Oncogene (2005) 24, 7301–7309
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# Fetal but not adult Leydig cells are susceptible to adenoma formation in response to persistently high hCG level: a study on hCG overexpressing transgenic mice

Petteri Ahtiainen[1,2], Susana B Rulli[1,3], Ramin Shariatmadari[1], Lauri J Pelliniemi[4], Jorma Toppari[1,5], Matti Poutanen[1] and Ilpo T Huhtaniemi*[,1,6]

[1]*Department of Physiology, University of Turku, FIN-20520 Turku, Finland;* [2]*Turku Graduate School of Biomedical Science, University of Turku, FIN-20520 Turku, Finland;* [3]*Institute of Biology and Experimental Medicine-CONICET, Vuelta de Obligado 2490, Buenos Aires 1428, Argentina;* [4]*Laboratory of Electron Microscopy, University of Turku, FIN-20520 Turku, Finland;* [5]*Department of Pediatrics, University of Turku, FIN-20520 Turku, Finland;* [6]*Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Campus, Du Cane Road, London W12 0NN, UK*

**We have previously demonstrated that male transgenic (TG) mice overexpressing human chorionic gonadotropin (hCG+) develop reproductive organ defects, but no tumors, in adult age. In this study, the effects of persistently elevated hCG were followed in TG males between day 5 postpartum and adulthood. Leydig cell (LC) adenomas were found in prepubertal mice, most prominently at the age of 10 days, but not in adult age. Serum testosterone concentrations were significantly increased in TG males at all ages studied. The phenotype of the prepubertal hCG+ males resembled that found in boys upon expression of constitutively activating luteinizing hormone (LH) receptor mutations. The temporal expression patterns of the fetal LC marker gene, *thrombospondin 2*, and those of adult LCs, *hydroxysteroid dehydrogenase-6*, *delta*[5]*-3-beta* and *prostaglandin D synthase*, were similar in wild-type and hCG+ males. Hence, the postnatal adenomas resemble functionally fetal LCs, and only these cells are susceptible to hCG-induced tumorigenesis. Our findings demonstrate a novel intriguing difference between the fetal and adult LC populations and provide further insight into the potential tumorigenic effects of gonadotropins.**
*Oncogene* (2005) **24,** 7301–7309. doi:10.1038/sj.onc.1208893; published online 11 July 2005

**Keywords:** fetal Leydig cell; adult Leydig cell; tumor; hCG

## Introduction

There are two temporally different populations of Leydig cells (LC) in the testis, that is, the fetal and adult LCs (Habert *et al*., 2001). The former are functionally responsible for the masculinization of male fetuses, and in rodents, some of the fetal LCs remain dormant until adulthood (Kerr and Knell, 1988; Ariyaratne and Mendis-Handagama, 2000). In the rat and mouse, fetal LCs start expressing the functional luteinizing hormone receptor (LHR) around day 16 postcoitum (p.c.) (Zhang *et al*., 1994; O'Shaughnessy *et al*., 1998), but LH action is not compulsory for the normal fetal masculinization (Cattanach *et al*., 1977; Kendall *et al*., 1995; O'Shaughnessy *et al*., 1998; Zhang *et al*., 2001; Ma *et al*., 2004). The activation of LHR provides a strong mitogenic stimulus for the fetal LCs (Kuopio *et al*., 1989). In contrast, human chorionic gonadotropin (hCG) treatment of adult male rats only leads to LC hyperplasia (Christensen and Peacock, 1980). The adult LCs appear after day 10 postpartum, and subsequently they proliferate and differentiate under the control of LH, producing the testosterone (T) needed to induce puberty and to maintain spermatogenesis and extragonadal male sex characteristics (Hardy *et al*., 1989). The crucial role of LH in LC proliferation in their different phases of life makes this hormone a likely candidate for promoter of tumorigenesis.

Indeed, several studies have provided evidence for the tumorigenic potential of LH. Epidemiological findings show high incidence of ovarian cancer after menopause, supporting the theory that elevated LH levels are involved in ovarian tumorigenesis (Wise *et al*., 1996). *In vitro* studies have demonstrated that LH stimulates the proliferation of human ovarian cancer cells (Simon *et al*., 1983; Parrott *et al*., 2001). Elevated LH levels in female transgenic (TG) mice have been shown to induce ovarian tumors in certain strains of mice (Keri *et al*., 2000), and the formation of gonadal tumors in inhibin-α-deficient mice is dependent on gonadotropins (Kumar *et al*., 1996). Recently, we have shown that LH accelerates the formation of granulosa and LC tumors in TG mice expressing the Simian virus 40 T-antigen under the inhibin-α promoter (Kananen *et al*., 1997; Mikola *et al*., 2003). However, when hCG was

*Correspondence: IT Huhtaniemi, Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Campus, Du Cane Road, London W12 0NN, UK;
E-mail: ilpo.huhtaniemi@imperial.ac.uk
Received 26 January 2005; revised 24 May 2005; accepted 31 May 2005; published online 11 July 2005

overexpressed in TG mice, females in adult age developed ovarian somatic cell tumors, but males only presented with mild LC hyperplasia and hypertrophy, but not tumors (Matzuk *et al*., 2003; Rulli *et al*., 2003). Hence, there seems to be a clear sex difference in the tumorigenic potential of chronically elevated LH/hCG stimulation.

Activating *Lhcgr* mutations are associated with LC hyperplasia (Themmen and Huhtaniemi, 2000), but LC adenomas have only been found in connection with a particular activating *Lhcgr* mutation, D578 H (Liu *et al*., 1999; Richter-Unruh *et al*., 2002). *In vitro* studies on this mutated receptor have shown that besides cAMP, it constitutively also activates the inositol phosphate and MAPK signaling cascades (Liu *et al*., 1999; Hirakawa *et al*., 2002). In addition, antiandrogens and estrogens have been shown to cause LC hyperplasia and adenomas, especially in mice, through an as yet unknown mechanism (Clegg *et al*., 1997).

The tumorigenic properties of elevated LH levels in males are thus poorly characterized and controversial. In the present study, we assessed the role of hCG, a highly potent LH analogue, in LC tumorigenesis in hCG-overexpressing TG mice. This model has relevance for the pathogenesis of human LC tumors, some of which occur in connection with activating *Lhcgr* mutations.

## Results

### *No signs of precocious puberty in hCG+ males despite elevated T levels*

The postnatal body and testis weights of the wild-type (WT) and hCG+ males are presented in Figure 1. While the hCG+ mice were slightly heavier at 21 days of age, the reverse was observed at 60 days. The testis weights were consistently lower in the 10-, 21- and 60-day-old hCG+ males compared with WT males ($P<0.05$–$0.001$). There was no difference in the age of balanopreputial separation between the WT and hCG+ males ($34.2\pm1.0$ vs $34.6\pm1.0$ days, respectively). Although serum hCG levels were high at all postnatal ages, they increased significantly between 10 and 60 days of age (Figure 2a). As expected, serum and testicular T were clearly increased ($P<0.001$) in the hCG+ males in all age groups (Figure 2b and c). Hence, the high hCG and T concentrations were not able to advance puberty in hCG+ males, as monitored with the external sign of balanopreputial separation.

### *LC adenomas in prepubertal hCG+ males*

In hCG+ males, both the volume density (Figure 3a) and the areas of largest LC islets (Figure 3b) were significantly increased as compared with WT littermate controls ($P<0.001$ in 5-, 10- and 21-day-old groups, $P<0.05$ in adults). Most prominent increase occurred in the proportion of LCs in the 10-day-old hCG+ males,

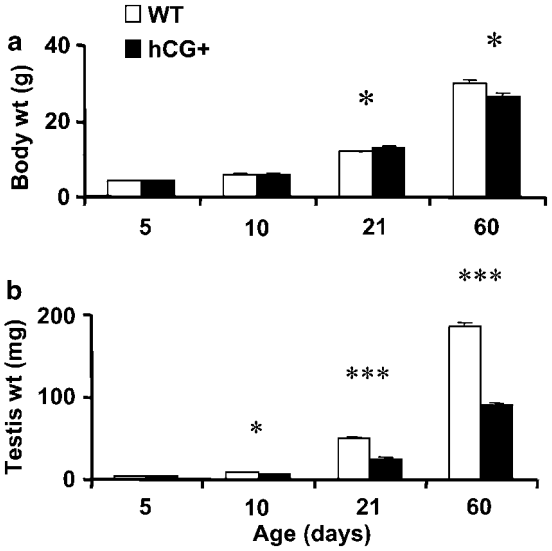


**Figure 1** Body (**a**) and testis (**b**) weights of WT and hCG+ males between 5 and 60 days of life. The asterisks denote significant difference between age-matched WT and hCG+ mice (*$P<0.05$, ***$P<0.001$). Mean±s.e.m.; $n=6$–9 animals/group

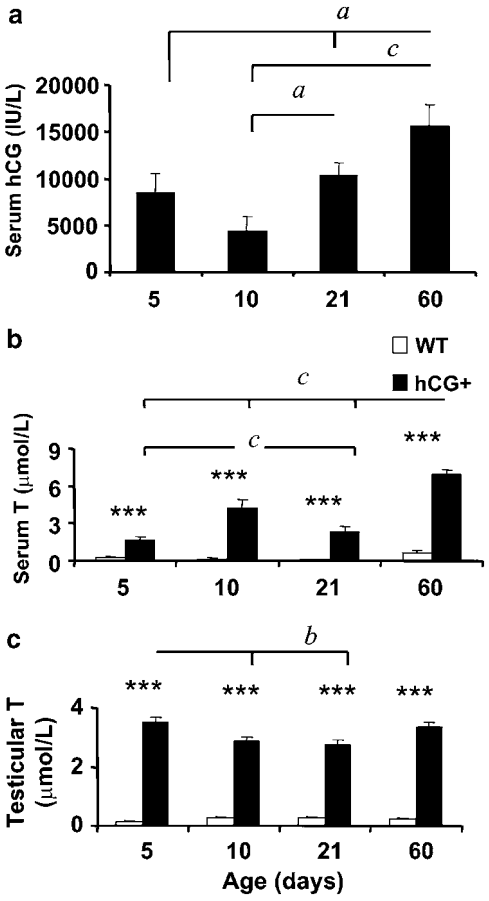


**Figure 2** Hormone profiles of WT and hCG+ males between days 5 and 60 of life. (**a**) serum hCG (TG mice only); (**b**) serum T; (**c**) testicular T. The results are mean±s.e.m.; $n=5$–7 animals/group. Asterisks denote statistically significant difference between age-matched WT and hCG+ groups (***$P<0.001$), and different letters denote differences across the ages ($^aP<0.05$, $^bP<0.01$, $^cP<0.001$)

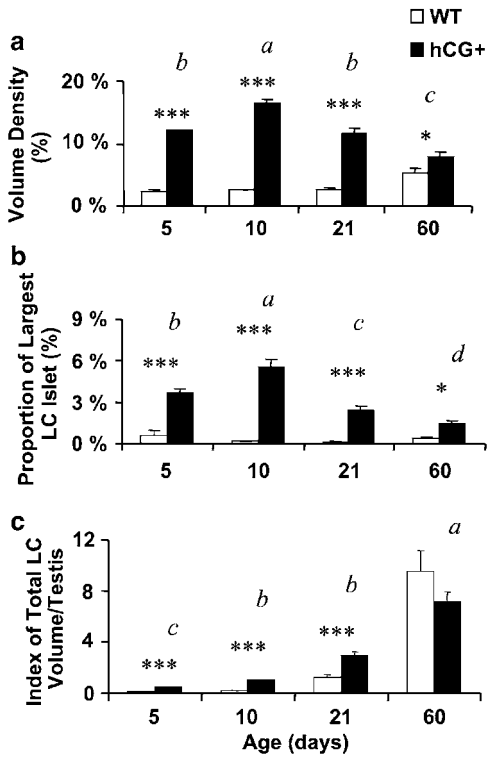


**Figure 3** Morphometric analysis of LC in WT and hCG+ testes of days 5–60 of life. (**a**) LC volume density; (**b**) proportion of the largest LC islet; (**c**) index of total LC volume (=volume density × testis weight). Mean±s.e.m.; $n=3$ animals/group. Asterisks denote significant differences between age-matched WT and hCG+ males (*$P<0.05$, ***$P<0.001$). Different letters indicate significant differences between the hCG+ male groups ($^{a}$ *vs* $^{b\text{–}d}P<0.001$, the other differences $P<0.05$ at least)

with a volume density of 16.5±0.8% (vs 2.5±0.3% in WT, $P<0.001$) and the largest LC islet areas of 5.5±0.6% (vs 0.2±0.1% in WT, $P<0.001$). Clearly, higher indexes of total LC volumes were measured in TG males at 5, 10 and 21 days, but not in adult age (Figure 3c). Histological evaluation revealed that the adenomatous LC islets were most prominent in 10-day-old hCG+ males. Furthermore, some mitotic figures were seen in LCs at this age indicating proliferative activity. Moreover, the diameter of the LC islets exceeded that of the seminiferous tubules (Figure 4) in all 10-day-old hCG+ animals studied, fulfilling the criterion for LC adenomas in rodents (Clegg *et al*., 1997; Cook *et al*., 1999). No overt changes were observed in the seminiferous tubular structure in any of the hCG+ groups, and the progression of spermatogenesis was similar in WT and hCG+ males (data not shown). Hence, the high postnatal T levels of hCG+ mice were unable to advance spermatogenesis.

Upon immunohistochemical analysis (Figure 4), we observed clear staining for HSD3B1 in the enlarged interstitial compartments of the prepubertal hCG+ males, indicating that they mainly originate from steroidogenically active LCs. The localization and expression level of AR appeared similar in WT and hCG+ testes. In the interstitial compartment of 5-, 10- and 21-day-old males, proliferating cell nuclear antigen (PCNA)-positive LCs were observed especially in hCG+ males, indicative of their proliferative activity. In adult hCG+ males, PCNA-positive cells were only occasionally found in the interstitial compartment, in keeping with the terminal differentiation of adult-type LCs.

### *LC hypertrophy in hCG+ males*

Electron microscopy revealed that the size of LCs at the age of 10 days was clearly increased in hCG+ males (Figure 5a and b). hCG overexpression also changed the morphology of LCs, which had large, round nuclei with prominent nucleoli, and their amount of agranular endoplasmic reticulum was increased (Figure 5c), indicating elevated functional activity. This correlated well with the elevated steroidogenic enzyme levels (see below) and T production found in the 10-day-old hCG+ males.

### *LC adenomas in hCG+ males are derived from fetal LCs*

Quantitative RT–PCR measurements revealed that the message of the fetal LC marker, *thrombospondin (Thbs2)*, was significantly higher in 5-day-old WT and hCG+ males as compared with older animals (Figure 6a) (O'Shaughnessy *et al*., 2002a, b). There was a significant difference in the *Thbs2* mRNA level between 5- and 10-day-old WT and hCG+ males, the levels being lower in the former group. The difference is even greater, if the greater volume density of the LCs in prepubertal hCG+ testes is taken into account. In 10-day-old WT males treated with hCG, *Thbs2* mRNA decreased by 41% (Figure 6b), indicating that high LH/hCG stimulation downregulates this message.

The mRNA levels of the adult LC markers, *hydroxysteroid dehydrogenase-6*, *delta$^{5}$-3-beta (Hsd3b6)* and *prostaglandin D synthase (Psgd)*, were significantly higher in the adult males as compared with other groups (Figure 6c and d) (O'Shaughnessy *et al*., 2002a, b). The 1-day treatment of 10-day-old WT males with hCG slightly but not significantly decreased the level of *Hsd3b6* mRNA, but did not affect the mRNA expression of *Psgd* (data not shown), indicating that these adult LC markers were not downregulated by hCG. Taken together, these data demonstrated that the temporal expression pattern of the fetal and adult LC marker genes was similar in hCG+ and WT testes.

The levels of several mRNAs for proteins involved in steroidogenesis were highly increased in the 5- and 10-day-old hCG+ testes (Figure 7). The expression patterns of mRNAs for *Lhcgr*, *Star*, *P450c17*, *P450scc* and *Hsd3b1* were similarly increased in the 5- and 10-day-old hCG+ males in comparison to WT males (Figure 7a–e). In addition, the amount of these mRNAs was significantly higher in the 10-day-old hCG+ males

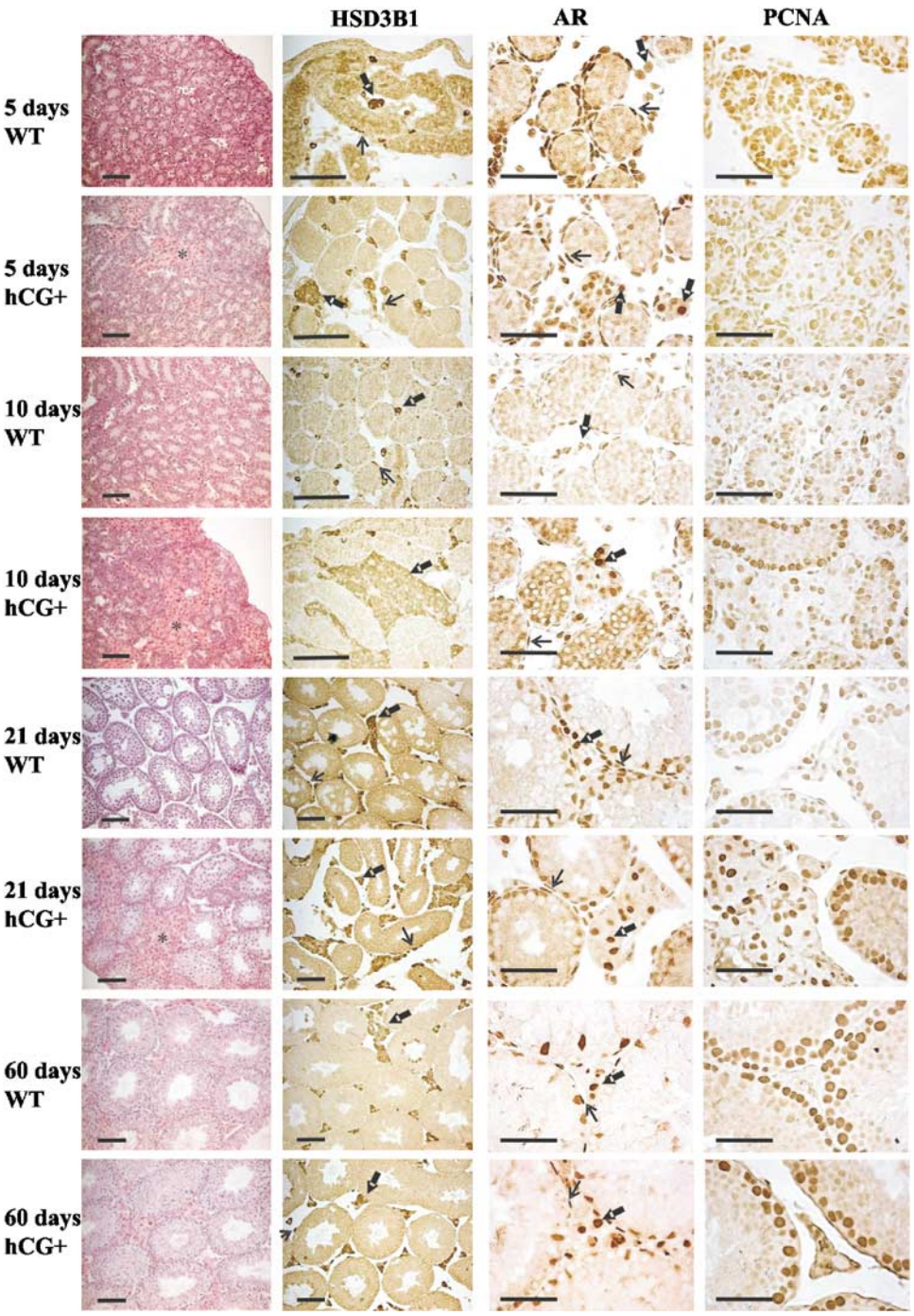


**Figure 4** Light microscopic and immunohistochemical analysis of the WT and hCG+ testes at ages days 5–60 days postpartum. *The left* vertical set of panels shows testicular histology. Asterisks denote the large hyperplastic LC islets regarded as adenomas. *The second* vertical set of panels to the left shows 3$\beta$HSD1 immunohistochemistry. Positive reaction for this enzyme is localized especially in LCs (large arrows), but also in peritubular Leydig precursor and mesenchymal cells (small arrows). *The third* set of vertical panels shows the immunolocalization of AR. The large arrows indicate LCs, small arrows peritubular myoid cells. *The fourth* set of panels presents immunolocalization of PCNA. Note that PCNA positive LCs are rare in 60-day-old WT and hCG+ interstitial tissue. Bar = 100 $\mu$m in the first and second vertical sets of panels, bar = 50 $\mu$m in the third and fourth vertical sets of panels

in comparison to other groups. At the adult age of 60 days, the mRNA levels were similar between the WT and hCG+ males. Altogether, when the differences in testis size and LC volume density were taken into account, clear increase in expression levels of the steroidogenic enzymes were only found in the two youngest age groups of the hCG+ mice, despite the persistently high level of serum hCG.

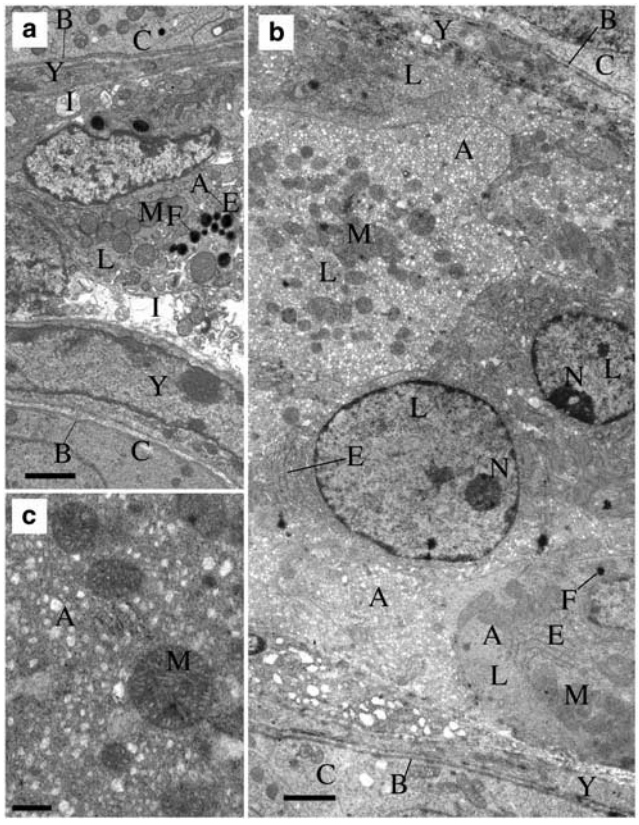


**Figure 5** A typical electron micrograph of the testis of a 10-day-old WT (**a**) and hCG+ (**b**) mouse. Testicular cords (C) are outlined by a basement membrane (B), which separates it from the interstitial tissue (I) between. Panel a: A Leydig cell (L) is seen in the middle, flanked by myoid cells (Y). The L cytoplasm is full of agranular endoplasmic reticulum (A), mitochondria (M), lipid droplets (F) and some cisternae of granular endoplasmic reticulum (E). Panel b: The cells (Y) in the interstitium are elongating to become myoid cells. Several large Leydig cells (L) are seen between the cords. Their cytoplasm is full of agranular endoplasmic reticulum (A), mitochondria (M), few lipid droplets (F) and large cistern complexes of granular endoplasmic reticulum (E). Several cells have large nucleoli (N). Scale bar 2 $\mu$m. (**c**) A high-power electron micrograph of the testis of a 10-day-old hCG+ mouse with typical view of LC cytoplasm, full of agranular endoplasmic reticulum (A), mitochondria (M), the key organelles of steroid synthesis. Scale bar 500 nm

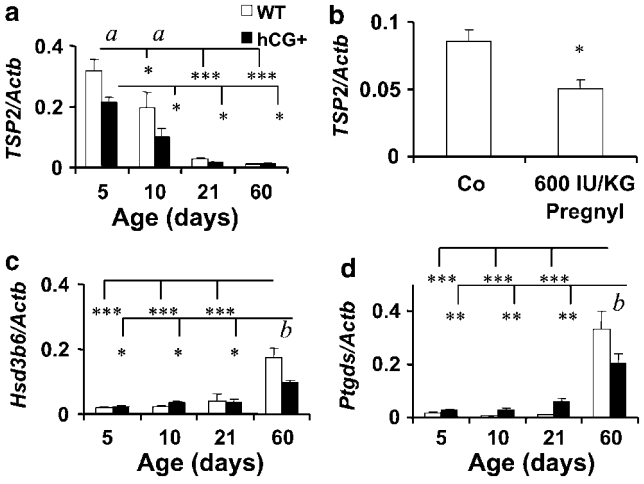


**Figure 6** mRNA expression of fetal and adult LC markers in the developing WT and hCG+ mouse testis at specified ages. Expression pattern of the *Thbs2* (**a**), *Hsd3b6* (**c**), and *Ptgds* (**d**) mRNAs are shown. (**b**) *Thbs2* mRNA levels of 10-day-old WT males after 1-day treatment with hCG. Mean±s.e.m.; $n=3$ (panels a, c and d) or 5 (panel b) animals/groups. The asterisks denote significant difference between the different age groups of WT and hCG+ males (*$P<0.05$, **$P<0.01$, ***$P<0.001$) and italic letters above the columns indicate significant difference between the age-matched WT and hCG+ mice ($^aP<0.05$, $^bP<0.01$)

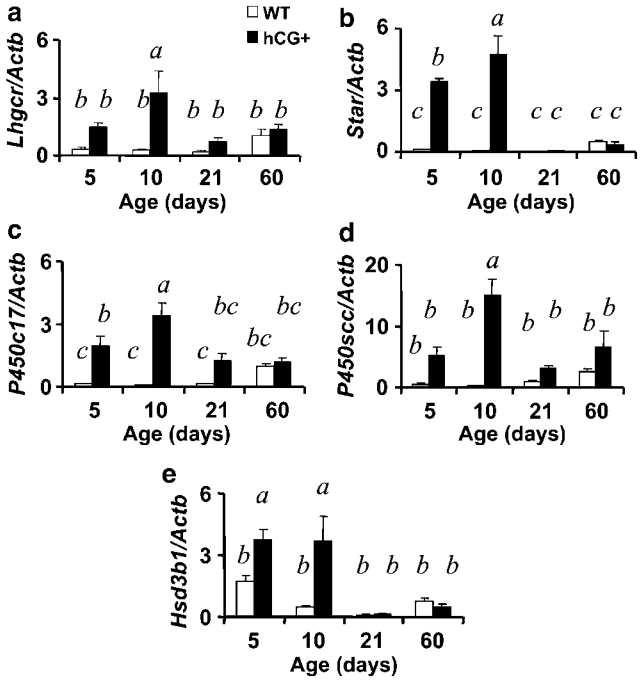


**Figure 7** The expression pattern of mRNAs of proteins involved in gonadotropin action and steroidogenesis. (**a**) *Lhcgr*; (**b**) *StAR;* (**c**) *P450c17;* (**d**) *P450scc*; and (**e**) *Hsd3b1*. Mean±s.e.m.; 3 animals/groups. Different superscript letters indicate significant differences between the groups ($P$ at least $<0.05$)

## Discussion

We have recently shown that adult hCG+ males display a clear phenotype with urinary tract obstruction, infertility and slight LC hypertrophy/hyperplasia, but no LC tumors (Rulli *et al*., 2003). Hence, in adult male mice, through an undefined mechanism, the testes are protected from tumorigenic effects of increased LH/hCG levels. This is in clear contrast with hCG+ females that develop an array of endocrine neoplasias in the ovary, pituitary and mammary gland in response to elevated hCG levels (Rulli *et al*., 2002, 2003). In the present study, we examined the temporal aspects of the potential of persistently high hCG/LH levels to induce testicular tumorigenesis.

Histological analysis revealed prominent LC hypertrophy and hyperplasia in prepubertal hCG+ testes, with a maximum effect on day 10 postpartum. LC volume density was increased in all hCG+ groups

studied, but the index of total LC volume was only increased prepubertally. In these animals, the diameter of the largest LC islet exceeded that of the seminiferous tubules, fulfilling the criterion of LC adenomas (Clegg *et al*., 1997; Cook *et al*., 1999). Most of the LCs of 10-day-old hCG+ mice were positive for PCNA, and also mitotic figures were observed, indicating increased proliferative activity. Electron microscopy revealed that these LCs were clearly larger than those of WT males. Therefore, the adenomatous growth of LCs represents a combination of hyperlasia and hypertrophy.

The temporal expression pattern of fetal and adult LC marker genes was similar in WT and hCG+ males. The expression of *Thbs2*, a fetal LC marker, was inhibited by 1-day hCG treatment of the 10-day-old WT males, suggesting that its decreased level in the 5- and 10-day-old hCG+ males was not due to fetal LC regression or advanced onset of adult LC proliferation. These results also indicate that the adenomas in prepubertal hCG+ testes originate from fetal LCs. Hence, the proliferation, but not the differentiation or the regression, of the fetal LCs is sensitive to elevated hCG levels. Although the normal function of fetal LCs is not LH/LHR dependent, fetal LCs do express functional LHR already several days before birth and thus they also respond to elevated LH/hCG levels with T synthesis and proliferation (Zhang *et al*., 1994; O'Shaughnessy *et al*., 1998). Furthermore, high hCG level accelerates the proliferation of adult LCs, as indicated by the increased number of PCNA-positive LCs in 3-week-old hCG+ males, without affecting the index of total LC volume/testis in adult age.

In humans, a specific activating mutation of the *Lhcgr*, D578H, was found to lead to the formation of LC adenomas (Liu *et al*., 1999; Richter-Unruh *et al*., 2002). Whether such adenomas are derived from fetal- or adult-type LCs remains unknown. Although the regression of fetal LCs in man starts normally already in fetal life (Chemes, 1996; Habert *et al*., 2001), we do not know how an activating *Lhcgr* mutation affects their lifespan. Nevertheless, the hCG+ mice provide a phenocopy of the human activating mutation of *Lhcgr* presenting with prepubertal LC adenomas and testotoxicosis (Liu *et al*., 1999; Richter-Unruh *et al*., 2002). Another phenotypic feature of the activating *Lhcgr* mutations is precocious puberty in boys between 1 and 4 years of age (Themmen and Huhtaniemi, 2000). We monitored the onset of pubertal development in the hCG+ males, but could not find any advancement in balanopreputial separation, body weights or spermatogenesis. Hence, the clearly elevated hCG and T levels in male mice did not advance puberty as is found in hCG$\beta$+ females (Rulli *et al*., 2003), or in boys with activating *Lhcgr* mutations (Themmen and Huhtaniemi, 2000). The duration of the spermatogenic wave of 35 days may impose a biological limit for the onset of spermatogenesis, which is already at its minimum in WT males and cannot be advanced by precociously elevated T production. It is also possible that some other factors than LH/T are permissive for the onset of male puberty.

Several studies have shown that elevated LH plays a pivotal role in the development of LC hyperplasia, adenomas and malignancies (Clegg *et al*., 1997; Cook *et al*., 1999). Chronic hCG and flutamide treatments, as well as other compounds decreasing T bioactivity and increasing LH levels have been shown to cause LC hyperplasia and adenomas in rodents (Christensen and Peacock, 1980; Prahalada *et al*., 1994; Clegg *et al*., 1997; Cook *et al*., 1999). Also, estrogens have been shown to cause LC adenomas by increasing LH or LHR levels in certain mouse strains (Huseby, 1980; Navickis *et al*., 1981). Our recent study of male TG mice overexpressing *P450 aromatase* (AROM+) demonstrated development of LC hyperplasia in the presence of normal circulating LH and low testicular T (Li *et al*., 2001). However, it is possible that the high prolactin levels in these mice have enhanced LC response to LH, because prolactin in rodents is able to upregulate testicular *Lhcgr* expression (Huhtaniemi *et al*., 1985b). Decreased androgen action has also been associated in rats with LC adenomas, in the form of treatment with the antiandrogen flutamide (Clegg *et al*., 1997; Cook *et al*., 1999). This finding indicates a functional role for AR blockade in the tumorigenic process. In our model, the AR content of adult LCs is high, which might protect them from adenomatous transformation. Therefore, it is possible that T has an autocrine inhibitory effect on adult LC proliferation and the lack, or decreased level of androgen action can permit the tumorigenic effect of LH/hCG. Further investigations are needed to confirm the role of androgens in the control of LC proliferation.

Curiously, the very high concentrations of circulating hCG were unable to induce even hyperplasia in adult LCs. In recent literature, the tumorigenic properties of elevated LH/hCG on LCs have been associated with some additional or synergistic factors, for example, inhibin-$\alpha$-deficiency, oncogene overexpression or decreased androgen action (Kumar *et al*., 1996). To our knowledge, this is the first report showing that increased LH/hCG alone promotes tumorigenesis, without additional contributing factors or alterations. Another important finding is that only the fetal LCs responded to hCG stimulation by adenomatous transformation. This response could be related to the numerous functional differences observed between fetal and adult LCs, such as the lack of LHR downregulation and steroidogenic desensitization in response to high LH/hCG stimulation in the former cells (Huhtaniemi *et al*., 1982, 1985b; Pakarinen *et al*., 1990).

In summary, the hCG-overexpresing TG mouse model studied here offers a good tool to investigate the effects of chronically elevated gonadotropin levels *in vivo*. The current findings demonstrate for the first time that increased LH/hCG stimulation alone is tumorigenic in male mice, even though this effect was limited to the fetal LC population. Since a genetically modified mouse model for activating *Lhcgr* mutations is not available, the hCG+ males can be used to evaluate the effects of precocious LHR activation and to understand the molecular pathogenesis underlying LC adenomas.

## Materials and methods

### *TG mice and determination of the onset of puberty*

Production, breedings and genotyping of double TG mice expressing the glycoprotein hormone common $\alpha$ and hCG$\beta$ subunits (hCG+ mice), driven by the ubiquitin C promoter, have been previously described (Rulli *et al*., 2002, 2003). Balanopreputial separation, an external sign of the onset of male puberty in rodents (Korenbrot *et al*., 1977), was monitored daily beginning on day 21 postpartum. The animals were housed in pathogen-free conditions at constant temperature (21°C) under the 12 h light–dark cycle. Tap water and commercial mouse chow were provided *ad libitum*. The animals were handled, and the experiments approved, in accordance to the institutional animal care policies of the University of Turku.

### *Hormone treatment, tissue collection and histological analysis*

WT males (10-days-old) were treated by one intraperitoneal injection of hCG, 600 IU/kg body weight, (Pregnyl, Organon, Oss, The Netherlands), and killed after 24 h. Blood was collected by cardiac puncture, and body and testes weights were recorded. Testes were frozen in liquid nitrogen, or fixed in 4% paraformaldehyde or Bouin's solution. Paraffin sections were stained with Harris' hematoxylin–eosin and used for light microscopy. Testicular sections were photographed by using the IM1000 microscope (Leica, Heerbrugg, Switzerland). LC areas were determined by using Leica IM 1000, version 1.02 software (Leica Microsystems AG, Heerbrugg, Switzerland). The whole testicular sections and LCs were manually outlined and the areas were determined. The volume density of LCs and the largest LC islets were calculated by relating the total or largest continuous LC area to the area of the whole section. The index of total LC volume per testis was calculated by multiplying the volume density by total testis mass, and the density of tissue was regarded as constant, 1 mg/mm$^3$. Three to eight testicular sections from three animals per group were analyzed.

### *Measurements of hormone levels*

Dimeric hCG levels were measured by immunofluorometric assay, using the Delfia hCG kit (Perkin-Elmer-Wallac, Turku, Finland), according to the manufacturer's instructions. The sensitivity of the assay was 0.5 IU/l. For measuring intratesticular and serum T, both the sera and testicular homogenates were extracted twice with 2 ml of diethyl ether, followed by conventional RIA (Huhtaniemi *et al*., 1985a).

### *Immunohistochemistry*

Sections (5 $\mu$m thick) of the testes fixed in paraformaldehyde were used. The following primary antibodies (dilutions in parentheses) were used: polyclonal rabbit anti-human AR (1 : 1000), (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); polyclonal rabbit anti-mouse hydroxysteroid dehydrogenase-1, delta$^5$-3-beta (HSD3B1) antibody (1 : 2000 in PBS) (kindly donated by Dr Anita H Payne, Stanford University, CA, USA); mouse monoclonal PCNA antibody (1 : 500) (NovoCastra, Newcastle, UK). For visualizing positive cells, the Vectastain Elite kit (Vector Laboratories Inc., Burlingame, CA, USA) was used according to the manufacturer's instructions.

**Table 1** Function, GeneBank accession number, primer sequences and annealing temperatures for the genes whose transcripts were measured by quantitative RT–PCR

| *Gene* | *Function* | *GeneBank* | *Primers (5′→3′)* | *Annealing* T (°C) |
|---|---|---|---|---|
| *Cytoplasmic actin beta (Actb)* | Housekeeping | NM_007393 | F:CGTGGGCCGCCCTAGGCACCA<br>R:TTGGGCTTAGGGTTCAGGGGG | 54 |
| *Thrombospondin (Thbs2)* | Fetal Leydig cell marker | NM_11581 | F:GCAGGATGTACGTGGCCAAA<br>R:GAGATCTGTGGTGATGTGAG | 54 |
| *Hydroxysteroid dehydrogenase-6, delta$^5$-3-beta (Hsd3b6)* | Adult Leydig cell marker | NM_013821 | F:GGAGGAGATCAGGGTCCTGG<br>R:TCTAGGATGGTCTGCCTGGG | 59 |
| *Prostaglandin D synthase (Ptgds)* | Adult Leydig cell marker | AB006361 | F:AGTGGTAGCCCCCTCCACAG<br>R:GAGTGGATGCTGCCCGAGTG | 61 |
| *Luteinizing hormone receptor (Lhcgr)* | Steroidogenesis | NM_013582 | F:TTGCCGAAGAAAGAACAGAAT<br>R:AGCCAAATCAACACCCTAAG | 54 |
| *Steroidogenic acute regulatory protein (Star)* | Steroidogenesis | NM_011485 | F:CAGGGAGAGGTGGCTATGCA<br>R:CCGTGTCTTTTCCAATCCTCTG | 56 |
| *Cytochrome P450, family 11, subfamily a, polypeptide 1 (P450scc)* | Steroidogenesis | NM_019779 | F:AGATCCCTTCCCCTGGCGACAATG<br>R:CGCATGAGAAGAGTATCGACGCATC | 64 |
| *Hydroxysteroid dehydrogenase-1, delta$^5$-3-beta (Hsd3b1)* | Steroidogenesis | NM_008293 | F:CAGGAGCAGGAGGGTTTGGT<br>R:GTGGCCATTCAGGACGAT | 54 |
| *Cytochrome P450, family 17, subfamily a, polypeptide 1 (P450c17)* | Steroidogenesis | NM_007809 | F:GGCCCCAGATGGTGACTCT<br>R:GGACTCCCCGTCGTATGTAA | 54 |
| *Hydroxysteroid (17-beta) dehydrogenase 3 (Hsd17b3)* | Steroidogenesis | NM_008291 | F:ATCAATGGGACAATGGGCAG<br>R:TCTAAGCCTTCAAGGTGTTC | 55 |

*Electron microscopy*

Pieces of testes of 10-day-old WT and hCG+ males, cut into pieces of approximately 1 $mm^3$, were first fixed in 5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.16 mol/L s-collidine buffer (pH 7.4) and postfixed in potassium ferrocyanide-osmium tetroxide. The pieces were embedded in epoxy resin (Glycidether 100, Merck, Darmstadt, Germany) and cut into sections as described earlier (Sundstrom *et al*., 1999). For light microscopic survey, 1-$\mu$m-thick sections were stained with 0.5% toluidine blue. The sections for electron microscopy were stained with 5% uranyl acetate and 5% lead citrate in Ultrostainer (Leica Corp., Wien, Austria) and examined in JEM 1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

*RNA isolation and quantitative RT–PCR*

Total testis RNA was extracted by Rneasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Prior to quantitative RT–PCR, all samples were treated by deoxyribonuclease I (Invitrogen, Carlsbad, CA, USA). Expression patterns of the genes presented in Table 1 were determined. The function, primer pairs, annealing temperatures and the Gene Bank Accession numbers are also listed in Table 1. Quantitative RT–PCR analysis was carried out by using the DNA Engine Opticon System (MJ Research Inc., Waltham, MA, USA) with continuous fluorescence detection. RT–PCR reactions were carried out using the Quantitect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA (100 ng) were used in all reactions. Standards and samples were run in triplicate, and three individual samples were analyzed per experimental group. The level of cytoplasmic $\beta$-actin mRNA was used to normalize the expression of the other mRNA species.

*Statistical analysis*

SigmaStat software (SigmaStat 2.03 for Windows; SPSS Inc., Chicago, IL, USA) was used for one-way analysis of variances and the differences between individual means were assessed by Student–Newman–Keuls test. *T*-tests were run for the comparison of two groups. The level of statistical significance was set as $P<0.05$, and all values are presented as mean $\pm$ s.e.m.

**Acknowledgements**
We thank Ms J Lahtinen and Ms T Laiho for their skilful technical assistance. This work was supported by grants from the Academy of Finland, The Finnish Cancer Society, the Sigrid Jusélius Foundation, the Turku Graduate School for Biomedical Sciences and the Wellcome Trust.

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