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Human Chorionic Gonadotropin (hCG) Up-Regulates *wnt5b* and *wnt7b* in the Mammary Gland, and hCGβ Transgenic Female Mice Present with Mammary Gland Tumors Exhibiting Characteristics of the Wnt/β-Catenin Pathway Activation

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Transgenic (TG) mice expressing human chorionic gonadotropin (hCG) β-subunit under the ubiquitin C promoter, presenting with a moderately elevated level of LH/hCG bioactivity develop multiple neoplasms secondary to the endocrine abnormalities, including mammary gland tumors after the age of 9 months. The increased levels of circulating estradiol, progesterone, and prolactin of the TG females after puberty boost the lobuloalveolar development in the mammary gland resulting ultimately in the formation of estrogen and progesterone receptor-negative, malignant tumors. These tumors have a similar histopathology with those observed in TG mice with activated wnt/β-catenin pathway, showing increased expression of β-catenin, also a common finding in human breast tumors. Transdifferentiation is observed in mammary tumors of the hCGβ TG mice, accompanied by abnormal expression of the *Wnt* genes in the tumorous and nontumorous mammary gland tissue. Specifically we found increased expression of *Wnt5b* in the TG mammary glands at the age of 3 months and up-regulation of *Wnt7b* and *-5b* in the subsequently appearing tumors. Importantly, hCG was found to up-regulate these *wnt* ligands in mouse mammary gland, independent of the changes in ovarian steroidogenesis. Thus, the hCGβ-overexpressing TG mice represent a novel model that links enhanced hCG action to dysregulated wnt signaling in the mammary gland, resulting in β-catenin-stabilizing mammary tumorigenesis. The novel finding of hCG up-regulating *wnt7b* and *wnt5b* could contribute to pregnancy-induced breast cancer in humans. (*Endocrinology* 148: 3694–3703, 2007)

HUMAN CHORIONIC GONADOTROPIN (hCG), secreted normally by the placenta, is a glycoprotein that is highly similar to LH but with a longer half-life than LH and binds to the LH receptor. Women are exposed to hCG during pregnancies and to LH throughout their reproductive years in a cyclic manner, but in menopause, a stable 4- to 5-fold increase in LH concentration is observed (1). LH and LH/hCG receptor is expressed in human breast cancer (2, 3), but studies on LH receptor or LH concentration and the risk of breast cancer have been largely controversial (4–10).

The wnt glycoproteins are highly conserved, secreted short-distance signaling molecules that bind to receptors of the Frizzeled and lipoprotein receptor-related protein families and induce β-catenin stabilization (canonical pathway) or act through the Wnt/Ca^{2+} or Wnt/polarity pathways (11). The intracellular wnt effector, β-catenin, is mutated or its degradation pathway is altered in many pathological conditions, including several types of cancers (12). β-Catenin is also found stabilized in human breast cancer (13, 14), but its mutations have not been found in this malignancy. However, deregulated *WNT* expression and epigenetic silencing of Wnt-pathway inhibitors Wif-1 and Sfrp1 have been found in human breast malignancies (15–19), and an autocrine mechanism resulting in constitutive activation of the wnt pathway in human breast cancer cells has been identified (20). Ectopic wnt signaling induces the accumulation of mammary progenitor cells and increases susceptibility to tumorigenesis (21).

*Wnt*s are involved in the normal development of the mammary gland (22, 23) and some of them are dependent on ovarian steroids. *Wnt* expression in the mammary gland is spatiotemporally regulated and altered in the induction of different developmental stages (23, 24). Classically, *Wnt*s encode transforming and nontransforming wnt proteins classified according to their potential to transform mammary epithelial cells *in vitro* (25). However, the classification does not necessarily correlate with transforming potential *in vivo* (26).

Studies on Wnt1- and Wnt10b-expressing transgenic (TG) mice have confirmed the role of these effectors as potent growth stimulators for the mammary gland epithelium (27, 28). Wnt4 has been shown to mediate progesterone receptor (Pgr) actions in the mammary gland (29). *Wnt2*, *-5a*, *-7b* and *-10b* are expressed during ductal development and *Wnt4*, *-5b*,

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Abbreviations: Esr1, Estrogen receptor-α; hCG, human chorionic gonadotropin; hCGβ, hCG β-subunit; IHC, immunohistochemistry; MIN, mammary intraepithelial neoplasia; P, progesterone; Pgr, progesterone receptor; q, quantitative; SMA, smooth muscle actin; TEB, terminal end bud; TG, transgenic; WT, wild type.

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and -6 during pregnancy. Expression of the wnt proteins is down-regulated during lactation, whereas *Wnt2*, *5a*, *5b*, and *7b* are reinduced upon involution (23, 24). The precise nature of hormonal regulation of the *Wnt*s still remains poorly understood. It has been shown that β-catenin functionally interacts with estrogen receptor-α (Esr1) (30) and that estrogen up-regulates *Wnt4* and *-5a* expression in an Esr1-independent manner in the mouse uterus (31). Recently *Wnt7b* mRNA was described to be enriched in terminal end buds (TEBs) in the mammary gland, and *Wnt5b* has been detected in both TEBs and mature ducts (32).

Several TG mouse models with activated Wnt/β-catenin pathway have been created. These include mouse mammary tumor virus reverse transcriptase-long-terminal repeat-driven ligands Wnt1, Wnt10b (27), and activated β-catenin mutants (33–35). These models present with similar mammary gland tumor histopathology, as do the TG mouse models stabilizing β-catenin by affecting its destruction complex (Apc^{min} and dominant-negative form of GSK3β, the dnGSK3β mice) (36, 37). Yet another mouse model recently described, Epimorphin/syntaxin-2, shares this phenotype (38). One of the most striking features of the wnt pathway tumors is the transdifferentiation of the mammary epithelium into epidermal structures and formation of pilar tumors (36, 39, 40).

We previously described the generation of the TG mouse model overexpressing hCG β-subunit (hCGβ) under the ubiquitin C promoter (41). Dimerization of the TG hCGβ subunit with endogenous α-subunit in the pituitary gland results in 20- to 30-fold elevated levels of LH/hCG bioactivity in circulation. In short, the mice develop precocious puberty and have elevated levels of estrogen during puberty. This is followed by marked ovarian luteinization, formation of pituitary prolactinomas, and subsequent progressive increase in serum levels of progesterone and prolactin. The hCGβ-expressing female mice are obese and infertile and develop mammary gland tumors from the age of 9 months onward, with 90% penetrance at the age of 12 months. All the gross phenotypes of these mice are initially directly or indirectly due to alterations of ovarian function because they all are abolished by ovariectomy at the age of 1–2 months.

In this study, we demonstrate that the endocrinologically induced mammary gland tumors in the hCGβ TG mice are histologically similar to those induced by activation of the wnt pathway and demonstrate a link between hCG action and wnt dysregulation in the mammary gland.

Materials and Methods

Transgenic mice and ovariectomy

Generation of the hCGβ TG mice has been previously described (41). The animals were housed in a specific pathogen-free environment under controlled conditions (12-h light, 12-h dark cycle; temperature 21 ± 1 C) and provided with tap water and commercial mouse chow *ad libitum*. All mice were produced and handled in accordance to the institutional animal care policies of the University of Turku. Genotyping of the hCGβ mice was carried out from tissue biopsies by PCR using primers and conditions described previously (41). The ovaries were removed from 3-, 5-, and 7-month-old TG and wild-type (WT) mice (n = 26) under isofluran Baxter, inhaled; Baxter, Deerfield, IN) anesthesia and buprenorphine (Temgesic, 3–5 μg/mouse sc; Schering-Plough, Pennyworth, NJ) analgesia from incision to the back.

hCG injections

Twelve-week-old FVB/N females were ovariectomized as above, and a pellet releasing estradiol (~0,19 mg/kg/d) and progesterone (P; ~4.8 mg/kg·d; Innovative Research of America, Sarasota, FL) or placebo was inserted under the skin. After 10 d, the animals were injected with 20 IU of recombinant hCG sc (provided by A. F. Parlow, National Hormone and Peptide Program, Torrance, CA) or saline for 5 consecutive days and then killed.

Histological and morphological analysis of the mammary gland

For histological studies, the fourth left inguinal mammary gland from the TG females and litter mates or tumor foci from the TG mice were dissected out at different ages between 1 and 12 months of age and fixed overnight in 4% paraformaldehyde. The tissues were dehydrated, embedded in paraffin, and cut into 5-μm-thick sections. Sections were stained with hematoxylin and eosin. For whole-mount preparations, the fourth right inguinal mammary gland from TG and WT litter mates was removed, spread on a glass slide, and fixed overnight with Carnoy's fixative (acetic acid-ethanol). The slides were then washed with ethanol and distilled water, stained with carmine-alum for 1–2 d, dehydrated in a series of ethanol and xylene, and finally mounted in Permount.

Immunohistochemistry

Five-micrometer-thick paraffin sections were mounted on slides, deparaffinized, and rehydrated in a series of xylene and ethanol. Antigen retrieval was performed by boiling the slides in antigen unmasking solution (Vector Laboratories, Inc., Burlingame, CA) for 15 min in a microwave oven (850 W) and keeping them for 20 min in hot solution. After washing with PBS + 0.1% Tween 20, the slides were treated with 1% H_2O_2 for 15 min. The sections were incubated in PBS + 3% BSA overnight at + 4 C with the antibodies against one of the following antigens: 1) estrogen receptor 1, dilution 1:200 (clone 1D5, Dako A/S, Glostrup, Denmark); 2) progesterone receptor, dilution 1:400 (Dako, A0098); 3) α-smooth muscle actin, dilution 1:1000 (clone 1A4, Sigma, St. Louis, MO); 4 and 5) keratin 5 and 6, dilution 1:200 (BabCo, Richmond, CA); and 6) β-catenin, dilution 1:100 (Transduction Laboratories Inc., Lexington, KY). As the secondary antibody, Dako EnVision antimouse or antirabbit secondary for primary antibodies 1–3 were used at room temperature for 30 min, followed by visualization with 3′3′-diaminobenzidine (Dako). Finally, the slides were stained with Gill's hematoxylin followed by dehydration and mounting. For antibodies 4–6 (Molecular Probes, Eugene, OR), AlexaFluor 594 antimouse and antirabbit secondary antibodies were used (1:400 dilution at 37 C for 1 h) followed by staining with 4′,6′-diamino-2-phenylindole for 15 min. Fluorescent slides were mounted in DakoCytomation fluorescent mounting medium. For fluorescent pictures, pseudocolors were created by Adobe Photoshop CS (San Jose, CA).

RNA isolation and quantitative (q) RT-PCR

The fourth mammary gland or mammary gland tumor was excised and snap frozen in liquid nitrogen. Total RNA was extracted with a lipid tissue minikit (QIAGEN, Valencia, CA) and treated with amplification grade DNase I (Invitrogen, Carlsbad, CA). For cDNA synthesis and subsequent qRT-PCR, the SYBR Green DyNAmo HS qRT-PCR kit (Finnzymes, Espoo, Finland) was used. For each qPCR, an aliquot of cDNA (diluted 1:20) was used except for the housekeeping genes in which 1:40 dilution was used. qRT-PCR analysis was performed using the DNA Engine Opticon system (MJ Research, Inc., Waltham, MA) with continuous fluorescent detection. Samples and standards were run in triplicates. The two housekeeping genes (L19 and cyclophilin) were analyzed to normalize the results between the samples. All primers used (Table 1) were intron spanning. The presence of a single, right-sized PCR product was confirmed by running the samples on 2% agarose gels.

Protein extraction and Western blotting

Mammary gland or tumor pieces were homogenized in radioimmunoprecipitation assay buffer with proteinase inhibitors and homoge-

TABLE 1. Primers used in the study

Primer	Primer sequence	Product size (bp)	$T_{annealing}$ (C)
L19 sense	CTGAAGGTCAAAGGGAATGTG	195	58
L19 anti	GGACAGAGTCTTGATGATCTC		
Cyclophilin 1	CATCCTAAAGCATACAGGTCCTG	165	58
Cyclophilin 2	TCCATGGCTTCCACAATGTT		
Wnt 4 sense	AACGGAACCTTGAGGTGATG	244	58
Wnt 4 anti	TCACAGCCACACTTCTCCAG		
Wnt 5b sense	TGGAGACAACGTGGAGTACG	166	60
Wnt 5b anti	GGCGACATCAGCCATCTTAT		
Wnt 7b sense	TACTACAACCAGGCGGAAGG	233	60
Wnt 7b anti	GTGGTCCAGCAAGTTTTGGT		
Wnt 6 sense	TTCGGGGATGAGAAGTCAAG	151	58
Wnt 6 anti	AAAGCCCATGGCACTTACAC		
β-Casein sens	GGACTTGACAGCCATGAAGG	197	60
β-Casein anti	CTTTAGCCTGGAGCACATCC		
Autotaxin sens	TCGAGGGCGAGAGAAGTTTA	153	60
Autotaxin anti	AGGGAAAGCCACTGAAGGAT		
Axin2 sense	CAAGACCAAGGAGGAGATCG	151	60
Axin2 anti	ACCTCTGCTGCCACAAAACT		
Cyclin D1 sens	TCTCCTGCTACCGCACAAC	168	59
Cyclin D1 anti	TTCCTCCACTTCCCCCTC		
Hig2 sense	ACGACCTGGTGTGACTGTGA	146	60
Hig2 anti	AACATGATGCCCAGCACATA		

nates were centrifuged twice followed by protein concentration measuring using the Bradford method. Twenty-five micrograms of protein were loaded on gel, and Western blotting was carried out using standard procedures. Antibody for *wnt7b* (Santa Cruz Biotechnology, Santa Cruz, CA) was used in 1:1000 concentration and actin (ICN Biomedicals, Aurora, OH) in 1:20,000 concentration. Secondary antibodies used were antigoat IgG-horseradish peroxidase (Santa Cruz Biotechnology) and antimouse IgG-horseradish peroxidase (Amersham Biosciences, Amersham, UK). The up-regulation of wnt7b protein in the TG tumors was confirmed by using six TG tumor samples, five TG nontumor samples, and six WT samples. Western blot analyses were quantified with the ImageJ program (http://rsb.info.nih.gov/ij/index.html) and normalized values were subjected to statistical analysis.

Statistical analysis

SigmaStat 3.1 (SPSS Inc., Chicago, IL) was used for the *t* test or the Mann-Whitney rank sum test. The limit of statistical significance was set at $P < 0.05$. The results are presented as mean ± SEM.

Results

Mammary glands of virgin hCGβ females show hyperplastic precursor lesions at 3 months of age and progression to wnt-type tumors

Whereas WT littermates presented with sleek ductal appearance at all ages (Fig. 1a), increased lateral side branching and budding was observed in mammary gland ducts of the TG females from the age of 1 month as a response to precocious hormonal activity (Fig. 1b). Budding was evident from primary and secondary ducts and hyperbranching was detected (Fig. 1, c and d). At 3 months of age, the TG mammary gland structurally resembled mid- to late-pregnancy mammary glands with apparent alveolar development.

Histological sections also demonstrated increased branching of the hCGβ mammary glands at the age of 1–2 months, compared with the WT (Fig. 2, a and b). The lobuloalveolar structures increasingly replaced the fat pad, but the organization of alveoli remained irregular with secretion trapped inside the glands. In contrast to diffuse alveolar hyperplasia, the first atypical focal lesions with no secretory activity were present from month 3 onward. These low- and high-grade

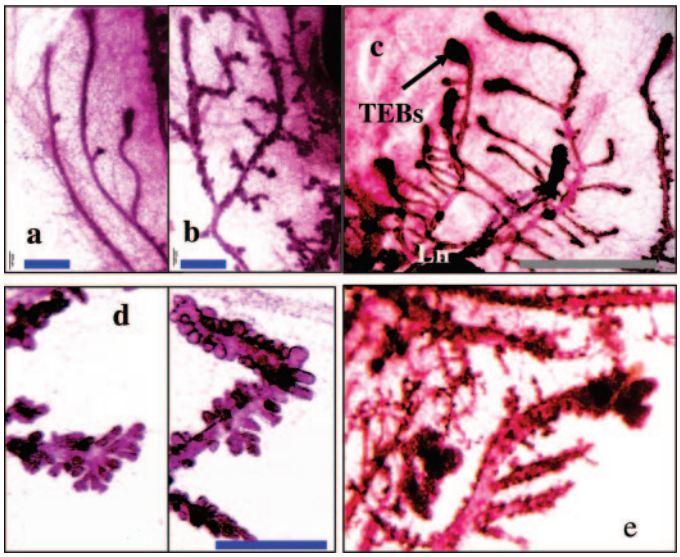

FIG. 1. Whole-mount analysis revealing precocious development, alveolar budding, and early pathological growth pattern of the TG mammary glands and irreversible changes in ovariectomized mice. a, One-month-old WT. b, TG mouse. c, One-month-old TG mouse with several progressing TEBs and abnormal branching pattern. d, One-month-old TG mouse with hyperbranching ducts and alveolar budding. e, Six-month-old TG mice, ovariectomized 1 month previously, showing ovarian-independent hyperplasia. Ln, Lymph node. *Gray bar*, 2 mm; *blue bar*, 0.5 mm.

foci of mammary intraepithelial neoplasias (MINs) presented with several layers of epithelium, filling the lumen with hyperplastic cells and loss of cell polarity (Fig. 2c). These masses of nonlactating epithelium became more abundant over time, and progressive keratinization was seen histologically in these areas at and beyond 5 months of age (Fig. 2d). The TG mammary glands expressed mRNA of the milk protein β-casein, but the secretory activity of the tumors was suppressed. Relative mRNA expression of β-casein is shown in Table 2.

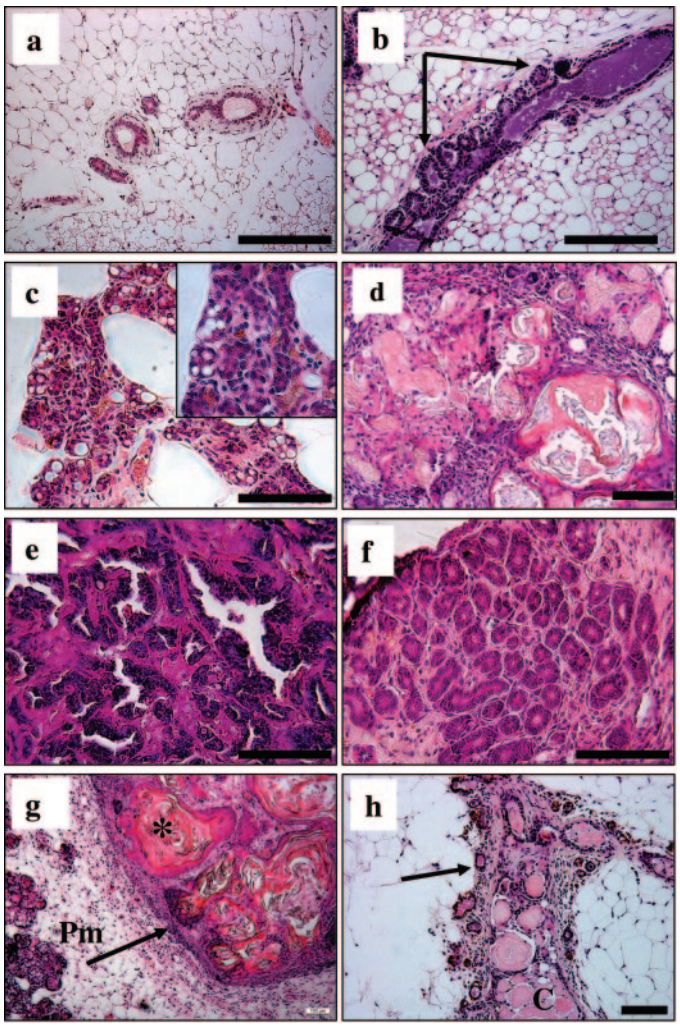

FIG. 2. Hematoxylin and eosin staining showing structural disturbances in mammary gland development of the hCGβ TG mammary glands and progression to tumors that show characteristic histological features of wnt pathway tumors. a, One-month-old WT mouse with normal duct histology. b, One-month-old TG mammary gland showing increased budding from the primary ducts indicated by *arrows*. c, MIN with several layers of epithelial cells in a 5-month-old TG mouse. d, Five-month-old TG with keratin nodule. e, A TG mammary gland tumor with papillary pattern. f, A TG tumor with glandular differentiation. g, A TG pilar tumor with keratin swirls (*asterisk*), inflammatory infiltrate, and pushing margin (Pm). H, Six-month-old TG mouse, ovariectomized 1 month previously, showing cystic structure (C) and persistent alveoli embedded in abnormal, fibrotic tissue. *Bar*, 100 μm.

Eventually, between 9 and 12 months, the hCGβ females developed aggressive mammary adenocarcinomas. Papillary (37%) (Fig. 2e), pilar (37%) (Fig. 2g), glandular (19%) (Fig. 2f), and acinar (7%) were the main subtypes in 27 independent tumor samples studied, but most often tumor areas presented with a combinations of these subtypes together with myoepithelial differentiation and abundant stroma. The tumor growth-induced inflammatory responses and pushing margins were observed at the growing borders of the tumors (Fig. 2g). Aggressive growth was apparent with rapidly growing tumor mass, and necrosis was frequently found in the center of the tumors. Keratinizing nodules preceded by ghost cell formation were often present in the tumors and precursor lesions (Fig. 2g). Based on histological criteria, solid tumors typical to ErbB/Ras TG pathways were never observed.

TABLE 2. Relative β-casein expression in the mammary gland

	Fold	*P* value
WT 3 months	1	
TG 3 months	40	0.002[a]
Tumor	10	0.026[a]
Lactation	163	

[a] Statistical significance.

The hCGβ mammary glands present with abnormal, inconsistent, and scattered myoepithelium layer

α-Smooth muscle actin (SMA) was used as the myoepithelial marker. It is normally expressed in the monolayer of the myoepithelium lining the ducts and alveoli, as shown in the mammary glands of WT mice (Fig. 3a). In TG mice, continuous α-SMA staining was detected around the secretory alveoli (Fig. 3c), but staining in the hyperplastic areas was either totally absent (Fig. 3b), increased, or inconsistently scattered (Fig. 3e). α-SMA staining also often lined the aberrant hyperplastic ductal structures (Fig. 3d). Myoepithelial differentiation was consistently found in tumor areas, which is a typical feature of tumors of the wnt pathway (Fig. 3f).

Mammary epithelium in the hCGβ-TG mice is Esr1 negative after 3 months of age but retains Pgr-positive foci

At 2 months of age, the Esr1 staining pattern of the TG mammary glands was similar to that of WT littermates (Fig. 3g). From the age of 3 months onward, Esr1 could not be detected by immunohistochemistry (IHC) in the TG mice (Fig. 3h) in contrast to WT mammary glands. Developing tumors were also Esr1 negative (Fig. 3i). Pgr was normally expressed in the TG mammary gland at 2 months of age (Fig. 3j), but thereafter the secreting alveolar structures did not express Pgr (Fig. 3k). However, Pgr expression could be detected in some of the nonsecreting MIN foci, and occasional Pgr positive areas were observed even in tumors, although most of the tumor cells were Pgr negative (Fig. 3l). No androgen receptor expression was found in the TG mammary glands or mammary tumors (data not shown).

Transdifferentiation of the mammary glands of the hCGβ-TG mice

Keratin 6 is normally expressed in the epidermis and some TEB structures but not in the mature mammary gland, as demonstrated by absent staining in WT controls (Fig. 4a). In TG mice, keratin 6 was expressed in single cells or small groups of cells at 3–5 months of age in 11 of 12 samples studied, well before the formation of apparent keratin nodules or tumors (Fig. 4, b and c). Keratin 6 was expressed as a uniform layer in all of the tumors studied by IHC in the keratin-forming tumor foci in the cytoplasms of the cells surrounding the acellular keratin core (Fig. 4, d and e). Thus, the tumors showed transdifferentiation into epidermal and pilar structures.

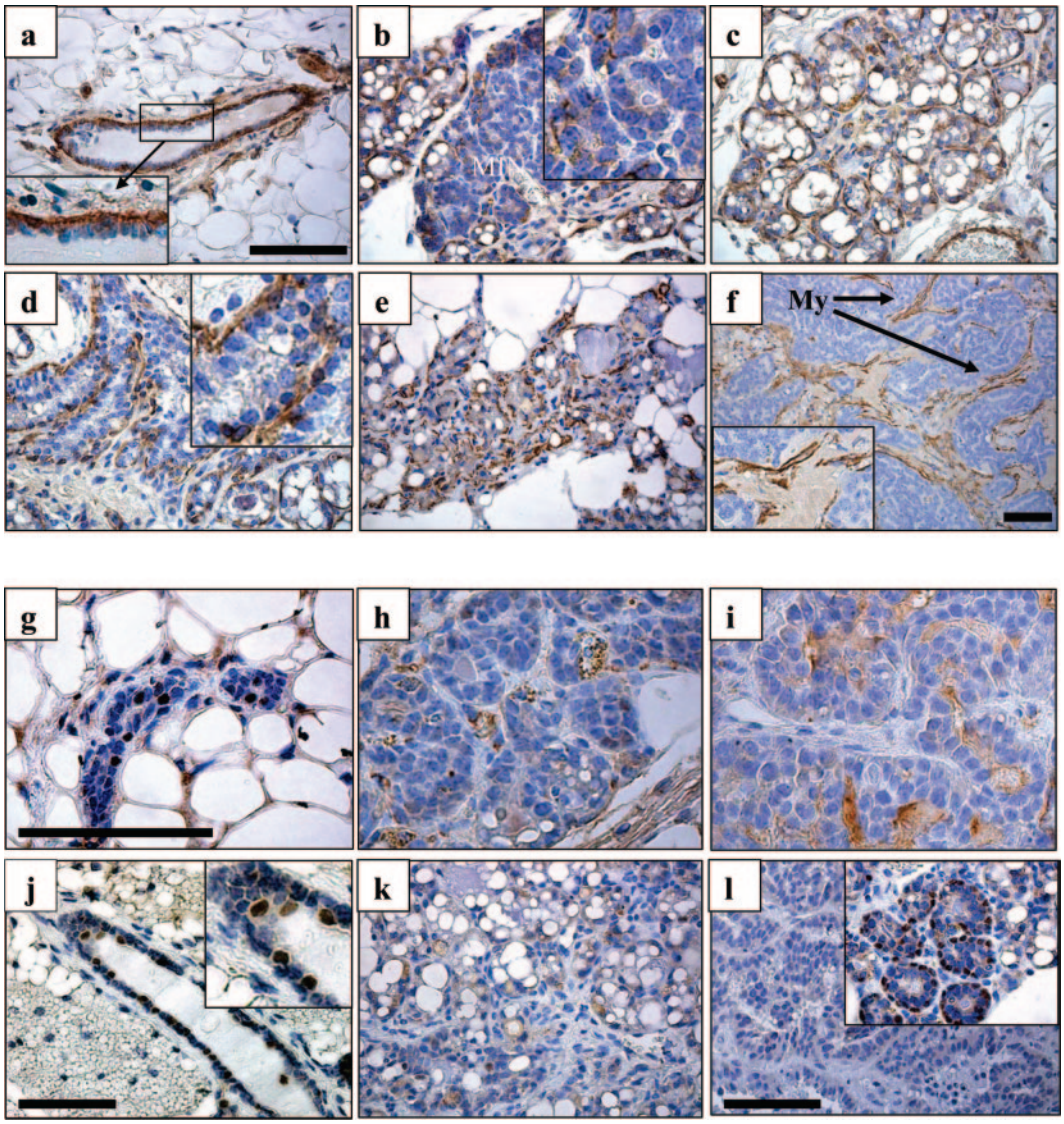

FIG. 3. Abnormal myoepithelial differentiation is observed (α-SMA, a–f) in hCGβ mammary gland that becomes Esr1 and Pgr negative but does retain some Pgr-positive foci. a, Normal periductal SMA staining in 3-month-old WT mammary gland, a detail in the *inset* showing apical side staining. b, A 3-month-old TG specimen with lacking SMA staining in the MIN focus. c, A 5-month-old TG sample with continuous SMA staining around the alveoli. d, A 5-month-old TG sample with staining around the aberrant ducts. e, 9-month-old TG, presenting with hyperplastic myoepithelial differentiation. f, TG tumor with abundant myoepithelial (My) differentiation. g, Esr1 present in WT in contrast to the 3 month-old TG (h) and TG mammary gland tumor (i) (nonspecific background staining). j, Pgr staining in 3-month-old WT mammary gland. Absent Pgr staining is shown in 3-month-old TG mammary gland (k) and TG tumor (l). *Inset*, Pgr + focus in 5-month-old TG mammary gland. *Bar*, 50 μm.

Keratin 5 expression was found in the myoepithelium of the WT mice (Fig. 4g, *inset*) and detected in TG mice in the same manner as SMA staining, scattered and disorganized and as a continuous lining in alveoli of the TG mice (Fig. 4, g and h). In the tumors, keratin 5 was expressed in an organized pattern lining the squamous metaplastic nodules in one or several cell layers (Fig. 4, i and j).

β-Catenin is stabilized in hyperplastic areas

By immunofluorescence, β-catenin staining was observed in the cell membranes of the TG and WT mice in which it is bound in catenin-cadherin complexes (Fig. 5, a and b). However, increased staining of β-catenin could be identified in the cytoplasm of the hyperplastic areas of 3-month-old TG mice (Fig. 5c), whereas the secreting alveoli did not stain strongly for this protein (data not shown). At later stages, strong β-catenin staining was observed in areas of squamous metaplasia (Fig. 5, d and e).

Late ovariectomy is not able to fully rescue the mammary gland phenotype

We performed ovariectomies for 3-, 5-, and 7-month-old TG and WT mice to clarify the ovarian dependency of the mammary gland changes. After 1 month of recovery, the mice were killed. In whole-mount sections, alveolar hyperplasia was diminished in all cases, but two thirds of the TG

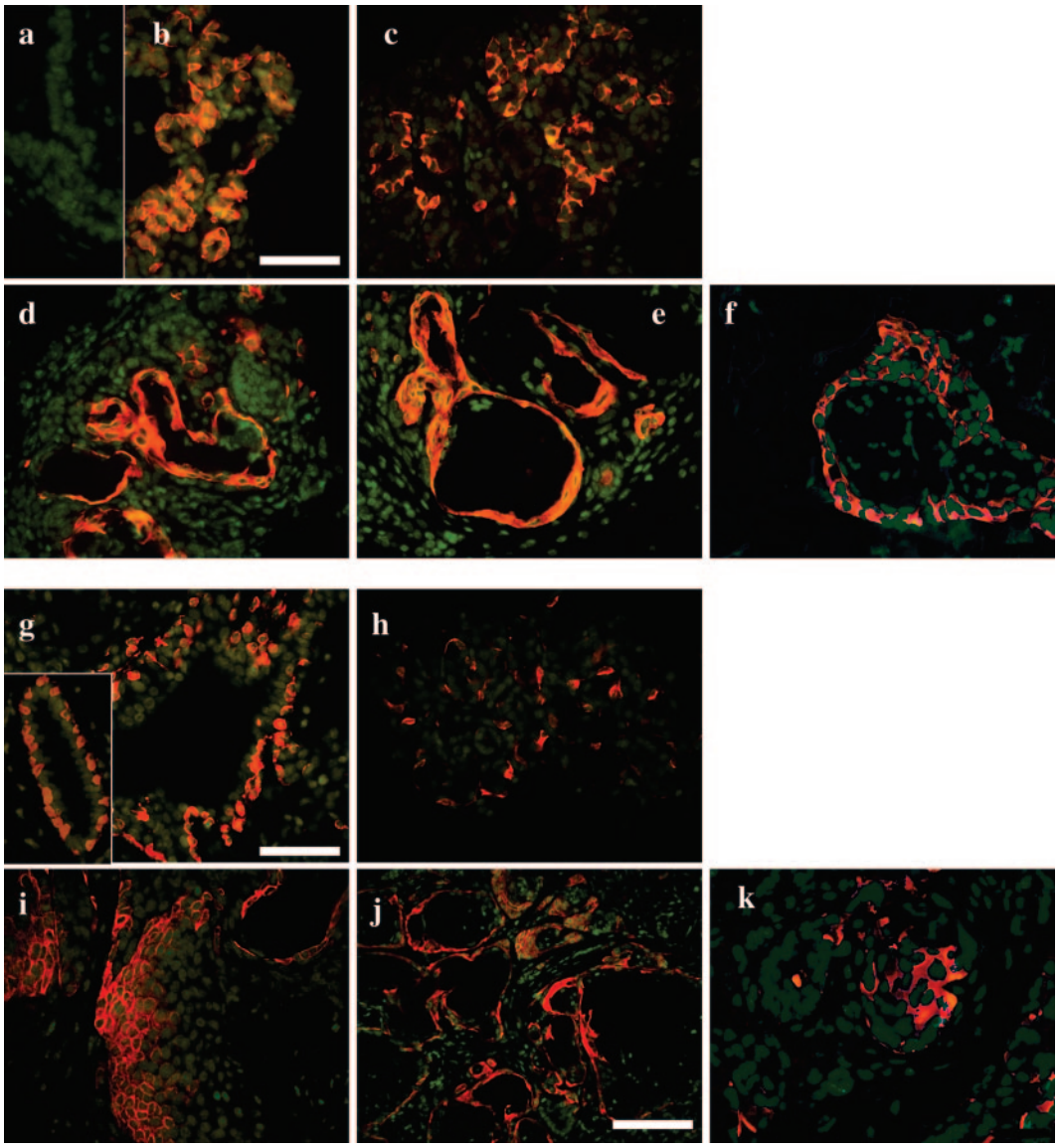

FIG. 4. hCGβ mammary gland expressed epidermal markers keratin 5 and 6 from 3 months of age onward. Pilar tumors have a continuous keratin margin. a–e, Keratin 6. g–j, Keratin 5. a, Absent staining in WT mammary gland. b, Three-month-old TG with abnormal abundant keratin 6 staining. c, Three-month-old TG with scattered keratin 6 staining. d, Five-month-old TG mammary gland with pilar differentiation and keratin 6 staining lining the periphery of the tumor. e, A TG mammary gland tumor with typical staining pattern of keratin 6-expressing cells of the wnt pathway tumors. f, Persistent keratin 6 expression in 6-month-old TG female, ovariectomized 1 month previously. g, Three-month-old TG with aberrant keratin 5 staining pattern, normal WT staining shown in the *inset*. h, Five-month-old TG with scattered keratin 5 staining. i, TG mammary gland tumor showing strong keratin 5 staining in several layers. j, Several pilar tumor swirls with peripheral keratin 5 expression. k, Multiple layers of keratin 5-expressing cells in 6-month-old mouse ovariectomized 1 month previously. *Bar*, 50 μm; 100 μm (j).

mice in all groups presented with hyperplastic nodules often together with multiple cystic structures in the mammary gland (Fig. 1e). Histological staining confirmed the presence of alveoli and collapsed structures filled with cellular debris (Fig. 2h). Furthermore, abundant fibrosis or epithelial to mesenchymal transformation was observed in histology. We performed keratin 5 and 6 IHC on these sections and observed reduced but persistent expression of keratin 5 and 6 in the TG mammary epithelium in single cells often lining the cysts or small groups (Fig. 4, f and k). However, pilar-type areas with excessive keratinization were absent. The pituitaries of the TG mice were regressed to normal size, suggesting reduced levels of prolactin in serum. Data on ovariectomized mice indicate that these potentially premalignant changes remained in the mammary gland and are ovarian independent. Thus, the damage previously caused by hormones cannot be fully reversed by withdrawal of ovarian hormones and prolactin at later stages.

hCGβ mice show disturbances in mammary gland Wnt expression

We next addressed the question whether any of the *Wnt*s would be up-regulated and potentially responsible for the activation of the canonical wnt pathway leading to the phenotype. We analyzed the mRNA expression of the *Wnt*s

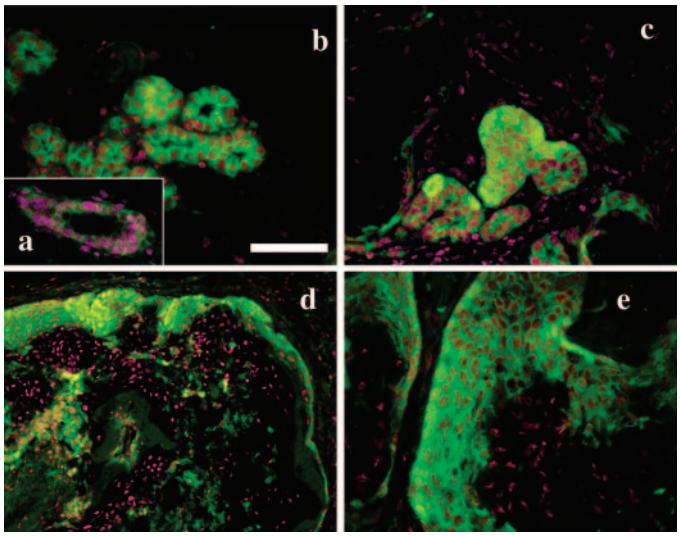

FIG. 5. IHC of β-catenin reveals stabilization of β-catenin in TG mammary gland and tumors. a, Three-month-old WT mammary gland, showing normal β-catenin staining in cell membranes. b, Three-month-old TG, with accumulation of β-catenin into cytoplasm. c, Five-month-old TG, displaying nuclear localization of β-catenin staining. d, TG mammary gland tumor, with β-catenin accumulation in borders of the tumor area. e, β-Catenin staining in TG tumor colocalized with keratin 6 expression (see Fig. 4i). *Bar*, 50 μm (a, b, and d); 100 μm in c and e.

naturally expressed in the mouse mammary gland, including *Wnt2*, *-4*, *-5a*, *-5b*, *-6*, *-7b* and *-10b*. *Wnt5b* was significantly up-regulated, reaching a 5-fold higher expression in TG animals at the age of 3 months, as compared with age-matched WT littermates. The expression was further increased in the TG tumors that expressed 9-fold higher levels of *Wnt5b* than the 3-month-old WT mice (Fig. 6A). *Wnt7b* is normally expressed in TEBs, and the expression is decreased in pregnancy. In our study, *Wnt7b* was up-regulated in the tumors, showing more than 2-fold higher mRNA expression as compared with WT mammary glands. Three-month-old TG mammary glands expressed *Wnt7b* mRNA at level similar to WT controls (Fig. 6A). Protein level of Wnt7b in tumors was increased 4-fold (Fig. 6C), compared with WT mice ($P = 0.002$), whereas its expression was similar in 3-month-old TG and WT mice. *Wnt*6 was not overexpressed in 3-month-old animals. In four of six tumor specimens studied, *Wnt*6 expression was nearly absent (Fig. 6A), but in two samples, it was clearly up-regulated.

Wnt4 was similarly expressed in 3-month-old TG mice and decreased in tumor tissues (Fig. 6A). The other *Wnt*s included in the study, *Wnt2*, *-5a*, and *-10b*, all produced in the stroma, exhibited decreased expression in the tumor samples and similar or decreased expression in 3-month-old TG mammary glands (not shown), a finding that could be attributed to a decreased epithelial/stromal ratio. *Wnt1* expression was not found in any mammary gland. Overall, the expression pattern of *Wnt*s in the hCGβ mammary glands did not resemble the natural expression pattern seen during virgin state, pregnancy, or lactation.

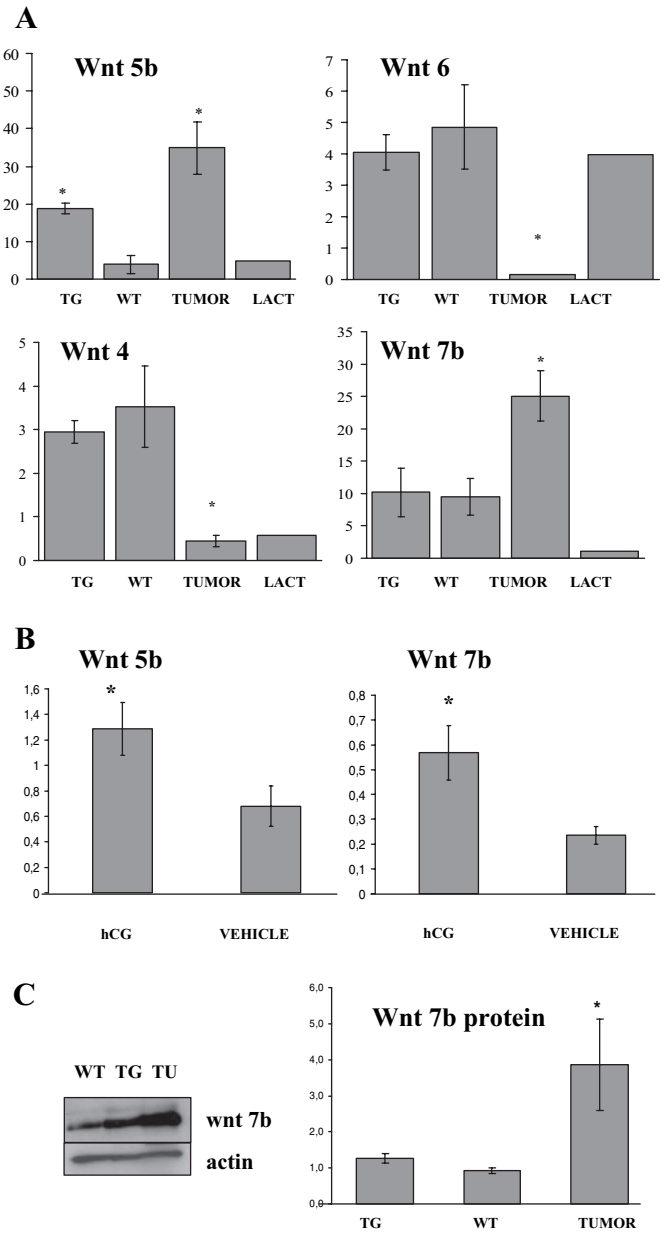

FIG. 6. Quantitative RT-PCR results, indicating the expression of wnt 5b, -6, -4, and -7b in TG mammary glands and tumors, compared with WT mammary gland expression (A) and in WT mammary gland in response to hCG or vehicle stimulation (B) (mean ± SEM). Protein level of wnt7b correlates with mRNA expression (C). TG, hCGβ mice (n = 6); WT, wild-type mice (n = 4) at 3 months of age; TUMOR, hCGβ mammary gland tumors (n = 6); LACT, expression of WT mammary gland in early lactation (n = 2) shown for comparison. *, Statistically significant change, compared with the WT ($P < 0.05$).

Wnt5b, Wnt7b, and classical wnt targets are up-regulated in the mammary gland in response to hCG stimulation without ovarian contribution

To elucidate the cause of up-regulation of *wnt5b* and *wnt7b* in the TG mice, we injected ovariectomized and estradiol- and P-treated WT mice with recombinant hCG or saline. Interestingly, we observed 2- and 2.5-fold up-regulation in *wnt5b* and *wnt7b* expression in the mammary gland, respec-

tively (Fig. 6B), without apparent histological changes. This suggests that hCG is directly responsible for regulation of *wnt5b* and *wnt7b* in the mouse mammary gland. However, it is possible that effects in the mammary gland are secondary changes due to other unknown systemic alterations because LH receptor expression in the mammary gland is low although present. Together with the induced expression of *wnt7b* and *wnt5b*, we observed a significant up-regulation of various well-characterized direct *wnt* target genes, such as autotaxin, axin2, cyclin D1, and Hig2 (Table 3). Injections with prolactin did not affect the expression of these wnts (data not shown). *Wnt5b* and *wnt7b* expression in the uterus was unchanged by hCG administration.

Discussion

Pregnancy causes breast epithelium expansion and differentiation by multiple hormonal pathways. In the present study, we identified two novel factors that are regulated by hCG in the mammary gland. These factors, *wnt5b* and *7b*, are also associated with TEB (and thus stem or progenitor cell) regulation by a yet-uncharacterized mechanism. Furthermore, we have shown that hCG administration up-regulates several wnt target genes in the mammary gland. In line with this, we showed that the hCGβ TG mice share a mammary gland tumor phenotype that is similar to those of other TG mouse models affecting the canonical β-catenin pathway. The tumors have areas of stabilized β-catenin, keratin 5 and 6 production, and typical histopathological features that are regarded as pathognomonic to activation of the wnt/β-catenin pathway. In contrast to other TG mouse models studied previously, the phenotype of the hCGβ mice is initially caused by elevated production of estrogen, progesterone, prolactin, and hCG expression by the transgene.

Ovariectomy at 6 wk of age abolishes the mammary gland phenotype at a gross morphology level in the hCGβ mice (41), thus mimicking endocrine induction of carcinogenesis in human breast tumors. Oncogenic damage cannot, however, be fully reversed by ovariectomy from the age of 3 months onward, showing the true ovarian independence of these lesions. In line with this, the subsequently appearing tumors are Esr1 and Pgr negative, which is also more typical for breast cancer associated with pregnancy (42, 43).

The hCGβ mice resemble most closely the TG mice expressing stabilized β-catenin and CK2α with the uniform keratin expression pattern and the extent of transdifferentiation (39). Keratin 6 expression has been demonstrated in the body cells of the terminal end buds (32, 44) and during lobuloalveolar growth of early pregnancy, whereas mature mammary epithelial cells rarely express it (45). Esr1 expression varies in mouse models with activated wnt/β-catenin and depends on the additional oncogenic mutations (46). Esr1 is, however, not definitively needed for wnt-induced tumorigenesis (47).

TABLE 3. Increased Wnt-target mRNA expression after hCG injections

	Fold	SEM	*P* value
Autotaxin	2.7	0.92	0.002[a]
Axin2	1.9	0.24	0.012[a]
Ccnd1	1.2	0.08	0.028[a]
Hig2	1.5	0.05	<0.001[a]

[a] Statistical significance.

Wnt5b and *-6* are up-regulated during pregnancy, secreted from the mammary gland epithelium, and show a similar expression pattern in normal pregnancy (24). The transformation potential of *Wnt5b* in mammary gland cell culture is controversial (25, 48). Furthermore, the ability to stabilize β-catenin is context dependent because classical noncanonical ligand *Wnt5a* has been shown to induce β-catenin stabilization in mammary epithelium (49). *Wnt5b* was overexpressed in the hCGβ TG mammary glands at 3 months of age and was still increased in tumors despite the lack of β-casein expression and nonsecreting tumor cell phenotype. This suggests a potential role for *Wnt5b* in mammary tumorigenesis of the hCGβ mice and differential mechanisms in the regulation of *Wnt5b* and *-6* expression. The expression of *wnt5b* and *wnt7b* early in the tumorigenesis suggests that additional genetic mutations are needed for malignant conversion. We have shown here a previously unknown mechanism of hCG promoting *wnt5b* and *-7b* expression in the mammary gland without changes in ovarian steroidogenesis. Furthermore, selective expansion of *Wnt5b* and *-7b*-producing cells during tumorigenesis was observed in TG females. *Wnt5b* and *Wnt7b* expression has been detected near the TEBs in virgin epithelium (23, 32), suggesting a role in progenitor cell regulation. In humans, the *WNT5B* gene could confer susceptibility to diseases because two common haplotypes show significant association with type 2 diabetes (50).

It has been suggested that *Wnt7b* could be involved in maintaining mammary cells in an uncommitted state (23). Interestingly, canonical ligand WNT7B has been found to be up-regulated in breast malignancies (16, 51). Because of the midpregnancy mammary phenotype observed in 3-month-old TG mice, *Wnt7b* was expected to be down-regulated (23), and therefore the presence of the *Wnt7b*-producing cell population was surprising. Wnt7*b* has been classified into the transforming class of wnt proteins, but it failed to induce hyperplasia *in vivo* when retrovirally expressed in mammary glands (26). Furthermore, *Wnt7b* expression is not regulated by ovarian steroids (23) but was found to be up-regulated by hCG in this study. Wnt7b is found up-regulated in 10% of breast carcinomas (16).

Wnt4 has been recognized as an essential mediator of Pgr function (29). *Wnt4* expression is induced by P and estrogen treatment (29), but because of the lacking Esr1 and Pgr expression in the TG mammary glands and in tumors, *wnt4* was not found to be up-regulated.

The data suggest a possible mechanism in which initial hCG stimulation drives the expression of *Wnt5b*, *Wnt7b*, and wnt target genes, thus contributing to β-catenin stabilization, transdifferentiation, and eventual tumorigenesis in the hCGβ females. Although the data show dysregulation of *wnt 5b* and *-7b* mRNAs, an alternative mechanism for β-catenin stabilization cannot be excluded in the presence of several hormonal disturbances because the canonical wnt pathway can also be activated through several G protein-coupled receptors without wnt contribution (52).

Activation of the wnt pathway has been shown to induce mammary gland tumors from progenitor cells (53). This is

consistent with the tumor histology, in which differentiation along the luminal and myoepithelial cell lineages is observed. Consistent myoepithelial differentiation in the wnt pathway tumors has a counterpart in human breast cancers because 17–37% of them are classified as the basal subtype, characterized by positive keratin 5/6 and vimentin staining but negative ESR1 and HER2 status. β-Catenin is stabilized in over 50% of human breast carcinomas and belongs therefore to an important molecular pathway involved in breast malignancies (13, 14). Mutations in the β-catenin signaling pathway are well documented in other cancers (12), but despite abundant β-catenin stabilization in human breast cancer, such mutations have not been found, indicating a functionally hyperactive pathway. Recent findings on epigenetic silencing of Wnt pathway inhibitors in breast cancer (15, 19) support this view, together with the studies showing a constitutive autocrine *Wnt* activation in breast cancer cell lines (20). Three-month-old TG mammary glands and the TG tumors in aged mice expressed a pattern of *Wnt* genes that did not resemble any normal developmental state of this organ.

In summary, hCG induces wnt signaling in the mammary gland. Accordingly, the hCGβ-expressing TG mice present with hormonally induced mammary tumors exhibiting all the typical characteristics of wnt pathway tumors and thus serve as a model of hormonally induced β-catenin-stabilizing breast cancers. The phenotype is initially hormone induced and is associated with precocious puberty and transiently increased circulating levels of estradiol, followed by high levels of serum P and prolactin. Hence, the mice are subjected to an induction mechanism in which altered endocrine factors and breast tumorigenesis are connected with abnormal wnt signaling and β-catenin stabilization, a common feature in human breast cancer. Disturbed wnt signaling is partially a direct consequence of elevated hCG, and this novel mechanism could mimic the poorly known mechanism of pregnancy-associated breast cancer and its induction. We can thus conclude that the current mouse model provides novel information about hCG action and hormonally induced basal-type tumorigenesis of the breast.

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