-box transcription factor) is necessary and sufficient to initiate the male specific gene expression cascade, as studies showed that ectopic expression of *Sry* in the XX gonad resulted in XY-specific gonadal differentiation (1-4). The differentiation occurs by a significant increase in proliferation and migration from the mesonephric cells, which give arise to peritubular myoid cells, testicular specific endothelial cells, and Leydig cells (1-4). Following expression of *Sry*, an increased *Sox9* expression in the Sertoli cells is observed. Studies have shown that ectopic expression of *Sox9* in XX gonad is sufficient to drive male development (1-4). In addition, expression of *Fgf9* occurs along the testis cord of the XY gonad and is implicated in Sertoli specification (1-4). In mice with homozygous null mutation of *Fgf9*, a male to female sex reversal is observed (10, 11). It is hypothesized that deficiency of *Fgf9* leads to reduced proliferation of the Sertoli cells (10, 11).

Although it is known that differentiation of the ovarian tissue occurs closer to birth, the detailed mechanism of the ovarian development is less well understood.

However, Wnt signaling related genes, specifically *Wnt4*, *Dax1*, and *Rspo1*, have been implicated in female development. First, Wnt4 expression is specific to XX gonads by e11.5. The analysis of *Wnt4* deficient mice revealed an absence of Mullerian ducts, but a development of testis-specific vasculature. It is hypothesized that Wnt4 functions to block mesonephric cell migration, which is responsible for forming testis-specific vasculature. In human duplication of the *DAX1*, which is a downstream target of Wnt signaling, results in phenotypic XY to XX reversal, which resembles the *WNT4* duplication phenotype (3, 4). *In vitro* studies have shown that *Dax1* is upregulated in response to *Wnt4* by Sf1/β-catenin-meditated gene transcription (12). Additional studies involving Dax1 knockout mice revealed deficiency in spermatogenesis and defects in early testicular development, implicating its contribution in XY to XX reversal in humans (3, 13). Lastly, a characterization of patients with XX to XY reversal identified *RSPO1* as the gatekeeper of female development (14). Rspo1 proteins are known to activate the Wnt/β-catenin pathway by competing against Dkk1 molecules to stabilize Lrp6 co-receptor leading to activation of the pathway (15). Mice deficient in *Rspo1* exhibited partial male to female sex reversal, similar to the *Wnt4* deficient mice, and this phenotype is rescued by expression of activated β-catenin (16, 17).

In addition to the role of Wnt signaling pathway in the normal gonadal development, the analysis of human gonadal tumors and studies involving stabilized β-catenin in stromal cells of the ovaries implicated dysregulation of the Wnt/β-catenin system in gonadal tumorigenesis (18, 19). Therefore, we hypothesized that constitutive activation of β-catenin in somatic gonadal cells, Sertoli and Leydig cells in the testis and thecal and granulose cells in the ovary, may contribute to abnormal gonadal development

and/or tumorigenesis. To test this hypothesis, we utilized Sf1-Cre transgenic mice, which expresses Cre-recombinase in the stromal and steroidogenic cells of the gonads, to conditionally activate β-catenin via ablation of *Apc*.

MATERIAL AND METHODS

Mice

All experiments involving mice were performed in accordance with institutionally approved and current animal care guidelines from the respective universities. The *Sf1/Cre*^{high} transgene targets high levels of expression of Cre recombinase to the urogenital ridge by E10.0 and activates a Cre-dependent reporter gene in the Sertoli and Leydig of testis and Granulosa and Thecal of ovaries (20); other sites of expression include pituitary gonadotropes, the ventromedial hypothalamic nucleus, adrenal cortex, and the spleen. The *Sf1/Cre*^{low} transgene is a single-copy transgene that is expressed at lower levels in the same sites. Mice carrying the floxed Apc allele (*Apc*$^{loxP/loxP}$) were purchased from Bart Williams Van Andel Institute (Grand Rapids, MI); this conditional allele contains *loxP* sites flanking exons 14, resulting in frameshift mutation generating a null product (21). The progenies were genotyped for *Sry* gene, as previously described.

Analysis of Gonadal Histology and Immunohistochemistry

Gonads were collected at the indicated ages and fixed for 2–3 h in 4% paraformaldehyde/phosphate buffered saline (PBS). Tissues were dehydrated in graded ethanol solutions and embedded in paraffin before sectioning. Sections were cut at 6 μm thickness and processed using standard procedures.

For immunohistochemical analyses, gonads were processed as above and washed in Tris-buffered saline/0.1% Tween-20 (TBST, pH 7.5). Antigen retrieval was performed by boiling rehydrated sections in 10 mM sodium citrate (pH 6.0) for 20 min, followed by one wash in deionized water and two washes in TBST at room temperature. Antibody

stainings were conducted using VECTASTAIN ABC kits and Vector Mouse on Mouse (M.O.M.) kits according to manufacturer's protocol (Vector Laboratories, Burlingame, CA). Tissue sections were blocked in antibody diluent solution for 1 h, and then incubated overnight at 4 C with anti-β-catenin (610154) (1:1000, BD Biosciences, San Jose, CA), anti-β-catenin (H-102) (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and anti-Sf1 (1:1000 dilution and generously provided by Dr. Ken Morohashi). The next day, sections were washed, exposed to secondary antibodies, and processed for signal detection according to the manufacturer's protocol

Real-Time PCR

Gonads were removed, cleaned, and snap frozen. Frozen tissues were lysed in Trizol reagent using an electric tissue homogenizer, and total RNA was prepared according to the manufacturer's protocol. Total RNA was treated with DNase (Ambion, Austin, TX) to remove residual genomic DNA and quantitated by UV spectrometry. 1 μg of total RNA was used to synthesize cDNA using the iScript kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The final cDNA products were purified and eluted in 50 μl of Tris-EDTA buffer using PCR purification columns (QIAGEN, Hilden, Germany) or directly diluted to final volume. Primer sequences for each gene are: Cyp19a1: Fwd-5' gagagttcatgagagtctggatca, Rev-5' catggaacatgcttgaggact; Fgf9: Fwd-5' tgcaggactggatttcatttag, Rev-5' ccaggcccactgctatactg; *Sox9*: Fwd-5' gaagctggcagaccagtacc, Rev-5' ggtctcttctcgctctcgttc; *Axin2*: Fwd-5' gcaggagcctcacccttc, Rev-5' tgccagtttctttggctctt; *Sf1*: Fwd-5' acaagcattacacgtgcacc, Rev-5'

tgactagcaaccaccttgcc; *Actb1* (β-actin): Fwd-5' ctaaggccaaccgtgaaaadg, Rev-5' accagaggcatacagggaca

For quantitative, real-time PCR (qRT-PCR) analyses of mRNA abundance, reactions were performed with a 2x SYBR Green PCR mastermix (Applied Biosystems, Foster City, CA) and gene-specific primers in the ABI 7300 thermocycler (Applied Biosystems, Foster City, CA). Each quantitative measurement was normalized to Rox dye as an internal standard and performed in triplicate. Transcript abundance was normalized in each sample to the average Ct value for β-actin (Livak et al., 2001). For mRNA quantitation, a minimum of three samples from differing genotypes was analyzed.

RESULTS

Normal sex determination in Apc^{low} KO mice but phenotypic sex-reversal in XY Apc^{high} KO mice

To conditionally stabilize β-catenin in the gonadal somatic cells, we bred the *Sf1-Cre^{low}* and the *Sf1-Cre^{high}* transgenic mice with the *Apc$^{loxP/loxP}$* transgenic mice to generate *Sf1-Cre^{low}/ Apc$^{loxP/loxP}$* (Apc^{low} KO) and *Sf1-Cre^{high}/ Apc$^{loxP/loxP}$* (Apc^{high} KO). Apc^{low} KO mice were born at an expected Mendelian ratio with equal numbers of males and females. The external genitalia of Apc^{low} KO mice at 9 weeks were indistinguishable from the WT littermate (Figure 4.1A).

Although the Apc^{high} KO mice were born at expected genotypic Mendelian ratios, inspection of the Apc^{high} KO mice at 9 weeks revealed generation of only female mice (Figure 4.1A). The comparison of the external genitalia of genotypic Apc^{high} KO females to WT revealed no discernable differences. However, examination of genotypic Apc^{high} KO male mice revealed a presence of an external genitalia that is indistinguishable from a WT female (Figure 4.1A). To determine the genetic sex of these mice, we genotyped the Apc^{high} KO mice for the presence of Y-chromosome using *Sry* primers, as described previously. The result of genotyping revealed a population of females to be positive for *Sry* (Figure 4.1B). Taken together, the data suggest that stabilization of β-catenin results in XY to XX reversal in Apc^{high} KO mice. While in Apc^{high} KO XY to XX phenotypic sex reversal was observed, we hypothesize that the Cre-mediated recombination of the *Apc* gene was not sufficient in the Apc^{low} KO, with subsequent lower levels of β-catenin stabilization, to induce phenotypic sex reversal.

Downregulation of Sox9 in Apc^{high} KO XY gonads

Previous studies have identified Sox9 and Fgf9 as factors important for XY determination (3, 4). In addition endocrine factors, such as estrogen, are critical for external genitalia development. Interestingly, the aromatase (Cyp19a1), an enzyme in the estrogen synthesis pathway, was recently found to be regulated by Sf1/β-catenin transcriptional complex.

To assess the gene expression profile of these factors in Apc KO mice, we performed quantitative PCR. *Axin2*, a control for activated β-catenin signaling, was elevated in the XY gonads of Apc^{high} KO mice (Figure 4.2). Also we observed an increase in *Sf1* transcript levels, a marker gene of somatic cells of the gonads, suggesting an expansion of somatic cells of the gonads following β-catenin stabilization (Figure 4.2). However, we found a downregulation of *Cyp19a1*, which we hypothesized to be upregulated a) because of a previous study showing regulation of *Cyp19a1* by Sf1/β-catenin mediated transcription and b) due to upregulation of *Sf1* and *Axin2* (22). Although the result was unexpected, we hypothesize that the regulation of *Cyp19a1 in vivo* requires finitely controlled levels of Sf1 and β-catenin. In addition, aromatase deficient XY mice do not undergo sex reversal, which led us to believe that downregulation of Cyp19a1 was not responsible for the Apc^{high} KO phenotype (23). Surprisingly, we observed an upregulation of *Fgf9*, which was inconsistent with our XY to XX reversal phenotype in Apc^{high} KO mice (Figure 4.2). Although we expected a downregulation of *Fgf9* transcript levels, a requirement for female-specific differentiation, we suspect that Apc^{high} KO XY gonadal cells are upregulating *Fgf9* in response to high β-catenin signaling. Moreover, with upregulation of *Fgf9*, we would

expect maintenance of male characteristics. Previous data and our analysis suggest that the sex-reversal in our model was independent of *Fgf9* expression. Interestingly, we observed a downregulation of *Sox9* in the XY gonads of the Apc^{high} KO mice (Figure 4.2). Previously, it has been shown that *Sox9* is downstream of *Sry* that determines male-specific differentiation. Therefore, we hypothesize that in our model, the XY to XX sex reversal may be due to downregulation of *Sox9* in response to stabilization and activation of β-catenin.

Development of gonadal tumors in Apc^{low} KO and Apc^{high} KO mice

Initially, we hypothesized that ablation of *Apc* and subsequent stabilization of β-catenin in the gonadal somatic cells would result in gonadal tumorigenesis. Although we observed tumors in both Apc KO mice, it was not until at least 30 weeks of age. At 30 weeks of age, all mice had developed gonadal tumors at a one hundred percent penetrance (Figure 4.1). In Apc^{low} KO mice, we observed development of both ovarian and testicular tumors (Figure 4.3). In Apc^{high} KO mice, we observed gonadal tumor development regardless of genotypic sex (Figure 4.1).

Examination of the testicular and ovarian tumors of Apc^{low} KO revealed an expansion of somatic cells that histologically resembled granulosa cell tumors (Figure 4.3). Moreover, we observed an expansion of Sf1 positive cells with high levels of nuclear β-catenin in both ovarian and testicular tumors (Figure 4.3). Through ablation of *Apc* in *Sf1* expressing somatic cells, we observed an expansion of Sf1 and β-catenin positive cells, which resulted in gonadal tumorigenesis in both sexes of Apc^{low} KO and Apc^{high} KO transgenic mice.

DISCUSSION

The initial goal of this study was to assess the role of activated Wnt/β-catenin signaling in gonadal tumorigenesis. However, we observed an intriguing XY to XX sex reversal phenotype within the Apc^{high} KO mice. Previous studies, involving *Wnt4*, *Dax1*, and *Rspo1*, had implicated Wnt signaling in female sex determination. However, this is the first study that utilized somatic cell specific Cre-transgenic mice to conditionally activate the main Wnt effector, β-catenin in gonadal cells. Utilizing *Sf1-Cre*^{low} to ablate *Apc*, we observed a normal gonadal development in both sexes. However, ablation of *Apc* utilizing *Sf1-Cre*^{high}, we observed preference towards female-specific differentiation in both genotypic sexes, resulting in XY to XX sex reversal. Because genotypic XX mice with Apc^{high} KO maintained normal female differentiation and previous data implicated a role of the Wnt pathway in female characteristic specification, we hypothesize that β-catenin mediates female-specific development.

In aged mice, gonads from Apc^{low} KO and Apc^{high} KO mice developed tumors regardless of their genetic sex. The gonadal tumors resembled granulosa cell tumors. However, further study is required to confirm the exact origin and the phenotype of the tumor cells. However, this work details the first model of the sex cord-stromal tumor of the testis and identifies β-catenin as one of the regulators of tumorigenesis.

While analyzing the molecular mechanism of XY to XX reversal, we identified Sox9 as a candidate responsible for the observed phenotype. While we were carrying out these studies, Maatouk et al published a similar observation in Human Molecular Genetic, 2008 (24). In this independent study, the authors utilized Sf1-Cre transgenic mice mated to mice harboring a conditionally activating β-catenin allele. The data

showed XY to XX sex reversal in those transgenic mice. In addition, the authors identified *Sox9* downregulation as a mechanism of this sex reversal. The data indicated that *Sox9* competed with β-catenin for target gene expression. In the presence of constitutively stabilized β-catenin, *Sox9* could no longer activate the transcription of male-specific target genes, resulting in the phenotypic XY to XX reversal. Independently but together, these data and our data support the role β-catenin in female sex-determination.

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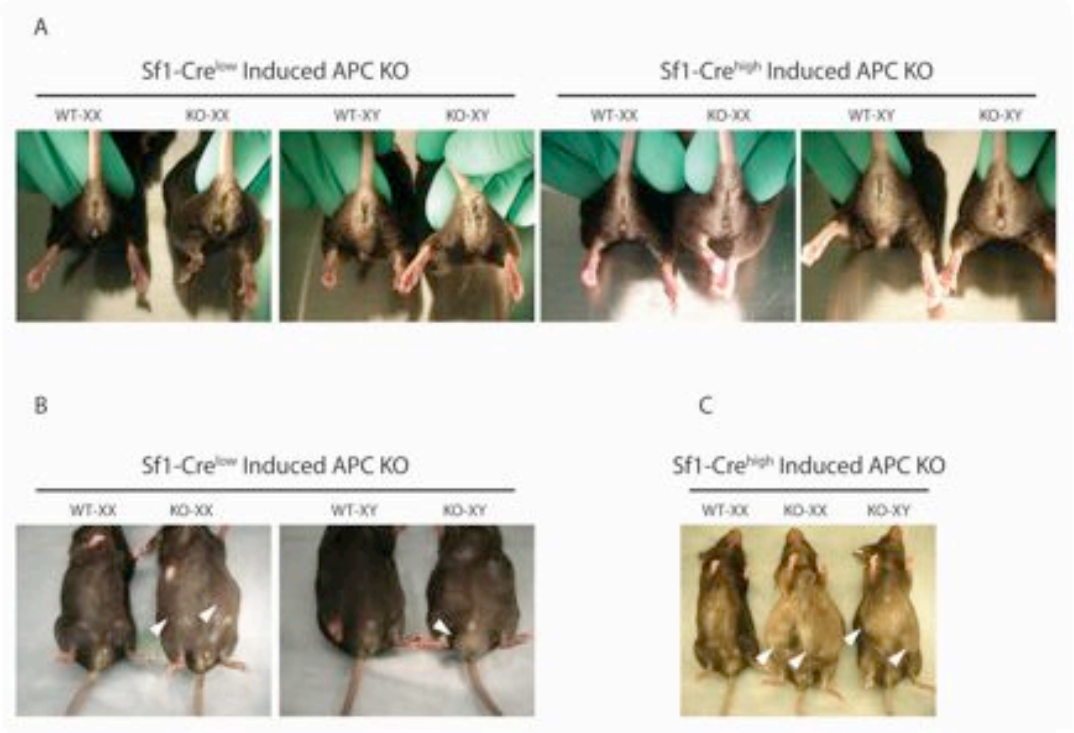
Figure 4.1 External Genitalia Examination of Apc^{low} KO and Apc^{high} KO Mice and Genotyping for Sry. A) Comparison of the Apc^{low} KO and Apc^{high} KO mice at 9 weeks. Apc^{high} KO underwent XY to XX reversal. B) Apc^{low} KO mice developed fully penetrant bilateral gonadal tumors by 45 weeks of age. C) Apc^{high} KO mice developed fully penetrant bilateral gonadal tumors either in both genotypic males and females. D) Genotyping for Cre, loxP, and Sry. Sry genotyping revealed that some of the phenotypic females Apc^{high} KO were genotypic males.


A
Sf1-Cre^low Induced APC KO
WT-XX
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Sf1-Cre^high Induced APC KO
WT-XX
KO-XX
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Sf1-Cre^low Induced APC KO
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WT-XY
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C
Sf1-Cre^high Induced APC KO
WT-XX
KO-XX
KO-XY

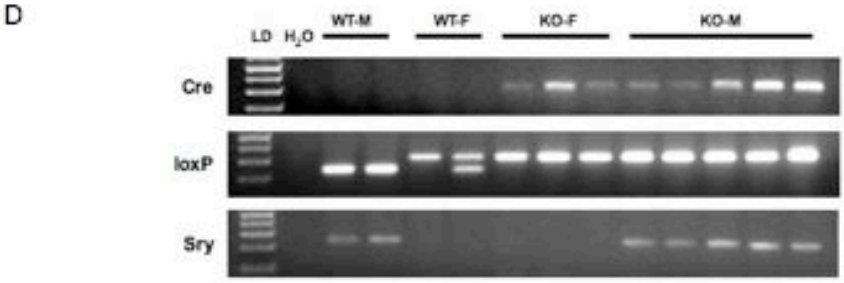

D
LD
H2O
WT-M
WT-F
KO-F
KO-M
Cre
loxP
Sry

Figure 4.2 Q-PCR Examination of Apc^{low} KO and Apc^{high} KO Gonads. Q-PCR was conducted in Apc^{low} KO and Apc^{high} KO and was compared to WT littermates at 9 weeks of age. Apc^{low} KO revealed moderate changes in gene expression Apc^{high} KO revealed expression changes in all genes assessed, compared to WT.

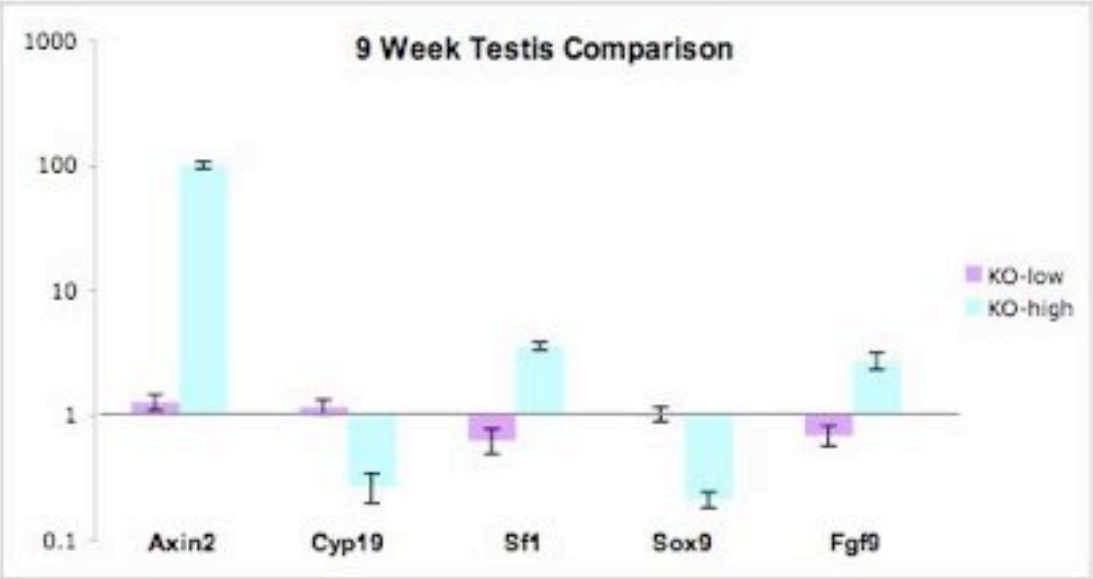
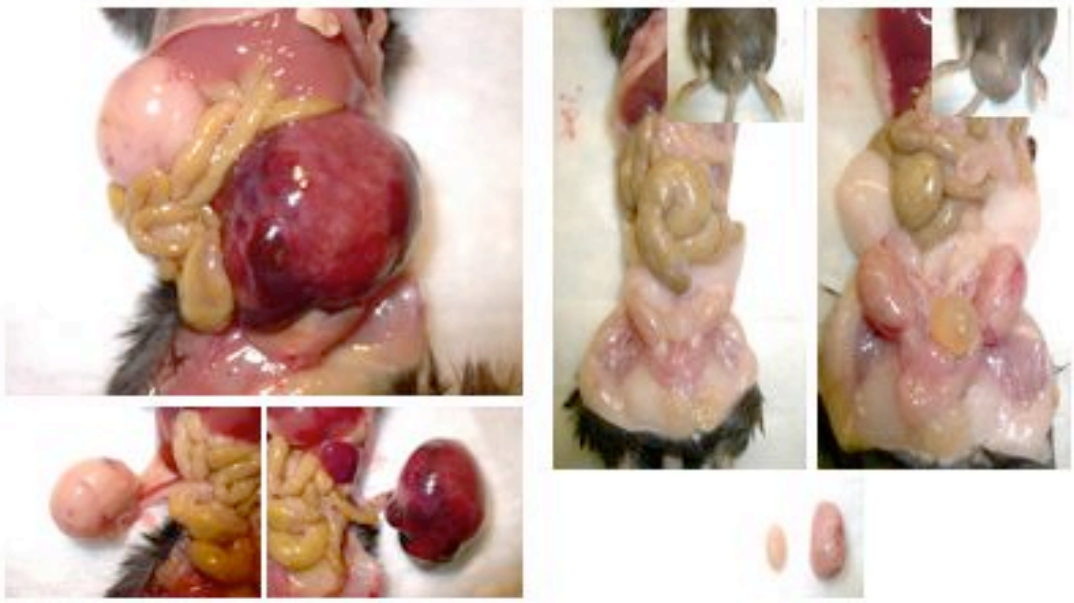
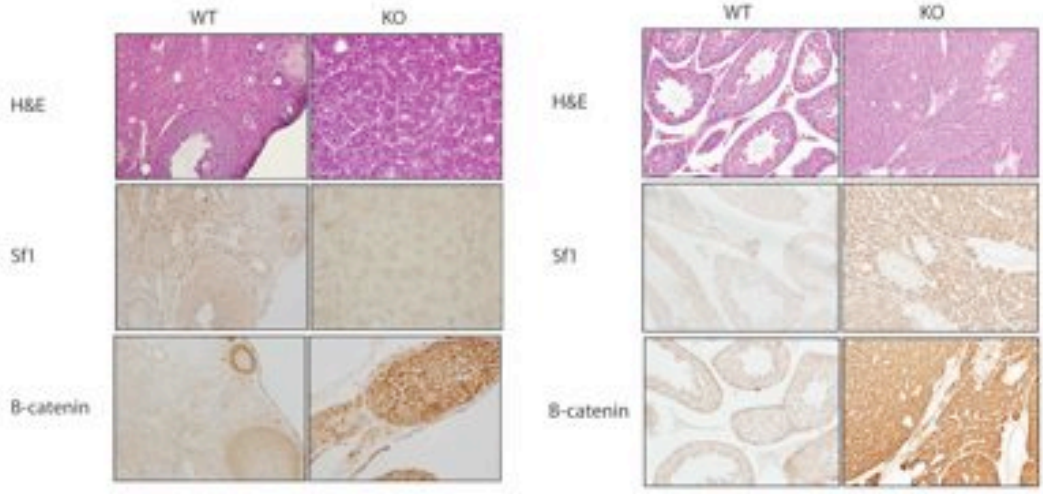

9 Week Testis Comparison
1000
100
10
1
0.1
KO-low
KO-high
Axin2
Cyp19
Sf1
Sox9
Fgf9

Figure 4.3 Histological Assessment of Gonadal Tumors of Apc^{low} KO. Both males and females developed bilateral gonadal tumors by 45 weeks of age. These tumors contained increased expansion of Sf1 cells and contained cells with high cytoplasmic and nuclear β-catenin. All sections were taken at magnification of 10x.




WT
KO
H&E
Sf1
B-catenin
WT
KO
H&E
Sf1
B-catenin

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CONCLUSION

The Wnt/β-catenin signaling pathway is known to regulate many different cellular processes within an organism. The balance of this delicate pathway, maintained by the central transcription factor, β-catenin, dictates the pathway's contribution to tissue homeostasis. The investigations conducted in this thesis examine the imbalance of the Wnt/β-catenin signaling pathway through manipulation of the β-catenin concentration in the adrenal gland. These experiments allowed us to analyze the role of Wnt/β-catenin in development, tissue maintenance, and tumorigenesis of the adrenal gland. Although many studies have implicated a role of Wnt/β-catenin signaling in the adrenal gland, the work described in this thesis is the first *in vivo* study that establishes the importance of this pathway in this organ system.

In Chapter 2, we examined the consequences of adrenocortical-specific ablation of β-catenin. The adrenal aplasia observed following an efficient ablation of β-catenin signified its crucial role in regulating adrenal gland development (1). In the adult organ, the β-catenin localization and signaling is specific to the subcapsular zone (SZ), the hypothesized residence of less differentiated adrenocortical stem/precursor cells, of the adrenal cortex (2). Through conditional knockout of β-catenin in only fifty percent of the adrenocortical cells, specifically within the cells of the SZ, utilizing the Sf1-Cre^{low} driver, we observed a normal development of the gland but an age-dependent depletion of the adrenal cortex, unraveling β-catenin's role in adult tissue maintenance (1). Recent

lineage tracing study by Zubair et al. concluded that adult cortex is derived from cells of the fetal cortex (3). Our study revealed the preferential localization of active β-catenin signaling in the developing and adult gland. From these data, it is reasonable to postulate that β-catenin expressing cells of the developing fetal gland established residence in the SZ as the progenitors of the adult cortex. However, the question of how the fetal cells transition into the adult cells still remains unanswered. We hypothesize that unknown downstream targets of the β-catenin signaling may mediate this process. Also, the question of deactivation of the β-catenin signaling during adrenocortical cell differentiation requires further investigations. It is known that Sf1 mediated transcription is responsible for expression of steroidogenic genes. It is also known that Sf1 and β-catenin, in presence and absence of Tcf/Lef transcription factors, regulate specific adrenocortical gene regulation. Therefore, it is possible that a) β-catenin expression is reduced during differentiation and b) β-catenin regulates gene expression independent of Tcf/Lef (as we do not observe active signaling in the inner differentiated cortex). We believe former to be true as we observe a gradient of β-catenin expression, highest in the periphery of the gland to lowest in the inner cortex. In any case, the data from Chapter 2 place β-catenin within the hierarchy of transcriptional regulators responsible for adrenal gland development. In addition, our data represent one of the few examinations of SZ cell biology using a genetics approach. Furthermore, we have established a conditional knockout system that serves as a platform to examine other signaling pathways and factors within the developing and adult adrenal gland.

In the field of cancer stem cell biology, it is believed that accumulation of mutations and subsequent dysregulation in somatic stem cells result in tumorigenesis.

Therefore, based upon the role of β-catenin in tissue homeostasis as detailed in Chapter 2 together with recent published data implying over active Wnt/β-catenin signaling in human adrenal tumorigenesis, we investigated the consequences of constitutive active β-catenin in the adrenal cortex. Our hypothesis was that constitutive stabilization and activation of β-catenin would result in adrenocortical tumorigenesis. Utilizing the transgenic mouse platform established in Chapter 2, we performed conditional ablation of *Apc* to stabilize β-catenin. In the Apc KO mice, we observed both developmental and adult gland maintenance phenotypes. Sf1-Cre^{high}-mediated, high efficiency excision of the *Apc* gene, resulted in a phenotypically immature adrenal gland at birth. The resulting gland was small, lacked clear concentric zones, and was devoid of an adrenal medulla. This adrenal did not undergo further differentiation with age and never developed tumors. On the other hand, *Apc* ablation utilizing the Sf1-Cre^{low} driver revealed an initial expansion of less differentiated aberrant adrenocortical cells. These cells were localized interspersed within the medulla of the adult adrenal gland. We hypothesized that the proliferation of these cells resulted in adrenocortical hyperplasia, which later became adrenocortical adenomas and carcinomas. To test that the observed phenotype was β-catenin-dependent, we performed a dual conditional knockout utilizing the Sf1-Cre^{low} driver. At 6 weeks, the dual *Apc* and β-catenin knockout adrenals were devoid of any hyperplastic cells and did not develop any tumors. Collectively, the data presented in this chapter confirm the importance of a delicate balance of Wnt/β-catenin pathway system. The question remains, as in Chapter 2, of what are the downstream targets of the β-catenin responsible for the phenotype. We hypothesize that these downstream target genes also play an important role in maintenance and regulation of adrenocortical

progenitor cell fate. Moreover, the mechanism of hyperplasia transition to tumors remains unclear. It is probable that the hyperplastic cells gain additional mutations resulting in tumorigenesis. However, the data presented in this chapter represent the first bona fide model of adrenal adenomas and carcinoma mice.

While assessing the consequences of stabilized β-catenin in the adrenal gland, we uncovered a gonadal phenotype, described in Chapter 4. Because Sf1 is expressed in the supporting and steroidogenic cells of the gonads, Sertoli and Leydig cells in testis and granulosa and theca cells in the ovary, we were also able to stabilize β-catenin in these cells. The result of Sf1-Cre^{low} mediated KO was the development of bilateral gonadal tumors, both testicular and ovarian, with an one hundred percent phenotypical penetrance by 30 weeks of age. These tumors were caused by expansion of Sf1-positive and β-catenin-positive cells. More interestingly, the Sf1-Cre^{high} mediated KO mice revealed a defect in male sex determination, resulting in XY to XX phenotypic sex reversal. We demonstrated that downregulation of the male-specific factor, Sox9, is the likely downstream effect and cause of the phenotype. As observed in the low driver, these gonads all developed bilateral tumors by 30 weeks of age, regardless of genotypic sex. However, our observation of phenotypic sex-reversal was confirmed in advance by Maatouk et al. 2008 (4). Nevertheless, our work presented in this chapter provides another model system to further examine the biology of sex-cord stromal tumors.

The studies conducted in this thesis raised questions that warrant future investigations. What are the adrenocortical specific targets of β-catenin-mediated transcription? As β-catenin-mediated transcription has been shown to activate transcription of adrenocortical-specific targets, we hypothesized that downregulation

and/or upregulation of these genes are most likely responsible for the phenotypes observed in our models. In a manuscript by Yochum et al. 2007, the authors utilize chromatin immunoprecipitation (ChiP) assays to identify β-catenin targets in colorectal carcinoma cells (5). Employment of such in-depth analysis for identification of specific target genes of the β-catenin-mediated transcription, Tcf/Lef and/or Sf1, together with our mouse models will allow us to further assess the role of these targets in adrenocortical development and tumorigenesis. Which Wnt ligands are responsible for activation of β-catenin in the adrenal gland? There are 19 Wnt ligands identified in the mouse genome. In the adrenal gland, Wnt2b expression is observed in developing adrenal gland, and Wnt4 is the only ligand described to participate in adrenal gland homeostasis (6, 7). Therefore, identification of Wnt ligands that activate β-catenin in the adrenal gland is critical. Is the role of β-catenin in adhesion involved in the adrenal gland development and homeostasis? While we performed ablation of the β-catenin in our models, only the signaling component of the pathway was examined. As β-catenin performs dual functions in signaling as well as in adhesion, it will be important to address such function in our system. Are there any signaling pathways that converge on Wnt/β-catenin pathway in the adrenal gland? In a recent work by our colleagues at University of Michigan revealed convergence of Wnt/β-catenin pathway with the hedgehog pathway in skin tumorigenesis (8). Interaction between the two pathways is particularly interesting with our novel observation of specific compartmentalization and localization of these pathways within the adrenal gland. The interplay between the two pathways may give new insights into organization of the adrenal gland as well as role of the hedgehog pathway in adrenocortical development and tumorigenesis. Moreover, utilizing our

established conditional knockout platform will allow us to examine other important developmental pathways, such as Notch, Tgfβ, Hippo, etc. What exactly are the SZ cells? As explained above, historical and our data in Chapter 2 implicate the importance of these cells in maintenance of adult gland. However, the investigation of SZ cell biology remains premature. Therefore, it will be crucial to isolate and further characterize these cells.

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