Gonadectomy in mice of the inbred strain CE/J induces proliferation of sub-capsular adrenal cells expressing gonadal marker genes

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Abstract

Mouse models of adrenal tumorigenesis have the potential to give insights in the dysregulation of adrenal growth and differentiation. The inbred mouse strain CE/J has been reported to develop adrenal tumors upon gonadectomy (GDX) similar to mice with targeted deletions of the inhibin alpha subunit (*Inh*^{-/-}). We performed a detailed morphological and molecular characterization of adrenal glands from CE/J mice 8–50 weeks of age to define the cellular origin of these tumors as well as the spatial and temporal expression of marker genes associated with tumor growth. In contrast to the induction of x-zone growth upon GDX in *Inh*^{-/-} mice, GDX in CE/J mice induced the appearance of sub-capsular nests of densely packed cells that progress into adrenal tumors. Staining for proliferative cell nuclear antigen confirms a substantial increase in cellular proliferation within this sub-capsular compartment and lack of apoptosis upon GDX. Induction of adrenal tumor growth was accompanied by

transcriptional changes that otherwise define gonadal endocrine cells. These regulated genes included transcription factors such as GATA-4, WT-1, FOG-1, and steroidogenic factor-1 (SF-1), peptide hormones such as Mullerian-inhibiting substance (MIS), hormone receptors including luteinizing hormone and MIS receptor, and steroidogenic enzymes such as P450c17 and P450 aromatase. The functional significance of steroid enzyme expression was demonstrated by a gradual increase of adrenal androgens after GDX. Taken together these data suggest that adrenal tumors in gonadectomized CE/J mice are direct derivatives from cells of the proposed sub-capsular stem cell zone. The distinct expression pattern of this cell population is consistent with a defect in the differentiation of these cells into a cell population with functional properties that otherwise define a gonadal endocrine phenotype.

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Introduction

Adrenal masses are one of the most common endocrine tumors diagnosed. Although most adrenal tumors are endocrine-inactive adenomas, a considerable proportion are associated with hormonal hyperfunction (Barzon *et al.* 2002) or malignancy (Mantero *et al.* 2000). Over the last decade, several molecular pathways involved in adrenal carcinogenesis have been identified, and mouse models of adrenal tumorigenesis have aided substantially in the understanding of basic mechanisms in the regulation of adrenocortical growth and differentiation. However, early steps in the pathogenesis of adrenal tumors have not been elucidated in detail.

Only recently, murine adrenal tumor models have provided evidence that luteinizing hormone (LH) and the transforming

growth factor- β (TGF- β) ligand family members inhibin and activin play opposite roles in adrenocortical hormone secretion and cellular proliferation (Rilianawati *et al.* 1998, Kero *et al.* 2000, Beuschlein *et al.* 2003). Transgenic overexpression of LH induces adrenal steroidogenesis (Kero *et al.* 2000) and high levels of gonadotropins are required for the induction of adrenal tumorigenesis in different mouse models (Matzuk *et al.* 1994, Rilianawati *et al.* 1998). One of these models, which has provided a significant amount of information on the mechanistic roles of inhibin and activin in the adrenal, is the inhibin null (*Inh*^{-/-}) mouse, which spontaneously develops activin-secreting gonadal tumors and adrenocortical carcinomas upon gonadectomy (GDX) (Matzuk *et al.* 1994). As we have demonstrated, the adrenal phenotype in *Inh*^{-/-} mice is indicative of x-zone growth dysregulation. Development of activin-secreting gonadal

tumors in $\text{Inh}^{-/-}$ mice is accompanied by a decrease in adrenal weight and regression of the x-zone. Activin, which plays a critical role as a paracrine and autocrine factor regulating cellular growth and differentiation, has been demonstrated to induce apoptosis and suppress proliferation in the human (Spencer *et al.* 1992, 1999) and murine adrenal cortex (Beuschlein *et al.* 2003). The ultimate cause of this regression in the murine adrenal cortex is the distinct x-zonal expression pattern of activin receptor subunits and the intracellular mediator Smad-2, which results in a particular responsiveness of the x-zone to activin. As a result, high levels of activin induce apoptosis specifically in the adrenal x-zone, thus preventing adrenal tumorigenesis in the presence of activin-secreting gonadal tumors. Conversely, the surgical removal of activin-secreting gonadal tumors in $\text{Inh}^{-/-}$ is followed by unopposed x-zone growth and ultimately by the development of adrenal tumors (Beuschlein *et al.* 2003).

The CE/J strain of mice, which bases on the *extreme dilution strain* established and maintained since 1920, has been characterized on a morphological basis in the 1940s. Intriguingly, the CE/J strain shares several phenotypical similarities with the $\text{Inh}^{-/-}$ mouse. Like $\text{Inh}^{-/-}$ mice, the CE/J strain is characterized by the occurrence of adrenocortical carcinoma when GDX is performed early in life (Woolley & Little 1945). In addition, morphological studies on accessory reproductive organs in gonadectomized CE/J mice have provided indirect evidence for the secretion of sex steroids from the adrenal tumors (Woolley & Little 1946a). Since adrenal tumors in gonadectomized $\text{Inh}^{-/-}$ mice secrete sex steroids (Beuschlein *et al.* 2003), the adrenal phenotype in CE/J mice points towards a dysregulation of x-zone growth after GDX as the basis of adrenal tumor growth. However, based on the early morphological studies (Woolley & Little 1946b) and recent reports on similar phenotypes in other inbred mouse strains (Bielinska *et al.* 2003, 2005), it seems likely that the precursor of tumor cells in CE/J mice are located in the sub-capsular zone of the adrenal cortex.

The adrenal cortex is a dynamic organ in which senescing cells are constantly replaced by newly differentiated daughter cells. According to the cell migration model, each zone of the adrenal cortex is derived from a common pool of progenitor cells located in the periphery of the cortex, which migrates centripetally and populates the inner cortical zones upon differentiation (Belloni *et al.* 1978, Spencer *et al.* 1999). The characteristic zonation of the adrenal cortex in cellular compartments with distinct functional properties is tightly controlled to ensure structural plasticity of the adrenal cortex and to maintain hormonal homeostasis. We hypothesized that dysregulation of adrenal stem cell proliferation and differentiation could be the basis of adrenal tumorigenesis in CE/J mice and undertook a detailed morphological and functional characterization of the adrenal phenotype of these mice.

Materials and Methods

Experimental animals

All experiments involving animals were performed in accordance with institutionally approved and current animal care guidelines. Male CE/J mice were obtained by breeding mice of the inbred CE/J background (Jackson Immuno-Research Laboratories, West Grove, PA, USA), while $\text{Inh}^{-/-}$ mice were generated by breeding of heterozygous animals ($\text{Inh}^{+/-}$) on an extensively outbred strain. Genotyping was performed as described earlier (Matzuk *et al.* 1994). All animals were maintained under standard conditions of temperature (22 °C) and lighting (12 h light:12 h darkness) and *ad libitum* with food and water. To obtain a time-course of the testicular and adrenal phenotype of gonadectomized and non-gonadectomized CE/J mice, a group of mice ($n=3-4$) was euthanized at 8, 14, 20, 26, 38, and 44 weeks of age, while another group of mice ($n=3-4$) was gonadectomized at 2 weeks of age following the standard procedures and euthanized at the same time points. Accordingly, $\text{Inh}^{-/-}$ and wild-type controls were gonadectomized at 2–4 weeks of age and euthanized at 19–27 weeks. Non-gonadectomized controls of each genotype were euthanized at 9 weeks of age. Trunk blood for hormonal measurements was taken within 60 s after initial mouse handling and adrenals and testes were collected. Following microdissection, adrenal and testicular weights were measured and the tissues were snap frozen for protein/RNA extraction or immersed in para-formaldehyde (PFA) or Bouin's fixative for histological studies.

Reverse transcription (RT)-PCR and real-time PCR

Individual adrenals from 44-week-old gonadectomized and non-gonadectomized CE/J mice ($n=2$ in each group) as well as the adrenals of the non-GDX and GDX $\text{Inh}^{-/-}$ knockout and $\text{Inh}^{+/+}$ controls ($n=2$ in each group) were used for RNA extraction using the Qiagen RNA mini kit (Qiagen) following the instructions of the manufacturer. cDNA was created from 1.0 µg of total RNA using a reverse transcription kit (Promega). Aliquots of the cDNA samples were subjected to the subsequent PCR for semi- and to real-time PCR for quantitative measurements of mRNA levels. RT-PCR and real-time PCR amplifications were performed with the corresponding primer pairs, as given in Table 1.

The PCR was carried out using the Advantage 2 Polymerase Mix (BD Biosciences, Heidelberg, Germany) with 32–35 cycles of denaturation at 94 °C for 20 s, primer annealing and extension at 68 °C for 20 s. Amplification products were separated on a 1% agarose gel and stained with ethidium bromide.

Real-time PCR was performed using the FastStart DNA Master^{plus} SYBR Green I reaction mix and the FastStart Taq DNA Polymerase (Roche) or the SYBR Premix Ex Taq (Cambrex, Baltimore, MD, USA) in an appropriate

Table 1 Primer sequences used in reverse transcription- and real time-PCR experiments

	Purpose	Sequence	Nucleotides	Annealing temp.	Reference
Amplification product					
β2Microgl (fwd)	Real-time PCR	GCTATCCAGAAAAACCCCTCAA	64–363	60 °C	
β2Microgl (rev)		CATGTCTCGATCCCACTAGACGGT			
18s rRNA (fwd)	Real-time PCR	GTAACCCGTTGAACCCCAATT	1579–1730	58 °C	
18s rRNA (rev)		CCATCCAATCGGTAGTAGCG			
SF-1 (fwd)	Real-time PCR	TGCACTGCAGCTGGACCCGAGGAGTT	1248–1638	61 °C	
SF-1 (rev)		AGGGCTCCTGGATCCCTAATGCAAGGA			
Dax-1 (fwd)	RT-PCR	GTCAAGTACTTGCCCTGCTTCCA	811–1323		
Dax-1 (rev)		GATGAATCTCAGCAGGAAAAGGGC			
	Real-time PCR	TCCTGTACCGCAGCTATGTG	604–941	58 °C	
		CTCGAAGTGCAGGTGATCTTG			
WT-1 (fwd)	RT-PCR	GGGGAAGCTTCCGCCATGGGTCCGAC-	477–910		(Wilhelm & Englert 2002)
WT-1 (rev)		GTGCGGGAC			
	Real-time PCR	GGATGGTAGGCTGGCTCTCCAG	271–704	62 °C	
		GCC TTC ACC TTG CAC TTC TC			
		CAT TCA AGC TGG GAG GTC AT			
GATA-4 (fwd)	RT-PCR	GCCTGTATGTAATGCCTGCG	1442–1938		
GATA-4 (rev)		CCGAGCAGGAATTTGAAGAGG			
	Real-time PCR	TGTGCCAACTGCCAGACTAC	1415–1859	60 °C	
		GCGATGTCTGAGTGACAGGA			
GATA-6 (fwd)	RT-PCR	GCAATGCATGCCGTCTCTAC	1487–2041		
GATA-6 (rev)		CTCTTGGTAGCACCAGCTCA			
	Real-time PCR	ATGCTTGCGGGCTCTATATG	1652–1872	59 °C	
		TGA GGTGGTCGCTTGTGTAG			
FOG-1 (fwd)	RT-PCR	CGGGATCCCGGCGCAGCGGGAACCCAC	2067–2504		(Robert <i>et al.</i> 2002)
FOG-1 (rev)		CGGAATCCCGCAGTAGTAGCGCTTGTC			
	Real-time PCR	GCTATATG GCGCCTTGTC	943–1098	59 °C	
		TTGATGACTGCGGTAGCAAG			
Sox-8 (fwd)	RT-PCR	TGGAGTCTGGTGCCTATGCCTGT	397–780		(Schepers <i>et al.</i> 2000)
Sox-8 (rev)		GCCGAGCACTGCATCAGCTTTGT			
	Real-time PCR	GCTCAATGGGCTCTCTATGC	1476–1821	58 °C	
		TATCCAAACCGGAGAGCAAC			
Sox-9 fwd)	RT-PCR	GCGTATGAATCTCTGGACC	369–902		
Sox-9 (rev)		GCGGCTGGTACTTGTAATCC			
	Real-time PCR	TACGACTACGCTGACCATCAGAACT	1673–2237	60 °C	
		GATTCTCCAATCGTCTCCATGT			
MIS (fwd)	RT-PCR	GAGCTCTTGCTGAAGTTC	426–658		(Arango <i>et al.</i> 1999)
MIS (rev)		CTGCTTGGTTGAAGGGTTAAG			
	Real-time PCR	GGGGGGTCTGAACAGCTATGACT	221–453	60 °C	
		GAGGCTCTTGGAACCTCAGCAA			
MIS-R (fwd)	RT-PCR	TCCAGCTGGCATCCTTTTGC	32–683		(Mishina <i>et al.</i> 1996)
MIS-R (rev)		TGACCTCCTTCTGGATTAC			
	Real-time PCR	CCTGGGAATGTTTCTCGTGT	507–818	60 °C	
		CGAACGATATGGTTCATGCTG			
LH-R (fwd)	RT-PCR	ATGGATCCCTCTCACTATCTCCCTGT	176–878		(Beuschlein <i>et al.</i> 2003)
LH-R (rev)		AGTCTAGATCTTCTTCGGCAATTCCTG			
	Real-time PCR	CTCGCCGACTATCTCTCAC	168–618	58 °C	
		AGATTAGCGTCGTCCCAATTG			
P450c17 (fwd)	RT-PCR	CGAAGCTTGGAACCTGTGGGTCTCTTGC	6–1513		(Beuschlein <i>et al.</i> 2003)
P450c17 (rev)		CGCTCGAGAACCCTCAACCTGTGCATCCT			
	Real-time PCR	CAAGCCAAGATGAATGCAGA	930–1367	60 °C	
		CATAAACCGATCTGGCTGGT			
P450arom (fwd)	RT-PCR	GACACATCATGCTGGACACC	575–1297		(Beuschlein <i>et al.</i> 2003)
P450arom (rev)		CAAAGCCAAAAGGCTGAAAG			
	Real-time PCR	ATGAACGATCCGTCAAGGAC	731–1216	63 °C	
		ACTCGAGCCTGTGCATTCTT			
MSTAR (fwd)	Real-time PCR	GACCTTGAAAGGCTCAGGAAGAAC	7–987	60 °C	
MSTAR (rev)		TAGCTGAAGATGGACAGACTTGC			

LightCycler. The real-time PCR conditions started with a preincubation at 95 °C for 10 min, followed by the amplification of 40–45 cycles at 95 °C for 10 s, the annealing temperature (primer dependent as given in Table 1) for 6 s and the extension at 72 °C, at which the time is calculated by

the product in bp divided by 25 (Roche) or was run as a three-step PCR following the instructions of the manufacturer (Cambrex).

The melting curve analysis was performed between 65 and 95 °C (0.1 °C/s) to determine the T_m of the amplified

product and to exclude undesired primer dimers. Furthermore, the products were run on a 1% agarose gel to verify the amplified product.

Quantification was adjusted using the house-keeping gene β 2-microglobulin as well as 18 s rRNA. In both instances, comparable results were obtained. To facilitate overall comparison, expression levels of the particular genes were set as 100% for non-GDX control animals (non-GDX CE/J mice and non-GDX $\text{Inh}^{+/+}$ respectively), even though in some cases, the expression levels were not distinct from the value of the water-negative control.

Histology, immunohistochemistry, and in situ hybridization

Adrenal glands and testes were rapidly dissected and placed in 4% PFA for 3 h and Bouin's fixative overnight respectively. Tissues for histochemistry ($n=3$ per group) were dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) following the standard protocols. H&E stained adrenal sections were examined with a standard light microscope using $50\times$ magnification. Areas of the total cortex, the adrenal x-zone, and tumor areas were quantified using the Spot software (Diagnostic Instruments, Sterling Heights, MI, USA) as described elsewhere (Beuschlein *et al.* 2003). In brief, to ensure a reliable comparison between the specimens, three adjacent sections from the middle portion of each individual adrenal were examined. In order to control for the spherical shape of the mouse adrenal gland, the x-zonal area and tumor area were normalized for the total cortical area and were expressed as the x-zone/total cortical area ratio and tumor/total area ratio, respectively.

For proliferative cell nuclear antigen (PCNA) and CYP17 immunohistochemistry, paraffin-embedded sections were rehydrated, blocked with 0.3% H_2O_2 in methanol for 10 min, and incubated with blocking buffer for 15 min. PCNA was immunolocalized overnight at 4°C by means of a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a dilution of 1:100 in blocking buffer containing 3% BSA (Roche), 5% goat serum (Jackson ImmunoResearch Laboratories), and 0.5% Tween 20, whereas CYP17 (courtesy of Dr Anita Payne, Stanford University, Palo Alto, CA, USA) was diluted 1:200 in blocking buffer. After rinsing for 15 min in PBS, secondary antibody (goat antirabbit biotinylated IgG (Vector Laboratories, Burlingame, CA, USA)) was applied for 30 min at room temperature. For the visualization of the bound CYP17 antibody, Vectastain Elite ABC system (Vector Laboratories) and Sigma Fast diaminobenzidine (Sigma) were used. Bound PCNA antibody was detected using the Santa Cruz Immunocruz Kit (Santa Cruz Biotechnology) according to the manufacturer's protocol. Presence of apoptotic cells was assayed using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) detection kit (Promega) as recommended by the manufacturer.

PCNA and TUNEL stained adrenal sections from both groups were examined with a standard light microscope using

$400\times$ magnification. Stained cell nuclei within the zona glomerulosa and outer zona fasciculata of three independent sections from two different animals per group were counted under standardized conditions. Cell counts are expressed as cell number/high power fields (HPF).

To analyze the mRNA expression of CYP17 and LH-receptor (LH-R), the paraffin-embedded sections were subjected to *in situ* hybridization as described previously (Beuschlein *et al.* 2004).

Plasma hormone measurements

Plasma steroid levels (dehydroepiandrosterone (DHEA), and testosterone) were measured using stable isotope dilution/gas chromatography–mass spectrometry as described earlier (Wudy *et al.* 2002).

Statistical analysis

All results are expressed as mean $\pm$ S.E.M. Statistical comparisons were analyzed by ANOVA and Fisher's protective least significant difference test. Statistical significance is defined as $P<0.05$ and is indicated as a star (*) in the figures.

Results

Male CE/J mice do not develop testicular tumors and do not suffer from wasting syndrome

As mice of the inbred CE/J strain have been reported to develop adrenal tumors upon GDX (Woolley & Little 1946b), they share phenotypical similarities with $\text{Inh}^{-/-}$ mice. Another characteristic of $\text{Inh}^{-/-}$ is the spontaneous development of activin-secreting gonadal tumors that result in the early death of the animals due to a wasting-like syndrome (Zajicek *et al.* 1986, Matzuk *et al.* 1994). However, unlike $\text{Inh}^{-/-}$ mice, testicular weight in CE/J mice did not change substantially during lifetime (8 weeks, 112.7 ± 5.0 mg; 14 weeks, 108.3 ± 0.7 mg; 20 weeks, 111.5 ± 5.2 mg; 26 weeks, 126.0 ± 11.0 mg; 38 weeks, 127.3 ± 7.6 mg; 44 weeks, 125.3 ± 3.8 mg; Fig. 1, lower panel) and no histological evidence of testicular tumor growth was detectable in any of the animals (Fig. 1, upper panel). CE/J animals did not suffer from wasting syndrome and premature death (body weight: 8 weeks, 19.3 ± 0.5 g; 14 weeks, 22.7 ± 0.5 g; 20 weeks, 25.0 ± 1.0 g; 26 weeks, 30.0 ± 0.9 g; 38 weeks, 25.6 ± 0.7 g; 44 weeks, 28.3 ± 2.5 g; Fig. 1, lower panel) consistent with the lack of elevated gonadal activin.

Gonadectomy in CE/J mice results in development of sub-capsular tumor nests and delayed regression of the x-zone

Histological examination of adrenals from non-gonadectomized (w/o GDX) male CE/J mice proved the expected x-zone regression after the onset of puberty with a loss of the x-zone

Figure 1 Male mice of the inbred strain CE/J do not develop testicular tumors and do not die from wasting syndrome. (A–C) Hematoxylin and eosin stained testicular sections from 8- to 44-week-old animals demonstrating regular morphology without evidence of tumor growth. (D) Time-course for body weight and testicular weight from male CE/J mice ($n=3-4$) without indication of (tumor-induced) increase of testicular weight. Bars in (A–C) = 20 μ m.

after 8 weeks of age (x-zone area/cortical area: 8 weeks, $21.6 \pm 3.7\%$; 14–44 weeks, $0.0 \pm 0.0\%$; Fig. 2A–E, L). In contrast, GDX was accompanied by a significantly larger x-zone area, compared to non-gonadectomized animals of the same age, that decreased gradually over a longer period of time (8 weeks, $29.3 \pm 0.9\%$, $P=0.011$; 14 weeks, $32.4 \pm 3.7\%$, $P<0.0001$; 20 weeks, $27.4 \pm 1.0\%$, $P<0.0001$; 26 weeks, $16.9 \pm 1.6\%$, $P<0.0001$; 38 weeks, $9.1 \pm 1.3\%$, $P=0.031$; 44 weeks, $6.7 \pm 1.4\%$, $P=0.067$; Fig. 2F–J, L). In addition, x-zone regression in gonadectomized animals was accompanied by the appearance of densely packed cell nests in the sub-capsular adrenal cortex that increased in size over time and eventually developed into a tumor (tumor area/cortical area: 20 weeks, $4.6 \pm 0.6\%$; 26 weeks, $7.0 \pm 0.4\%$; 38 weeks, $19.6 \pm 0.4\%$; 44 weeks, $31.5 \pm 2.1\%$; Fig. 2H–J). These results demonstrate that GDX-induced adrenocortical tumors in CE/J mice are situated within the sub-capsular zone, unlike adrenal tumors in $Inh^{-/-}$ mice, which are situated within or contiguous to the x-zone. Overall, GDX resulted in a small, albeit significant higher adrenal weight at each time point (8 weeks, 5.8 ± 0.4 mg, $P=0.02$; 14 weeks, 6.6 ± 0.3 mg, $P=0.0008$; 20 weeks, 6.4 ± 0.0 mg, $P=0.009$; 26 weeks, 6.7 ± 0.7 mg, $P=0.003$; 38 weeks, 8.5 ± 0.6 mg, $P<0.0001$; 44 weeks, 8.8 ± 0.5 mg, $P<0.0001$) as compared to non-gonadectomized animals (8 weeks, 4.4 ± 0.5 mg; 14 weeks, 4.6 ± 0.4 mg; 20 weeks, 4.8 ± 0.2 mg; 26 weeks, 4.2 ± 0.2 mg; 38 weeks, 4.7 ± 0.1 mg; 44 weeks, 4.8 ± 0.2 mg; Fig. 2K). Moreover, tumor - development in gonadectomized mice was accompanied by a significant increase in adrenal weight in comparison to earlier time points (adrenal weight 8 weeks, 5.8 ± 0.4 mg vs 38 weeks,

8.5 ± 0.6 mg, $P=0.0003$ and 44 weeks, 8.8 ± 0.5 mg, $P<0.0001$ respectively).

Gonadectomy in CE/J mice is followed by an increase in sub-capsular adrenocortical proliferation and lack of adrenocortical apoptosis

To further define the underlying growth dysregulation in the adrenal cortices of gonadectomized CE/J mice and in an attempt to delineate the cellular origin of the developing adrenal tumors, we determined the number and distribution of proliferating and apoptotic cells in adrenals from non-gonadectomized (w/o GDX) and gonadectomized (GDX) CE/J mice. In accordance with the observed tumor development in the sub-capsular zone, PCNA-positive cells were mainly restricted to the sub-capsular cells (Fig. 3C and D) with a significant higher number of proliferating cells (20 weeks, 200.3 ± 16.6 ; 38 weeks, 810.0 ± 72.7) in comparison to non-gonadectomized animals (20 weeks, 41.7 ± 4.6 , $P=0.0008$; 38 weeks, 56.0 ± 11.2 , $P<0.0001$; Fig. 3A and B). In contrast, the number of apoptotic cells per high power field was not different after GDX (20 weeks, 10.0 ± 1.5 ; 38 weeks, 8.7 ± 7.2 ; Fig. 3G and H) as compared to non-gonadectomized animals at these time points (20 weeks, 7.7 ± 2.7 , $P=0.74$; 38 weeks, 3.3 ± 0.7 , $P=0.47$; Fig. 3E and F). Intriguingly, the tumor cell population which is characterized by high proliferation showed virtually no apoptotic cells (Fig. 3H, insert). Taken together, these results indicate that GDX is followed by high proliferation of the sub-capsular cell population, which results in the unopposed growth of tumor cells.

Adrenal tumors in gonadectomized CE/J and *Inh*^{-/-} mice express gonadal marker genes and secrete sex steroids

To further define the functional properties of adrenal tumors in gonadectomized CE/J mice, we determined the expression pattern of a variety of marker genes. Interestingly, adrenal tumors in CE/J mice express a distinct pattern of steroidogenic enzymes, including 17 α -hydroxylase (P450c17; non-GDX $100.0 \pm 7.1\%$ vs GDX $3756.3 \pm 75.4\%$, $P < 0.0001$) and aromatase (P450c19; non-GDX $100.0 \pm 31.7\%$ vs GDX $1800.0 \pm 200.0\%$, $P = 0.001$), peptide hormone such as Mullerian-inhibiting substances (MIS; $P = 0.0012$), and hormone receptors including LH-R ($P = 0.0008$) and MIS receptor ($P < 0.0001$) that otherwise define gonadal endocrine function. In addition, transcription factors such as GATA-4 ($P = 0.0005$), Wilms tumor gene-1 (WT-1; $P < 0.0001$), and steroidogenic factor-1 (SF-1; $P = 0.0029$) were significantly up-regulated after GDX, while other transcription factors such as FOG-1 ($P < 0.0001$) and GATA-6 ($P = 0.0004$) were down-regulated. In contrast, Sox-8 and DAX-1 did not significantly different between the groups (Fig. 4A and B).

As demonstrated in Fig. 4C, a similar expression profile was present in adrenal tumors from gonadectomized *Inh*^{-/-} animals with significant increase of GATA-4, MIS receptor, CYP17, and CYP19. In contrast, no significant changes in the expression pattern of these genes were evident in gonadectomized wild-type controls, indicating that in addition to GDX, transcriptional activation of these genes is dependent on adrenal tumorigenesis.

To localize some of the up-regulated genes, *in situ* hybridization and immunohistochemistry were performed, which demonstrated expression of P450c17 and LH-R expression restricted to the tumor cells (Fig. 5C and I) and lack of expression in adjacent areas of morphologically normal adrenal cortex (Fig. 5B and H). To investigate the functional significance of the expression pattern of steroidogenic enzymes, hormonal profiles of gonadectomized CE/J mice were determined. The detected increase of the adrenal androgen DHEA (Fig. 5J) as well as testosterone (Fig. 5K) during the time-course experiment in gonadectomized CE/J mice is in line with the presence of functional active 17 α -hydroxylase/17,20 lyase activity in adrenal tumors.

Figure 2 Time-course for adrenal morphology, weight, and zonal distribution in non-gonadectomized (w/o GDX; A–E) and gonadectomized (GDX; F–J) CE/J mice at 8 weeks (A, F), 14 weeks (B, G), 20 weeks (C, H), 26 weeks (D, I), and 44 weeks (E, J) of age. Whereas the x-zone regresses in non-gonadectomized mice within the first 14 weeks of age (A, B, L), it is retained in gonadectomized animals for a longer period of time before it gradually regresses (F–I, L). In gonadectomized mice, dense, sub-capsular tumor nests appear (H), which eventually develop into adrenocortical tumors (I, J). Bars in (A–J) = 100 μ m. D, definitive zone; X, x-zone; M, medulla; T, tumor. Stars in (L) denote significant differences in x-zone area between gonadectomized and non-gonadectomized animals from the same age group.

Figure 3 Distribution of proliferating (PCNA immunohistochemistry; A–D) and apoptotic (TUNEL staining; E–H) cells in adrenals from non-gonadectomized (A, B, E, F) and gonadectomized (C, D, G, H) CE/J mice at different time points. Gonadectomy is followed by an increase in the number of PCNA, mainly found in the sub-capsular tumor cell nests (C, D). In contrast, while the number of TUNEL-positive cells after GDX (G, H) is comparable with non-gonadectomized mice (E, F), the tumor cell population, which is characterized by high proliferation, shows no apoptotic cells (magnified tumor area in H insert). Bars in (A–H) = 100 μ m; for high magnification inserts = 50 μ m; D, definitive zone; X, x-zone; M, medulla; T, tumor.

Taken together, these data demonstrate the expression of distinct transcription factors, receptors, and steroidogenic enzymes otherwise expressed in gonadal endocrine cells suggesting a differentiation defect as part of the adrenal phenotype in gonadectomized CE/J mice.

Discussion

The overall similar adrenal phenotype of *Inh*^{−/−} mice and CE/J mice—induction of adrenal tumorigenesis by GDX—prompted us to compare morphological and molecular alterations initiated by surgical GDX in adrenal glands in these two animal models. As we demonstrate, GDX in CE/J mice is accompanied by development of sub-capsular tumor nests that are characterized by a high proliferation index as measured by PCNA staining. These findings provide evidence that in contrast to *Inh*^{−/−} mice, which are characterized by tumor growth within or contiguous to the adrenal x-zone (Beuschlein *et al.* 2003), adrenal tumors in CE/J mice derive from the sub-capsular zone of the adrenal cortex similar to other susceptible strains of mice (Bielinska *et al.* 2003, 2005). The shared gonadal-like phenotype of the adrenal tumors in both strains, and the clear involvement of the x-zone in *Inh*^{−/−} mice indicate that both tumors arise from defects in the differentiation and maturation of x-zone cells. However, the results from our present study suggest that adrenal tumorigenesis in CE/J mice reflects dysregulation at an earlier stage that also includes a migration defect of cells descent to be x-zone cells. Indeed, an increasing body of evidence indicates that a common pool of stem cells that reside in this sub-capsular cellular compartment is likely

to play a key role for adrenal physiology as distributors of all adrenocortical cells that migrate centripetally upon differentiation (Zajicek *et al.* 1986, Kataoka *et al.* 1996). Thus, adrenal tumorigenesis in CE/J mice might be of particular interest as a model of adrenal stem cell dysregulation.

The physiologic role of the murine adrenal x-zone, which becomes evident histologically at 10–14 days of age and subsequently begins to degenerate in males, coinciding with sexual maturity (Howard-Miller 1928), is not defined. In accordance with earlier findings demonstrating prevention of x-zone degeneration in male mice by castration (Howard-Miller 1928, Beuschlein *et al.* 2003), GDX delays x-zone degeneration and induces adrenal tumor formation in both strains of mice. In addition, at later time points, when adrenal tumors develop, the x-zone cross-sectional area gradually decreases, indicating that the development of adrenal tumors might directly or indirectly affect x-zone growth. Indeed, in *Inh*^{−/−} mice, the pathological regression of the x-zone is induced by high levels of tumoral activin that result in selective apoptosis of x-zone due to their unique expression of activin receptors and the downstream effector Smad-2 (Beuschlein *et al.* 2003). In contrast, adrenal tumors in CE/J mice do not display elevated activin mRNA levels (data not shown), indicating that in this mouse, model high levels of activin are not required for the observed tumor-induced x-zone regression. In fact, the role of activin for physiological x-zone regression during puberty in male mice and during first pregnancy in female animals remains unproven. Other potential candidates involved in this process include sex steroids and gonadotropins (Howard-Miller 1928). Since the murine adrenal cortex is deprived of a functional equivalent of the zona reticularis and lacks expression of P450c17, the mouse

adrenal does not secrete adrenal androgens. However, adrenal tumors in CE/J mice not only express steroid enzymes required for sex-steroid production, but also adrenal tumor development is accompanied by increasing levels of serum DHEA and testosterone. Thus, sex steroids secreted by the adrenal tumors or secondary suppression of gonadotropins could be responsible for x-zone regression in these animals.

During development, numerous transcriptional cascades are utilized to ensure tight control over cellular proliferation and proper spatio-temporal expression of target genes. A gonadal specific target gene which is expressed in adrenal tumors of CE/J mice is the Mullerian-inhibiting substance (MIS), a glycoprotein dimer that—like inhibin and activin—belongs to the TGF- β super-family (Lee & Donahoe 1993). Secretion of MIS by fetal sertoli cells is essential for normal male sex differentiation, since it induces regression of the Mullerian ducts in the developing male embryo. The specific expression in sertoli and granulosa cells has provided even diagnostic usefulness of the MIS gene for sub-classification of gonadal tumors (Rey *et al.* 2000). The gonadal restricted expression of the MIS gene requires a specific combination of transcription factors, including the zinc finger factor GATA-4 and the nuclear receptor SF-1, as well as Sox-9 and WT-1 (De Santa Barbara *et al.* 1998, Nachtigal *et al.* 1998, Viger *et al.* 1998).

WT-1 is a zinc finger-containing transcription factor, which has been implicated in the development of the indifferent gonad prior to sexual differentiation as well as in the etiology of certain neoplasia. Interestingly, GATA-4 together with WT-1 is up-regulated in adrenal tumors from gonadectomized CE/J mice. Thus, adrenal tumors in gonadectomized CE/J mice resemble the expression pattern of transcription factors required for the expression of MIS in sertoli cells. *In vivo* and *in vitro* data suggest a pathway in which the products of the WT-1 and Lhx-9 genes activate expression of SF-1 and thus mediate early gonadogenesis (Wilhelm & Englert 2002). As SF-1 levels are slightly up-regulated in adrenals from gonadectomized CE/J mice, these data are in line with the concept of transcriptional activation of the SF-1 promoter by WT-1 as part of the dedifferentiation in these adrenal tumors.

The friend of GATA proteins (FOG: FOG-1 and FOG-2) can act as either enhancers or repressors of GATA transcriptional activity, depending on the cell and promoter context. It has been reported that the FOG proteins are co-expressed with GATA factors in testicular cells in which they differentially repress the promoter activities of several GATA-dependent target genes (Robert *et al.* 2002).

Intriguingly, FOG-1 expression is down-regulated in adrenal tumors from gonadectomized CE/J mice, in line with the concept of loss of repression of GATA-dependent transcriptional activation of target genes such as LH-R, CYP17, and CYP19 (Bielinska *et al.* 2003, 2005).

Although CE/J mice carry a polymorphism in the gene encoding SF-1 (SF-1^{S172}, data not shown), which has been associated with lower steroidogenic capacity and possibly higher susceptibility of GDX-induced adrenal tumorigenesis (Bielinska *et al.* 2003), it remains unclear from the data presented herein, which steps are the initial dysregulated events that induce adrenal tumor formation in these animals. GDX induces both a decrease of gonadal hormones (including inhibin and sex steroids) and an increase of gonadotropins (including LH). Chronically elevated levels of LH have been demonstrated to induce adrenal LH-R expression (Kero *et al.* 2000, Beuschlein *et al.* 2003) and boost adrenal tumor growth (Beuschlein *et al.* 2003, Mikola *et al.* 2003), depending on the strain background or targeted genetic alterations. As we could demonstrate, activin, which is secreted from gonadal tumors in *Inh*^{-/-} mice, leads to the induction of x-zone apoptosis, thus preventing the growth of x-zone derived adrenal tumors (Beuschlein *et al.* 2003). Since CE/J animals are not prone to spontaneous gonadal tumor growth, activin as a repressor of adrenal tumor growth is not likely to play a similar key role in the pathophysiology of CE/J mice. However, high levels of LH might be sufficient to cause the adrenal phenotype in CE/J mice, as seen after GDX, a hypothesis which will be tested by introducing transgenic LH overexpression on the CE/J background.

In conclusion, the dysregulation of adrenal growth in gonadectomized CE/J, DBA/2J (Bielinska *et al.* 2003), nude mice (Bielinska *et al.* 2005), and *Inh*^{-/-} mice (Beuschlein *et al.* 2003) is accompanied by a differentiation defect that drives adrenocortical cell differentiation towards a gonadal phenotype. The LH dependency of adrenal tumorigenesis in these tumor models is reminiscent of the clinical situation in patients with chronically elevated levels of proopiomelanocortin peptides due to steroid hydroxylase deficiencies, who develop adrenal rest tumors in the gonads (Stikkelbroeck *et al.* 2001). It is tempting to speculate that the ability of the adrenal stem cell population to mimic gonadal endocrine function might reflect the common embryological origin of adrenocortical and gonadal cells from adjacent areas of the urogenital ridge during early development (Smith & Mackay 1991). In fact, expression of P450c17 has been demonstrated in a

Figure 4 (A) Semi-quantitative RT-PCR panel of adrenals from gonadectomized (GDX) and non-gonadectomized (w/o GDX) CE/J mice. (B) Expression levels by means of real-time PCR analysis for CE/J mice with non-gonadectomized mice set as 100%. In accordance with the semi-quantitative RT-PCR analysis, a significant increase in the expression of transcription factors such as SF-1, WT-1, and GATA-4 is evident, whereas GATA-6 and FOG-1 expression is down-regulated in adrenals from gonadectomized animals. In addition, Mullerian-inhibiting substance (MIS) and MIS receptor (MIS-R) as well as LH-R and steroidogenic enzymes such as 17 α -hydroxylase (CYP17) and aromatase (CYP19) are significantly up-regulated. (C) Real-time PCR analysis from gonadectomized and non-gonadectomized *Inh*^{-/-} and wild-type controls (with non-gonadectomized wild-type animals set as 100%) demonstrate similar expressional changes for GATA-4, MIS-R, CYP17, and CYP19 only in adrenal tumors from gonadectomized *Inh*^{-/-} animals, whereas gonadectomy in wild-type animals does not significantly affect transcriptional levels. Stars denote significant differences in expression levels between GDX and non-GDX animals, whereas a cross indicates significant differences between gonadectomized inhibin knockout mice (*Inh*^{-/-}) and wild-type controls (WT).

Figure 5 *In situ* hybridization (A–C) and immunohistochemistry (D–F) for 17 α -hydroxylase (CYP17) and *in situ* hybridization for LH-R (G–I) in adrenals from gonadectomized CE/J mice at 8, 38, and 50 weeks of age, demonstrating staining specifically in tumorous areas. High magnification of immunohistochemical staining (D–F) and control reactions for *in situ* hybridization with sense probes (A–C, G–I) are shown as inserts. Bars including low magnification inserts = 100 μ m; bars in high magnification inserts (D–F) = 20 μ m; D, definitive zone; X, x-zone; M, medulla; T, tumor. Time-course of steroid profile in gonadectomized CE/J mice suggesting functional significance of up-regulated expression of CYP17 in adrenal tumors with increase of the adrenal androgen DHEA (J) as well as testosterone (K).

specific spatio-temporal pattern in a distinct cell population of the developing mouse adrenal (Keeney *et al.* 1995), highlighting the concept of closely related progenitor cells in adrenal and gonadal development.

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