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Genetics

Development of cancer cachexia-like syndrome and adrenal tumors in inhibin-deficient mice

(tumor suppressor/activins/hepatocellular necrosis)

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ABSTRACT **Activins and inhibins, members of the type β transforming growth factor superfamily of growth regulatory proteins, are produced in multiple tissues and affect diverse physiologic processes. Using embryonic stem cell technology, we previously demonstrated that inhibin can function as a gonadal tumor suppressor. In this study, we show that development of gonadal tumors is rapidly followed by a cancer cachexia-like wasting syndrome. Cachectic inhibin-deficient mice develop hepatocellular necrosis around the central vein and parietal cell depletion and mucosal atrophy in the glandular stomach, are anemic, and demonstrate severe weight loss. The liver pathology is consistent with studies demonstrating an effect of elevated activins on rat hepatocytes. In inhibin-deficient mice with tumors, activins are >10-fold elevated in the serum and are likely causing some of the cachexia symptoms. In contrast, inhibin-deficient mice gonadectomized at an early age do not develop this wasting syndrome. However, these gonadectomized, inhibin-deficient mice eventually develop adrenal cortical sex steroidogenic tumors with nearly 100% penetrance, demonstrating that inhibin is also a tumor suppressor for the adrenal gland.**

The inhibins (α:β heterodimers) and activins (β:β dimers) are related proteins that share common β subunits (either βA or βB subunits) (1). These proteins, members of a large family of structurally related growth regulatory proteins, which includes the type β transforming growth factors (TGF-β), bone morphogenetic proteins, and Mullerian inhibiting substance (MIS) (1–4), have been shown to have diverse functions in a variety of assay systems including antiproliferative effects on carcinoma cell lines [i.e., MIS and TGF-β (2, 3)].

The activins and inhibins also have diverse functions in mammalian physiology and development. Activin and inhibin subunit mRNAs and proteins are synthesized in a variety of cell types and embryonic and adult tissues in different species (1, 5–10). In the adult mammal, although activins were initially discovered for their ability to stimulate pituitary follicle-stimulating hormone secretion, they have also been shown to influence other functions including liver metabolism and glucose regulation. For example, activin A can stimulate glycogenolysis (11) and inhibit DNA synthesis in isolated rat hepatocytes *in vitro* (12). In addition, infusion of activin A into rats caused hepatocellular necrosis around the central vein of the liver (13). These physiologic effects of activin on hepatocytes are consistent with findings that a major site of ^{125}I-labeled activin A binding is the rat liver (14) and that the type II activin receptor is expressed in the mouse liver (ref. 15; R. Towns and M.M.M., unpublished data).

Using embryonic stem cell technology, we have generated inhibin-deficient mice (16), which develop sex cord stromal tumors at an early age with nearly 100% penetrance, demonstrating that inhibin functions *in vivo* as a tumor suppressor in the gonads of mice (16). In the present study, we show that a severe wasting syndrome, which mimics the human cancer cachexia syndrome (17), accompanies the development of gonadal sex cord stromal tumors in inhibin-deficient mice. Furthermore, we demonstrate that inhibin also functions as a tumor suppressor in the adrenal cortex.

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MATERIALS AND METHODS

Mice and Histologic Analysis. Generation of the inhibin-deficient mice has been described (16). Inhibin-deficient and littermate control mice were weighed weekly on the same day. Tissues were processed and analyzed as described (16, 18).

Blood Analysis. For hematocrit analysis, periorbital blood from anesthetized mice was withdrawn into heparinized microhematocrit capillary tubes, spun for at least 5 min in a microhematocrit centrifuge, and read on a microcapillary reader. For the cytokine and growth factor (activin) assays, whole blood from anesthetized mice was isolated by cardiac puncture and allowed to clot in Microtainer serum separator tubes (Becton Dickinson) before centrifugation and separation of the serum. Serum was frozen at −20°C before analysis. Serum activin A and B levels were determined by an ELISA method as described (19).

RESULTS

Inhibin-Deficient Mice Develop a Severe Wasting Syndrome. The first overt sign of ovarian and testicular tumor development in inhibin-deficient (homozygote) mice is severe weight loss (Fig. 1). Initially, the growth curves of homozygote and heterozygote mice are indistinguishable from wild-type mice (Fig. 1*A*; data not shown). However, after 6–7 weeks of age, both male (Fig. 1*A*) and female (data not shown) inhibin-deficient mice begin to lose weight and eventually die. These inhibin-deficient mice develop a hunchback and sunken-eye appearance. They have a pale periphery and exhibit severe thoracic kyphoscoliosis as the cachexia progresses. This severe wasting syndrome is secondary to the gonadal tumor formation because inhibin-deficient male and female mice gonadectomized before they are 6 weeks old continue to gain weight, although at later time points they weigh less than controls (Fig. 1*B*; data not shown).

To determine the cause of this weight loss, biochemical, histological, and morphological analyses were performed on the cachectic inhibin-deficient mice, which revealed several major abnormalities (Fig. 2). The livers of the cachectic mice were uniformly micronodular with diffuse, confluent hepatocellular necrosis around the central vein (zone 3 necrosis) and showed foci of chronic lymphocytic inflammation (Fig. 2*B*). Furthermore, the hepatocytes and the nuclei of the cachectic inhibin-deficient mice (Fig. 2*B*) appeared larger compared to the noncachectic control mice (Fig. 2*A*). Con-

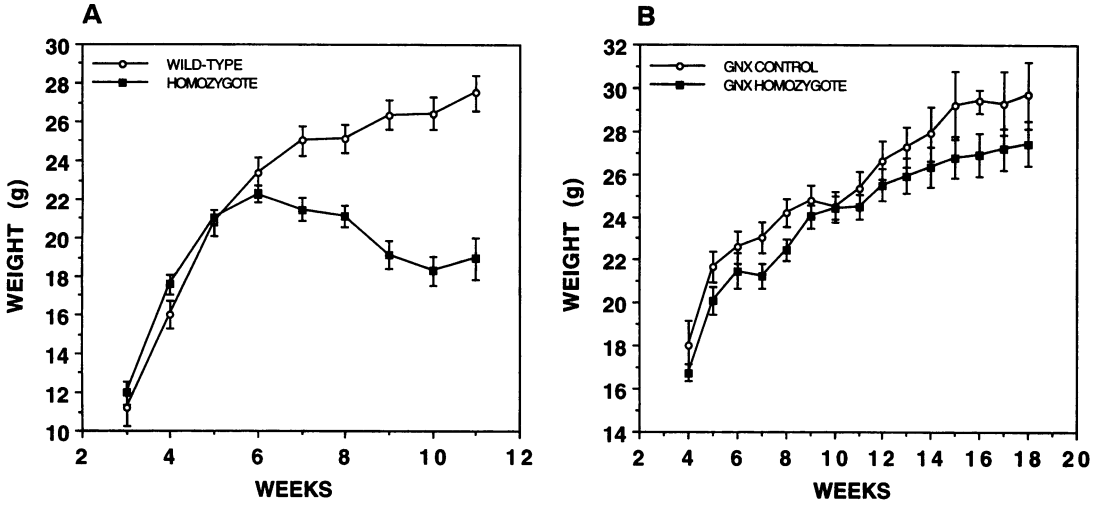

FIG. 1. Weights of intact (*A*) and gonadectomized (GNX) (*B*) male mice. Mice in *B* were gonadectomized when they were ≤6 weeks old. Of the homozygote intact male and female (data not shown) mice, >95% succumb to the wasting syndrome by 12 and 17 weeks, respectively. Since these mice die or were sacrificed secondary to the wasting syndrome, fewer mice are included at the later time points. Control mice in *B* were both wild type and heterozygotes. Numbers of mice used for this study were as follows: intact wild type, $n = 22$; intact homozygote, $n = 38$; GNX control and GNX homozygote, $n = 10$. Values at each point are means ± SEM.

sistent with the hepatic damage, pronounced elevations in the serum levels of several liver enzyme markers (i.e., aspartate and alanine aminotransferases and alkaline phosphatase) were noted (data not shown). The glandular stomach from the cachectic inhibin-deficient mice displayed mucosal atrophy with parietal cell depletion (Fig. 2*D*). These findings contrast with the normal liver and glandular stomach of gonadectomized inhibin-deficient mice at similar ages (Fig. 2 *A* and *C*) or wild-type or heterozygote control mice (data not shown).

The hematocrits of male inhibin-deficient mice 4–6 weeks old (44.4% ± 2.4%; $n = 7$) were indistinguishable from age-matched control mice (44.0% ± 2.4%; $n = 11$). However, as the inhibin-deficient male mice became cachectic (7–12 weeks old), they also became anemic (28.8% ± 2.6%; $n = 7$) compared to controls (46.7% ± 1.4%; $n = 5$). Gonadectomy of inhibin-deficient males before 6 weeks of age prevents this anemia and proves that the anemia is related to the development of the tumors. Similar findings are seen in the inhibin-deficient females (data not shown). Complete blood cell analysis in a limited number of mice revealed that these cachectic mice also have reduced leukocyte and platelet numbers (data not shown). This suggests that these cachectic mice have pancytopenia, which is contributing to the wasting syndrome.

Activin Levels Are Elevated in Cachectic Inhibin-Deficient Mice. Cachexia can be induced in animals by production or injection of a number of cytokines (17, 20–22). The cachexia and histologic findings associated with the relatively small and confined sex cord stromal tumors in these inhibin-deficient mice contrast with the minimal systemic effects of the tumors in the tumor-prone p53-deficient mouse model or mice with other gonadal tumors (18, 23, 24). We hypothesized therefore that the sex cord stromal tumors in these inhibin-deficient mice might be producing one or more cytokines, which were causing these pronounced endocrine effects. Since the tumors are derived from cells of the granulosa/Sertoli cell lineage, which synthesize activins, and the liver pathology in the inhibin-deficient mice is similar to the effects of treatment with recombinant activin A (13), we speculated that elevated activins were causal. By using an ELISA (19), the levels of activin A (βA:βA homodimer) in the serum of these mice were found to be elevated 13-fold (males) and 20-fold (females) (Table 1). Similar elevations of serum activin B (βB:βB homodimers) were also observed (Table 1). Consistent with these findings, we have observed elevated activin βA subunit mRNA in tumor tissue (V. Trudeau and M.M.M., unpublished data), and tumor cell lines derived from these mice also produce high levels of activins (25). The serum levels of other proteins (tumor necrosis factor α, interferon γ, and interleukin α), known to be produced in the gonads and which can cause cachexia (20–22), were found not to be elevated (data not shown). Thus, these results suggest that the cachexia and at least the liver pathology in the inhibin-deficient mice may be directly or indirectly due to secretion of activins from the gonadal tumors. This does not rule out the involvement of other cytokines in these processes.

Gonadectomized Inhibin-Deficient Mice Develop Adrenal Tumors. The primary gonadal sex cord stromal tumors developed rapidly and were invariably lethal with >95% penetrance in inhibin-deficient male and female mice by 12 and 17 weeks, respectively (Fig. 1). To address the potential tumor suppressor functions of inhibin in other organs, we gonadectomized the inhibin-deficient mice. These gonadectomized inhibin-deficient mice also eventually became cachectic, had elevated serum activin A and B levels, and displayed identical histological changes in the livers and stomachs compared with inhibin-deficient mice developing gonadal sex cord stromal tumors (Fig. 2; data not shown).

When cachectic, gonadectomized, inhibin-deficient mice were examined, they invariably had adrenal tumors. To date, 99% of gonadectomized inhibin-deficient [male (41/42) and female (25/25)] mice have developed adrenal tumors (56 unilateral, 10 bilateral) and the median ages of death were 33 and 36 weeks for females and males, respectively. The earliest tumors were observed at 21 weeks of age. In contrast, only 1 heterozygote female mouse of 77 gonadectomized control heterozygote and wild-type mice (38 males and 39 females) developed an adrenal tumor over the same time period.

Histological and ultrastructural examination of these adrenal tumors revealed that they were derived from the adrenal cortex. The tumors completely altered the architecture of the gland (Fig. 3 *A–C*). Some of the cells in each tumor were well-differentiated and had steroidogenic features by light and electron microscopy consistent with a cortical origin (data not shown). Elevated serum estradiol levels in 5 of 9 female and in 3 of 6 male gonadectomized inhibin-deficient mice with adrenal tumors (data not shown) supported the pathologic findings and suggested that these tumors were

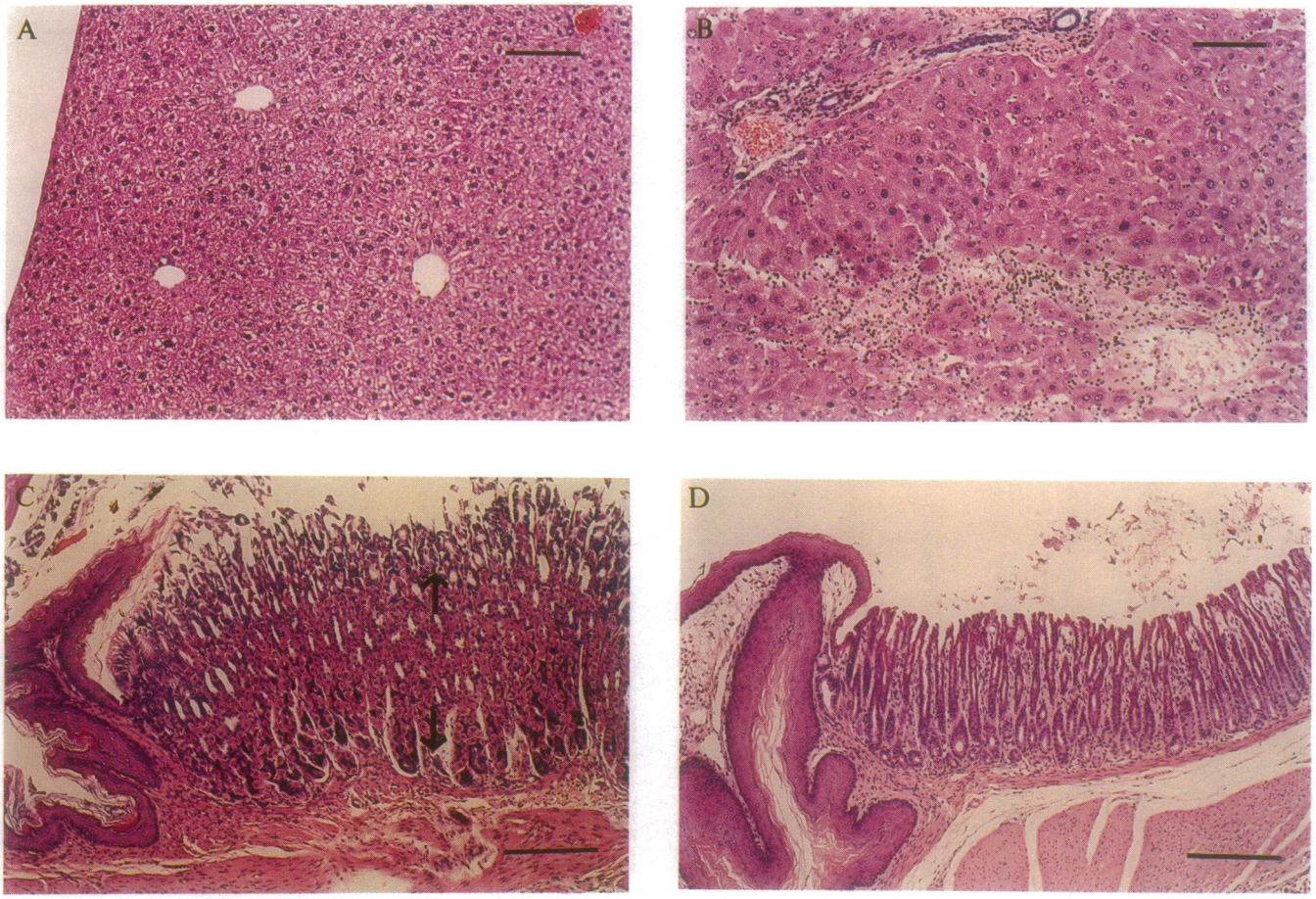

FIG. 2. Histology of livers and stomachs from inhibin-deficient mice. (*A*) Normal liver of a 15-week-old inhibin-deficient male mouse gonadectomized at 7 weeks. Portal tract is to the left and two central veins are above and to the right. Hepatocytes are uniform and contain plentiful glycogen. Liver architecture and cytology are indistinguishable from a normal mouse of the same age. (Bar = 10 μm.) (*B*) Liver from a 9-week-old inhibin-deficient male mouse with a large testicular sex cord stromal tumor. Hepatocytes around the portal tract (top) are essentially normal. In contrast, there is necrosis and chronic lymphocytic inflammation along the course of the central vein (bottom). In addition, there is a decrease in glycogen content of all hepatocytes. All hepatocytes and hepatocyte nuclei appear to be larger than the control (see *A*). The increase in size of these hepatocytes may be secondary to high levels of activins and/or the loss of many hepatocytes. (Bar = 10 μm.) (*C*) Glandular stomach of the 15-week-old inhibin-deficient male mouse in *A* gonadectomized at 7 weeks. Stomach is normal with >70% of the glandular epithelial lining cells being large, eosinophilic parietal cells (layer of cells between arrows). For orientation and comparison to *D*, the area photographed shows the junction between the squamous epithelial-lined forestomach (left) and the glandular region (right) and was photographed at the same magnification as *D*. (Bar = 20 μm.) (*D*) Glandular stomach from a 33-week-old cachectic gonadectomized female that had developed bilateral adrenal tumors. There is mucosal atrophy and total absence of parietal cells. Identical findings are seen in cachectic inhibin-deficient mice with gonadal tumors (data not shown). (Bar = 20 μm.)

derived from sex steroidogenic cells in the zona fasciculata and zona reticularis of the adrenal cortex (26). In contrast, serum cortisol levels were not elevated in these mice (data not shown). In addition, these tumors were malignant in 4 mice as demonstrated by nests of malignant cells that invaded the liver (data not shown) and lung (Fig. 3*D*) and resulted in a more rapid death of these mice.

Table 1. Serum activin levels (mean ± SD)

	WT or +/− (*n*)	Homozygote (*n*)
Activin A		
Females		
4–6 weeks	NE (5)	NE (5)
10–20 weeks	6.9 ± 1.3 (5)	137.0 ± 78.0 (10)
Males		
4–6 weeks	17.0 ± 1.2 (5)	20.0 ± 0.5 (5)
7–12 weeks	9.4 ± 1.0 (5)	122.4 ± 61.1 (5)
Activin B		
Females		
4–6 weeks	NE (5)	15.3 ± 23.2 (4)
10–20 weeks	NE (5)	123.2 ± 73.2 (9)
Males		
4–6 weeks	NE (5)	1.4 ± 1.4 (5)
7–12 weeks	NE (5)	99.5 ± 82.1 (8)

WT, wild type; +/−, heterozygote; NE, serum activin levels not elevated above the blank control.

DISCUSSION

We have previously shown that inhibin-deficient male and female mice develop sex cord stromal tumors at an early age, which are relatively small and confined to the gonads (16). However, as the tumors developed, the mice demonstrated severe weight loss, appeared cachectic, and eventually died. Gonadectomy prevents the death of these mice from the gonadal tumor-induced cachexia. However, these mice ultimately succumb to a similar wasting syndrome secondary to the development of adrenal cortical tumors, which occur at a high penetrance similar to the gonadal tumors. Thus, inhibin can also function as a tumor suppressor in the adrenal cortex. Since previous studies have demonstrated that inhibin is produced in and can bind to the adrenal gland (14, 27–29), our studies suggest an important local and/or endo-

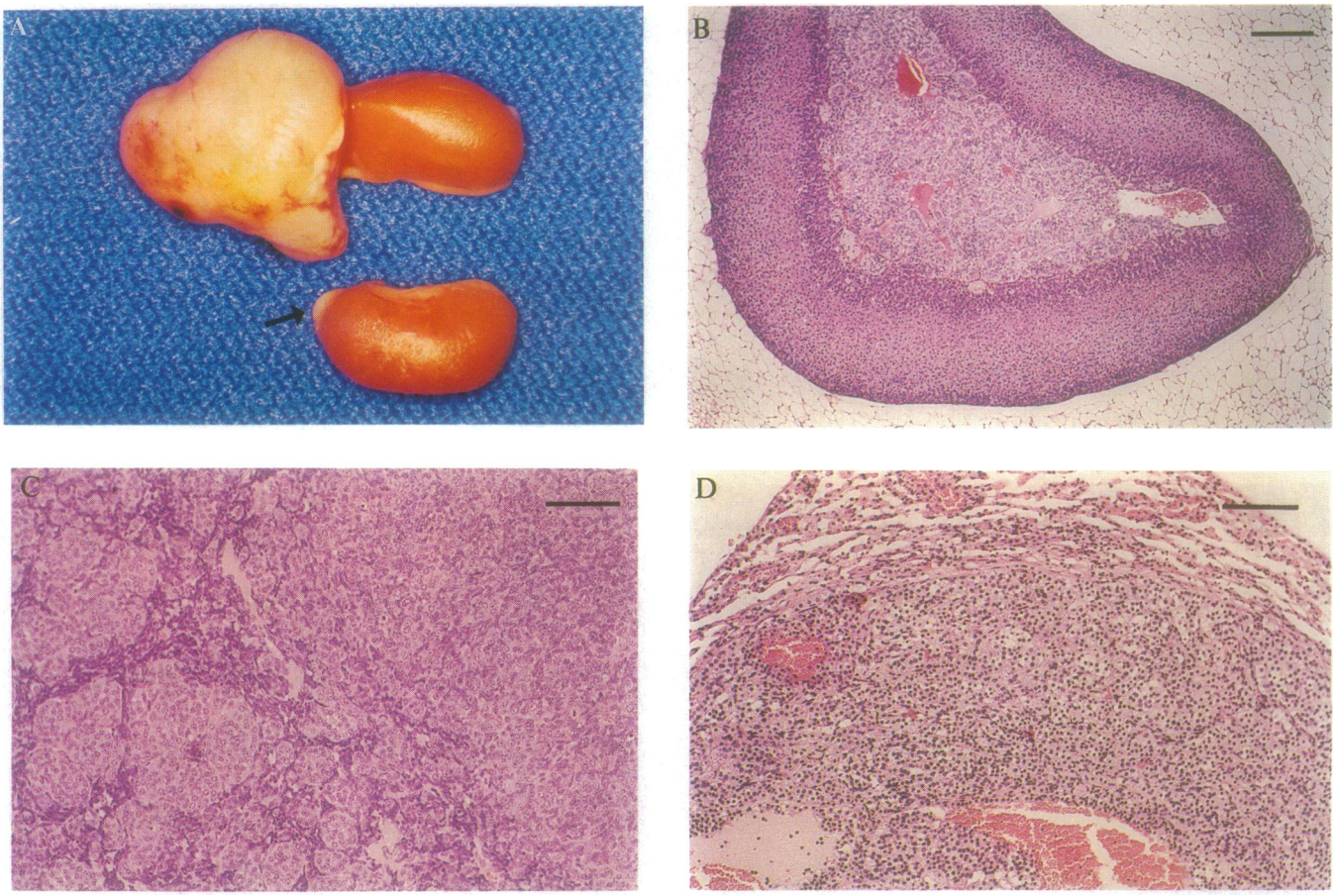

FIG. 3. Histological analysis of primary and metastatic adrenal tumors. (*A*) Adrenal carcinoma from a 41-week-old inhibin-deficient male mouse gonadectomized at 6 weeks (top). Normal adrenal gland is visible at the upper pole of the right kidney (bottom, arrow). The solid tumor (top) is encapsulated, focally hemorrhagic, and necrotic. Most of the tumor is soft, light ivory-yellow, and well-vascularized. (*B*) Histology of a normal adrenal gland from an 18-week-old inhibin-deficient male mouse gonadectomized at 9 weeks. Cells in the cortex (outer layer) are very eosinophilic and arranged in defined zones compared to the medulla (inner layer). Normal mouse adrenal gland is ≈2 mm in diameter. (Bar = 20 μm.) (*C*) Histology of an adrenal tumor (1 × 1.6 cm) derived from a 41-week-old inhibin-deficient male mouse gonadectomized at 6 weeks. Most of the tumor is composed of solid nests of polygonal cells with uniform, central round nuclei and modest amounts of eosinophilic cytoplasm. There are scattered mitoses. Another minority population of small cells of uncertain origin can be seen between the nests of endocrine cells. Elsewhere, many tumors (including other regions of this tumor) had foci of cystic degeneration, which created a pseudotubular appearance. (Bar = 10 μm.) (*D*) Metastatic adrenal carcinoma in the lung of a 15-month-old inhibin-deficient female mouse gonadectomized at 5 weeks. These cells are relatively uniform and polygonal with eosinophilic cytoplasm consistent with adrenal cortical origin and are identical to the primary adrenal tumor in this case (data not shown). There was no primary tumor in the ovaries that were removed from this mouse. (Bar = 10 μm.)

crine antiproliferative role for inhibin on sex steroidogenic cells in the adrenal cortex.

The differential temporal sensitivity of the adrenal and gonadal cells with respect to tumorigenesis is interesting. This might reflect other polypeptide hormones such as follicle-stimulating hormone or activin, which are upregulated in the absence of inhibin and may exert positive growth effects on only the gonadal cells (16, 30). Increased follicle-stimulating hormone or activins probably have little effect on the adrenal where follicle-stimulating hormone or activin receptors are absent (14). It is also unclear why tumor development is limited to only the gonads and adrenals, while other inhibin-responsive tissues, such as the pituitary, remain intact.

Inhibin-deficient mice with gonadal and adrenal tumors develop a severe wasting syndrome that mimics human cancer cachexia syndrome and cytokine-induced cachexia in animals. At very early time points, inhibin-deficient mice develop liver pathology that mirrors the known effects of exogenous activins on the liver (13), suggesting that the elevated serum activins, secreted from the tumors, may be causing these aberrations. It is unclear whether the parietal cell depletion and mucosal atrophy are a direct result of the elevated activins or are secondary to the changes in the liver. However, we have recently demonstrated the presence of activin type II receptor mRNA in the glandular stomach (R. Towns and M.M.M., unpublished data). It is also unclear what is causing the weight loss and anemia. Intrinsic factor, a parietal cell product, is required for vitamin B_{12} absorption (31), and iron absorption requires an acid environment produced by the parietal cells (32). Analysis of the erythrocyte profile in a limited number of mice demonstrates that the anemia in these cachectic mice may be secondary to a combined vitamin B_{12} and iron deficiency (data not shown). Thus, it appears that the stomach and liver pathology may be causing nutritional deficiencies, which are indirectly causing the severe weight loss and anemia. A way to further test these hypotheses would be to generate compound homozygote mice deficient in both inhibins and type II activin receptors; if activins are causing their effects through this receptor, the pathologic effects of activins may be blocked in these receptor-deficient mice.

The findings described here have important clinical implications in humans. Until now, no tumor suppressor with high specificity and high penetrance for the adrenal cortex has been discovered. Clearly, the analysis of inhibin production and/or inhibin binding to similar adrenal cortical tumors (since inhibin receptors may also be altered) will be a

prerequisite for future studies in humans. In addition, cancer cachexia syndrome in humans, including anorexia and weight loss, can severely limit the ability of a patient to combat cancer. As observed in this study, destruction of the liver architecture and mucosal atrophy in the glandular stomach may be contributing to the malnutrition. Since activin subunit mRNA and/or protein is produced in many tissues, and since activin has effects on a large number of cell types where activin receptors are present (1, 5, 9, 11–15, 27–29), activin production from some human tumor types may contribute to human cancer cachexia syndrome. Evaluation of activin concentrations in the serum of cancer patients may reveal a similar function of activins in human cachexia syndrome.

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