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Adrenal hyperplasia and tumours in mice in connection with aberrant pituitary-gonadal function

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Abstract

Gonadectomy induces in certain inbred strains of mice adrenal hyperplasia and tumorigenesis, originating from the putative subcapsular stem/progenitor cell layer. This response is apparently triggered by the elevated post-gonadectomy levels of luteinising hormone (LH), followed by ectopic upregulation of adrenal LH/chorionic gonadotrophin (CG) receptors (*Lhcgr*). The clear strain dependence of this adrenal response to gonadectomy prompted us to study its genetic basis. Tumorigenic DBA/2J and non-tumorigenic C57BL/6J mice, as well as their F2 and backcrosses, were studied by whole genome linkage analysis. Gonadectomy induced similar up-regulation of adrenal *Lhcgr* in both parental strains and their crosses, irrespective of the tumor status, indicating that ectopic expression of this receptor is not the immediate cause of tumours. Linkage analysis revealed one major significant quantitative trait locus (QTL) for the tumorigenesis on chromosome 8, modulated by epistasis with another QTL on chromosome 18. Hence, postgonadectomy adrenal tumorigenesis in DBA/2J mice is a dominant trait, not a direct consequence of adrenal *Lhcgr* expression, and is driven by a complex genetic architecture. A promising candidate gene in the tumorigenesis linkage region is *Sfrp1* (secreted frizzled-related protein 1), a tumour suppressor gene, which was down-regulated in the neoplastic tissue. Our findings may have relevance to the human pathogenesis of macronodular adrenal hyperplasia and postmenopausal adrenocortical tumors. A distinctly different adrenal response was observed in TG mice overexpressing LH or CG, or a constitutively activated form of the *follicle-stimulating hormone receptor* (*Fshr*). These mice developed perimedullary hyperplasia of foamy multinucleated cells, reminding of macrophages and filled with lipofuscin. Similar response was observed in TG mice overexpressing *aromatase* (*CYP19*). The cause of this response is not related to direct LH/CG action, but merely to adrenal response to chronically elevated oestrogen levels. This phenotype is reminiscent of the rare ‘black adenomas’ of the human adrenal cortex.

Keywords: adrenal cortex, adrenal tumours, adrenal adenomas, adrenal hyperplasia, black adenoma, gonadotrophins, luteinising hormone (LH), chorionic gonadotrophin (CG), LH/CG receptor (Lhcgr), gonadectomy, lipofuscin

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1. Introduction

We became interested in the possibility that hormones of the reproductive axis could exert modulatory effects on adrenal function through a serendipitous finding. We were studying tumorigenesis of transgenic (TG) mice expressing the *SV40 virus T antigen* under the *inhibin- α subunit (Inha)* promoter, with the expectation that the mice would develop tumours in gonadal somatic cells, the site of strong *Inha* expression (Kananen et al., 1996a; Kananen et al., 1995). In addition to the expected gonadal tumours, we also found neoplasms in the lungs and liver of the TG mice. To find out whether the latter were primary tumours or metastases from gonads, we gonadectomised the animals prepubertally, i.e. before the age of appearance of the gonadal tumours. Neither liver nor lung tumours were found in the gonadectomised mice but, surprisingly, their adrenal glands developed large malignant cortical tumours (Kananen et al., 1996b), which were subsequently found to express high level of *Lhcgr*, and hCG was able to stimulate their growth and steroidogenesis (Rilianawati et al., 1998).

Subsequent reading of old literature from the 1940's – 70's revealed that post-gonadectomy adrenal hyperplasia and tumorigenesis was a well-established phenomenon specific for certain inbred strains of mice and totally absent in others (Fekete et al., 1941; Hofmann et al., 1960; Huseby and Bittner, 1951; Murthy et al., 1970; Tullos et al., 1961; Woolley et al., 1941). This prompted us to embark on a genetic study with the purpose to identify by linkage analysis the quantitative trait locus/loci (QTL) containing the genetic polymorphism(s) responsible for the susceptibility to post-gonadectomy adrenal tumorigenesis. This study was considered important because a subclass of human adrenocortical tumours is also gonadotrophin dependent, and even the persistently elevated postmenopausal levels of gonadotrophins could be responsible for adrenocortical stimulation in some women (Bernichtein et al., 2008a).

In our other studies on mice with genetically modified gonadotrophin or gonadal steroid production we came across a different type of adrenal hyperplasia, which, unlike the subcapsular post-gonadectomy tumours, is located at the perimedullary junction and appears as large, pigmented, cloudy and often multinucleated cells, resembling macrophages. This type of response was found in LH (Kero et al., 2000), CG (Rulli et al., unpublished observations) and aromatase (Li et al., 2004) overexpressing TG mice, as well as mice expressing a constitutively activating mutation of *Fshr* (Peltoketo et al., unpublished findings). Although elevated LH/CG levels are found in some of these models, it is not their common feature, but rather the chronically elevated levels of oestrogen. In fact, similar adrenal response has been described in the older literature as a response of mice to ageing, oxidative stress and oestrogen treatment (Frith, 1996). Hence, there appear to be two distinct hyperplasias in the mouse adrenal gland in response to dysregulation of the pituitary-gonadal function, i.e. the subcapsular response to direct LH/CG action, and the perimedullary response to elevated oestrogen levels.

The purpose of this article is to review in more detail our findings on these two types of adrenocortical pathologies.

2. Adrenal hyperplasia and tumours following gonadectomy

Although older literature has reported in mice the appearance of adreno-cortical neoplasms in response to prepubertal ovariectomy (see above) the molecular mechanisms involved have remained unknown. Since this pathological condition only occurs in specific susceptible inbred strains of mice (e.g. DBA/2J, CE, C3H, NH, Balb/c), and is absent in others (e.g. C57BL/6J, FVB/N), it was suggested that a strong genetic component could predispose mice to these adrenal responses to gonadectomy.

When mice are gonadectomized, a set of changes occurs within months. First, the mice rapidly exhibit elevated levels of serum LH, followed by, in susceptible strains (e.g. DBA/2J), subsequent development of adrenocortical tumors with abnormal overexpression of *Lhcgr* and the transcription factor *Gata-4* (Bielinska et al., 2003). These neoplasms originate as non-steroidogenic small spindle-shaped cells (termed A cells) in the subcapsular cortex. With time, A cells proliferate and extend within the zona fasciculata, forming wedge-shape areas. Thereafter, foci of lipid-laden, LH-responsive steroidogenic cells (termed B cells) appear within the A cell patches. As a result, a clear tumorigenic pattern of islands containing A and B cells is observed with complete disorganization of the normal adrenocortical architecture (Figure 1). Interestingly, the B cells resemble those of gonadal stroma and express several gonad-specific genes, including *Sf1*, *Gata-4*, *Inha*, *Lhcgr*, *Cyp 17*, *Cyp19a*, *Esr1* (Bielinska et al., 2005). Although this transformation of the adrenal cells can potentially be explained by the common ontogenic origin of the adrenal and gonad (Looyenga and Hammer, 2006), direct evidence for the origin and relationship of A and B cells is still missing, likewise whether the A cells are derivatives from the putative subcapsular stem/progenitor cell zone (Mitani et al., 2003). Interestingly, some other recent data show that a subset of adrenocortical cells retain the developmental plasticity to respond to pituitary gonadotropic stimuli and that these cells become dysregulated due to convergent gonadectomy and chronic LH exposure, thus leading to hyperproliferative clusters of cells in the adrenal cortex culminating in tumorigenesis. These observations arise from two genetically modified mouse models, i.e. the of *Inha* KO mice (Looyenga and Hammer, 2006) and the *Inha/Tag* TG mice, both developing adrenocortical tumorigenesis after gonadectomy (Kananen et al., 1996b). In these models, in addition to altered inhibin and/or activin levels, the gonadectomy-induced LH elevation is proposed to trigger the pathological adrenocortical growth response.

Altogether, these observations converge to the hypothesis of the post-gonadectomy adrenocortical tumours as a strain-specific phenomenon, with a genetic component, responsive to elevated LH secretion and subsequent *Lhcgr* overexpression in the adrenal gland. We therefore decided to examine the genetic component of this condition as an approach to unravel its molecular basis. We carried out the first genetic approach related to adrenocortical tumorigenesis in two selected strains of mice by performing a genome-wide screen in segregating populations derived from the nonsusceptible C57BL/6J and the neoplastic DBA/2J strains (Bernichtein et al., 2008b). Our results showed that both parental strains and their crosses responded to gonadectomy with similar LH rise, but unexpectedly this increase was associated with similar upregulation of adrenal *Lhcgr* expression in both parental strains and their crosses, irrespective of their tumor status. Thus, the ectopic adrenal *Lhcgr* expression could not explain the tumorigenesis, indicating that *i*) the ectopic *Lhcgr* expression is not the immediate cause of the tumorigenesis, and *ii*) the different genetic backgrounds influenced differently some downstream events beyond *Lhcgr* activation leading to induction of the neoplastic phenotype.

Linkage analysis identified a major highly significant QTL (LOD 5.6) score on chromosome 8 for the tumorigenesis, with no evidence for linkage to *Lhcgr* (chromosome 17) or *Gata-4* (chromosome 14) genes, known to be highly expressed in the tumour cells (Bernichtein et al., 2008b; Bielinska et al., 2005). Results from a 2-D genome scan revealed that the adrenocortical tumorigenesis likely involves a polygenic component, suggesting that the main locus identified on chromosome 8 is modulated by epistasis with at least another major quantitative trait locus on chromosome 18. Altogether, we propose the postgonadectomy adrenal tumorigenesis to be regarded as a genetic trait driven by a complex genetic architecture.

Analysis of candidate genes within the major locus on chromosome 8 identified *Sfrp1* (secreted frizzled-related protein 1), a negative modulator of the Wnt signaling pathway and a tumor suppressor gene, as a positional candidate gene that could be proposed as one of the biological candidate genes in the induction of the adrenal tumors. Indeed, *Sfrp1* protein is expressed in the normal adrenal cortex whereas its expression is clearly suppressed in the neoplastic areas. We found differential expression of *Sfrp1* within the tumor areas since the subcapsular A cells, *i.e.* the first stage of adrenal tumors, did not express this gene at detectable level, whereas cortical B cells did. This finding may be of significance with regard to the hypothesis of Looyenga and Hammer (2006), proposing that the A cells are derived from the same cortical zone as the pluripotent stem/progenitor cells, and that the adrenal tumorigenesis could thus be an example of stem cell metaplasia. This putative adrenal stem cell dysregulation could be a good example of how reproductive hormones modulate adrenal functions. In fact, one possibility is that after gonadectomy and LH rise, *Lhcgr* is induced in the stem cells, along with the transcription factor *Gata-4*. The activation of genes normally expressed in gonads is in turn stimulated, leading to the adrenal dysregulation. Combined with susceptible genetic factors, tumor suppressor genes (*i.e.* *Sfrp1* gene) can then be affected, leading to excessive proliferation of the cells and culminating in adrenocortical tumours.

3. Adrenal hyperplasia following exposure to high oestrogen levels

As described above, gonadectomy triggers in susceptible strains of mice the formation of adrenocortical tumorigenesis that is linked with the concomitant elevation of serum LH concentration (Bernichtein et al., 2008b). A different type of adrenal response develops in several other TG mice with abnormal pituitary-gonadal function, including mice expressing high levels of LH and hCG (Kero et al., 2000; Rulli et al., 2002). At variance with the post-

gonadectomy tumorigenesis type, the adrenocortical hyperplasia occurs in deeper layers at the cortico-medullary junction. It is found in intact TG mice, but can be, conspicuously, prevented by gonadectomy (Table 1), thus suggesting different mechanisms of pathogenesis, related to ovarian function.

TG female mice expressing an analogue of LH, chimeric bovine LH β /C-terminal peptide of CG β (bLH β -CTP)(Kero et al., 2000), CG β (Rulli et al., unpublished results) or constitutively active *Fshr* (Const-Fshr) (Peltoketo et al., unpublished results) show dramatically increased formation of pigmented and foamy multinucleated cells in the cortico-medullary junction of the adrenal gland (Figure 2). A similar layer of cells is also observed in adrenal glands of male TG mice overexpressing *aromatase* (AROM+) with increased oestradiol production (Li et al., 2001). The pathological cell layer is positive for periodic acid schiff (PAS) staining suggesting it to be lipofuscin (data not shown), called lipogenic or ageing pigmentation, or ceroid if pathological in origin (Seehafer and Pearce, 2006).

The accumulation of lipofuscin also occurs at low frequency in adrenal glands of wild-type mice as a normal process of senescence (Frith, 1996). The accumulation of the ageing pigmentation, a mixture of oxidized proteins and lipids, carbohydrates and trace amounts of metals, mainly takes place within postmitotic cells of a variety of organs (Yin, 1996). In the adrenal gland the deposition of lipofuscin initially occurs at the cortico-medullary junction and eventually encircles the medulla. When the amount of lipofuscin increases, nuclei may become pyknotic and the cells fuse to form multinucleated giant cell resembling macrophages (Figure 2).

Increased oxidative stress during ageing is suggested to be the main reason for the formation of lipofuscin; in addition the disruption in autophagy and altered lysosomal function may lead to lipopigment accumulation (Seehafer and Pearce, 2006; Yin, 1996). As early as 1937, and in several studies thereafter, it has been shown that chronic oestrogen treatment also induces the accumulation of lipofuscin to adrenal glands (Alpert, 1953; Cramer and Horning, 1937; Frith, 1996; Schardein et al., 1967), while testosterone has been shown to have an opposite effect (Alpert, 1953; Heiman, 1944). In all the four TG mouse models with adrenal lipofuscin accumulation, serum oestradiol concentration is elevated, though in the CG β mice only temporarily in peripubertal age (Table 1). The presence of oestrogen receptor in the mouse adrenal cortex (Kuiper et al., 1998) supports the direct involvement of oestradiol in the pathogenetic response. In addition, gonadectomy and antioestrogen treatment prevent the premature lipofuscin accumulation in the bLH β -CTP, CG β +, and AROM+ females. In contrast, increased testosterone is not able to counteract the response (Table 1).

Prolactin (PRL) secretion, due to the known stimulatory effect of oestradiol on the secretion of this hormone, is also elevated in all TG lines with elevated oestrogen production. PRL receptor is abundantly expressed in the adrenal cortex (Ouhtit et al., 1993), and it is known to upregulate *Lhcgr* expression (Gafvels et al., 1992; Huhtaniemi and Catt, 1981), and may thus also contribute to the formation of lipofuscin deposits. However, high LH is not a requirement for their development since, in addition to bLH β -CTP and CG β +, females, they also develop in the AROM+ and Const-Fshr mouse lines with unchanged or even suppressed LH levels.

Accumulation of lipopigment in the adrenal gland and other tissues is a sign of excessive oxidative stress and ageing or disturbed cellular autophagy and lysosomal function. Little

is known about its effects on general function of the adrenal gland. Corticosterone synthesis is elevated in bLH β -CTP females and AROM+ males, and the former line even develops a Cushing's syndrome-like phenotype (Kero et al 2003). However, adrenocortical hyperfunction in the CG+ TG mice has not been a consistent finding (Rulli et al., 2002). Altogether these TG lines are useful in further studies on the mechanisms leading to the accumulation of lipofuscin and adrenal gland degeneration. This phenomenon is clinically relevant, because lipofuscin accumulation also occurs in the human adrenal gland, with the extreme state of rare 'black adenomas' (Ueda et al., 1997) (Figure 3).

4. Conclusions and future directions

Concerning the gonadectomy-induced adrenocortical hyperplasia in specific strains of mice, our study was the first attempt to unravel the genetic background of this intriguing phenomenon. It turned out to be a complex genetic trait, with a major QTL on chromosome 8 but with a strong epistatic component, at least from chromosome 18. Interestingly, enhanced *Lhcgr* expression did not seem to be responsible for the phenotype. The QTL on chromosome 8 has one particularly interesting candidate gene, *Sfrp1*, a negative modulator of the Wnt signaling pathway. This gene is down-regulated in the tumorous A cells, but direct demonstration of its involvement in the pathological process is not yet available. The other adrenal phenotype that was detected in LH/CG, constitutively activated *Fshr* and *aromatase* overexpressing TG mice included perimedullary deposition of lipofuscin in foamy giant cells. The most likely cause of this response is chronically elevated oestrogen action, in agreement with some earlier findings. It is important to recognize that the two adrenal phenotypes of the mice with dysregulated pituitary-gonadal function represent totally different pathogenetic mechanisms. They both are clinically relevant, because analogous adrenal pathologies are known in humans (Bernichtein et al., 2008a; Ueda et al., 1997). Hence, unraveling the molecular

pathogenesis of these responses using the available mouse models may prove clinically important in our attempts to improve the diagnostics and treatment of adrenal hyperplasias and tumours.

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Figure legends

Figure 1. Histological view of the adrenal gland of a gonadectomised F1 (DBA/2J x C57BL/6J) mouse killed at 6 mo. The tumours start as wedge-shaped subcortical islets of basophilic A cells (A) with spindle-shaped nuclei and scanty cytoplasm. Subsequently, A cells start migrating centripetally while larger pale B cells (B) appear within the A cell patches. N = normal adrenocortical tissue.

Figure 2. Adrenal glands of transgenic female mice expressing *bLH β -CTP* (A), *hCG β* (B), and a constitutively active form of *Fshr* (C), as well as of a male mouse overexpressing *ubiquitin-C* promoter- driven *aromatase* (D). The lipofuscin layer is situated at the corticomedullary junction and consists of foamy, multinuclear cells (arrow in panel B and insert in panel C). m, medulla, ZG, zona glomerulosa, ZF, zona zasciculata, ZR, zona reticularis. Scale bars in the figures are 20 μ m.

Figure 3. High power magnification image of human black adenoma stained with HE. Cells are filled with lipofuscin-like pigment (arrows) and also multinuclear cells are seen (arrow head). (Courtesy of Drs. T. J. Giordano and G. D. Hammer, Comprehensive Cancer Clinic, Ann Arbor, MI)

Table 1. The different mouse models with dysregulated pituitary-gonadal function presenting with adreno-cortical hyperplasia or tumours.

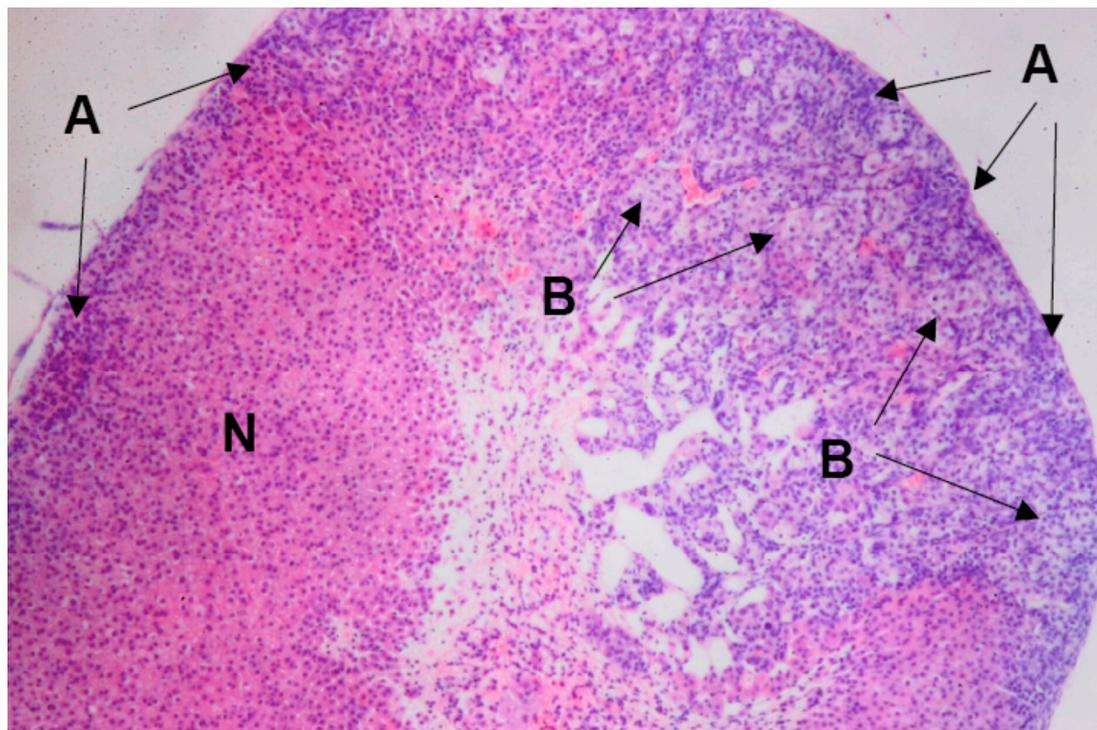
Models	Contributing to neoplasia	Tumours/Hyperplasia	Reference(s)
Wild type mice (non-susceptible strain)			Bernichtein et al. (2008b)
Intact	None	-	
Gonadex	LH↑	-	
Wild-type mice (susceptible strains)			Bernichtein et al. (2008b)
Intact	Genetic susceptibility+	-	
Gonadex	Genetic susceptibility+, LH↑	+	
Inhibin- α /SV40Tag TG			Kananen et al. (1996), Rilianawati et al. (1998)
Intact	Tag+	-	
Gonadex	Tag+, LH↑	+	
Inhibin- α KO			Looyenga & Hammer (2006)
Intact	No inhibin (+activin↑?)	-	
Gonadex	No inhibin, LH↑	+	
bLH β -CTP TG			
Intact	LH↑, PRL↑, E2↑, T↑	+	Kero et al. (2000)
Gonadex	LH↑	-	
hCG β TG			
Intact	LH↑, PRL↑, E2↑*, T↑	+	Rulli et al. (2002)
Gonadex		-	
Const-Fshr TG			
Intact	PRL↑, E2↑, T↓	+	Peltoketo et al. (unpublished)
Gonadex		?	
AROM+ TG, male			
Intact	PRL↑, E2↑, T↓	+	Li et al. (2001, 2004)
Gonadex		?	

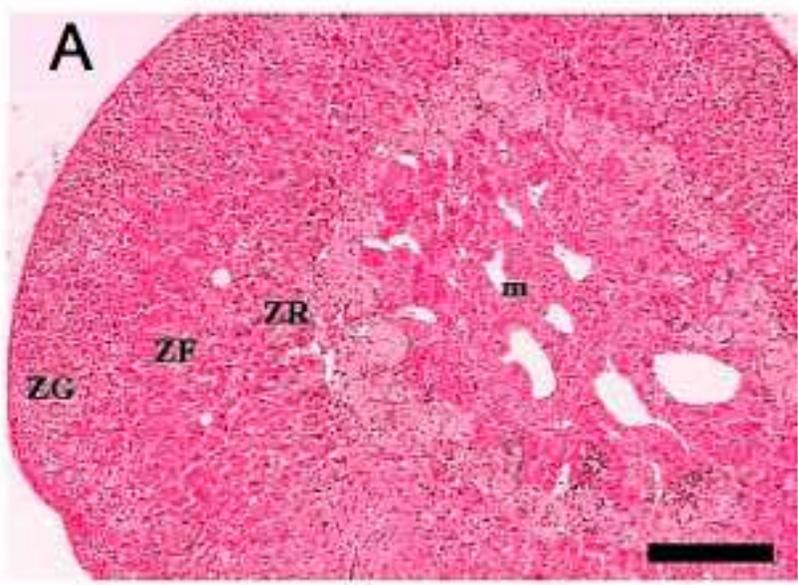
* Oestradiol (E2) concentration transiently elevated at age of 1 month

Tag = SV40 T antigen, PRL = prolactin, E2 = Oestradiol

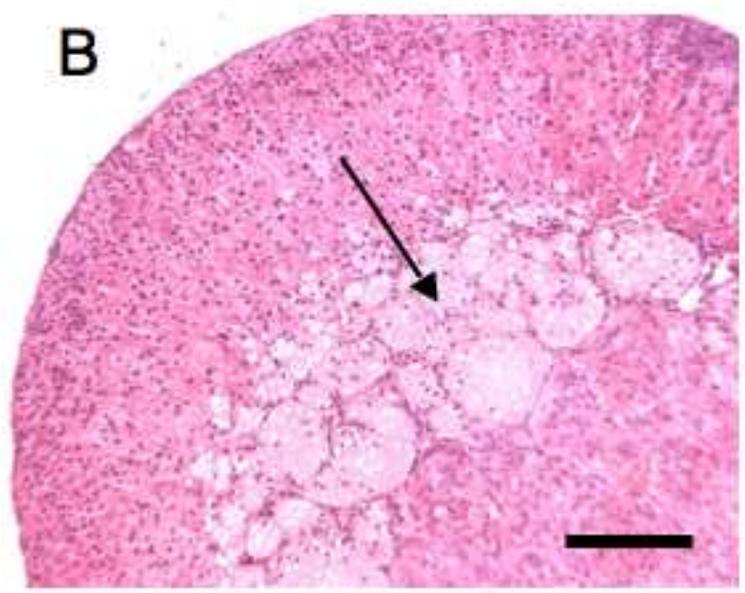
-, no tumour, + tumour, ?, no information, ↑ circulating level increased, ↓ circulating level decreased

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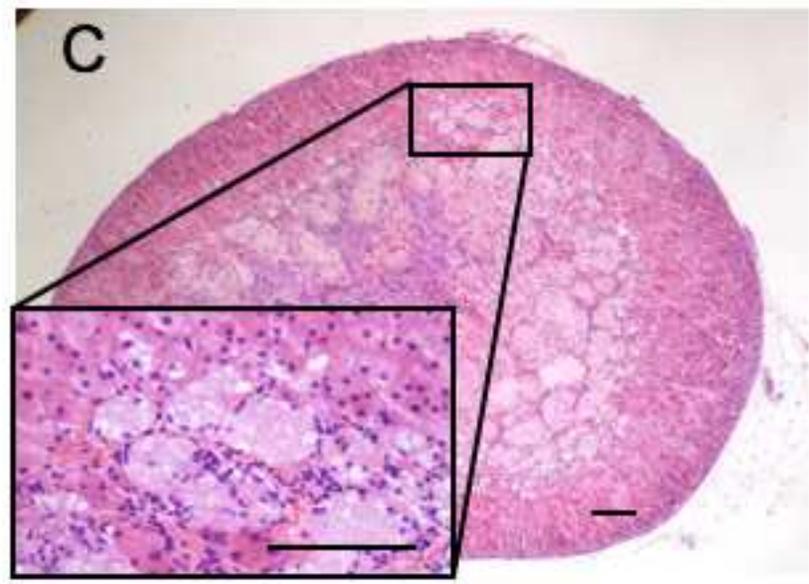




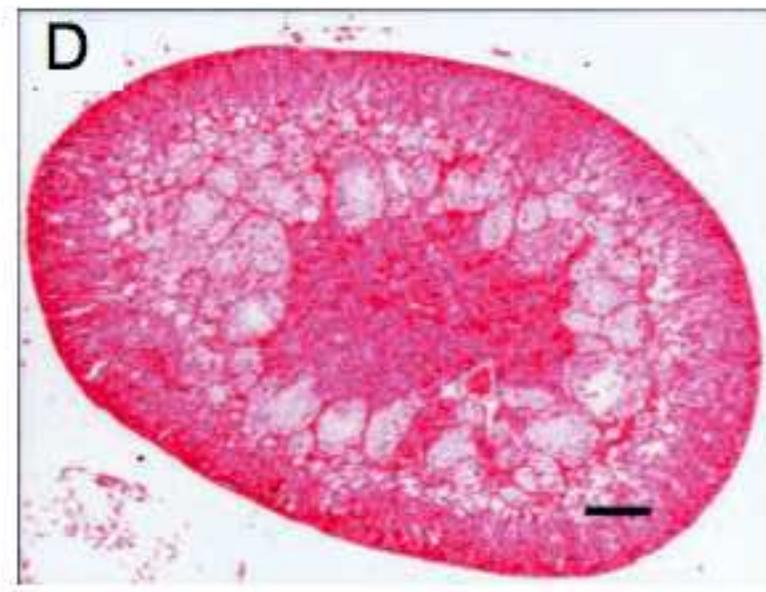
bLHβ-CTP TG



hCGβ+ TG



Constit. act. FSHR TG



Ubiqu. C-Arom + TG

