Aldosterone-Producing Adenoma Formation in the Adrenal Cortex Involves Expression of Stem/Progenitor Cell Markers

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Aldosterone producing adenoma (APA) is the most common form of surgically curable hypertension. To further understand mechanisms involved in APA formation, we investigated the expression of molecules linked to adrenal stem/precursor cells [β-catenin, Sonic hedgehog (Shh), CD56], and nuclear receptors that play key roles in adrenocortical development and function steroidogenic factor 1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1) in six control adrenal glands and 14 adrenals with APA and compared their expression with that of specific markers of zona glomerulosa (ZG) [CYP11B2, Disabled 2 (Dab2)]. Both Dab2 and CD56 were expressed in ZG. Although Dab2 associates uniquely with differentiated ZG cells and its expression is lost when cells transdifferentiate to zona fasciculata (ZF) cells, CD56 was also expressed in ZF and in aldosterone-producing cell clusters, confirming that these structures possess an intermediate phenotype between ZG and ZF cells. Shh was barely detectable in cells located to the outer part of the ZG in the control adrenal; in contrast, its expression was detected in the entire APA and was dramatically increased in the hyperplastic peritumoral ZG. Transcriptome profiling revealed differential expression of components of Shh signaling pathway in a subgroup of APA. Similarly, Wnt/β-catenin signaling was activated in the majority of APA as well as in the entire peritumoral adrenal cortex; however, no mutation was identified in the *CTNNB1* gene that could account for β-catenin activation. Our data suggest that both APA and adjacent ZG present characteristics of stem/precursor cells; the reexpression of genes involved in fetal adrenal development could underlie excessive ZG cell proliferation and APA formation. (*Endocrinology* 152: 4753–4763, 2011)

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Abbreviations: APA, aldosterone-producing adenoma; APCC, aldosterone-producing cell clusters; COMETE, Cortico- et Medullo-surrénale, les Tumeurs Endocrines; Dab2, Disabled 2; DAX-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1; DIG, digoxigenin; GSK-3, glycogen synthase kinase-3LRP, low-density lipoprotein receptor-related protein; HES, hematoxylin-eosin safran; MABT, maleic acid, NaCl, and NaOH and Tween 20; PA, primary aldosteronism; PCCA, principal component analysis in correlations space; SF-1, steroidogenic factor 1; Shh, Sonic hedgehog; SSC, saline sodium citrate; TNE, Tris HCl, NaCl, and EDTA; ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis.

Primary aldosteronism (PA) is the most common form of endocrine hypertension with a prevalence of 7–10% of hypertensive patients (1). PA is due to autonomous aldosterone production by the adrenal gland leading to hypertension with hypokalemia and suppressed renin activity. The two major causes of PA are unilateral aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia, together accounting for about 95% of cases (2). Although recent evidence indicates that a subset of PA is due to KCNJ5 mutations that alter the function of the potassium channel Kir3.4 (3), the mechanisms involved in APA formation and development of bilateral adrenal hyperplasia remain elusive.

The adrenal cortex is composed of three distinct morphological zones with specific functions (4). The zona glomerulosa (ZG) is located immediately underneath the capsule and produces mineralocorticoids involved in sodium and potassium homeostasis and in the regulation of blood pressure. The zona fasciculata (ZF), the thickest zone of the cortex, produces glucocorticoid hormones that play important roles in stress response and energy homeostasis, whereas biosynthesis of sexual steroids takes place in the inner zona reticularis (ZR). During development, steroidogenic activity takes place in the human fetal adrenal cortex, which is composed of four structures: the capsule, the definitive zone, the fetal zone, and the transitional zone, which becomes apparent later in gestation (5). Just before birth some cells from the definitive zone take on characteristics of the adult ZG and produce aldosterone (6). The outer definitive zone of the fetal adrenal cortex contains a pool of proliferating progenitor cells from which the inner cortical cells, populating the intermediate and fetal zone, are derived. Intense postnatal remodeling of the adrenal gland with involution of the fetal zone, leads to the development of adult cortical zones from their primordial structures (7–10).

β-Catenin and Sonic hedgehog (Shh) play a key role in adrenocortical development in the mouse. Expression of Shh was found in the developing mouse adrenal (11), and recent studies have shown that conditional inactivation of Shh in the adrenal cortex produces severe hypoplasia and histological disorganization, with defects in encapsulation of the adrenal medulla (8–10). β-Catenin signaling is active in the subcapsular zone of the mouse adrenal cortex, in which precursor cells are located, and is required for the development and maintenance of this zone (12). Constitutive activation of β-catenin, as shown by its nuclear localization by immunohistochemistry, has been implicated in the pathogenesis of adrenocortical tumors (13). In addition to Shh and β-catenin, CD56 (also known as neural cell adhesion molecule), a glycoprotein of the immunoglobulin superfamily, has been shown to be expressed in the definitive zone of the human fetal adrenal gland, in which progenitor cell populations are localized (6). CD56 is developmentally associated with the ZG, suggesting that it may be specifically linked to aldosterone production (6, 14). The adult adrenal gland maintains organ homeostasis by replenishing adrenocortical cells throughout life, suggesting that stem-like cells may be present in the outer compartment of the gland (15). It has been suggested that the precursor cells are located underneath the capsule and differentiate within the ZG before migrating centripetally to gain zone-specific characteristics (16).

We have recently shown that ZG hyperplasia and adrenal cortex remodeling are major features of adrenals with APA (17). The aim of the present study was to further improve our understanding of the mechanisms involved in APA formation and to investigate the link with peritumoral tissue remodeling and ZG hyperplasia. To do this, we have analyzed the expression of molecules linked to adrenal stem/precursor cell function (β-catenin, Shh) and nuclear receptors that play a key role in adrenocortical development and function [steroidogenic factor 1 (SF-1), dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1 (DAX-1)] in control adrenal glands and adrenals with APA and compared their expression with that of specific markers of ZG differentiation (CYP11B2) and function [Disabled 2 (Dab2), CD56].

Subjects and Methods

Patients

We obtained formalin-fixed and paraffin-embedded adrenals through the COrtico- et MEdullo-surrénale, les Tumeurs Endocrines (COMETE) network from 14 patients who had undergone surgery for APA at Hôpital Européen Georges Pompidou between 2001 and 2007. The clinical and biological characteristics of the patients are summarized in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. The study of β-catenin localization was extended to 12 additional formalin-fixed and paraffin-embedded adrenals obtained through the COMETE Network. Methods for screening and criteria for diagnosing PA and APA were in accordance with institutional guidelines and have been described recently (18). Six control adrenals were obtained from enlarged nephrectomies (kindly provided by Dr. M. Sibony, Hôpital Tenon).

Transcriptome analyses were performed on 122 APA obtained through the COMETE network and 11 control adrenals obtained from enlarged nephrectomies (kindly

provided by the Department of Pathology, University Hospital of Rouen). All patients gave written informed consent to participate to the study.

In situ hybridization

In situ hybridization was performed on the entire adrenal gland (APA or nodules and peritumoral adjacent tissue) from PA patients and on control adrenals. Paraffin-embedded tissue samples were cut in serial sections of 4 μm and mounted on Super Frost Plus slides (Dutscher, France). The sections were deparaffinized with xylene, rehydrated through graded ethanol, and treated with proteinase K (Sigma, St. Louis, MO) 8 μg/ml in PBS for 10 min. Prehybridization was performed by incubating the sections with preheated (at 85 C for 5 min) hybridization buffer (1× salt, 50% formamide, 10% dextran sulfate, yeast RNA 10 mg/ml, Denhardt's solution) for 4 h at 65 C in an humidified chamber [50% formamide, 2× saline sodium citrate (SSC)]. The preheated hybridization solution containing hybridization buffer and digoxigenin (DIG)-labeled probe was applied on the sections, covered with Parafilm, and put in a humidified chamber at 65 C overnight. The slides were rinsed in SSC 5× at 65 C, washed in a solution containing SSC 2× and 50% formamide for 30 min at 65 C and then in 10 mM Tris HCl (pH 7.6), 500 mM NaCl, and 1 mM EDTA (TNE) for 10 min at 37 C. Slides were treated with ribonuclease A (20 μg/ml; Roche, Indianapolis, IN) diluted in TNE for 30 min at 37 C and washed once in TNE 10 min at 37 C, once in SSC 2× 20 min at 65 C, twice in SSC 0.2× 20 min at 65 C, and twice in 100 mM maleic acid, 150 mM NaCl, and 192 mM NaOH and 1.1% Tween 20 (MABT) for 10 min at room temperature. The sections were covered with blocking solution (MABT, 20% normal goat serum) at room temperature for 1.5 h and subsequently incubated with anti-DIG antibody (Roche) diluted 1:2000 overnight at 4 C. After washing the sections twice in MABT for 5 min and once in a solution of 100 mM NaCl, 100 mM Tris HCl (pH 9.5), 50 mM $MgCl_2$, and 1% Tween 20 (NTMT) for 10 min, they were revealed using BMP purple (Roche), 0.1% Tween 20 for 3–5 d for CYP11B2, or 2 wk for Shh and mounted in aqueous medium.

The 3′-untranslated region of CYP11B2 (nucleotides 2727–2931 of CYP11B2, GenBank accession no. NM_000498) and a fragment of 173 bp of Shh (nucleotides 780–952 Shh, GenBank accession no. NM_000193) were subcloned into pGEMT-Easy (Promega, Madison, WI). Antisense and sense probes were synthesized using the DIG RNA labeling kit (Roche) according to the manufacturer's protocol. No specific hybridization was observed for Shh and CYP11B2 sense probes (Supplemental Fig. 1). All microscopic examinations were done on a Leica microscope (Wetzlar, Germany).

Immunohistochemistry

Immunohistochemistry was performed on serial sections of the same tissues used for *in situ* hybridization. Sections were deparaffinized in xylene and rehydrated through graded ethanol. For antigen unmasking, the slides were incubated in antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 30 min at 98 C. Endogenous peroxidases were inhibited by incubation in 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) in water for 10 min and nonspecific staining blocked with normal goat serum. Primary antibodies [Dab2 (Santa Cruz, sc-13982; Santa Cruz Biotechnology, Santa Cruz, CA); 1:1000; CD56 (555514; Becton Dickinson, Franklin Lakes, NJ); 1:100; β-catenin (Becton Dickinson (559046), 1:100; SF-1 (19), 1:5000; DAX-1 (20), 1:1000] were incubated overnight at 4 C. Sections were washed, incubated 30 min with affinity-purified horse antimouse (1:400; Vector laboratories) or goat antirabbit (1:400; Vector Laboratories) antibodies, washed, and incubated with an avidin-biotin-peroxidase complex (Vectastain ABC Elite; Vector Laboratories) for 30 min. The slides were developed using diaminobenzidin (Vector Laboratories) and counterstained with hematoxilin (Sigma). In the negative control reactions, the primary antibodies were omitted from the dilution buffer, which in all instances resulted in a complete absence of staining (Supplemental Fig. 1). All microscopic examinations were done on a Leica microscope.

Histological examination

Histological examination was performed on 4-μm sections stained with hematoxylin-eosin safran (HES). Nuclei are stained in blue with hematoxylin, cytoplasm in pink with eosin, and collagen in yellow with safran. The peritumoral cortex was defined as the entire cortex from glands harboring an APA (see Supplemental Fig. 2).

Nucleic acid extraction and mutation analysis of the *CTNNB1* gene

Somatic DNA from 41 APA was coextracted with RNA using a modified Trizol protocol (Invitrogen, Paisley, UK). Exons 3 and 5 of *CTNNB1*, coding for regions of β-catenin containing phosphorylation sites that are mutated in adrenocortical tumors (21, 22) were studied. Primers used to amplify exons and the intron-exon flanking regions were: intron 2 forward, 5′-TCTTGGCTGT CTTTCAGATT-3′, and intron 3 reverse, 5′-TCACTATC CACAGTTCAGCA-3′; intron 4 forward, 5′-TCAAGG GGAGTAGTTTCAGA-3′, and exon 6 reverse, 5′-TGGT

TGCCATAAGCTAAAAT-3′. PCR was performed on 100 ng of DNA in a final volume of 50 μl containing 1.5 mM of $MgCl_2$, 400 nM of each primer, 200 μM of deoxynucleotide triphosphate, and 1.25 U of Taq Polymerase (Sigma-Aldrich). Direct sequencing of PCR products was performed using the ABI Prism Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) on an ABI Prism 3700 DNA analyzer (Applied Biosystems).

Transcriptome analysis

Transcriptome data from 122 aldosterone-producing adenomas and 11 control adrenal cortex were generated using standard procedures and will be described elsewhere (Boulkroun, S., *et al.*, manuscript in preparation). For the purposes of investigating the activity of the Shh pathway, data were median centered, log2 transformed, and model adjusted. Interarray and intergroup normalizations were carried out using the NeONORM method described previously (23–25). Multiple probes for a single gene and cross-reactivity of a single probe to several genes as well as the resolution of probe-ID annotations were accounted for as defined previously (26). Specific analysis for Shh and WNT/β-catenin pathways were performed for 12 of the 14 APA samples analyzed in the present study. A subset of 37 APA samples were selected from the pool of APA transcriptome profiles according to their overall similarity in gene expression, with four of the APA samples analyzed histologically for Shh expression, and presenting homogenous expression in the entire APA. Complete-linkage hierarchical clustering was done using Euclidean distances using the R-packages hclust and gplot. Principal component analysis in correlations space (PCCA) was used, indicating the fraction of information contained in the first two principal components using custom tools.

A Shh signature, which is composed of 141 genes that participate in the Shh pathway or are well-described transcriptional targets of this pathway, was obtained from Ingenuity (www.ingenuity.com) and used to develop a complete-linkage hierarchical clustering in the Euclidean distance space. In addition, a Wnt/β-catenin signature composed of 431 genes including Wnt-β-catenin signaling pathway genes and their known main transcriptional targets was assembled from Panther (www.pantherdb.org) based on Kyoto Encyclopedia of Genes and Genomes/Gene Ontology (KEGG/GO) annotations. This signature was used for the clustering analysis under the same parameters as the previous signature.

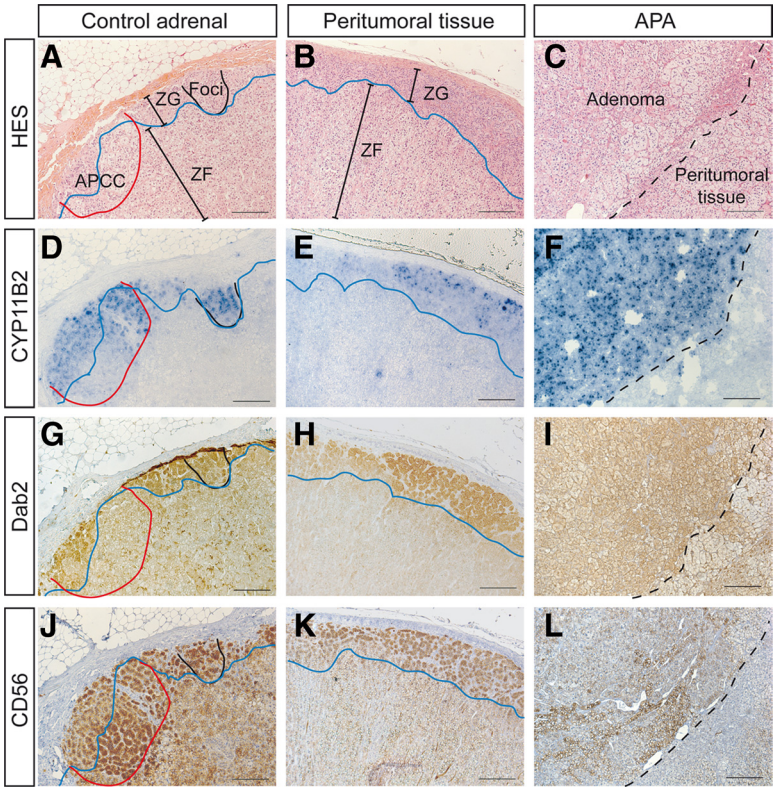

FIG. 1. Expression of CYP11B2, Dab2, and CD56 in control adrenal cortex, peritumoral tissue, and APA. In A, B and C, HES staining of the control adrenal (A), peritumoral tissue (B) and APA (C) is presented *In situ* hybridization is presented for CYP11B2 (D–F) and immunohistochemistry for Dab2 (G–I), and CD56 (J–L). In control adrenal glands (A, D, G, and J), expression of CYP11B2 (D), Dab2 (G), and CD56 (J) in foci (delineated by *black line*) and APCC (delineated by *red line*) is presented in relation to the ZG (delineated by *blue line*, A and B). Foci and APCC are distinguishable by their cellular composition and specific gene expression (A, D, G, and J). CYP11B2, Dab2, and CD56 are expressed in ZG cells. However, whereas Dab2 is restricted to ZG, CD56 presents also a gradient of expression throughout the ZF. Moreover, CYP11B2 and CD56 are expressed in the entire APCC, whereas Dab2 only in its ZG cells. In peritumoral tissue (B, E, H, and K), ZG hyperplasia is observed in HES (*blue line*, B). As in control adrenal, Dab2 (H) is restricted to ZG, whereas CD56 (K) is expressed in ZG but also in ZF. CYP11B2 is expressed in the entire hyperplasic ZG. CYP11B2 (F) and Dab2 are expressed in all the APA as well as CD56, despite the patchy appearance of its labeling due to higher level of expression in some cell clusters. APCC, foci, ZG, ZF, adenoma, and peritumoral tissue are labeled. *Bar*, 50 μm.

Statistical analysis

Pairwise comparisons were done using Student's unpaired *t* test or nonparametric Mann-Whitney test, and contingency analysis was done using the χ^2 test. All analyses were performed using the GraphPad Prism version 5.01 Software (GraphPad Software, San Diego, CA). Results are presented as mean ± SD.

Results

Dab2 and CD56 identify different features of aldosterone-producing structures

We have previously shown that in normal human adrenal, CYP11B2 mRNA is not homogeneously distributed in the ZG, with CYP11B2-expressing cells being organized as foci, megafoci (Fig. 1, A and D), and aldosterone-producing cell clusters (APCC) located in a subcapsular position (17, 27). While in control adrenal, Dab2 localizes to all the histologically recognizable ZG (Fig. 1G), foci, and megafoci. APCC represent particular structures composed of an outer layer of morphological ZG cells expressing Dab2 and inner, Dab2-negative, columnar ZF-like cells (17, 27). In control adrenals, CD56 was rather uniformly distributed throughout the ZG (Fig. 1J), with positive staining also observed in foci and megafoci. In contrast to Dab2, however, CD56 presented a gradient of expression along the ZF and was strongly expressed in the entire APCC (Fig. 1J). These results suggest that Dab2 is a marker of highly differentiated ZG cells whose expression is lost as soon as the cells transdifferentiate to become ZF cells. APCC express both CYP11B2 and CD56, supporting the view that these structures possess an intermediate phenotype between ZG and ZF cells and indicating that CD56 is a marker of not only ZG cells but also cells that acquire some characteristics of ZF cells.

In peritumoral adrenal cortex, CYP11B2 was expressed in the hyperplastic ZG (Fig. 1, B and E), foci, megafoci, and APCC, consistent with recent findings (17). The pattern of the expression of Dab2 and CD56 in peritumoral cortex was comparable with that of control adrenals (Fig. 1, H and K). As expected (14, 17, 27), despite the ZF-like phenotype of cells composing the APA (Fig. 1C), they strongly expressed CYP11B2 (Fig. 1F) as well as Dab2 (Fig. 1I). CD56 was expressed throughout the entire APA, although with a patchy appearance due to higher expression in some cell clusters. (Fig. 1L).

The nuclear receptors SF-1 and DAX-1 have an essential role in the development and function of the adrenal cortex. In the mouse a complete overlap exists between SF-1 and DAX-1 expression during development and in the adult (28). In human control adrenals, SF-1 and DAX-1 were distributed throughout the different layers of the adrenal cortex and were primarily localized to the nucleus (Fig. 2, A, D, and G). Although SF-1 was similarly expressed in APA and the peritumoral cortex (Fig. 2, B, C, E, and F), DAX-1 expression was lower in APA compared with peritumoral tissue in all, except one, APA, which is consistent with previously published data (29) (Supplemental Table 2). In contrast, DAX-1 expression was comparable with that of control adrenals in the peritumoral cortices of all patients investigated (Fig. 2, H and I).

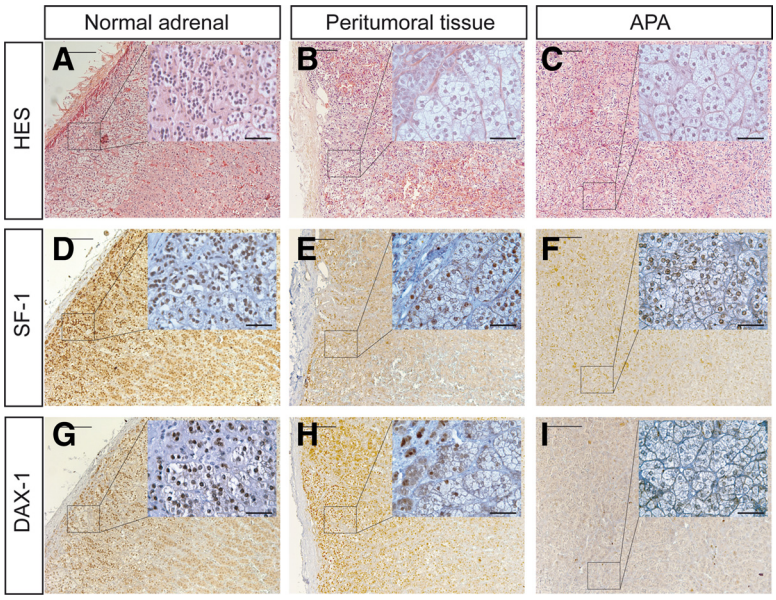

FIG. 2. Localization of SF-1 and DAX-1 in control adrenal, peritumoral tissue, and APA. In control adrenal (A, D, and G) as in peritumoral tissue (B, E, and H), both SF-1 (D and E) and DAX-1 (G and H) were expressed throughout the cortex. In APA, SF-1 was expressed (F), whereas DAX-1 showed only poor expression compared with peritumoral tissue. Higher magnification revealed predominant nuclear localization of SF-1 (D–F) and DAX-1 (G–I). In A, B, and C HES staining of control adrenal (A), peritumoral tissue (B), and APA (C) is presented. *Bar*, 200 μm on low magnification and 50 μm on high magnification.

Increased Shh expression in APA and peritumoral adrenal cortex

Analysis of Shh in control adrenals revealed that its expression is restricted to few cells of the outer part of the ZG (Fig. 3, A and D); this localization is consistent with that of the stem/precursor cells of the adrenal cortex. In contrast, expression of Shh was weak but detectable in APA, with four of 14 presenting homogeneous expression in the entire nodule (Fig. 3, C and F), whereas the remaining 10 APA presented heterogeneous expression (Supplemental Table 3). Given the recent description of somatic KCNJ5 mutations in a subset of APA (3), we asked whether the expression of Shh could be correlated to the presence of KCNJ5 mutations. Although KCNJ5 mutations were found in 10 of 13 APA (either G151R or L168R mutation), no statistical analysis could be performed due to the high frequency of KCNJ5 mutations in this small sample set (data not shown). Re-

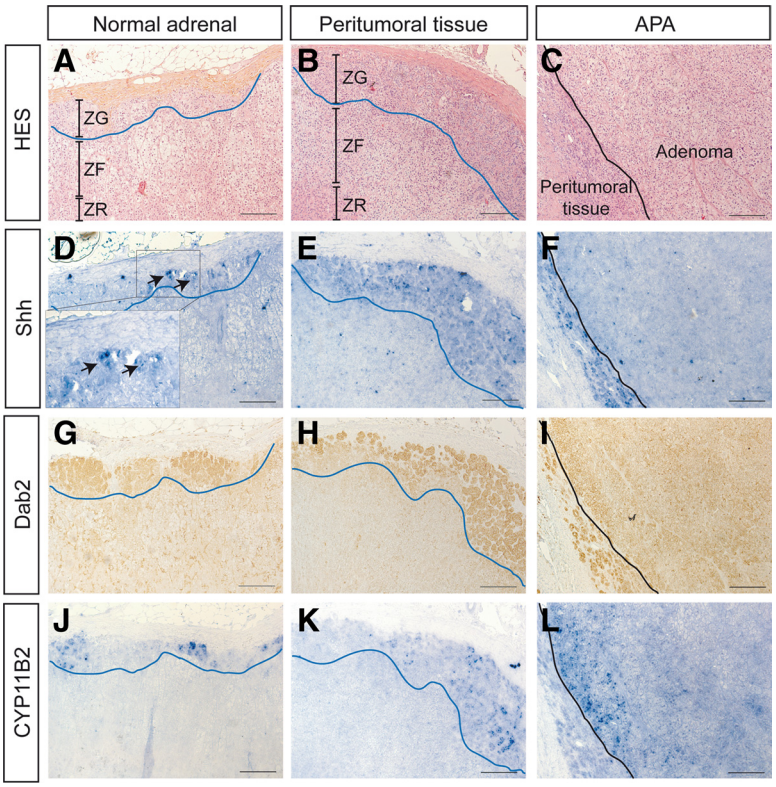

FIG. 3. Localization of Shh in control adrenal, peritumoral tissue, and APA. In control adrenal (A, D, G, and J), Shh expression (D) is barely detectable and restricted to the subcapsular region as indicated by HES (A), whereas Dab2 is expressed in the entire ZG (G) and CYP11B2 in some clusters of cells (J). In peritumoral tissue (B, E, H, and K), Shh is expressed in the entire ZG (B and E) sharing the same pattern of expression with Dab2 (H) and CYP11B2 (K). In APA (C, F, I, and L), Shh shows lower expression compared with peritumoral tissue (F). Dab2 (I) and CYP11B2 (L) are both expressed in APA. *Black arrows* indicate Shh-positive cells. The boundary between APA and peritumoral adjacent tissue is indicated by a *black line*, the boundary between ZG and ZF by a *blue line*. ZG, ZF, ZR, tumor, and peritumoral tissue are labeled. *Bar*, 200 μm.

markably, in peritumoral tissue, Shh expression was dramatically increased and extended to the entire hyperplasic ZG (Fig. 3, B and E), presenting the same pattern of expression as CYP11B2 (Fig. 3K) and Dab2 (Fig. 3H).

Activation of the Shh pathway was investigated on the expression profiles obtained from the 12 APA studied by *in situ* hybridization and 11 control adrenals. The discrimination between APA and control adrenals, revealed by hierarchical clustering and PCCA, demonstrates that a large number of genes composing the Shh signature are differentially expressed in APA *vs.* control adrenals (Fig. 4, A and B, and Supplemental Table 4). Moreover, this analysis allows us to partially separate APA with homogeneous or heterogeneous expression revealed by *in situ* hybridization (Fig. 4A). The canonical Shh pathway signature was analyzed using PCCA and revealed 10 genes (two isoforms of HMGA1, NR4A1, TUBB3, GATA2, ZNF9, UNG, ITGB1, CCND2, and STAT1) that are most likely to contribute to the first principal component separating control adrenals and APA samples (Fig. 4C). Given the small number of APA investigated by *in situ* hybridization presenting homogeneous Shh expression and the molecular heterogeneity of the disease, we extended our analysis by investigating activation of the Shh pathway on expression profiles obtained from 122 APA and 11 control adrenals. Separate clustering of control adrenals and APA samples could be obtained using the canonical Shh pathway signature on a subgroup of 37 samples, most closely related in terms of gene expression to the APA samples presenting homogeneous Shh expression (Supplemental Fig. 3 and, Supplemental Table 5), indicating significant changes in Shh pathway activity at the transcriptome level with few outliers. These results were confirmed using PCCA (Supplemental Fig. 3B) and the Shh gene signature was analyzed again using PCCA to reveal seven genes (*HMGA1*, *TUBB3*, *GATA2*, *ZNF9*, *UNG*, *CCND2*, and *STAT1*) that contribute most to the first principal component separating control adrenals and APA samples (Supplemental Fig. 3C). Altogether these results indicate that Shh, a precursor/stem cell marker, is expressed and active in APA. Importantly, the peritumoral adrenal cortex has also acquired similar characteristics of precursor/stem cells.

The Wnt-signaling pathway is activated in peritumoral adrenal cortex

In control human adrenal cortex, β-catenin was detected in the entire cortex (Fig. 5, A and B) including ZG, ZF, and ZR. Interestingly, in ZG we observed activation of β-catenin, as shown by cytoplasmic and nuclear accumulation, whereas β-catenin was mainly located at the cell membrane in ZF (Fig. 5, A–C). In peritumoral tissue adjacent to APA, β-catenin was expressed in the entire adrenal cortex and was activated not only in ZG but also in ZF and ZR (Fig. 5, D–F). In APA, despite a similar histological aspect (Fig. 6, A–C), different patterns of β-catenin expression were observed (Fig. 6, D–F). In 50% of the APA analyzed (seven of 14), β-catenin was expressed only at the cell membrane (Fig. 6D), reflecting a nonactivated state; the other 50% of cases displayed abnormal cytoplasmic and/or nuclear accumulation of β-catenin (Fig. 6, E and F), evidence for activation of the Wnt signaling pathway (Sup-

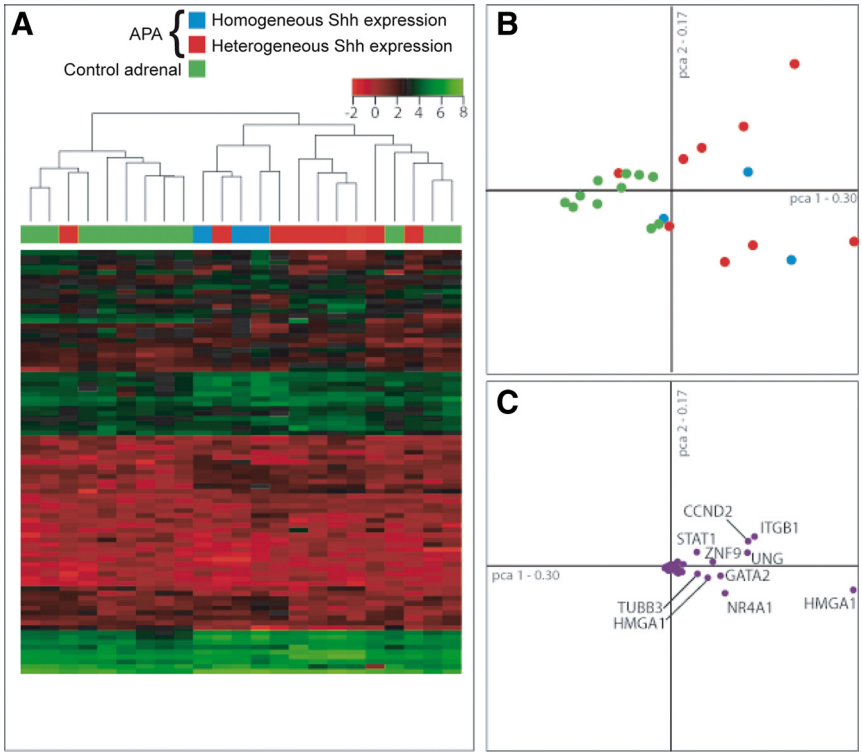

FIG. 4. Transcriptome analysis of the Shh signaling pathway. A, Complete linkage hierarchical clustering of transcriptome profiles from 12 APA and 11 control adrenal samples using Euclidian distances for the pathway of 141 selected Shh signaling pathway genes and their known main transcriptional targets. Most of the control adrenals are clustered apart from APA. Among APA, adenomas presenting homogeneous Shh expression in *in situ* hybridization clustered together. B, PCCA of the same samples as in A. The first two principal components and their inertia are displayed. PCCA confirmed difference observed in hierarchical clustering between control adrenals and APA. C, PCCA in gene space of the samples from A. The first two principal components and their inertia are displayed. Particularly highly contributing genes that most contribute to the first principal component separating control adrenals and APA samples are identified.

plemental Table 3). The analysis of 12 additional samples confirmed this heterogeneity with β-catenin activation in 66% of APA in total (17 of 26). However, this difference in the β-catenin activation was not associated with any biological and clinical parameters measured in patients with APA (Supplemental Table 6). Mutations of exons 3 and 5 of the β-catenin gene (*CTNNB1*), resulting in constitutive activation of the protein, are frequently found in a variety of human cancers including adrenocortical tumors (13, 30, 31). Sequencing of these two exons in somatic DNA from 41 patients did not show any mutation that could account for β-catenin activation.

Transcriptome profiles of the Wnt-β-catenin pathway genes were investigated in 12 of the 14 APA samples studied by immunohistochemistry (Fig. 7). Interestingly, we observed separate clustering of control adrenals and APA samples (Fig. 7A), indicating changes in Wnt/β-catenin pathway gene expression between these two groups (Supplemental Table 7). These results were confirmed by PCCA analysis (Fig. 7B). Among the 50 genes differentially regulated between APA and control adrenals, 36 (72%) are down-regulated and only 14 up-regulated. Using PCCA, the Wnt-β-catenin gene signature was analyzed to reveal genes (*CTNNAL1*, *TTBK1*, *ACTR1B*, *DVL1*, *AES*, *CSNK2B*, *GNG10*, *CCND2*, *PCDHGB5*, *MYC*, and *PPP2CA*) that contribute most to the first principal component separating control adrenals and APA samples (Fig. 7C). Moreover, among APA we could separate those presenting membrane localization of β-catenin (nonactive) from those presenting nuclear localization (active, right part of the clustering). Surprisingly, APA-presenting cytoplasmic accumulation of β-catenin, which is considered to reveal an activated form, clustered with those presenting membrane localization of β-catenin (Fig. 7, A and B), possibly indicating activation of distinct pathways, depending on intracellular β-catenin localization.

Discussion

The primary goal of the present study was to investigate the molecular phenotype of APA and the link with adrenal cortex remodeling in the peritumoral adrenal gland. Despite their ZF-like cellular phenotype, APA are composed of cells expressing ZG markers (CYP11B2, Dab2, and CD56) in addition to steroidogenic markers (SF-1 and DAX-1). Remarkably, whereas in control adrenals, Shh was poorly detectable in a restricted number of subcapsular cells, its expression was detected in the entire APA and dramatically increased in the hyperplastic peritumoral ZG. Similarly, Wnt/β-catenin signaling was activated in the majority of APA as well as in the entire peritumoral adrenal cortex.

We have previously characterized different structures expressing CYP11B2: 1) foci or megafoci that are Dab2 positive and CYP11B1 negative; and 2) APCC that present a mixed cellular composition of outer ZG and inner ZF cells (17, 27). Expression of CYP11B2 throughout the entire APCC suggested that these structures possess an intermediate phenotype between ZG and ZF cells. These results are now further supported by showing the expression of CD56 in ZG, ZF, and also in APCC. In contrast,

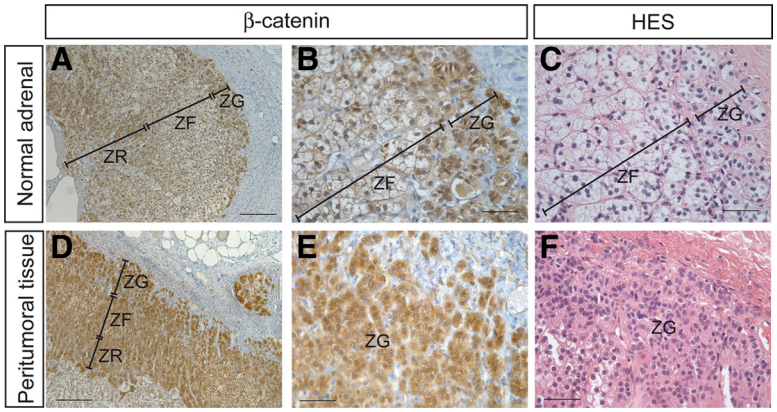

FIG. 5. Localization of β-catenin in control adrenal and peritumoral tissue. Immunohistochemistry of β-catenin (A and B) shows expression throughout the control adrenal cortex (A). In control adrenal, immunostaining revealed membranous, cytoplasmic, and nuclear localization of β-catenin in ZG, whereas in ZF β-catenin presented predominantly membranous expression (B). In peritumoral tissue, β-catenin is also found in the entire adrenal cortex (D). Immunostaining revealed membranous, cytoplasmic, and nuclear localization in ZG, ZF, and ZR (E). HES staining (C and F) showed ZG hyperplasia in peritumoral adjacent tissue. ZG, ZF, and ZR are labeled. *Bar*, 200 μm (A and D) or 50 μm (B, C, E, and F).

Dab2 expression is restricted to the morphological ZG, associated or not with aldosterone production, but disappears as soon as cells transdifferentiate to ZF. Its expression in APA clearly indicates that adenoma cells derive from ZG despite their morphological appearance.

It is remarkable that by immunohistochemistry we found decreased expression of DAX-1, a known repressor of steroidogenesis (32), in APA compared with the peritumoral tissue. These findings confirm previous gene expression data (29) and suggest that this nuclear receptor may be involved in the definition of the steroid secretion phenotype of APA.

The Wnt/β-catenin pathway plays an important role in embryonic development, stem cell maintenance, and differentiation in many tissues (31). Briefly, in the absence of Wnt, casein kinase 1 and glycogen synthase kinase-3 (GSK-3) sequentially phosphorylate β-catenin in the axin complex, resulting in its ubiquitination and degradation by the proteasome. Activation of the Wnt/β-catenin pathway occurs when a Wnt ligand binds to its cell surface receptor, which consists of a seven-pass transmembrane Frizzled receptor and its coreceptor, low-density lipoprotein receptor-related protein (LRP) 6 or its close relative LRP5. The formation of a Wnt-Frizzled-LRP6/5 complex together with the recruitment of the scaffolding protein Dishevelled, which antagonizes the action of GSK-3 through an unknown mechanism, results in the inhibition of β-catenin phosphorylation, dissociation from the Axin complex, accumulation in the cytoplasm, and translocation to the nucleus, thus stimulating the expression of various Wnt target genes (31). Loss of Wnt4 results in the aberrant migration of adrenocortical cells into the developing gonad (33). Furthermore, a role for β-catenin signaling in the regulation of steroidogenesis is supported by the observation that in Wnt4-deficient mice a decreased number of ZG cells leads to a decrease in aldosterone production (34). In addition, studies in human adrenocortical cells have indicated that Wnt signaling molecules have multiple actions on steroidogenesis, particularly in regulating aldosterone biosynthesis (34, 35). Our results demonstrate that in human, β-catenin is expressed throughout the adrenal cortex in both control adrenal as well as in peritumoral adjacent tissue; however, activated β-catenin was found only in ZG, suggesting that, similarly to rodents, restriction of β-catenin activation to subcapsular regions, and in particular ZG is necessary for the development and/or maintenance of functional zonation in the human adrenal cortex (31).

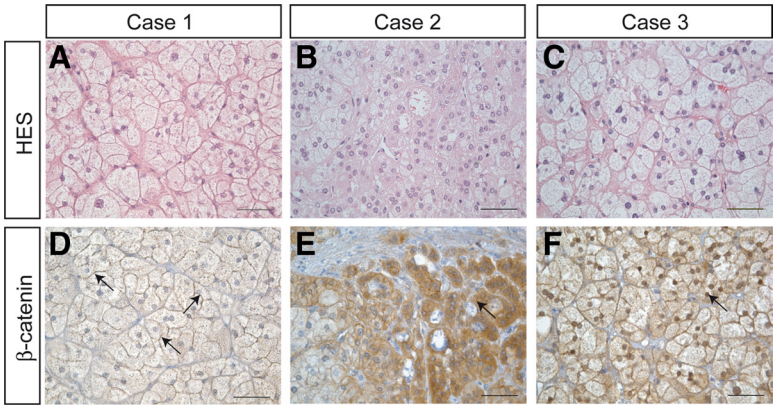

FIG. 6. Localization of β-catenin in APA. Three different cases of APA are presented. In case 1 (A and D), immunostaining of β-catenin (D) reveals only membranous localization. In case 2 (B and E), staining of β-catenin shows strong accumulation in the cytoplasm (E). In case 3 (C and F), β-catenin is accumulated into the cytoplasm and the nucleus (F). HES staining are presented in A, B, and C. *Black arrows* indicate β-catenin localization. *Bar*, 50 μm.

β-Catenin dysregulation has been observed in a subset of sporadic adrenocortical adenomas and carcinomas (13, 21, 30). Mice expressing constitutively active β-catenin in the adrenal gland develop hyperaldosteronism at the age of 10 months (36), similarly to mice expressing a defective APC allele (37). However, at 17 months of age, these animals develop malignant adrenal tumors (uncontrolled neovascular-

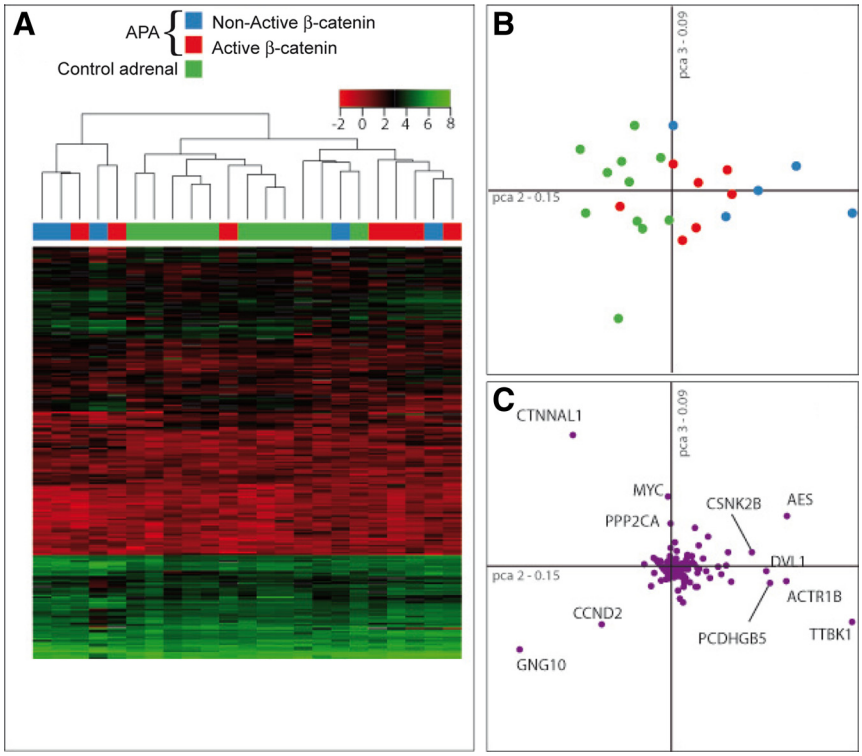

FIG. 7. Transcriptome analysis of the Wnt/β-catenin signaling pathway. A, Hierarchical clustering of transcriptome profiles from APA and control adrenal samples using Euclidian distances for the pathway of 431 selected Wnt/β-catenin signaling pathway genes and their known main transcriptional targets. B, PCCA of the same samples as in A. The first two principal components and their inertia are displayed. C, PCCA in gene space of the samples from A. The first two principal components and their inertia are displayed. Particularly highly contributing genes that most contribute to the first principal component separating control adrenals and APA samples are identified.

ization and locoregional metastatic invasion) (36), features only very rarely observed in APA. Similarly, up-regulation of targets of the Wnt/β-catenin pathway in adrenocortical carcinoma is associated with poor prognosis (38, 39). Thus, Wnt/β-catenin activation may play distinct roles in APA compared with adrenal cortex carcinoma, contributing to aldosterone hypersecretion rather than to autonomous cell proliferation. Our results indicate that activation of β-catenin occurs in approximately two thirds of APA, heterogeneity that is reflected by the expression of downstream target genes. Among the differentially expressed genes identified by the transcriptome analysis, specific β-catenin target genes (cyclin D1, v-Myc) and some genes involved in Wnt/β-catenin signaling (WNT7b, GSK3B, TCF7L2) are down-regulated in APA. Given the absence of somatic mutations in the *CTNNB1* gene in APA, it is possible that Wnt/β-catenin activation in APA is not related to tumor development but reflects the cellular nature of the tumor deriving from the ZG and may not translate into activation of a subset of its canonical target genes.

Similarly to the Wnt/β-catenin signaling pathway, Shh signaling is essential for adrenal gland development and maintenance, and Shh has been characterized as a stem/precursor cell marker (8–10). In rodent adult adrenals, Shh is expressed exclusively in the subcapsular region of the cortex in cells also expressing SF-1 (8, 9, 40), indicating their commitment to steroidogenic cells. Mice invalidated for Shh specifically in SF-1-positive cells present reduced proliferation of capsular cells and a significant reduction of adrenocortex thickness and adrenal size but no modification of adrenal zonation, indicating that Shh is essential for expansion of the adrenal cortex but not for zonation and differentiation (10). In a previous work, we have demonstrated that adrenals with APA present large cortical remodeling and functional ZG hyperplasia (17). Here we show that in the human adrenal cortex Shh is specifically expressed in a restricted number of cells of the subcapsular region, in which stem/precursor cells are supposed to be localized (9, 16, 40). In contrast, Shh was weakly expressed but detectable in all APA, with homogeneous expression in 29% of samples. Despite this heterogeneity in Shh expression in APA, some components of this pathway clearly differed in their expression levels between a subgroup of APA and control adrenal cortex. Among them, some have been previously described to play a role in cell cycle progression, cell proliferation, and tumorigenesis (CCND1, TSC22D3, GATA2, PAX9, HMGA1), suggesting that activation of Shh signaling pathway may be in part related to cell proliferation and thus to APA formation. Importantly, a dramatic increase in Shh expression was observed in the entire hyperplastic peritumoral ZG, with a similar pattern of expression than CYP11B2 and Dab2, suggesting that both APA and the adjacent ZG had acquired some characteristics of stem/precursor cells or, alternatively, that reexpression of fetal markers from the definitive zone in the adrenal cortex could underlie excessive proliferation and APA formation.

Conclusion

Acquisition of some characteristics of stem/precursor cells and reexpression of fetal markers may underlie ZG expansion and formation of autonomous buds. Identification of the mechanisms involved in Shh and Wnt/β-catenin activation will lead to a better understanding of

the processes involved in both APA formation and in the development of bilateral adrenal hyperplasia.

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References

1. **Plouin PF, Amar L, Chatellier G** 2004 Trends in the prevalence of primary aldosteronism, aldosterone-producing adenomas, and surgically correctable aldosterone-dependent hypertension. Nephrol Dial Transplant 19:774–777
2. **Conn JW, Louis LH** 1955 Primary aldosteronism: a new clinical entity. Trans Assoc Am Physicians 68:215–231; discussion 231–233
3. **Choi M, Scholl UI, Yue P, Björklund P, Zhao B, Nelson-Williams C, Ji W, Cho Y, Patel A, Men CJ, Lolis E, Wisgerhof MV, Geller DS, Mane S, Hellman P, Westin G, Åkerström G, Wang W, Carling T, Lifton RP** 2011 K+ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. Science 331:768–772
4. **Arnold J** 1866 Ein beitrag zur feineren struktur und dem chemismus des nebennieren. Virchows Arch Patholog Anatomie Physiol Klin Med 35:64–107
5. **Kempn á P, Flück CE** 2008 Adrenal gland development and defects. Best Pract Res Clin Endocrinol Metab 22:77–93
6. **Muench MO, Ratcliffe JV, Nakanishi M, Ishimoto H, Jaffe RB** 2003 Isolation of definitive zone and chromaffin cells based upon expression of CD56 (neural cell adhesion molecule) in the human fetal adrenal gland. J Clin Endocrinol Metab 88:3921–3930
7. **Ishimoto H, Jaffe RB** 2010 Development and function of the human fetal adrenal cortex: a key component in the feto-placental unit. Endocr Rev 32:317–355
8. **Ching S, Vilain E** 2009 Targeted disruption of Sonic Hedgehog in the mouse adrenal leads to adrenocortical hypoplasia. Genesis 47:628–637
9. **King P, Paul A, Laufer E** 2009 Shh signaling regulates adrenocortical development and identifies progenitors of steroidogenic lineages. Proc Natl Acad Sci USA 106:21185–21190
10. **Huang CC, Miyagawa S, Matsumaru D, Parker KL, Yao HH** 2010 Progenitor cell expansion and organ size of mouse adrenal is regulated by sonic hedgehog. Endocrinology 151:1119–1128
11. **Bitgood MJ, McMahon AP** 1995 Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Dev Biol 172:126–138
12. **Kim A, Giordano TJ, Kuick R, Serecky K, Hammer GD** 2009 Wnt/β-catenin signaling in adrenocortical stem/progenitor cells: implications for adrenocortical carcinoma. Ann Endocrinol (Paris) 70:156
13. **Tissier F, Cavard C, Groussin L, Perlemoine K, Fumey G, Hagner é AM, René-Corail F, Jullian E, Gicquel C, Bertagna X, Vacher-Lavenu MC, Perret C, Bertherat J** 2005 Mutations of β-catenin in adrenocortical tumors: activation of the Wnt signaling pathway is a frequent event in both benign and malignant adrenocortical tumors. Cancer Res 65:7622–7627
14. **Caroccia B, Fassina A, Seccia TM, Recarti C, Petrelli L, Belloni AS, Pelizzo MR, Rossi GP** 2010 Isolation of human adrenocortical aldosterone-producing cells by a novel immunomagnetic beads method. Endocrinology 151:1375–1380
15. **Mesiano S, Jaffe RB** 1997 Developmental and functional biology of the primate fetal adrenal cortex. Endocr Rev 18:378–403
16. **Kim AC, Barlaskar FM, Heaton JH, Else T, Kelly VR, Krill KT, Scheys JO, Simon DP, Trovato A, Yang WH, Hammer GD** 2009 In search of adrenocortical stem and progenitor cells. Endocr Rev 30:241–263
17. **Boulkroun S, Samson-Couterie B, Dzib JF, Lefebvre H, Louiset E, Amar L, Plouin PF, Lalli E, Jeunemaitre X, Benecke A, Meatchi T, Zennaro MC** 2010 Adrenal cortex remodeling and functional zona glomerulosa hyperplasia in primary aldosteronism. Hypertension 56:885–892
18. **Letavernier E, Peyrard S, Amar L, Zinzindohou é F, Fiquet B, Plouin PF** 2008 Blood pressure outcome of adrenalectomy in patients with primary hyperaldosteronism with or without unilateral adenoma. J Hypertens 26:1816–1823
19. **Doghman M, Karpova T, Rodrigues GA, Arhatte M, De Moura J, Cavalli LR, Virolle V, Barbry P, Zambetti GP, Figueiredo BC, Heckert LL, Lalli E** 2007 Increased steroidogenic factor-1 dosage triggers adrenocortical cell proliferation and cancer. Mol Endocrinol 21:2968–2987
20. **Tamai KT, Monaco L, Alastalo TP, Lalli E, Parvinen M, Sassone-Corsi P** 1996 Hormonal and developmental regulation of DAX-1 expression in Sertoli cells. Mol Endocrinol 10:1561–1569
21. **Polakis P** 2000 Wnt signaling and cancer. Genes Dev 14:1837–1851
22. **Kikuchi A** 2003 Tumor formation by genetic mutations in the components of the Wnt signaling pathway. Cancer Sci 94:225–229
23. **Noth S, Brysbaert G, Pellay FX, Benecke A** 2006 High-sensitivity transcriptome data structure and implications for analysis and biologic interpretation. Genomics Proteomics Bioinformatics 4:212–229
24. **Noth S, Brysbaert G, Benecke A** 2006 Normalization using weighted negative second order exponential error functions (NeONORM) provides robustness against asymmetries in comparative transcriptome profiles and avoids false calls. Genomics Proteomics Bioinformatics 4:90–109
25. **Wilhelm E, Kornete M, Targat B, Vigneault-Edwards J, Frontini M, Tora L, Benecke A, Bell B** 2010 TAF6Δ orchestrates an apoptotic transcriptome profile and interacts functionally with p53. BMC Mol Biol 11:10
26. **Noth S, Benecke A** 2005 Avoiding inconsistencies over time and tracking difficulties in Applied Biosystems AB1700/Panther probe-to-gene annotations. BMC Bioinformatics 6:307
27. **Nishimoto K, Nakagawa K, Li D, Kosaka T, Oya M, Mikami S, Shibata H, Itoh H, Mitani F, Yamazaki T, Ogishima T, Suematsu M, Mukai K** 2010 Adrenocortical zonation in humans under normal and pathological conditions. J Clin Endocrinol Metab 95:2296–2305
28. **Ikeda Y, Swain A, Weber TJ, Hentges KE, Zanaria E, Lalli E, Tamai KT, Sassone-Corsi P, Lovell-Badge R, Camerino G, Parker KL** 1996 Steroidogenic factor 1 and Dax-1 colocalize in multiple cell lineages:

potential links in endocrine development. Mol Endocrinol 10:1261–1272
29. **Reincke M, Beuschlein F, Lalli E, Arlt W, Vay S, Sassone-Corsi P, Allolio B** 1998 DAX-1 expression in human adrenocortical neoplasms: implications for steroidogenesis. J Clin Endocrinol Metab 83:2597–2600
30. **Tadjine M, Lampron A, Ouadi L, Bourdeau I** 2008 Frequent mutations of β-catenin gene in sporadic secreting adrenocortical adenomas. Clin Endocrinol (Oxf) 68:264–270
31. **El Wakil A, Lalli E** 2011 The Wnt/β-catenin pathway in adrenocortical development and cancer. Mol Cell Endocrinol 332:32–37
32. **Lalli E, Sassone-Corsi P** 2003 DAX-1, an unusual orphan receptor at the crossroads of steroidogenic function and sexual differentiation. Mol Endocrinol 17:1445–1453
33. **Jeays-Ward K, Hoyle C, Brennan J, Dandonneau M, Alldus G, Capel B, Swain A** 2003 Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. Development 130:3663–3670
34. **Heikkil ä M, Peltoketo H, Leppäluoto J, Ilves M, Vuolteenaho O, Vainio S** 2002 Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. Endocrinology 143:4358–4365
35. **Schinner S, Willenberg HS, Krause D, Schott M, Lamounier-Zepter V, Krug AW, Ehrhart-Bornstein M, Bornstein SR, Scherbaum WA** 2007 Adipocyte-derived products induce the transcription of the StAR promoter and stimulate aldosterone and cortisol secretion from adrenocortical cells through the Wnt-signaling pathway. Int J Obes (Lond) 31:864–870
36. **Berthon A, Sahut-Barnola I, Lambert-Langlais S, de Joussineau C, Damon-Soubeyrand C, Louiset E, Taketo MM, Tissier F, Bertherat J, Lefrançois-Martinez AM, Martinez A, Val P** 2010 Constitutive β-catenin activation induces adrenal hyperplasia and promotes adrenal cancer development. Hum Mol Genet 19:1561–1576
37. **Bhandaru M, Kempe DS, Rotte A, Rexhepaj R, Kuhl D, Lang F** 2009 Hyperaldosteronism, hypervolemia, and increased blood pressure in mice expressing defective APC. Am J Physiol Regul Integr Comp Physiol 297:R571–R575
38. **Ragazzon B, Lib é R, Gaujoux S, Assi é G, Fratticci A, Launay P, Clauser E, Bertagna X, Tissier F, de Reyniès A, Bertherat J** 2010 Transcriptome analysis reveals that p53 and β-catenin alterations occur in a group of aggressive adrenocortical cancers. Cancer Res 70:8276–8281
39. **Ragazzon B, Assi é G, Bertherat J** 2011 Transcriptome analysis of adrenocortical cancers: from molecular classification to the identification of new treatments. Endocr Relat Cancer 18:R15–R27
40. **Guasti L, Paul A, Laufer E, King P** 2011 Localization of Sonic hedgehog secreting and receiving cells in the developing and adult rat adrenal cortex. Mol Cell Endocrinol 336:117–122