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NOVEL ROLES OF HCG/LH SIGNALLING IN THE MOUSE MAMMARY GLAND AND ITS TUMORIGENESIS

by

Aino Rönnblad

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From the Department of Physiology
Institute of Biomedicine
University of Turku
Turku, Finland and
Turku Graduate School of Biomedical Sciences

Supervised by

Professor Matti Poutanen, PhD
Department of Physiology
Institute of Biomedicine
University of Turku
Turku, Finland

Professor Ilpo Huhtaniemi, MD, PhD
Department of Reproductive Biology
Imperial College
London, UK and
Department of Physiology
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Professor Pirkko Härkönen, MD, PhD
Department of Laboratory Medicine
Lund University
Malmö, Sweden

Docent Robert Winqvist, PhD
Department of Clinical Genetics
Faculty of Medicine
University of Oulu
Oulu, Finland

Dissertation opponent

Professor Jan Brosens, MD, PhD
Institute of Reproductive and Developmental Biology
Imperial College
London, UK

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To Sebastian and Sienna

ABSTRACT

Aino Rönnblad. Novel roles of HCG/LH signalling in the mouse mammary gland and its tumorigenesis.

Department of Physiology, Institute of Biomedicine, Faculty of Medicine, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland

Breast cancer is the most common cancer in women, and its development is intimately related to hormonal factors, but how hormones affect breast physiology and tumorigenesis is not sufficiently known. Pregnancy elicits long-term protection from breast cancer, but during the first ten years after pregnancy, breast cancer risk is increased.

In previous studies, there has been conflicting data on the role of human chorionic gonadotropin (HCG) and the functionality of its receptor in extragonadal tissues. The aim of this study was to elucidate the role of chronically elevated HCG in mouse physiology. We have created a transgenic (TG) mouse model that overexpresses HCG. HCG is similar to lutenizing hormone (LH), but is secreted almost solely by the placenta during pregnancy. HCG and LH both bind to the LH receptor (LHR). In the current study, mammary gland tumors were observed in HCG TG mice. We elucidated the role of HCG in mammary gland signalling and the effects of LHR mediated signalling in mouse mammary gland gene expression. We also studied the effects of HCG in human breast epithelial cell cultures.

Several endocrine disturbances were observed in HCGβ TG female mice, resulting in precocious puberty, infertility, obesity and pituitary and mammary gland tumors. The histology of the mammary gland tumors of HCGβ TG females resembled those observed in mouse models with activated Wnt/β-catenin signalling pathway. Wnts are involved in stem cell regulation and tumorigenesis, and are hormonally regulated in the mammary gland. We observed activated β-catenin signalling and elevated expression of Wnt5b and Wnt7b in TG tumors and mammary glands. Furthermore, we discovered that HCG directly regulates the expression of Wnt5b and Wnt7b in the mouse mammary gland. Pharmacological treatment with HCG also caused upregulation of several Wnt-pathway target genes in ovariectomized wild type (WT) mice in the presence of physiological concentrations of estradiol and progesterone. In addition, differential expression of several metabolic genes was observed, suggesting that HCG affects adipocyte function or glucose metabolism. When WT mice were transplanted with LHR deficient or wild type WT mammary epithelium, differential expression of several genes affecting the Wnt-signalling pathway was observed in microarray analysis. Diminished expression of several genes associated with LHR function in other tissues, such as the ovary, was observed in mammary glands deficient of epithelial LHR. In cultured human mammary epithelial cells HCG upregulated the expression of WNT5B, WNT7B similar to mouse, suggesting that the observations found are relevant in human physiology. These studies suggest that HCG/LHR signalling affects gene expression in non-gonadal tissues, and that Wnt-signalling is regulated by HCG/LH in human and mouse mammary glands.

Key words: human chorionic gonadotropin, mammary gland, wnt-signalling, transgenic mouse

TIIVISTELMÄ

Aino Rönnblad. Istukkahormoni hiiren rintarauhasen soluviestinnässä ja kokeellisen rintasyövän muodostuksessa.
Fysiologian osasto, Biolääketieteen laitos, Lääketieteellinen tiedekunta, Turun Yliopisto, Kiinamyllynkatu 10, 20520 Turku, Suomi.

Rintasyöpä on naisten yleisin syöpä ja sen synnyn tiedetään olevan läheisessä yhteydessä hormonaalisiin tekijöihin. Hormonien vaikutuksista rinnan fysiologiaan ja kasvainten syntyyn ei tiedetä riittävästi. Raskauden tiedetään suojaavan rintasyövältä pitkäaikaisseurannassa, mutta ensimmäisten kymmenen vuoden aikana raskauden jälkeen, rintasyöpäriski on lisääntynyt.

Aiemmissa tutkimuksissa on saatu ristiriitaisa tuloksia istukkahormonin (human chorionic gonadotropin, HCG) toiminnasta muissa kudoksissa kuin sukurauhasissa. Tämän tutkimuksen tavoitteena oli tutkia HCG:n vaikutusta hiiren fysiologiaan. Kehitimme siirtogeenisen hiirimallin, joka tuottaa HCG:tä. HCG on lutenisoivan hormonin (LH) kaltainen, istukasta erittyvä hormoni, joka vaikuttaa LH reseptorin (LHR) kautta. HCG-hormonia ylimäärin tuottaville siirtogeenisille hiirille muodostuu rintarauhaskasvaimia. Keskityimme tutkimaan erityisesti rintarauhaskasvainten muodostumiseen johtavia seikkoja tässä eläinmallissa. Selvitimme LH/HCG vaikutuksia hiiren rintarauhasessa ja ihmisen rintarauhasepiteelissä ilmenevien geenien säätelyyn. Lisäksi tutkimme, johtaako LHR:n poisto hiiren rintarauhasepiteelistä muutoksiin rintarauhasen soluviestinnässä.

HCG:ta ylituottavilla naarailla havaittiin useita hormonaalisia poikkeavuuksia, joista seurasi aikaistunut puberteetti, hedelmättömyys, lihavuus sekä rintarauhasen ja aivolisäkkeen kasvainten muodostuminen. Rintarauhaskasvainten histologian havaittiin muistuttavan sellaisiin hiirimalleihin ilmaantuvia kasvaimia, joille on yhteistä Wnt/β-kateniini -signalointireitin aktivoituminen. Wnt-perheen geenien tiedetään olevan hormonien säätelemiä rintarauhasessa sekä säätelevän kantasolujen toimintaa ja vaikuttavan kasvainten muodostukseen. HCG:ta ylituottavien siirtogeenisten hiirten rintarauhaskasvaimissa todettiin β-kateniin signaloinnin poikkeava aktivoituminen, sekä Wnt5b ja Wnt7b lisääntynyt ilmentyminen kasvaimissa sekä jo ennen kasvainten muodostumista.

HCG:n farmakologisen annon normaalille hiirelle havaittiin säätelevän Wnt5b:n ja Wnt7b:n geenien sekä useiden β-kateniini signaloinnin kohdegeenien ilmentymistä rintarauhasessa, kun hiiren munasarjat olivat poistettu ja hiiren estradioli ja progesteroni korvattu normaalille tasolle. Näillä hiirillä havaittiin mikrosiruanalyysissä myös useiden metabolisten geenien muuntunut ilmentyminen. Tämä viittaa siihen, että HCG:llä on suoria vaikutuksia rasvasoluihin tai glukoosiaineenvaihduntaan. Kun normaaleihin hiiriin istutettiin rintarauhasepiteeliä, josta puuttui LHR, havaittiin mikrosiruanalyysillä useiden Wnt-signalointiin vaikuttavien geenien muuttunut ilmentyminen. Havaitsimme lisäksi viljellyissä ihmisen rintaepiteelisoluissa HCG:n lisäävän WNT5B:n ja WNT7B:n tuottoa. Yhteenvetona voidaan todeta, että LHR-signaloinnilla on vaikutuksia geenien ilmentymiseen myös muissa kudoksissa kuin sukurauhasissa ja että HCG säätelee Wnt-signalointireitin toimintaa rintarauhasessa sekä hiirellä että ihmisellä.

Avainsanat: istukkahormoni, rintarauhanen, wnt-signalointi, siirtogeeninen hiiri

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ABBREVIATIONS

Gene/mRNA/protein symbols in the text refer to the human counterpart when capital letters (MMP7) are used in contrast to mouse symbols written with only initial capital letter (Mmp7).

ACTH	adrenocorticotropin
ADPN	adiponutrin
ANGPTL4	angiopoietin-like 4
APC	adenomatosis polyposis coli
AR	androgen receptor
BCR-ABL	Breakpoint cluster region and v-abl Abelson murine leukemia viral oncogene homolog 1 fusion product
BMD	bone mineral density
BMI	body mass index
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
cAMP	adenosine-3´-5´-cyclic monophosphate
CHEK2	CHK2 checkpoint homolog
CK	cytokeratin
COMT	catechol-O-methyltransferase
CYP17	cytochrome P450, family 17, subfamily A, polypeptide 1
CYP19	cytochrome P450, family 19, subfamily A, polypeptide 1
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1
DAG	diacylglycerol
DKK1	dickkopf homolog 1
DMBA	dimethylbenzanthracene
DSH	dishevelled
E2	estradiol
EGFR	epidermal growth factor receptor
ERK1/2	extracellular signal-regulated protein kinase 1/2
ESR1	estrogen receptor 1 (ERα)
ESR2	estrogen receptor 2 (ERβ)
FRL1/CRIPTO	teratocarcinoma-derived growth factor
FSH	follicle stimulating hormone receptor
FSHR	follicle stimulating hormone
FZ	frizzled homolog
GH	growth hormone
GHR	growth hormone receptor

GNRH	gonadotropin-releasing hormone 1
GPC3	glypican 3
GPCR	G protein coupled receptor
GSK3	glycogen synthase kinase 3
GSTP1	glutathione S-transferase pi
HCG	human chorionic gonadotropin
HCGβ	human chorionic gonadotropin, β-subunit
HER2	ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
HIG2	hypoxia-inducible protein 2
HRT	hormone replacement therapy
HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1
IGF1	insulin-like growth factor 1
IGFBP	insulin-like growth factor binding protein
IHC	immunohistochemistry
IL6	interleukin 6
INOS	nitric oxide synthase, inducible
INT3	notch gene homolog 4
IP3	inositol triphosphate
JAK	janus kinase
JNK	jun N-terminal kinase
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KO	knock-out
LDLR	low density lipoprotein receptor
LEF1	lymphoid enhancer-binding factor 1
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LIF	leukemia inhibitory factor
LRBP	LH/HCG receptor mRNA –binding protein
LRP5/6	low density lipoprotein receptor-related protein 5/6
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MMP7	matrix metallopeptidase 7
MMTV	mouse mammary tumor virus
mRNA	messenger ribonucleic adic
MTOR	FK506 binding protein 12-rapamycin associated protein 1
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)

NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)
NEU	Erbb2, HER2 homolog in mouse
NOD/SCID	non-obese diabetic / severe combined immunodeficiency
P	progesterone
PCP	planar cell polarity
PDE5	phosphodiesterase 5A, cGMP-specific
PGR	progesterone receptor
PI3K	phosphoinositol 3-kinase
PL	placental lactogen
PLP	proteolipid protein (myelin)
PMP22	peripheral myelin protein 22
PON	paraoxonase
PPAR	peroxisome proliferator-activated receptor gamma
PRL	prolactin
PRLR	prolactin receptor
PTHRP	parathyroid hormone-like peptide
PYMT	polyoma middle T
RANK	tumor necrosis factor receptor superfamily, member 11a
RAS	Harvey rat sarcoma virus oncogene 1
ROR2	receptor tyrosine kinase-like orphan receptor 2
RT-PCR	reverse-transcriptase polymerase chain reaction
RYK	receptor-like tyrosine kinase
SCA1	ataxin 1
SERPINA3	serpin peptidase inhibitor, clade A
SFRP	secreted frizzled-related protein
SMA	smooth muscle actin
SNP	single nucleotide polymorphism
SORBS1	sorbin and SH3 domain containing 1
SOX9	SRY-box containing gene 9
STAT5	signal transducer and activator of transcription
TCF/LEF	transcription factor 3
TEB	terminal end bud
TG	transgenic
TGFB	transforming growth factor 1, β1
TNXB	tenascin XB
TP53	tumor protein p53 (Li-Fraumeni syndrome)
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor

UHRF1	ubiquitin-like, containing PHD and RING finger domains, 1
VEGF	vascular endothelial growth factor
WIF	WNT inhibitory factor 1
WISP2	WNT1 inducible signaling pathway protein 2
WNT	wingless-related MMTV integration site
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by roman numerals (I-III). Some unpublished data on gene expression studies and animal experiments is also included.

I Rulli SB, **Kuorelahti A**, Karaer O, Pelliniemi LJ, Poutanen M, Huhtaniemi I (2002). Reproductive disturbances, pituitary lactotrope adenomas, and mammary gland tumors in transgenic female mice producing high levels of human chorionic gonadotropin. *Endocrinology 143: 4084-95.*

II **Kuorelahti A**, Rulli S, Huhtaniemi I, Poutanen M (2007). 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/beta-catenin pathway activation. *Endocrinology 148: 3694-703.*

III **Rönnblad A**, Wärri A, Huhtaniemi I, Poutanen M (2008). LHR signalling elicits multiple metabolic effects on mouse and human mammary gland and regulate Wnt-signalling pathway. *Submitted.*

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INTRODUCTION

Breast cancer is the most common cancer in women in the Western countries, and the incidence is rising continuously. Five to ten percent of the breast cancer cases are known to be due to hereditary predisposition, but most breast cancers appear to be sporadic. Although some non-genetic risk factors have been identified, a substantial proportion of the underlying predisposing factors are currently unrecognized. Tumorigenesis in the breast is significantly influenced by hormonal factors, but the precise mechanisms of tumor induction and promotion are still poorly known. Pregnancy has a dual effect on breast cancer risk: after a ten-year period of heightened risk, breast cancer risk is permanently dimished compared to nulliparous women if the pregnancy occurred before the age of 35 years (Schedin, 2006). However, pregnancy diminishes only ESR1+/PGR+ breast cancer without having an effect on hormone receptor negative subtype (Ma *et al.*, 2006). During pregnancy, women are exposed to a myriad of hormones, for example, to human chorionic gonadotropin (HCG), which is exclusively secreted during pregnancy in normal human physiology. HCG and lutenizing hormone (LH) bind to the same receptor, luteinizing hormone receptor (LHR). In addition to its well-characterized actions in the ovaries, LH/LHR signalling has been proposed to be involved in several pathophysiolgical conditions in non-gonadal tissues, although the data is controversial (Casadesus *et al.*, 2007; Mikola *et al.*, 2003; Pakarainen *et al.*, 2007; Tanaka *et al.*, 2000). The human breast is reported to express LHR (Funaro *et al.*, 2003; Meduri *et al.*, 1997), suggesting that it might be a possible target tissue of LH/HCG actions, but little is known of the possible effects LH/LHR signalling might elicit in the mammary gland. Since all women are exposed to LH/HCG during their reproductive years and in menopause, possible signalling by LH in the breast also has clinical relevance.

The objective of this thesis was to elucidate the role of HCG/LHR signalling in mouse physiology and mammary gland tumorigenesis. We studied the consequences of chronically elevated HCG in a transgenic (TG) mouse model, with special focus on mammary gland biology, and studied the role of HCG in cultured human mammary epithelial cells. Furthermore, we studied the role of LHR-signalling in the mouse mammary gland *in vivo* by deleting LHR from the mammary epithelium by utilizing a tissue transplantation technique.

REVIEW OF THE LITERATURE

1. Normal breast development and anatomy

1.1. Anatomy

The mammary gland is the organ that distinguishes mammals from other animals. Its function is to deliver an adequate amount of milk, which is the only food a newborn human can consume. Unlike most of the other organs, its development takes place mostly post-natally, and its closely regulated functions are dependent on hormonal stimulation. The mouse mammary gland is composed of epithelium and loose stroma. In a rudimentary mammary gland, a specialized club-shaped structure terminal end bud (TEB) is responsible for ductal elongation and grows from the nipple to the surrounding fat pad. TEB is composed of two highly proliferating cell types: cap cells and body cells. Cap cells, at the tip of the TEB, differentiate into myoepithalial cells and body cells become ductal cells. In mature ducts and alveoli, one layer of the myoepithelium surrounds the epithelium and continuous basement membrane layer embeds two layered ductal structures. Growing ducts form secondary and tertiary branches in response to hormonal and paracrine stimulation. In pregnancy, alveoli are formed from branches. Alveoli, that are the basic units of milk production, fill the mammary fat pad progressively during pregnancy. In humans, the mammary gland divides into 15-20 lobules and each lobule is supported by septae of connective tissue. Milk ducts from lobules collect the milk during lactation and converge into 6-10 collecting ducts in the nipple area. In rodents, ducts empty into a single primary duct.

1.2. Breast development from embryo to adolescence

There are differences between rodent and human mammary gland development, but they share the majority of mechanisms of branching morphogenesis and regulation of function. The hormonal control of mammary gland development and function is presented in Figure 1. In humans, breast development begins *in utero.* Approximately at the gestational week 12, the milk line has shrunk to two placodes and future mammary region has primary buds that consist of two different cell populations, central and peripheral, supported by mesencyme. In the following weeks, the primary bud forms indentations, from which mammary projections start to grow and penetrate into the specialized mesenchyme that surrounds the mammary bud (Jolicoeur, 2005). Prenatal mammary development can be divided into 10 stages (Russo and Russo, 2004). In the last stage, the primitive mammary gland produces secretion and ductal structures appear dilated, due to stimulation by maternal hormones (Russo and Russo, 2004). Growth during childhood is mostly allometric and mammary gland development is enhanced only in puberty, when estradiol and progesterone boost the ductal elongation and bifurcation. Ovarian and pituitary hormones are absolutely essential for proper mammary gland growth and function after birth, but prenatal development occurs without these hormonal requirements. A knock-out (KO) mouse model demonstrated the requirement for estrogens and estrogen receptor α (Esr1) in adolescent growth (Mallepell *et al.*, 2006), but estrogen receptor β (Esr2) is not needed for normal morphogenesis of the mammary gland (Forster *et al.*, 2002). Esr1 deficient mammary cells can grow in the presence of wild-type mammary cells, demonstrating that Esr1 is needed in epithelium and mediates its functions in a paracrine manner (Mallepell *et al.*, 2006). Progesterone (P) and

its receptor (Pgr) are required for tertiary branching, but not for adolescent growth (Brisken *et al.*, 1998; Soyal *et al.*, 2002). Mammary gland transplantation studies with Pgr KO mice demonstrated that a simple ductal system develops without epithelial or stromal Pgr, but side-branching is minimal. Pgr is indispensable for alveolar growth (Brisken *et al.*, 1998). Pgr operates in a paracrine manner, but the precise mechanism by which progesterone elicits its signals is not known (Brisken *et al.*, 1998). Wnt4 and Rank have been suggested to participate in downstream signalling of Pgr. They are also both regulated by P (Brisken *et al.*, 2000; Conneely *et al.*, 2003). In hypophysectomized rats, estradiol (E2) and prolactin (Prl) alone cannot rescue post-natal mammary growth. Thus, growth hormone (Gh) and its downstream signal insulin-like growth factor 1 (Igf1) are essential for adolescent mammary growth, as also demonstrated by impaired mammary gland development in mice lacking the GH receptor (GHR) or Igf1 (Gallego *et al.*, 2001; Kleinberg *et al.*, 2000).

The fate of epithelium is largely determined by cross-talk between epithelium and stroma. For example, the embryonic mammary gland can turn into salivary gland epithelium, but retain the capacity for milk protein production in response to prolactin, when combined with salivary gland mesenchyme and grafted under the renal capsule in mice (Sakakura *et al.*, 1976). Even the non-mammalian epidermis can be instructed by mammalian mesenchyme to form glandular mammary epithelium (Propper and Gomot, 1973), further emphasizing the role of paracrine signalling by stroma for the identity of epithelium. Stromal factors direct the density of the ducts, which varies between different mouse strains (Naylor and Ormandy, 2002). The spacing between ducts is controlled by a growth factor gradient so that branching occurs at the site of minimal concertration of inhibitory morphogens, such as transforming growth factor beta 1 (TGFβ) (Nelson *et al.*, 2006).

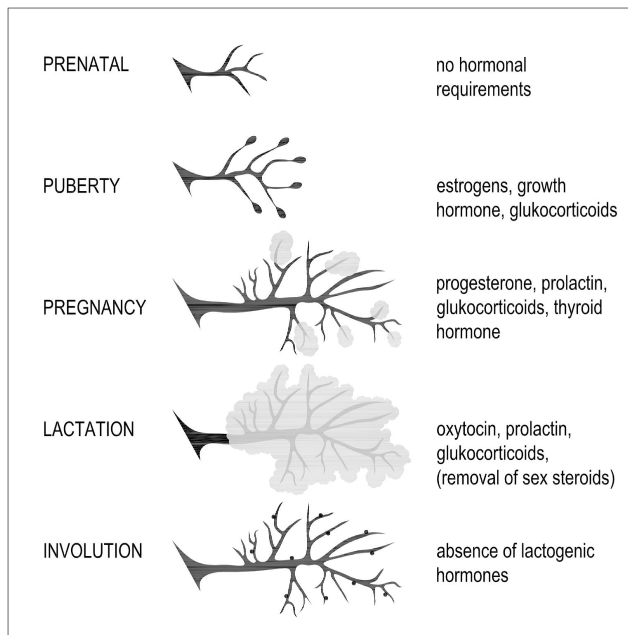

Figure 1. Hormonal control of mammary gland development and function

There are differences between the mouse and human mammary gland. *In utero,* mammary gland development begins in the first trimester in humans, whereas it begins after mid-gestation in mice. Only one pair of mammary buds develops in humans compared to five pairs in mice. The secretory phase observed in humans in late gestation is not present in mice. One important difference is that in humans, males also develop mammary glands that are indistinguishable until puberty. The human male mammary gland does not involute but remains capable for growth and differentiation. In male mice, the mammary rudiment involutes in response to androgens in mid-gestation (Kratochwil and Schwartz, 1976). Mouse and human stroma are also substantially different. In humans, ducts are surrounded by dense, fibroblastic stroma, whereas the mouse periductal stroma is scarce and the fat pad is more adipose than its human counterpart. Subgross and histological images of the normal human breast are shown in Figure 2, A (the images in Figure 2 are from http://tgmouse.compmed.ucdavis.edu/srwtxt/, with the permission of prof. Robert D. Cardiff, University of California, Davis).

1.3. Breast in pregnancy and lactation

During pregnancy, the number of alveoli and their differentiation increase. In response to pregnancy hormones, the breast epithelium acquires the capacity to produce milk proteins (lactogenesis 1). Glucocorticoids, thyroid hormone and prolactin are required for milk secretion (Brisken *et al.*, 1999; Capuco *et al.*, 1999; Cowie *et al.*, 1980), but hormonal requirements for lactogenesis 1 have not been fully elucidated. In mouse, Prl receptor (Prlr) signalling is needed for the terminal differentiation of mammary gland, synthesis of milk proteins and maintenance of a differentiated state (Brisken *et al.*, 1999; Cui *et al.*, 2004; Liu *et al.*, 1997). Prlr KO mice are unable to form lobuloalveolar units, or to express a milk protein gene, β-casein. Stat5a deficient mice have a similar phenotype with Prlr KO mice, suggesting that Stat5a is the main signalling mediator responsible for the lactogenic effects of Prlr. Stat5 is needed for the maintenance of a differentiated state, as conditional the KO model resulted in the premature cell death of differentiated mammary epithelium, when Stat5 signal was discontinued (Cui *et al.*, 2004). In line with the animal data, women with low PRL concentration fail to breast-feed (Martin and Oakey, 1982).

Pgr is needed in epithelium for alveolar growth in lactogenesis 1 as demonstrated by tissue transplantation experiments with Pgr KO mice (Brisken *et al.*, 1998). In the absence of Pgr, epithelium could not undergo alveolar development in the presence of pregnancy hormones. Pgr elicits its functions in a paracrine manner, since Pgr KO epithelium was able to contribute to alveolar growth in the presence of wild type (WT) epithelium. Thus, Pgr is needed in epithelium but not in stroma. Pgr expression before lactation is needed for the proliferation of the mammary gland, but removal of P signalling in lactation enables terminal differentiation and the emergence of a functional lactating mammary gland (Ismail *et al.*, 2002).

Placental lactogen (PL) and GH have also been suggested to play a role in lactation. PL is not necessary in human for lactation (Gaede *et al.*, 1978), despite its abundant secretion during pregnancy. The requirement of GH in lactation varies between species. GH or GHR deficiency in humans and mice does not impair lactation (Rimoin *et al.*, 1968; Rosenbloom *et al.*, 1999; Zhou *et al.*, 1997), but inhibition of GH secretion during lactation decreased

milk synthesis in rat (Flint and Vernon, 1998). GH also increased milk yield in dairy cows and it can be synthesized in mammary gland in addition to the pituitary (Mol *et al.*, 2000).

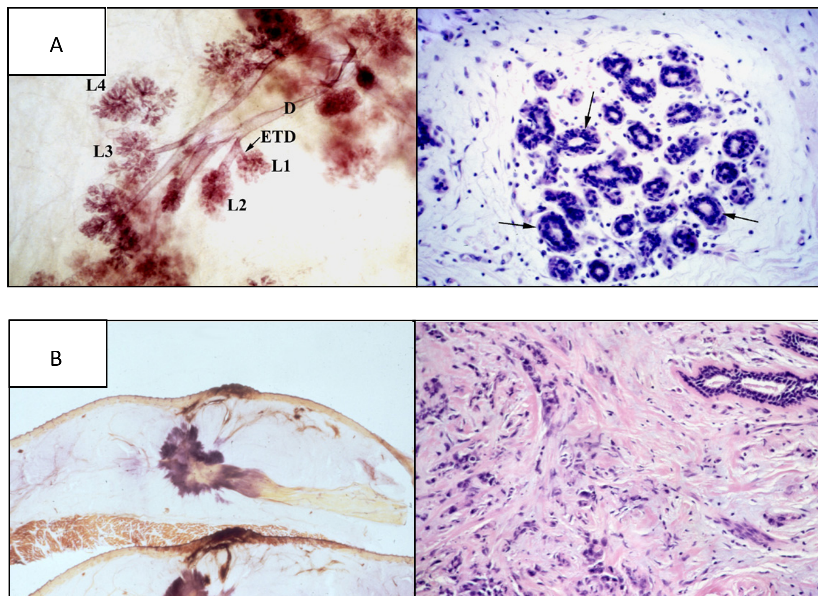

Figure 2. Normal breast (A) and infiltrating breast carcinoma (B).

During pregnancy, a high concentration of circulating progesterone (P) prevents lactation. After parturition, sex steroid concentrations rapidly drop, allowing full milk production and ejection upon oxytocin stimulation (lactogenesis 2). Oxytocin is released from the posterior pituitary in response to the suckling stimulus. Oxytocin is produced in the *supraoptic and paraventricular nuclei* of the hypothalamus, transported to and released from the posterior pituitary. The hypotalamus regulates the release of oxytocin from the pituitary, and hypothalamic neurons can be activated (or inhibited) by the higher brain centers. The sensory stimulus from suckling of the nipple conveys afferent information to the central nervous system and causes oxytocin release from the axon terminals. Other simuli, such as stress or fear, may interfere with the system and inhibit it. The sound or sight of the offspring can result in release of oxytocin. During the first days post partum in humans, the mammary gland goes through a series of rapid physiological and anatomical changes after which the breast secretes substantial amounts (approximately 600-1000 ml) of milk daily. Myoepithelium surrounding the ducts and the alveoli contract pushing milk to the nipple. Larger ducts do not secrete milk but passively transfer milk towards the nipple. Other cell types also contribute to in milk production. Stromal fibroblasts and adipocytes supply with growth factors and B cells residing in the mammary gland become plasma cells providing immunoglobulins to the milk (Anderson *et al.*, 2007). (1998).

1.4. Involution

After the cessation of suckling, the mammary gland epithelium turns apoptotic and rapidly involutes to its pre-lactation state. In humans, milk removal must begin by day 3-4 after

parturition in order to initiate lactation (Neville *et al.*, 2001), in other species such as mouse, involution begins within hours in the absence of suckling stimulus. Local signals can inhibit milk synthesis and begin apoptosis even in the presence of lactogenic hormones (Li *et al.*, 1997). During involution, T and B cells are activated and macrophages recruited into the mammary gland to perform remodelling. The collagen content of the mammary gland changes (Schedin *et al.*, 2007).

1.5. Mammary gland stem cells

Mammary gland stem cells have been investigated with greast interest in recent years. The importance of mammary gland stem cells lies within their longevity, and thus, their putative role in carcinogenesis. The existence of mammary gland stem cells has been suspected since the 1950s, when DeOme et al. developed the mammary gland tissue transplantation technique for experimental animals (Deome *et al.*, 1959), that has been widely used since. In this technique, the prepubertal mammary gland fat pad is cleared from the endogenous epithelium that has not yet grown to fill the mammary fat pad. Pieces from the donor mammary gland can then be transplanted into the remaining fat pad and it will repopulate the remaining fat pad, demonstrating the existence of stem cells in an adult mammary gland. Any portion of the mammary gland is able to repopulate the fat pad and repopulation capability does not diminish with aging (Smith and Medina, 1988). Mouse mammary stem cells have been purified and it was recently shown that one single stem cell was able to reconstitute a functional, lactating mammary gland in which all of the cell types (luminal, alveolar, myoepithelial) were present (Shackleton *et al.*, 2006). These outgrowths were further transplanted into a secondary host and were able to regrow into a mammary gland. In this study, mammary gland stem cells were characterized by expression of cell surface markers Cd24, and Cd29 (β1-integrin) or Cd49f (α6-integrin). There has been disagreement over the surface markers of the stem cells. Hoechst dye exclusion (due to increase in membrane transporter proteins), or Sca1 expression that characterize some stem cells and has been used as markers for mammary gland stem cells (Liu *et al.*, 2004), did not characterize these cells (Stingl *et al.*, 2006). Mammary cell differentiation is thought to happen hierarchically from long-term repopulating stem cells first producing common progenitors and further lineage specific progenitors (luminal and myoepithelial) finally resulting in production of ductal, alveolar and myoepithelial cells (Visvader and Lindeman, 2006). The important question of possible Esr1 expression in the stem cell and progenitor cell compartments are yet without definitive proof due to uncertainty in stem cell markers, but it appears that the most primitive stem cells and part of the progenitor cells do not express Esr1 or Pgr (Asselin-Labat *et al.*, 2006; Sleeman *et al.*, 2007; Visvader and Lindeman, 2006).

2. Breast cancer

2.1. Epidemiology of breast cancer

Breast cancer is the most common cancer and a leading cause of cancer death in women worldwide (1996). Twelve to thirteen percent of women born now in USA will develop breast cancer during their lives. Incidence rates are highest in the developed countries, such as the USA and most of Europe, where incidence varied from 61 to 97 / 100000 women

in 1993-1997. In Latin America and Asia, the incidence is currently substantially lower (Althuis *et al.*, 2005). The incidence of breast cancer is, however, rising everywhere. In 10 out of 18 countries studied, the increase in incidence between 1973-1977 and 1993-1997 was 20-40% and the most dramatic increases were seen in Japan (89%) and Finland (81%) (Figure 3).

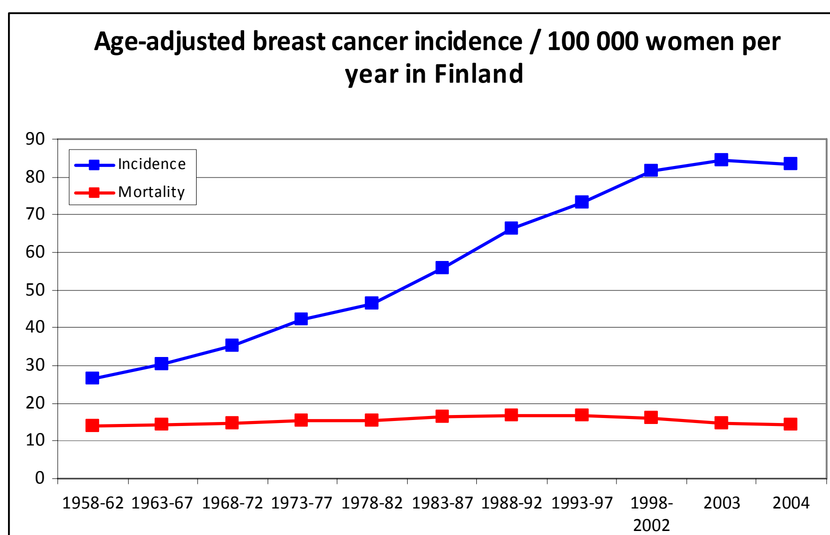

Figure 3. Breast cancer incidence and mortality in Finland

In countries where breast cancer screening programs have been implemented, an approximately 30% rise in breast cancer incidence has been observed. This increase consists mostly of small localized breast cancers (Althuis *et al.*, 2005). Breast cancer incidence rises dramatically with age, less than 1% of all breast cancer cases are detected before the age of 30 (National Cancer Institute, Cancer Surveillance Research Program). In the USA, the incidence of breast cancer increases approximately 100-fold between ages 30 and 50 years.

The discrepancies observed in the incidence rates between countries are attributed to different prevalence of risk factors, such as age at menarche and menopause, obesity and age at first birth (Althuis *et al.*, 2005). Genetic, environmental and behavioural and occupational factors also modify the breast cancer rates (Brody *et al.*, 1996; Hansen, 2001; Neuhausen, 1999; Shaham *et al.*, 2006). Despite the increasing incidence, breast cancer mortality has been stable or has decreased in most countries. Mortality goes parallel with incidence between countries: in high incidence countries, mortality is between 17-27/100000 and in low incidence areas in Latin American and Asia 7-14/100000 (Althuis *et al.*, 2005). Ductal carcinoma (65-90%) is the most common breast cancer type, followed by lobular carcinoma (10%). Inflammatory, medullary, papillary and metaplastic breast cancer account for the rest of the cases. Subgross and histological images of human invasive ductal carcinoma are shown in Figure 2, b.

Benign breast lesions are more common than malignant ones, but they have not been extensively studied. Benign breast lesions are a heterogenous group of developmental abnormalities (ectopic breast, hypoplasia), inflammatory lesions (mastitis, fat necrosis), epithelial (cysts, adenosis, hyperplasias, papillomas) and stromal proliferations (pseudoangiomatous stromal hyperplasia) and expansions (fibroadenoma, lipoma, adenoma). These lesions are not considered premalignant, although the breast cancer risk is slightly increased in individuals with a history of some of these benign lesions (Guray and Sahin, 2006)

2.2. Risk factors of breast cancer

Risk factors for breast cancer (1996; 2001; Beral, 2003; Brinton *et al.*, 1988; Dupont *et al.*, 1994; Key *et al.*, 2002; Meijers-Heijboer *et al.*, 2002; Monninkhof *et al.*, 2007; Nagy *et al.*, 2004; Renehan *et al.*, 2004; Shannon and Smith, 2003; Veronesi *et al.*, 2005; Wooster and Weber, 2003) are presented in Table 1. Factors not related to susceptibility to breast cancer are presented in Table 2. There are a number of well-documented risk factors for breast tumorigenesis that range from hormonal and genetic factors to lifestyle and environmental factors. However, it has remained difficult to predict which women will be affected by the disease, reflecting the complex interplay between genetic and environmental factors in breast tumorigenesis. The effect of a single risk factor is often diluted or lost when breast cancer is considered as one disease entity – for example, one risk factor could contribute only to ESR1-negative cancers while others to ESR1-positive cancers (Ursin *et al.*, 2005).

Only 5-10% of breast cancer cases are considered to have a hereditary disease background, and only a part of the involved susceptibility genes are currently known. Most of the breast cancer cases appear sporadic: eight out of nine women who develop breast cancer do not have affected 1st degree relatives. With one affected relative, the lifetime excess of breast cancer incidence is only 5.5% (2001). The characteristic features of heritable predisposition for a particular cancer include younger age at diagnosis, positive family history (several generations affected), and patients with multiple primary tumors (Nagy *et al.*, 2004).

Constitutional mutations in the classical breast cancer susceptibility genes BRCA1 and BRCA2 confer a substantially increased risk to their carriers (approximately 60% and 85% lifetime risk of developing breast cancer). However, only 2-3% of all breast cancer cases are attributable to these mutations (Wooster and Weber, 2003). BRCA1 and BRCA2 mutations account for 20% of the familial cases each, CHEK2 mutation for 5%, TP53 mutation (Li-Fraumeni syndrome) for <1%, leaving half of the alterations connected to breast cancer predisposition currently unknown (Wooster and Weber, 2003). Multiple genes displaying genetic polymorphisms (low-penetrance alleles) that translate into considerably milder increases in breast cancer risk have also been identified. These include PALP2 (Erkko *et al.*, 2007), CYP19, GSTP1 COMT, CYP17, CYP1A1, NAT1, NAT2) (Dunning *et al.*, 1999).

Table 1. Breast cancer risk factors

	Relative risk	
Intrinsic hormonal factors		
Age at menarche	1.2	<12 years vs >15 years
Age at menopause	1.8	>50 years vs >40
Age at first full time pregnancy	3	after age 40 years
Height		per height increase of 5 cm
in premenopause	1,02	
in postmenopause	1,07	
Elevated blood levels of estradiol	2, - 2,6	highest vs lowest quintile
Elevated blood levels of estrone	2,-2,2	highest vs lowest quintile
Elevated blood levels of IGF-1	1,7	premenopausal breast cancer
Increased levels of IGFBP-3	1,5	premenopausal breast cancer
Exogenous hormones		
Oral contraceptives	1,2	current user
Hormone-replacement therapy	1,3-2,0	current user, E2 ±P
Diethylstillbestrol	2	use during pregnancy
Life style and environment		
High BMI		
premenopause	0,7	
postmenopause	2	
Location	5	developed countries
Socioeconomic group	2	high vs low
Alcohol consumption	1,07	per every daily drink
Physical exercise, postmenopause	0,9	each hour/week increase of exercise
Inonizing radiation	3	elevated exposure to girls > 10 years
Saturated fat, 5% increase	1,09	
Breast biology		
Age	>10	
Family history		
one 1st degree relative	1,8	
two 1st degree relatives	2,9	
three or more 1st degree relatives	3,9	
Breast density	>5	
Benign breast disease		
atypical hyperplasia	4-5	
fibroadenoma	2,17	
complex fibroadenoma	3,1	
Cancer in other breast	<4	
Parity	0,93	7% decrease for every birth
Breastfeeding	0,96	per 12 months of breastfeeding
Genetic factors		
BRCA1	6,2	
BRCA2	3,5	
CHEK2	2	
TP53	7,1	

Table 2 Factors found not to be associated with breast cancer

Abortion (induced or sponteneous)
The use of meat or dairy products
Fruit and vegetable consumption
Smoking
Exposure to chlorine pesticides

Age at menarche and menopause are well-documented factors contributing to breast cancer risk. Young age at menarche is correlated with elevated risk of breast cancer and the risk is especially strong if the woman is genetically susceptible (Hamilton and Mack, 2003). Menarche at the age of 15 compared to < 12 years results in risk ratio of 0.81 for breast cancer (Pike *et al.*, 2002). Later age at menopause is a risk factor, the risk ratio being 1.31 for women having their menopause at the age of 55 years or more compared to the women having menopause at the age of 44 or under (Pike *et al.*, 2002). It is believed that prolonged exposure to estrogens is the biological variable behind the risk of early menarche and late menopause. This is a rational biological assumption because higher postmenopausal blood levels of E2 and estrone translate into higher breast cancer risk (Key *et al.*, 2002). In the same analysis, high testosterone (T) was also found to increase breast cancer risk (RR 2.22 in the highest vs lowest quintile). Breast density in mammographic screening have been identified as a relatively high risk factor (Boyd *et al.*, 2007; Wolfe, 1976). Mammographic breast density reflects the ratio between epithelium and stroma versus fat. Breast density of 75% versus 10% has an odds ratio between 3.4 and 5.7 in different datasets (Boyd *et al.*, 2007). High density has a masking effect on breast cancer detection and results in a higher amount of false negative screenings, but this effect is additive to the true higher incidence in dense breasts (Whitehead *et al.*, 1985). The biological mechanism mediating this clear clinical risk factor is discussed below.

In recent years, diet and especially dietary phytoestrogens have gained attention as possible risk factors and as a means of breast cancer prevention. Epidemiological studies have suggested that a phytoestrogen-rich diet is associated with lower risk of breast cancer, but direct evidence is lacking (Gikas and Mokbel, 2005). The influence of diet on breast cancer risk is likewise unclear. There is no clear, strong association of any single dietary factor on breast cancer risk, except for alcohol, which has a positive correlation with breast cancer incidence (Michels *et al.*, 2007). This may reflect a true lack of association, or the dilution of a true, but complex association of dietary factors and genetic background.

2.3. Outlines of breast cancer treatment

Breast cancer treatment options depend on the size of the primary tumor, involvement of lymph node metastases, existence of distant metastases (TNM-staging) and ESR1, PGR and HER2 (ERBB2) status at the time of diagnosis. Breast conserving surgery is recommended when applicable. Patients with invasive tumors that can be resected cosmetically acceptably with proper healthy tissue margins are subject to conserving surgery. Small (< 3 cm) intraductal carcinomas can also be removed this way. When resection margins are clear of tumorous tissue, conservative surgery has the same survival rate as with total mastectomy. When tumor diameter is large (> 3-4 cm), the tumor is multifocal or of inflammatory type, total mastectomy is recommended. If resection margins in the primary operation are not tumor-free, a total mastectomy regime should be applied. The axillary lymph node resection or sentinel lymph node biopsy is performed in order to clarify the potential lymph node invasion of the primary tumor. Lymph node infiltration is an important prognostic marker and resection of metastatic lymph nodes has therapeutic importance. Neoadjuvant treatment is given if the tumor is large (or breast-conserving therapy is desired but the original tumor is too large) or if the tumor is of an inflammatory type. After maximal tumor shrinkage, operation and radiotherapy are performed. Radiotherapy is given after breast

conserving surgery to the whole breast and it diminishes local recurrences by 68% and also has an effect on prognosis. After mastectomy, radiotherapy is recommended in lymph node positive cancers and with primary tumors larger than 5 cm (T3-4), because it is shown to diminish breast cancer mortality. A total of 50 Gy is usually given in 1.9-2.0 Gy fractions. (2005)

Ductal carcinoma in situ is treated with mastectomy or breast-conservative surgery with radiotherapy. Lobular carcinoma in situ has an increased risk of developing breast cancer (although it is not regarded as cancer itself), and mammography and clinical follow-up is recommended. The manifestation of Paget´s disease is a skin abnormality in the mamilla area and is treated with resection and radiotherapy, since the underlying cause of Paget´s disease is intraductal or invasive carcinoma.

Adjuvant chemotherapy is recommended for patients whose estimated 10-year recurrence is >10%. This includes patients with 1) lymph node invasion and 2) patients without lymph node invasion, but a tumor diameter < 5cm if the nuclear gradus is 2-3 and the tumor is hormone negative. In women under 65-70 years, adjuvant systemic chemotherapy is recommended, but evidence is lacking in women over 70 years. In estrogen and /or progesterone receptor positive breast cancers, adjuvant hormone therapy is recommended regardless of other variables. Antiestrogen therapy (anti-estrogen or aromatase inhibitor) is given for five years after chemotherapy, or ovarian ablation is used (ovariectomy, GNRH analogs). If the tumor expresses HER2, trastuzumab can be used in adjuvant therapy (Carlson *et al.*, 2006; Kaufmann *et al.*, 2006; Pestalozzi *et al.*, 2005).

2.4. Molecular profiling of breast tumors

Breast cancer is a multifaceted disease, and the disease can be divided into both poor- and good-prognosis diseases depending on multiple variables. In terms of clinical decision making, tumor size, lymph node status and ESR1, PGR and HER2/ERBB2 positivity / negativity, grade (I-III) and proliferation index are validated and widely used as predictive markers (Donegan, 1992). Currently, they also guide treatment protocols. There has been a strong effort to define more detailed disease entities beyond these classical variables, because there is still considerable under- and over-treatment of breast cancer. Molecular profiling has offered a possibility to divide breast cancers into several subtypes and elucidate patterns of gene expression associated with different prognoses and risk of distant metastases (Hu *et al.*, 2006; Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003; van de Vijver *et al.*, 2002; van ‘t Veer *et al.*, 2002; Wang *et al.*, 2005a). Human breast cancer can be divided into 5 subtypes identified by hierarchical clustering in several microarray gene expression profiling studies: Basal epithelial –like, ERBB2 over-expressing, luminal A and B, and normal breast like (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003). Luminal A has a significantly longer disease-free time interval, whereas basal and ERBB2 have the worst prognoses (Sorlie *et al.*, 2003). In clinical use, there is currently a validated multigene prognostic assay (Oncotype DX®) commercially available in the USA that has been shown to stratify the risk of ESR1-positive patients (Paik *et al.*, 2004) and several predictive gene sets predicting treatment responses are under development (Andre and Pusztai, 2006). Decision-making does not currently rely on gene expression on a large scale, but predictive tests can guide treatment options in the future. The existence of several

molecular subtypes can also be interpreted as potentially several differing mechanisms of disease induction and several differing sets of risk factors for a particular cancer subtype. The epidemiological connection might be lost between some true risk factors in specific subtypes, since epidemiological studies made so far have not taken into account different molecular subgroups.

Molecular profiling could contribute to basic research in elucidating different mechanisms of tumor induction, varying oncogenic pathways and the target cells present. The information networks present in cancer cells are complex and until recently, it has been difficult to draw conclusions from underlying mechanisms based on profiling studies. Importantly, new therapeutic targets could be identified, by gaining information about aberrant molecular pathways. It is believed that tumor cells in general are heavily dependent on stimulation by specific oncogenes that vary by tumor type ("oncogene addiction"), unlike normal tissue that utilizes a broader range of signals (Weinstein, 2002). This phenomenon can be utilized therapeutically: abolishing the specific signalling cascade would damage the tumor tissues´ capability of maintaining a malignant phenotype. Currently, there are drugs in clinical use targeting ESR1 (anastorozole, exemestane, tamoxifen) and ERBB2 (trastuzumab) in breast cancer, and several other target molecules in other cancers (VEGF, EGFR, BCR-ABL) and a growing number of new targets in clinical trials (RAS, mTOR, PI3K, KIT) (Bild *et al.*, 2006).

An extensive comparison analysis between mammary tumors from 13 TG mouse models and human breast cancer revealed several similarities but also some important differences (Herschkowitz *et al.*, 2007) between mouse and human mammary cancer. Mouse mammary tumor models were divided into four main categories and ten subgroups in unsupervised hierarchical cluster analysis. Mouse models such as WAP-Myc, MMTV-Neu, MMTV-PyMT produced homogenous tumor signatures, but other widely used models (MMTV-Wnt1, Brca1$^{co/co}$, p53$^{+/-}$) were more heterogenous as a group and fell into several categories. Human basal-like tumor signature was recapitulated in several mouse tumors, as in Brca deficient and MMTV-Wnt1 mouse tumors. Importantly, mouse models (Neu, PyMT, Myc, Int3), reminiscent of human luminal tumors, lacked the expression of Esr1 and Esr1-regulated genes. Interestingly, MMTV-Neu tumors did not resemble HER2/ESR1 negative human tumors, but merely clustered into luminal tumors. Despite these obvious and important differences, both murine and human mammary cancers clustered into basal/luminal subgroups and showed proliferation and interferon signalling signatures. The initiating oncogenic event determines the gene expression signature in mouse models (Desai *et al.*, 2002), suggesting that the disease induction mechanism is reflected also in the tumor tissue.

2.5. Endocrine regulation of breast carcinogenesis

The endocrine environment profoundly affects mammary gland growth and function. Mammary carcinogenesis is virtually dependent on ovarian hormones: ovariectomy before sexual maturity reduces the relative risk of mammary carcinomas in dogs to 0.005 (Schneider *et al.*, 1969). The first indication of the connection between hormones and human breast cancer was made already in 1896 when Beatson removed ovaries from breast cancer patients and observed remission (Beatson, 1896). Since then, a plethora of correlations between breast cancer and different hormones have been made. Current knowledge of hormonal factors and breast cancer association is described below.

2.5.1. Estrogens

A large body of evidence indicates that exposure to estrogen increases the risk of breast cancer, in both the induction and promotion of breast tumors. However, the mechanisms are complex and surprisingly poorly known. Estrogen is thought to exert its adverse effects on the breast by directly stimulating tissue growth and by its mutagenic, genotoxic metabolites (Clemons and Goss, 2001; Yager and Davidson, 2006).

Anti-estrogen treatment has been shown to diminish the risk of invasive breast cancer in pre- and postmenopausal women at risk for breast cancer by 49%. A reduction of 69% was seen in ESR1 positive cancer but tamoxifen did not reduce the risk of ESR1 negative cancer (Fisher *et al.*, 1998). Ovariectomy decreases both ESR1 negative and positive cancers and the incidence of (primarily ESR1 negative) breast cancers of BRCA1/BRCA2-mutation carriers (Rebbeck *et al.*, 2002). Estrogens could, however, have more complex role in breast cancer initiation and promotion than simple ESR1 activation in breast epithelium. Recently, experimental data supporting this concept was published, showing that estrogen systemically promotes tumorigenesis by a mammary cell independent mechanism. Estradiol promotes angiogenesis and recruits bone-marrow derived cells to contribute to the enhanced growth of Esr1 negative cells (Gupta *et al.*, 2007).

The epidemiological studies on serum estrogen concentration and the breast cancer susceptibility are controversial (Clemons and Goss, 2001), but the data indicates a positive correlation between these variables (Cauley *et al.*, 1999; Toniolo *et al.*, 1995). The relative risk for current users of estrogen replacement therapy is 1.2-1.4 and combined with progestin, the relative risk is 1.4 (Beral, 2003; Clemons and Goss, 2001). Not all of the studies have found a correlation between an estrogen-only regime of HRT (hormone replacement therapy) and breast cancer risk. The risk returns to base level within a year after discontinuation of HRT (Yager and Davidson, 2006). Current use of oral contraceptives (estrogen combined with progestin) elicits a small 1.24-1.07 increase in relative risk of breast cancer. After 10 years of cessation, there is no exess risk (1996). Bone density has been used as a surrogate marker of estrogen exposure. In several studies, a positive correlation between bone mineral density and the risk of breast cancer has been observed (Clemons and Goss, 2001). The relationship between breast cancer and polymorphisms in enzymes synthesizing or catabolizing estrogens is controversial despite many studies (Clemons and Goss, 2001).

Finally, estrogens could induce mutations leading to the tumor formation, since they are metabolized into quinones that can react with DNA and create apurinic sites (Cavalieri *et al.*, 2006). *In vitro* data indicates that 17β-estradiol is able to induce neoplastic transformation in a human mammary gland cell line (MCF-10F, ESR1 negative cell line) in a non-receptor mediated manner. Estrogen was able to cause chromosomal losses and gains, induce anchorage-independent growth and invasiveness and tumor cells derived from transformed cell lines were able to form tumors when injected into SCID mice (Russo *et al.*, 2006).

2.5.2. Progestins

Progestins have long been thought to elicit a differentiating and protecting effect on the mammary gland – largely based on observation of uterine response to progestins.

Proliferation in the breast is highest in the luteal phase (high progesterone) (Graham and Clarke, 1997). However, endogenous progestin levels have been shown not to correlate with breast cancer risk (Eliassen *et al.*, 2006) or to inversely correlate (Kaaks *et al.*, 2005). The largest epidemiological studies on HRT and breast cancer has been conflicting, since some studies have found an increased risk of breast carcinoma in women using a combination of estrogen and progestin vs estrogen only HRT (Beral, 2003; Ross *et al.*, 2000), whereas others have not (1997). Whether these discrepancies could be attributed to different progestin compounds, doses and administration routes is not known.

In rodents, Pgr clearly mediates tumorigenic response. Only 15% of Pgr KO mice developed DMBA induced tumors whereas 60% of WT mice were affected (Lydon *et al.*, 1999). *In vitro* studies on the effects of progestins on mammary gland cells have been less clear, since progestins has been shown to increase and decrease cell proliferation (Lamb *et al.*, 1999; Lanari *et al.*, 2001; Lange *et al.*, 1999). There is evidence that progestins up-regulate growth factor receptors and Pgr crosstalk with growth factors (Lamb *et al.*, 1999), thus making the progesterone effect context dependent. Furthermore, Pgr has two isoforms (PgrA and PgrB), which are regulated and expressed differentially during mammary gland development and elicit different responses (Kariagina *et al.*, 2007). In conclusion, progesterone has complex and context dependent effects on the breast and these effects depend on tissue dynamics, timing and the amount of progesterone stimulation (Lange *et al.*, 1999).

2.5.3. Prolactin

Epidemiologic data suggests that higher serum prolactin is associated with a moderately increased risk of breast cancer, and the risk is strongest in post-menopause for ESR1+/PGR+ cancers (Tworoger *et al.*, 2006). In rodents, PRL is clearly a tumorigenic hormone in the mammary gland: expression of prolactin in mammary epithelium resulted in mammary carcinomas in 65-80% of the mice (Rose-Hellekant *et al.*, 2003).

Prolactin receptor is expressed in six isoforms and its expression is found in up to 98% of breast carcinomas (Clevenger *et al.*, 2003). PRL is mainly produced in the pituitary gland, but it can be synthesized locally. This makes the paracrine or autocrine effects of this hormone likely, since the majority of breast cancers express PRL mRNA (Clevenger *et al.*, 2003). Experimentally, prolactin has been shown to promote cell survival and proliferation as well as tumor vascularization and motility. Production of PRL in breast cancer cells line increased proliferation 1.5 times and a prolactin producing cell line grew 2-4 times faster when injected in mouse (Tworoger and Hankinson, 2006). PRLR inhibition impairs proliferation and cell cycle progression in cell lines (Clevenger *et al.*, 2003). There is currently parallel experimental and epidemiological data on prolactin and breast cancer, although further mechanistic studies on signalling pathway interactions are needed.

2.5.4. Androgens

Androgens in women are produced in the ovaries, adrenals and by the peripheral metabolism of androgen precursors (Somboonporn and Davis, 2004). Androgens are precursor hormones for estrogen biosynthesis, but also act on the breast directly. 60% of invasive breast carcinomas and 82% of ductal carcinoma in situ express androgen receptor (AR)

and AR expression was present even in ESR1 negative cancers (Moinfar *et al.*, 2003). Higher androgen levels have consistently been found to associate with higher breast cancer risk (Eliassen *et al.*, 2006; Kaaks *et al.*, 2005; Somboonporn and Davis, 2004) possibly by increased peripheral conversion of androgens to estrogens. Higher testosterone also predicts breast cancer recurrence (Berrino *et al.*, 2005). In contrast, animal studies with mice, rat and monkeys have suggested a decrease in the proliferation or progression of mammary tumors when androgens have been supplied (Somboonporn and Davis, 2004). *In vitro* studies on the effects of androgen on proliferation and apoptosis have largely been conflicting (Somboonporn and Davis, 2004).

2.5.5. Growth hormone and growth pattern

There is growing amount of human, animal and *in vivo* evidence that growth hormone and its down-stream effector IGF1 are associated with breast cancer development. Given its important role in mammary gland formation, it is not surprising that GH can act as a mitogen in the breast. Transgenic mice over-expressing Gh develop mammary adenocarcinomas (Tornell *et al.*, 1992) and mice having a mutation in the growth hormone releasing hormone receptor with low Gh and Igf1 levels show reduced tumor growth (Yang *et al.*, 1996). Pharmacological inhibition of Gh-Igf-1 axis also impairs xenograft tumor growth (Buchholz *et al.*, 2007; Divisova *et al.*, 2006). However, human studies on hormone concentrations of GH/IGF1 axis have been controversial, but a positive association between breast cancer and high IGF1 and IGFBP3 levels has been suggested. IGF1 and IGFBP3 also correlate with increased breast density (Wagner *et al.*, 2006). Several single nucleotide polymorphisms (SNPs) in the GH-IGF1 axis and their relation to breast cancer have been described, but the effects of these variations are small (Wagner *et al.*, 2006).

Growth pattern *in utero* as well as later in life has been described to influence an individuals breast cancer risk. The GH-IGF1 axis has been partially suggested to mediate the risk observed between high birth weight and breast cancer (Michels and Xue, 2006). There are over 20 studies on the effect of birth weight on subsequent breast cancer risk and the estimated relative risk from several studies for high (> 4000g) vs low (<2500g) birth weight is 1.23 (Michels and Xue, 2006). This association is seen in pre-menopausal but not postmenopausal breast cancer. In addition to the GH-IGF1 axis, intrauterine exposure to estrogens has been suggested to mediate the effect of high birth weight in breast cancer risk. However, a small study with monozygotic twins indicated that in the presence of similar hormonal environment *in utero*, the heavier twin is at greater risk of breast cancer (Hubinette *et al.*, 2001). Hormonal exposure *in utero* and during puberty has been suggested to alter epigenetic programmingin mammary gland, and thus, affect subsequent breast cancer risk (De Assis and Hilakivi-Clarke, 2006). In addition to birth weight, other anthropometric variables associate with breast cancer risk. Adult height correlates with breast cancer risk with an 11% increase of breast cancer incidence with each 5 cm increment of adult height (Baer *et al.*, 2006; Lahmann *et al.*, 2004; van den Brandt *et al.*, 2000). Furthermore, the timing and dynamics of pubertal growth affects breast cancer risk. Taller height at younger age and earlier peak growth was found to increase breast cancer risk (Ahlgren *et al.*, 2004). Small body mass index (BMI) during puberty and pre-menopause increases breast cancer risk, whereas high BMI in post-menopause associates with increased risk (Ahlgren *et al.*,

2004; Key *et al.*, 2003; Ursin *et al.*, 1995). Higher BMI in post-menopause is associated with higher bio-available estrogens (Key *et al.*, 2003).

2.5.6. LHR polymorphism and breast cancer risk

A specific polymorphism in the LHR gene, an insertion of 6 bp upstream of the proposed signal peptide cleavage site (LHR insLQ), with a frequency of 44% in the normal population, has recently been associated with adverse outcome in breast cancer patients. Clinically, the carriers present with earlier onset of breast cancer (52 vs 60 years) and significantly worse overall and disease-free survival with a tendency for lymph node involvement and larger tumor size (Piersma *et al.*, 2006; Powell *et al.*, 2003). The insertion did not affect the overall breast cancer risk. Biologically, this polymorphism increased LHR receptor sensitivity and cell surface expression, and it was suggested that the adverse outcome was due to altered synthesis of ovarian hormones, although a more direct mechanism of the variant LHR affecting in breast tissue specifically could not be excluded. The effect of the polymorphism on ovarian function was not studied. Another single nucleotide polymorphism (SNP) in LHR (LHR312Asn) was found to be a breast cancer susceptibility allele, increasing the disease risk from 15% to 26% without affecting clinico-pathological characteristics (Piersma *et al.*, 2007). Interestingly, no functional changes in the LHR312Asn variant were observed despite the clear increase in breast cancer incidence. In contrast, another polymorphism in LHR, 291Ser LHR, resulted in altered glycosylation status and increased receptor sensitivity without affecting breast cancer risk or clinico-pathological findings. Thus, polymorphisms in LHR affect the risk, survival and the clinico-pathological features of breast cancer by mechanisms that are not yet fully understood.

2.6. Pregnancy and breast cancer

If breast cancer is diagnosed during pregnancy or within one year of delivery, it is defined as a pregnancy-induced breast cancer (Michel *et al.*, 2007). Although pregnancy is generally considered to be a protective factor from breast cancer, it transiently promotes breast cancer incidence in all age groups. Increase in breast cancer risk lasts for a decade and increased risk peaks 6 years after parturition. Furthermore, pregnancy permanently increases breast cancer risk in women whose first full time pregnancy was at age of more than 35 years (Schedin, 2006). Pregnancy-induced breast cancer has overall worse survival rates (Daling *et al.*, 2002) and the survival rate is especially decreased if breast cancer is diagnosed within a year of parturition (15-year survival rate 38% vs 65% of that of age matched nulliparous women (Schedin, 2006)). Recently, breast tumor stroma of pregnancy-induced breast cancer was found to recruit fetal derived mesenchymal and epithelial cells, whereas normal breast tissue did not have fetal cells present (Dubernard *et al.*, 2008), suggesting that these cells contribute to carcinogenesis of the breast during pregnancy. The mechanism of initial promotion of pregnancy-induced breast cancer incidence is not known, but immune modulation and profound changes in the extra-cellular matrix have been suggested to contribute to the risk (McDaniel *et al.*, 2006; Shakhar *et al.*, 2007). High hormonal load during pregnancy could also contribute to the promotion of pre-malignant cells. Direct stimulation of HCG is also possible, and the mechanism has been suggested to involve increased angiogenesis by HCG upregulating VEGF (Michel *et al.*, 2007). Pregnancy-induced breast tumors tend to be PGR negative, have lymph node involvement and a higher

histological grade (Daling *et al.*, 2002). Ultimately, parity and early first pregnancy only diminishes ESR1+, PGR+ cancers, without having an effect on receptor negative cancer types, suggesting that pregnancy contributes to carcinogenesis by a different mechanism in ESR1 positive and negative subtypes (Britt *et al.*, 2007; Ursin *et al.*, 2005). Importantly, ESR1 negative cancers are not diminished by parity. Long term protection from ESR1 positive cancer has been proposed to result from altered hormonal levels of parous women, increased epithelial differentiation, decreased number of mammary stem cells, or due to changes in estrogen responsiveness (Britt *et al.*, 2007).

3. Lutenizing hormone and human chorionic gonadotropin

Gonadotropins LH and FSH are the master regulators of steroidogenesis and reproductive fuctions. Their release from the anterior pituitary is controlled by hypothalamic gonadotropin-releasing hormone (GNRH) that is secreted in a pulsatile fashion.

GNRH is released to the portal circulation and binds to GNRH receptors on the surface of the gonadotroph. The pulse frequency of GNRH varies from 60 minutes in the late follicular phase to 200 min during the late luteal phase. More rapid frequency favours LH secretion and slower frequency FSH secretion. Continuous GNRH or GNRH agonist administration inhibits LH and FSH secretion in animal models by uncoupling of the GNRH signalling. Gonadal steroid hormones and inhibin elicits a negative feedback signal on gonadotrophs and hypothalamic GNRH secretion, but estrogen elicits positive feedback on LH and FSH secretion prior to ovulation. Recently, kisspeptin (which binds to GPR54) acting in the central neural system was recognized as an essential activator of the GNRH-gonadotropin axis. It has an important role in puberty onset and in controlling ovulation. It is involved in integrating the metabolic status of the body with appropriate timing of puberty, and also integrates other environmental cues to reproductive function (Dungan *et al.*, 2006; Roa *et al.*, 2007). Other neurotransmitters such as dopamine, serotonin and norepinephrin also modulate GNRH secretion. The inhibitory and stimulatory feedback loops are, however, complex and context dependent. For example, estrogen can either inhibit or stimulate both GNRH and gonadotrophin synthesis and secretion. Interactions between GNRH, FSH, LH secretion and ovarian hormones are presented in Figure 4. (1998).

Human chorionic gonadotropin (HCG) is a pregnancy hormone (Larsen PR, 2002), secreted from the trophoectoderm prior to implantation and subsequently from the placenta (syncytiotrophoblasts) later on (Keay *et al.*, 2004). It is essential for maintaining progesterone production from the corpus luteum in early pregnancy, and prepares endometrium for implantation (Berndt *et al.*, 2006). In males, it is needed for stimulation of fetal testicular testosterone production. The level of HCG rises rapidly in early pregnancy, peaking in maternal circulation at 7-9 weeks of gestation and then declines until week 20, after which the level is constant. The mechanism of regulation of HCG secretion is not well known, but GNRH is produced in syncytiotrophoblasts and it is able to stimulate HCG secretion *in vitro* and *in vivo*. Trophoblastic tumors also secrete HCG dimers.

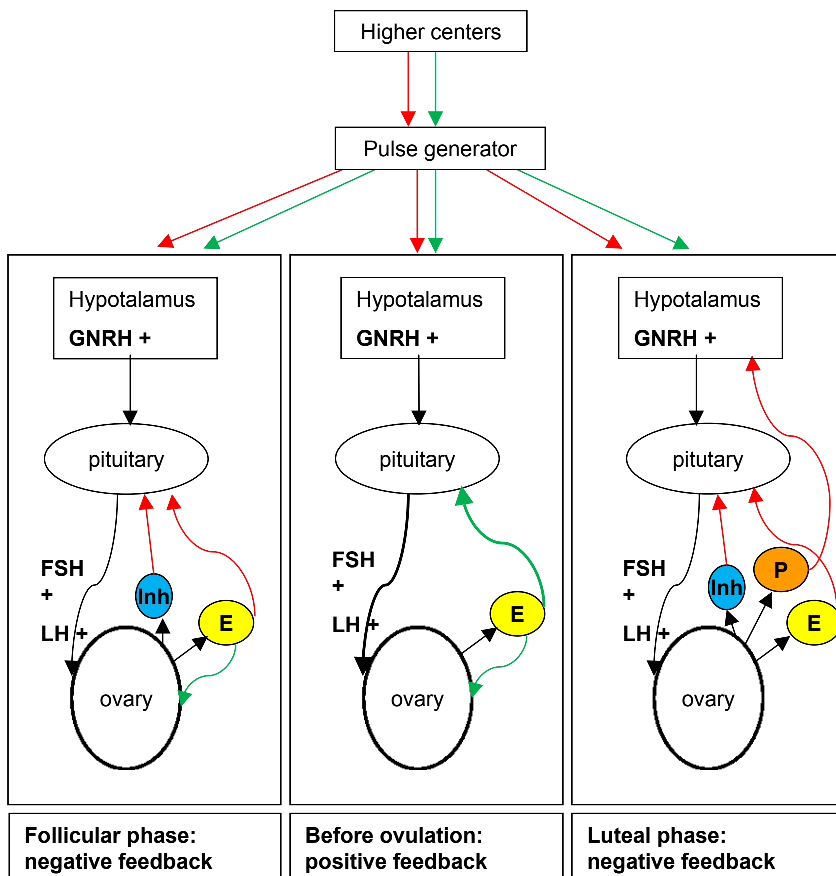

Figure 4. Hormonal control of LH secretion in females

3.1. Structure of LH, HCG and LH receptor

3.1.1. Structure of LH and HCG

Luteinizing hormone (LH) is a glycoprotein hormone, secreted from the anterior pituitary gland. It is a heterodimer, composed of two subunits: the α-subunit is common with other glycoprotein hormones (FSH, TSH, HCG) and the β-subunit is hormone specific. The subunits are noncovalenty linked by disulfide bonds and glycosylated at specific residues. Heterodimerization is obligatory for LH to elicit its actions. LHβ and HCGβ genes are both located on 19q13.32, in a chromosomal region that also harbours six pseudo-HCGβ-genes (Talmadge *et al.*, 1983). LHβ mRNA has 3 exons. The 121-amino-acid protein is cleaved from 145-amino-acid precursor peptide. LH is conserved across species, unlike human chorionic gonadotropin (HCG), that is present only in primates and equine species. HCGβ gene codes a 145-amino-acid peptide that shares 82% homology with the LHβ subunit. HCG is glycosylated at specific sites, making its half-life longer compared to LH (24 hours vs 20 minutes). LHβ and HCGβ both bind to LH receptor (LHR) and elicit the same biological responses, although there has been speculation about the existence of a separate HCG receptor (Keay *et al.*, 2004).

3.1.2. Hyperglycosylated HCG and free β-subunit

Hyperglycosylated HCG (HCG-H) has the same peptide structure as HCG but is glycosylated at different residues, resulting in altered structure and function of the protein. The structure of HCG-H resembles that of the TGFβ family with a unique cystine knot structure and its functions poorly mimic classical HCG actions. HCG-H has only 1/25 capacity for binding to LHR, and thus, an alternative method of action has been proposed (Cole, 2007). Invading cytotrophoblast cells secrete HCG-H, whereas it is not produced in mature syncytiotrophoblasts. It is essential in the early invasion and a low amount of HCG-H predicts poor outcome of the pregnancy (Cole and Khanlian, 2007). About 90% of total HCG is hyperglycosylated at the 3rd week of gestation, but in contrast, only about 15% of total HCG is hyperglycosylated in the 7th week (Cole and Khanlian, 2007). Hyperglycosylation is observed in invading trophoblastic diseases but not in the quiescent forms, indicating also that HCG-H has a direct role in the invasion. HCG-H has been suggested to act in autocrine rather than endocrine manner, involving blockage of TGFβ-mediated apoptosis (Cole, 2007).

HCG β-subunit is ectopically secreted in many epithelial cancers, such as bladder, lung, breast, cervical, ovarian, colorectal and ovarian cancers and has been found to have prognostic significance (Butler and Iles, 2003). The role of free β-subunit, which is not capable of binding to LHR, is controversial. It is able to inhibit apoptosis, and thus, increase the growth rate in bladder cell culture setting (Butler *et al.*, 2000) and the effect was abolished by antibodies against a free β-subunit. Again, evidence for TGFβ-RII antagonism was found and suggested as a mechanism. In a study utilizing HCG β-subunit transfected mouse mammary carcinoma cells transplanted into host mammary glands, inhibition of tumor growth and angiogenesis was observed without effect on endocrine function (Shi *et al.*, 2006).

3.1.3. Structure and interactions of LH receptor

LHR is a G protein coupled receptor (GPCR) belonging to the rhodopsin-like GPCR family together with TSHR and FSHR. The gene for LHR is located on chromosome 2 in humans and has 11 exons. LHR mRNA has several splice-variants depending on species, varying in size from 1.8 kb to 7.8 kb (Menon *et al.*, 2004; Vihko *et al.*, 1992), not all of which are functional. The transcription initiation site varies greatly by tissue and species (Huhtaniemi *et al.*, 1992; Tsai-Morris *et al.*, 1991; Tsai-Morris *et al.*, 1993). LHR protein contains seven transmembrane spanning α-helices, a large extracellular domain and an intracellular domain. LHR elicits its signal mainly by increasing intracellular adenosine-3´-5´-cyclic monophosphate (cAMP) and in some cases, by stimulating phospholipase C, thus, leading to production of inostitol triphosphate (IP3) and diacylglycerol (DAG). Stimulation of MAPK and JAK –pathways has also been described (Dos Santos *et al.*, 2007; Menon *et al.*, 2004). Despite the close homology between HCG and LH, their interactions with LHR are not identical. Deletion of the exon 10 of the LHR results in an inability of LH to bind to LHR and evoke response, whereas HCG is able to bind to the mutant LHR and produce the appropriate cAMP-response (Muller *et al.*, 2003). It has recently become more apparent that GPCRs form both homo- and heterodimers and that signal transduction is affected by dimerization (Bai, 2004). Ligand-binding-deficient TSHR could induce an appropriate cAMP signal upon TSH stimulation when dimerized with signal transduction-deficient TSHR. Dimerization of TSHR and heterodimers between TSHR and LHR has been

demonstrated in living cells (Urizar *et al.*, 2005). Heterodimerization happens primarily between the transmembrane segments of the receptors (Urizar *et al.*, 2005).

There is substantial sequence homology between β-subunits of glycoprotein hormones and their respective receptor binding sites. Although the affinity of LH/HCG to its receptor is high, in higher ligand concentrations, LH/HCG is able to bind to other rhodopsin-like GPCRs (Schubert *et al.*, 2003). Studies with HCG yoked with LHR, FSHR and TSHR indicate that cAMP production was increased by 13- (FSHR) and 4-fold (TSHR) compared of the basal level, whereas HCG/LHR complex yielded a 20-fold increase in cAMP production. This interaction does not, however, prevent binding of the receptors cognate ligands to its receptor (Schubert *et al.*, 2003). Cross-reactivity of this hormone receptor family is observed to some degree even at physiological hormone concentrations. Gestational hyperthyroidism is caused by high HCG stimulating TSHR, and in hypothyroidism, high TSH can cause precocious puberty by stimulating LHR. This confirms that cross-reactivity has relevance in pathophysiological conditions in humans (Niedziela and Korman, 2001; Rodien *et al.*, 2004).

3.2. Distribution and regulation of LHR

LHR is mainly expressed in the gonads. In the ovary, LHR is expressed in theca, granulosa, luteal and interstitial cells. LHR mRNA levels in follicles vary by maturation state and during the reproductive cycle. In primary and secondary follicles, primarily FSH stimulates granulosa cells. In mature preovulatory follicles LHR is located in theca and granulosa cells, usually in humans this occurs only in one follicle (England *et al.*, 1981). After ovulatory doses of HCG, LHR mRNA decreases, but expression is regained in the formation of the corpus luteum (Liu *et al.*, 1998; Peng *et al.*, 1991). However, treatment with lower doses of LH results in an increase in LHR. In testis, LHR is expressed in Leydig cells where LH stimulates T synthesis. LHR mRNA is expressed already in fetal Leydig cells from E16, although prenatal Leydig cell function is normal in the absence of gonadotrophins in mice (O'Shaughnessy *et al.*, 1998). In contrast, LHR is needed for normal masculinization in human male fetuses (Latronico, 2000).

Expression of LHR in non-gonadal tissues has been detected in the adrenal gland (Apaja *et al.*, 2005; Pabon *et al.*, 1996b), blood vessels (Lei *et al.*, 1992; Reshef *et al.*, 1990), brain (Apaja *et al.*, 2004; Lei *et al.*, 1993a), cervix (Lin *et al.*, 2003), fetal tissues (Abdallah *et al.*, 2004), kidney (Apaja *et al.*, 2005), lymphocytes (Lin *et al.*, 1995), mammary gland (Funaro *et al.*, 2003; Tao *et al.*, 1997b), human breast cancer cells (Funaro *et al.*, 2003; Meduri *et al.*, 1997; Meduri *et al.*, 2003), oviduct (Han *et al.*, 1996; Lei *et al.*, 1993b; Zhang *et al.*, 2001b), placenta (Reshef *et al.*, 1990), prostate (Reiter *et al.*, 1995; Tao *et al.*, 1997a), seminal vesicle (Tao *et al.*, 1998), skin (Pabon *et al.*, 1996a; Venencie *et al.*, 1999), sperm (Eblen *et al.*, 2001), spinal cord (Rao *et al.*, 2003), umbilical cord (Rao *et al.*, 1993) and uterus (Han *et al.*, 1997; Reshef *et al.*, 1990; Zhang *et al.*, 2001b). The protein has been detected by Western blotting, immunohistochemistry and immunoprecipitation, while others have reported mRNA by RT-PCR, Northern blotting or *in situ* hybridization. The functionality of the observed LHR is not documented and methodolocially the studies concerning non-gonadal LHR are rather weak (Stewart, 2001). LHR protein has not often been of the expected size of 75 kD and mRNA molecules were smaller than that observed in the ovaries, possibly representing alternative splice variants. Moreover, in some mRNA studies, the authors have not been able to amplify the extracellular domain, while the transmembrane

domain has been detected, thus questioning the functionality of the possible protein product (Stewart *et al.*, 1999). Even in the presence of full length mRNA of right size protein in a given tissue, it has not been always possible to detect binding of the ligand to the LHR and the subsequent intracellular events, such as increase in cAMP or phosphorylation of ERK1/2 (Viswanath *et al.*, 2007; Yarram *et al.*, 2003). However, alternatively spliced LHR variants appear to be a conserved phenomenon in several species and several extragonadal sites. cAMP response has been reported in extragonadal sites, such as rat prostate (Reiter *et al.*, 1995) and human sperm (Eblen *et al.*, 2001). Extragonadal activation of alternative LHR signalling pathways after HCG stimulation have been observed in human adipose cells (MAPK/c-fos-pathway) (Dos Santos *et al.*, 2007)

LHR mRNA has been found in human reduction mammoplasty samples (Popnikolov *et al.*, 2001). With immunohistochemistry, LHR has been found in human breast samples where LHR localized mainly in the basal cells of the lobular structures (Funaro *et al.*, 2003). Occasionally, epithelial and stromal LHRs were also observed. In the lactating and postmenopausal breast, only occasional staining was observed, suggesting that LHR expression in human breast requires functional ovaries. Fetal breast tissues do not express LHR. In contrast, intense staining for the LHR protein in the membranes of the basal cells was observed in fibroadenomas and papillomas. LHR expression has been demonstrated in 72% of breast cancer biopsies by immunocytochemistry (Meduri *et al.*, 2003), and expression was found to be more frequent in the tumors of premenopausal women. LHR expression associated with better tumor cell differentiation and longer metastasis free survival, but not with other clinical or histopathological features. Functional LHR has also been found in commonly used breast cancer cell lines (Lojun *et al.*, 1997). LHR mRNA and protein expression as well as HCG-binding has been reported in the lactating rat mammary gland (Tao *et al.*, 1997b), suggesting the functionality of the receptor.

LHR expression is regulated at many levels, including gene transcription, RNA processing, mRNA decay, translation and post-transcriptional modification. Although regulation of transcription is thought to be the main regulatory level, substantial post-transcriptional modifications of the mRNA also occurs. Increase of cAMP after pharmacological doses of HCG stimulates expression of LH/HCG receptor mRNA –binding protein (LRBP), resulting in decay of LHR mRNA. LRBP was identified as mevalonate kinase (Menon *et al.*, 2004; Nair *et al.*, 2002). Recently, it was described that truncated receptor variants form complexes with full-length LHR and target them for degradation, resulting in a decreased number of LHR in the plasma membrane (Minegishi *et al.*, 2007). FSH, PRL, E2 and P participate in LHR regulation in the gonads (Casper and Erickson, 1981; El-Hefnawy and Huhtaniemi, 1998; Goxe *et al.*, 1992) and LHR is downregulated by its ligand (Hoffman *et al.*, 1991). At least in some extragonadal sites, LHR protein expression is regulated by estrogens and progesterone (Gawronska *et al.*, 2000) *in vivo*. E2 and P together could induce LHR expression in porcine oviducts, but it was not induced with either of them alone or without these hormones.

3.3. Effects of LH in gonads

Schematic presentation of LH-ovary –interactions are presented in the Figure 4. LH levels are low in the follicular phase of the menstrual cycle, when FSH and estrogen -stimulated growth of follicles slowly increase estradiol concentration. FSH, estradiol and activin

stimulate the appearance of LHR on granulosa cells. In the midcycle, rising estradiol triggers positive feedback in the pituitary, releasing a surge of LH. This results in ovulation 34-36 hours after the LH peak. Subsequently, the corpus luteum is formed from granulosa and theca cells. In the ovary, follicles are able to develop up to the antral stage in the absence of LHR, but development beyond that is blocked (Pakarainen *et al.*, 2005b). Although estrogen and progesterone elicit negative feedback on LH and FSH secretion in the follicular and luteal phases, the LH peak is induced by a positive feedback effect in the midcycle prior to ovulation. Positive feedback is caused by increasing levels of estradiol after a certain threshold level, which is essential for the timing for the ovulation. LH is furthermore needed for maintaining the corpus luteum in the luteal phase, and withdrawal of LH results in premature regression of corpus luteum. The corpus luteum regresses after 14 days in a normal cycle despite the steady level of circulating LH, unless pregnancy occurs, which maintains corpus luteum stimulation with HCG. The aging corpus luteum becomes insensitive to LH stimulation, but the precise mechanism is not known. Interestingly, even large amounts of LH cannot rescue the corpus luteum in a normal cycle whereas HCG (or other hormonal factor secreted in pregnancy) can. LHR are found in theca-interstitial cells in the follicular phase, where LH promotes androgen production, and thus, indirectly also estrogen production. This happens by aromatization of androgen-precursors in granulosa cells that do not express LHR in the early phase of the cycle. Theca cells do not possess aromatase activity and cannot convert androgen-precursors to estrogens on a large scale. In the luteal phase of the menstrual cycle, LHR is expressed in the corpus luteum. Under stimulation, it produces large quantities of progesterone and estrogens. (1998).

Women with missense mutation in LHR present with anovulation and increased levels of FSH and LH in the absence of negative feedback (Themmen and Huhtaniemi, 2000). This is in line with animal studies which have shown that LH is absolutely required for ovum development and cannot be replaced by other signals (Pakarainen *et al.*, 2005b). In males, LH stimulates sex steroid (mainly testosterone) synthesis in Leydig cells. Mice lacking LHR develop normally in the prenatal period, but sexual development thereafter is arrested (Zhang *et al.*, 2004). Although infertile, these mice are able to recover spermatogenesis in the presence of very low production of testosterone (Zhang *et al.*, 2003), demonstrating that LH is not absolutely required for mouse spermatogenesis. Furthermore, the fertility of LHR KO mice is partially restored by testosterone treatment (Pakarainen *et al.*, 2005a), demonstrating that the absence of LHR inhibits spermatogenesis by secondary events. In humans, LHR is needed in the masculinisation of male foetuses, and inactivating mutation in LHR results in Leydig cell hypoplasia and pseudohermafroditism (Latronico, 2000).

3.4. Effects of LH and HCG in non-gonadal tissues

LH and HCG induced effects have been reported in several non-gonadal tissues, including breast, uterus, adrenal gland, central neural system, and adipocytes. Although the data has been contradictory and not always convincing, increasing evidence currently suggests that LH/HCG elicit actions in non-gonadal tissues. In the mammary gland and breast, LH/HCG actions are poorly characterized and the results have been variable. However, caution is needed in interpreting both negative and positive results in non-gonadal tissues, because of the probable differences between species in LH/HCG –biology.

The experimental data on LH/HCG effects on breast tumorigenesis is scarce and comes from a limited number of laboratories. Although it has been suggested that HCG would elicit anti-tumorigenic effects in rat after DMBA exposure (Russo *et al.*, 1990), epidemiological data does not support the protective role of HCG on breast cancer risk. Women that have suffered from a hydatiform mole (producing high amount of HCG) had a risk ratio of 1.3 in developing breast cancer in a prospective study with 57 000 person-years of follow-up (Erlandsson *et al.*, 2000). In a recent small-scale study with breast cancer patients, recombinant HCG given 2 weeks prior to surgery significantly reduced the proliferation and the expression of ESR1 and PGR. The effect of 500 ug recombinant HCG every other day on metastatic breast cancer was evaluated in 13 cases without consistent effects, although partial remissions were reported (Janssens *et al.*, 2007). Elevated levels of HCG have also been found in breast cyst fluids (Abney *et al.*, 1988). It has been suggested that HCG would stimulate VEGF, and thus promote angiogenesis in tumors, resulting in worse prognosis observed in breast cancer associated with pregnancy (Michel *et al.*, 2007).

Experimentally, HCG was found to increase the proliferation of normal human breast epithelium transplanted into nude mouse in a dose dependent manner (Popnikolov *et al.*, 2001), where it had an additive effect on proliferation upon estradiol and progesterone stimulation. The proliferation effect was abolished by ovariectomy, demonstrating that HCG requires ovarian steroids in order to elicit effects on breast epithelium. However, in this study, changes in ovarian function could contribute to the increase in proliferation since ovariectomy was not performed. In *in vitro* studies, HCG has been reported to decrease proliferation, increase apoptosis and protect from mammary carcinogenesis (Srivastava *et al.*, 1997). MCF-7 cells exhibit dynamic changes in genes regulating apoptosis and proliferation in response to treatment with HCG (Guo *et al.*, 2004). It has been reported that the same cells were able to convert dihydroepiandrosterone to estrogens in the presence of HCG, and thus, increase proliferation (Tanaka *et al.*, 2000). A small 10% enhancement in radiosensitivity of MCF-7 was reported in response to HCG (Pond-Tor *et al.*, 2002). Together the data on HCG exposure and human breast cancer is scarce and inclonclusive, and the cell culture data suggests that HCG effects are highly dependent on cell culture conditions.

HCG has a paracrine role in the embryonic implantation site and prepares the endometrium for pregnancy (Licht *et al.*, 2007). HCG elicits angiogenic signals on mouse endometrial endothelium by enhancing Vegf production from endometrial epithelium (Berndt *et al.*, 2006). HCG has been shown to induce morphological and gene expression changes in baboon uterus (Srisuparp *et al.*, 2001), where it was shown to regulate several dozens of genes, including SerpinA3, Mmp7, Lif, Il6 and Sfrp4 (Sherwin *et al.*, 2007). HCG was also found to regulate Lif and Il6 expression in decidual and endometrial epithelium in other studies (Perrier d'Hauterive *et al.*, 2004; Ren *et al.*, 1997). Myometrium and fetal membranes are also target tissues of HCG, in which HCG regulates the expression of Pde5 and iNos and elicits pregnancy-promoting actions (Ticconi *et al.*, 2007).

LHR is expressed in fetal and adult, normal and abnormal adrenal tissues (Carlson, 2007). There are several reports of LHR aberrantly stimulating the production of adrenal hormones: glucocorticoids, aldosterone, androgens and estrogens. Conditions with elevated LH or HCG (postmenopause, pregnancy) have been documented to clinically provoke some of these cases. In animal models with elevated Lh-action, adrenal hyperplasia, adenomas and carcinomas

have been observed. HCG does not however normally stimulate glucocorticoid production, nor do all of the LHR expressing adrenal tumors respond to HCG stimulation, making the association more complex (Carlson, 2007). An animal study utilizing Lhr-tranfected cells transplanted into immunodeficient mice demonstrated, however, that at least in some cases, the overexpression of the functional receptor alone is sufficient to induce hypersecretion of cortisol and hyperplasia of the transfected cells (Mazzuco *et al.*, 2006).

Lh has been speculated to be involved in neuronal differentiation (Lei and Rao, 2001), and the pathogenesis of neurogenerative diseases, such as Alzheimer´s disease (Barron *et al.*, 2006). Recently, a direct LHR mediated mechanism of HCG stimulating neuronal differentiation was described *in vitro* (Meng *et al.*, 2007). Studies *in vivo* confirmed the proliferatory effect of Lh on neurons in the mouse olfactory bulb and hippocampus and confirmed Lh´s role in mediating responses to pheromone exposure (Mak *et al.*, 2007). A previously unknown role of HCG in preadipocyte differentiation and function was also recently described. In preadipocytes, HCG was shown to elicit its action by the MAPK-signalling pathway (Dos Santos *et al.*, 2007). HCG was shown to enhance angiogenesis in several *in vivo* and *in vitro* assays by stimulating VEGF secretion (Berndt *et al.*, 2006).

3.5. Transgenic mouse models for studying LH action

Several genetically modified mouse models for studying Lh-action have been created by different groups. These include two models for inactivating mutation for Lhr (Huhtaniemi *et al.*, 2006) with similar phenotypes. A mouse model for overexpression of LHβ, and two models overproducing HCG have been created. Both HCG-models (producing αβ or β alone) resemble each other. An activating HCG-Lhr complex has been described with essentially the same findings on the phenotype (Huhtaniemi *et al.*, 2006). Table 3 summarizes phenotypes found in mouse models and respective human spontaneous mutations.

Table 3. Phenotypes of the human LH and LHR mutations and corresponding mouse models.

FEMALE	**Activating LHβ**	**Activating LHR**	**Inactivating LHβ**	**Inactivating LHR**
HUMAN	*ND*	*No phenotype*	*ND*	*Normal pubertal development, anovulation, infertility*
MOUSE	*TG for LHβ, HCGβ or HCGαβ: Precocious puberty, ovarian luteinization /teratomas, multiple secondary phenotypes*		*Normal at birth, hypogonadal as adults, anovulation*	*Normal at birth, no puberty, anovulation, infertility*

<table>
<tr><th>MALE</th><th>Activating LHβ</th><th>Activating LHR</th><th>Inactivating LHβ</th><th>Inactivating LHR</th></tr>
<tr><td>HUMAN</td><td>ND</td><td>Gonadotrophin independent precocious puberty</td><td>Normal fetal masculinization, no puberty, infertility</td><td>From hypogonadism to pseudo-hermaphrodism</td></tr>
<tr><td>MOUSE</td><td colspan="2">TG for LHβ, HCGβ or HCGαβ: No phenotype/enhanced androgen production and infertility, secondary phenotypes</td><td>Normal fetal masculinization, no puberty, infertility</td><td>Normal fetal masculinization, no puberty, infertility</td></tr>
</table>

Briefly, the female phenotype of Lhr knock-out mice models include blockage of the follicular maturation beyond the antral stage, and thus, anovulation and hypogonadism together with subsequent secondary phenotypes due to lack of ovarian hormones (Pakarainen *et al.*, 2005b). Lhr is thus essential for ovulation, although some discrepancies exists, since in some studies, Fsh was reported to induce ovulation in hypophysectomized mice (Tapanainen *et al.*, 1993) whereas others could not induce ovulation with Fsh (Pakarainen *et al.*, 2005b). Overexpression of LHβ or HCG in female mice results in precocious puberty and ovarian changes, including increased luteinization, cyst development and anovulation. This results in either infertility (activating Lhr mutation, overexpression of the ligands) or subfertility (ligand-mediated constitutively active Lhr) (Meehan and Narayan, 2007). These phenotypes are in contrast with human female phenotype, where activating mutation of LHR does not elicit any phenotype (Latronico, 2000; Rosenthal *et al.*, 1996). Activating mutations of LHβ have not been yet found in humans.

4. Wnt signalling

4.1. Wnt proteins and their receptors

The Wnt protein family is a highly conserved growth factor family and its members are important in developmental processes and tissues homeostasis. Dysregulation of Wnt signalling pathways is involved in several malignancies, such as cancer. Recently, Wnt signalling has been discovered to influence stem cell fate.

In humans, there are 19 members of Wnt family genes that can be divided in 12 subfamilies (Gordon and Nusse, 2006). Wnts act as short-distance (20-30 cell diameters) signalling molecules that are mostly found in extra-cellular matrix and cell membranes. Crystal structures for Wnts have not been elucidated. They are hydrophobic and include signal sequence for secretion. Wnts share also defined spacing of cysteine residues, suggesting common structure in protein folding. Wnts are modified by glycosylation and by lipid modification (palmitoylation), and these modifications affect Wnt function heavily. Although Wnt genes were classically dived into canonical and non-canonical ligands, individual Wnt ligands can operate in both canonical and non-canonical signalling, depending on the presence of the receptors and various inhibitory proteins (Liu *et al.*,

2005; Mikels and Nusse, 2006b). Furthermore, final target gene transcription is affected by interactions with other pathways and the presence of co-factors, creating a cell context specific response upon Wnt stimulation (Gordon and Nusse, 2006).

There are several receptors capable of Wnt signal transduction. Best known are the members of the Frizzled (Fz) family, that are seven-pass transmembrane proteins possessing a large extracellular domain with several potential Wnt binding sites. Other receptors mediating Wnt signals are Ryk and Ror2, both of which are structurally non-related to Fz receptors. Ror2 has a similar binding domain (cysteine residue domain CRD) to Fz receptor but RYK utilizes an alternative binding domain (WIF) (Mikels and Nusse, 2006a). Additional co-receptors (LRP5/6, FRL1/Cripto) are recruited for intracellular signal transduction.

4.2. Wnt signalling cascades

4.2.1. Canonical Wnt signalling cascade

A simplified presentation of the canonical Wnt-signalling pathway is presented in Figure 5. In an unstimulated state, cytoplasmic β-catenin is phosphorylated by destruction complex, composed of Axin, GSK3, and APC, and thus targeted for degradation. The hallmark of activated canonical Wnt signalling is the accumulation of β-catenin into cytoplasm. In an activated state, following Wnt binding to Fz receptor, Fz and its co-receptor LRP5/6 interact to bind the destruction complex in a process recruiting Dishevelled (Dsh). Binding of destruction complex to Fz-LRP-Dsh -complex leaves cytosolic β-catenin unphosphorylated, leading to its accumulation. Increase in cytosolic β-catenin leads to its translocation into nucleus, where it forms a complex with TCF/LEF transcription factor by replacing transcriptional repressor Groucho. This initiates target gene transcription. Several additional important regulatory proteins not mentioned above are involved in the regulation of canonical pathway and its transcription complex, but the exact physical interactions between these proteins remain to be elucidated (Gordon and Nusse, 2006; Miller, 2002).

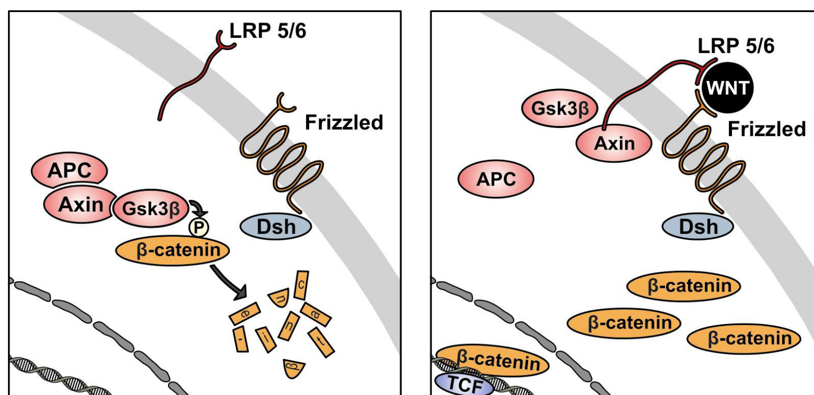

Figure 5. The canonical wnt-signalling pathway

Aberrant activation of the canonical Wnt-pathway is one of the most common themes in human cancers. Recently, canonical Wnt signalling has been discovered to influence stem cell fate not only in embryos, but in adult tissues as well. Wnt regulation of stem or progenitor cell proliferation and differentiation is found at least in hair follicles, intestine, neural progenitors and hematopoietic stem cells (Reya and Clevers, 2005). Wnts regulate also oncogenesis in these tissue types, indicating that disturbed Wnt-signalling shifts the delicate balance between proliferation and differentiation. Disturbances in canonical Wnt-pathway are indicated behind several pathological conditions, including many types of cancer, kidney diseases and osteoarthritis, among others. The disease prototype is colon cancer, where inactivating mutation in the APC gene, encoding a member of β-catenin regulatory destruction complex, causes colon polyposis and subsequent colon carcinoma both in familial and sporadic cases. APC is mutated in substantial amount of the cases, and in the absence of germline or somatic APC defects, other genes from the canonical pathway are often somatically mutated or epigenetically dysregulated causing constitutive activation of the pathway (Segditsas and Tomlinson, 2006). In human breast cancer, 50% display β-catenin accumulation (Lin et al., 2000). Mutations in genes of the Wnt-pathway have not been found in breast cancer although the pathway is frequently activated, suggesting functional over-activity. Importantly, activation of the canonical Wnt-pathway has been reported to increase the number of progenitor cells in the mammary gland, and increase the susceptibility to mammary gland tumorigenesis (Li et al., 2003; Liu et al., 2004).

4.2.2. Non-canonical Wnt signalling cascades

Non-canonical Wnt cascades and their functions are thus far poorly characterized. While most of the studies concerning Wnt signalling have been done with Drosophila, there are some major differences in vertebrate and Drosophila non-canonical signalling, such as the planar cell polarity (PCP) pathways independence of ligands in Drosophila. In vertebrates, non-canonical pathway can signal through several downstream pathways, including Ca-signalling, JNK-pathway and Rho-signalling. These pathways govern processes such as gastrulation, dorsoventral patterning, neuronal migration, heart induction and even cancer (Veeman *et al.*, 2003). These pathways utilize also Fz receptors and Dsh, but are independent on β-catenin signalling.

4.3. Wnt signalling in the mammary gland

Wnt signals are required for mammary gland morphogenesis and are regulated in the induction of different developmental stages. Wnt proteins have unique expression patterns and sites in virgin, pregnant, lactating and involuting glands. Wnts mediate communication between mammary epithelium and stroma. Some Wnt proteins are able to induce or inhibit lobuloalveolar development and ductal branching in absence of hormonal stimulus, suggesting that Wnts are at least partially responsible for mediating hormone actions in breast. Precise hormonal regulation of Wnts is not known, although Wnt4 has been suggested to signal downstream of progesterone receptor (Brisken *et al.*, 2000). Wnt signalling can also initiate carcinogenesis in mammary gland, as demonstrated by several mouse models with activated Wnt/ β-catenin signalling.

Wnt signalling is needed in the formation of mammary anlagen and prenatal mammary formation. Mice lacking the transcription factor lymphoid enhancer-binding factor 1 (Lef1) fail to form mammary glands. Lef1 induces expression of parathyroid hormone-like peptide (Pthrp) and its receptor, that are needed for proper mesenchyme condensation and further epithelial programming (Brennan and Brown, 2004). Interfering the activation of canonical pathway by over-expression of Wnt antagonist Dkk1 results in failure of mammary bud formation as well. Wnt10b is expressed also in mammary rudiments. In postnatal mouse development, RNA expression of Wnt2, Wnt5a and Wnt7b are strongly induced in the phase of ductal development, while other Wnts are absent (Weber-Hall *et al.*, 1994). Wnt2 and Wnt5a are exclusively expressed in stroma, while Wnt7b is expressed in epithelial compartment and localized to TEB structures (Kouros-Mehr and Werb, 2006), suggesting a potential role in the stem cell regulation. Wnt5a was recently reported to be a downstream component of TGFβ signalling, and thus regulates branching morphogenesis (Roarty and Serra, 2007). In Wnt5a KO mammary gland, increased lateral branching and proliferation of mammary epithelium is observed together with larger TEBs. During pregnancy, the above-mentioned Wnts are downregulated early, and the expression of epithelially expressed Wnt4, Wnt5b and Wnt6 are strongly induced, connecting these Wnts to lobuloalveolar development in mouse. In lactation, only low Wnt6 expression has been detected, but upon involution, several Wnts reappear. Wnt5b and Wnt7b are expressed only 2 days after cessation of weaning and Wnt2 and Wnt5a reappeare by day 7. *In situ* analysis localized the expression of Wnt5b both in ductal and alveolar epithelium. Hormonal regulation of Wnt expression is not well studied, but ovariectomy has been reported not alter the expression of Wnt5a and Wnt7b (Weber-Hall *et al.*, 1994), showing that these Wnts are regulated by other factors than ovarian hormones. In contrast, Wnt2, Wnt4 and Wnt5b mRNA levels were reduced but not abolished following ovariectomy, showing that these Wnts are to some level under steroid hormone control. The localization, expression pattern and hormonal control suggest that in mouse, Wnt7b may be involved in maintaining cells in an uncommitted state and that Wnt5b initiates lobular development from ductal cells.

In humans, the reports of the expression pattern of Wnts in breast and breast cancer have been controversial. Some reports have shown WNT2, WNT3, WNT4, WNT7B expression in normal human breast tissue and expression of WNT3, 4 and 7B in breast cell lines (Huguet *et al.*, 1994; Kirikoshi and Katoh, 2002), while other reports have indicated strong expression of WNT1 and WNT6 both in benign and malignant breast tissue and down-regulation of WNT7B in breast cancer (Milovanovic *et al.*, 2004). Also expression of WNT1, WNT2B, WNT5A, WNT5B, WNT9A, WNT19B and WNT 16 has been reported in normal human breast cell line (Benhaj *et al.*, 2006). Low expression of WNT5A has been associated with shortened recurrence-free survival of breast cancer patients (Jonsson *et al.*, 2002). Frizzled receptors (except for 9 and 10), and co-receptors LRP5 and LRP6 were shown to be expressed in immortalized normal human breast cells and maintained in breast cancer cell lines (Benhaj *et al.*, 2006). Receptors Fz1 and Fz2 have been reported to be upregulated in breast cancer (Milovanovic *et al.*, 2004). Furthermore, frequent inactivation of WIF1 (antagonist of Wnt-signalling) has been observed in human breast cancer cell lines (Ai *et al.*, 2006). In summary, consensus of the importance of the expressed Wnt-ligands and other Wnt-pathway components in human breast cancer has not been reached, but importance of the pathway in breast tumorigenesis is emphasized by the fact that β-catenin

activity correlates with poor prognosis of the breast cancer patients (Lin *et al.*, 2000). An autocrine mechanism leading to a constitutively active Wnt-pathway has been described in human breast cancer cell lines, suggesting that β-catenin stabilization is dependent on functionally overactive, ligand dependent Wnt-pathway and not a result of genetic mutations or some other stimulus (Bafico *et al.*, 2004).

4.3.1. Mammary gland phenotype of mouse models with activated Wnt-pathway

Several TG mouse models with activated Wnt/β-catenin pathway have been created. These include MMTV-LTR driven ligands Wnt1, Wnt10b, and activated β-catenin mutants. TG mouse models stabilizing β-catenin by affecting its destruction complex have been created as well (Apc^{min} and dominant negative form of GSK3β, the dnGSK3β mice) and some other interacting genes from the pathway have been transgenically overexpressed (casein kinase II α, keratinocyte growth factor, fibroblast growth factor). These models present with similar mammary gland tumor histopathology that is distinct from the phenotypes of other widely studied mammary tumor models, such as TG mice for ErbB2, c-Neu and Ras (Rosner *et al.*, 2002). Yet another mouse model recently described, Epimorphin/syntaxin-2 shares Wnt-pathway phenotype (Bascom *et al.*, 2005), suggesting that it might have a role in Wnt-signalling. One of the most striking features of the Wnt-pathway tumors is the transdifferentiation of mammary epithelium into epidermal structures and formation of pilar tumors (Miyoshi *et al.*, 2002). Other common histological features are myoepithelial differentiation and organization into ductules or acini, inflammatory response and abundant stroma. ErbB2, c-Neu and Ras TG mammary tumors form more solid nodules with abundant cytoplasm. Histology of Wnt-pathway tumors with differentiation along epithelial and myoepithelial cell lineages suggests that the target cell for transformation is different between different tumor models activating different pathways. It was later shown that activating Wnt-pathway results in accumulation of progenitor cells (Liu *et al.*, 2004), and subsequent mammary tumorigenesis from progenitor cells (Li *et al.*, 2003). Abundant transdifferentiation or formation of pilar tumors is rarely observed in the human breast tumors, but this could reflect merely the intensity or the tissue location of the (Wnt-) signal or may be due to differences between species. Importantly, the tumors induced by activated wnt-pathway share the molecular profile with the human subgroup of basal breast cancers (Herschkowitz *et al.*, 2007). As a result of analyzing several TG mouse models, Wnt-pathway was discovered as a strong oncogenic signal in mammary gland, since many of the models exhibit early tumorigenesis with strong penetrance. Wnt signals are able to drive tumorigenesis even in the absence of Esr1, overdriving the need for endocrine support for tumor induction and maintenance (Bocchinfuso *et al.*, 1999).

4.4. Wnt 7b and Wnt5b

Wnt7b and Wnt5b has been retrovirally expressed in mouse mammary epithelium, but although Wnt7b activates canonical signalling, it failed to induce hyperplasia upon transplantation in contrast to Wnt1 (Naylor *et al.*, 2000). Expression of Wnt7b has been found in human breast and breast cell lines, and it is highly upregulated in 10% of breast cancer tissues studied (Huguet *et al.*, 1994). Wnt7b is overexpressed also in other malignant tissues, including endometrial carcinoma cell lines (Bui *et al.*, 1997) and in superficial bladder tumors, where its expression was 4-fold elevated (Bui *et al.*, 1998). Wnt7b is expressed in

normal pancreas, placenta, prostate, thyroid and bladder tissues among others. In placenta, Wnt7b is involved in chorion-allantois fusion (Parr *et al.*, 2001). Knock-out study showed that it is also needed in the lung morphogenesis: its deletion results in lung hypoplasia and respiratory failure (Shu *et al.*, 2002). Specifically, Wnt7b regulates vascular development in lungs by regulating vascular smooth muscle cells via canonical Wnt-pathway (Wang *et al.*, 2005b). Wnt7b is also secreted by macrophages. This can initiate apoptosis in target cells by activating canonical signalling, suggesting that macrophages use Wnt –ligands to alter cell fate of other cells (Lobov *et al.*, 2005).

WNT5B is found usually downregulated in breast cancer cell lines (Benhaj *et al.*, 2006), but upregulated in MCF-7 cells in response to estradiol (Saitoh and Katoh, 2002). A polymorphism in the human WNT5B gene has been associated with susceptibility to type 2 diabetes (Kanazawa *et al.*, 2004). WNT5B is expressed in several tissues in humans, including prostate, ovary, uterus, pancreas, liver and adipose tissues. It has been found to regulate preadipocyte differentiation in mouse cells and promote adipogenesis (Kanazawa *et al.*, 2005), providing further assiociation with the pathogenesis of type 2 diabetes. In humans, WNT5B is also specifically upregulated in chronic lymphocytic leukaemia (Lu *et al.*, 2004), in leiomyomas (Mangioni *et al.*, 2005) and in several other human cancer types, such as embryonal and esophageal tumor cell lines (Saitoh and Katoh, 2002). Wnt5a and Wnt5b regulate bone growth by coordinating chondrocyte proliferation and differentiation (Yang *et al.*, 2003).

AIMS OF THE PRESENT STUDY

Breast cancer is the most common cancer type in women. The vast majority of breast cancers arise without apparent hereditary predisposition and involve gradual accumulation of genetic defects that eventually triggers the malignant phenotype. The contribution of hormonal factors in breast cancer initiation and progression is strong. However, detail about endocrinologically contributing factors and particularly the way in which they confer susceptibility to tumorigenesis, with or without genetical input, are not well recognized. The importance and complex nature of endocrine factors are emphasized by the fact that pregnancy elicits a dual role in breast cancer risk. Although it has overall protective effects on breast cancer risk, pregnancy also elicits a period of heightened risk of breast tumorigenesis lasting for a decade, and does not protect from ESR1 negative breast cancer. In this study, we aimed to study the role of LH/HCG signalling in physiology of the female mice, and study its contributions to regulating mammary gland gene expression and its role in mammary tumorigenesis.

The specific aims of the study were:

1. To characterize the phenotype of the transgenic mice overproducing HCG (HCGβ TG mice) with special interest in the mammary phenotype.

2. To specify the signalling cascades associated with mammary gland tumorigenesis in HCGβ TG mice.

3. To study the role of HCG/LH in mouse and human mammary gland gene expression.

4. To study the changes in gene expression caused by lack of LHR in mouse mammary epithelium *in vivo.*

MATERIALS AND METHODS

1. Generation of the HCGβ TG mice

A 579-bp cDNA fragment of the ß-subunit of HCG, corresponding to gene 5 (provided by Dr. J. C. Fiddes, California Biotechnology Inc.) was subcloned downstream of the 1.2-kb human ubiquitin C promoter, into the *Hind*III site of pUB6/V5-HisA plasmid (Invitrogen, Carlsbad, CA). The vector included the ubiquitin C promoter consisting of the nontranslated first exon and intron and the bovine growth hormone polyadenylation signal. A 2.8-kb ubiquitin C/HCGß fragment (UbiC-HCGß) was released from the vector by digesting with *Bgl*II and *Nsi*I enzymes. The fragment was resolved in 1% agarose gel, isolated by electroelution, and purified with Elutip-D columns (Schleicher, Schuell, Inc., Keene, NH). The fragment was diluted in TE buffer (10 mM Tris-HCl; 5 mM EDTA, pH 7.5) at a concentration of 2 ng/µl for microinjection.

Before microinjection, the activity of the UbiC-HCGß vector was confirmed *in vitro*. For this purpose, HEK-293 cells (300,000 cells/35-mm^2 culture plate) were transiently transfected with the UbiC-HCGß plasmid using Lipofectamine (Life Technologies, Inc., Grand Island, NY). The cells were incubated for 30 h, and the medium was tested for the presence of HCGß by a specific immunofluorometric assay (IFMA), using time-resolved fluorometry for signal detection (Delfia, Wallac, Inc., Oy, Turku, Finland). Microtitration wells from the HCG Delfia kit containing an immobilized antibody against HCGß were used in combination with an europium-labeled mouse monoclonal antibody against the ß-subunit of HCG from the hLH Delfia kit. The reference preparation from the National Institues of Diabetes and Digestive and Kidney Disorders (NIDDK) (NIH, Bethesda, MD); HCGß CR-121 was used as standard. The detection limit of the assay was 40 µg/liter. The transfection assay demonstrated that the transgene was capable of encoding the production of high amounts of HCGß *in vitro*.

Founder TG mice were generated by microinjecting the transgene into the pronuclei of fertilized oocytes from FVB/N mice, and the microinjected embryos were implanted into the oviducts of pseudopregnant female mice of the NMRI strain. PCR analyses of genomic DNA from tail biopsies were used to identify TG animals. One microgram genomic DNA was added to the 50 µl PCR containing 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM deoxynucleotide triphosphate mix, 0.2 µM primers, and 2.5 U DNA polymerase. An 830-bp DNA fragment of the transgene was amplified using primers specific for the ubiquitin promoter (5'-CGCGCCCTCGTCGTGTC-3') and the HCGß-cDNA (5'-AAGCGGGGGTCATCACAGGTC-3'). The DNA was denatured at 97 C for 4 min, followed by PCR at 96 C for 0.5 min, 58 C for 1 min, and 72 C for 1.5 min for 32 cycles. The resulting PCR products were analyzed by electrophoresis on 2% agarose gel, and the fragments were UV visualized with ethidium bromide.

Five founders were obtained after pronuclear microinjection of the transgene, four males and one female. Two of the four males were fertile, whereas two males and the female were infertile. Consequently, two independent mouse lines overexpressing the HCGß subunit (HCGß+mice) were established by breeding the fertile males with WT FVB/N female mice.

Female TG mice derived from both lines presented with identical phenotypes. Transmission of the transgene was followed by PCR analysis, as described for the TG founder mice. The animals were housed in a specific pathogen-free environment under controlled conditions of temperature and light, and tap water and commercial mouse chow were provided *ad libitum*. All mice were handled in accordance to the institutional animal care policies of the University of Turku and with appropriate permissions.

For the expression analysis of the transgenic lines, total RNA was isolated by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). One microgram total RNA was reverse transcribed using the avian myeloma virus reverse transcriptase, (Promega Corp., Madison, WI) and the resulting cDNAs were PCR amplified in the same tube.

2. Hormone assays

Animals were sacrified in the morning (1000–1200 h) by cervical dislocation, and blood was collected from the heart. Serum samples were separated by centrifugation and stored at -20 C until hormone measurements. Serum HCGß concentration was measured by IFMA, as described above. FSH levels were measured by IFMA. Prolactin (PRL) was measured by RIA, using a mouse PRL antibody and mouse reference preparation provided by NIDDK. Estradiol levels were measured by IFMA after diethylether extraction, using the human estradiol Delfia kit (Wallac, Inc.) adapted for mouse samples. Serum testosterone and progesterone levels were measured by conventional RIAs after diethylether extraction. The bioactivity of circulating HCG was determined by the mouse interstitial cell *in vitro* bioassay.

3. Fertility studies and estrous cycle

For testing fertility, female TG mice were bred with wt FVB/N mice for up to 6 months. For analyzing the onset of puberty, females were examined daily for vaginal opening from 20 days of age. Puberty was defined as the day of vaginal opening. The duration of the estrous cycle was determined by analyzing the vaginal smears from the onset of vaginal opening until the age of 8 wk.

4. Histological and immunohistochemical analyses, mammary gland whole mount analysis

For histological studies, tissues from the TG females and WT littermates 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 mammary gland whole mount preparations, the fourth right inguinal mammary gland from TG and WT littermates 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 days, dehydrated in a series of ethanol and xylene, and finally mounted in Permount. For the electronmicroscopy study of the pituitary glands, tissue pieces of 1-mm^3 size were cut and pieces were fixed first in 5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.16 mol/

liter s-collidine buffer (pH 7.4) and postfixed in potassium ferrocyanide-osmium tetroxide, embedded in epoxy resin (Glycidether 100, Merck) and cut into sections. For the light microscopic survey, 1-µm-thick sections were stained with 0.5% toluidine blue, and the thin sections for electron microscopy were stained with 5% uranyl acetate and 5% lead citrate in Ultrostainer (Leica Corp., Wien, Austria) and examined under a JEM-100SX transmission electron microscope (JEOL, Tokyo, Japan).

For immunohistochemistry, five-µm 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 by 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 if peroxidase-based secondary antibodies were used. 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), 4-5) Keratin 5 and 6, dilution 1:200 (BabCo, Richmond, CA), 6) β-catenin, dilution 1:100 (Transduction Laboratories Inc. Lexington, KY). As the secondary antibody, Dako EnVision anti-mouse or anti-rabbit secondary for primary antibodies 1-3 were used at room temperature for 30 min, followed by visualization with 3´3´-diaminobenzidine (Dako). The slides were counterstained with Gill´s hematoxylin followed by dehydration and mounting. For antibodies 4-6, Molecular Probes (Eugene, OR, USA) AlexaFluor 594 anti-mouse and anti-rabbit secondary antibodies were used (1:400 dilution at 37˚ for 1h) followed by staining with DAPI for 15 minutes. Fluorescent slides were mounted in DakoCytomation Fluorescent Mounting Medium. For fluorescent pictures, pseudocolors were created by Adobe Photoshop CS. Immunohistochemical localization of the pituitary hormones was performed using the streptavidin-biotin-peroxidase technique. Primary antisera against mouse PRL, mouse GH, rat TSHß, human adrenocorticotropin (ACTH), and HCGß (NIDDK) were used at dilutions 1:10,000–1:15,000. After overnight incubation at 4 C, the reactions were visualized using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA), and 3,3’-diaminobenzidine (Zymed Laboratories, Inc. Corp., San Francisco, CA).

5. RNA isolation and quantitative RT-PCR analyses

For gene expression analysis, tissue pieces were excised and snap frozen in liquid nitrogen. Total RNA was extracted with the Qiagen RNEasy Mini or Qiagen Lipid tissue mini kit (Qiagen) and treated with Amplification Grade DNase I (Invitrogen). For cDNA synthesis and subsequent quantitative (q) RT-PCR, the SYBR Green DyNAmo HS qRT-PCR kit (Finnzymes) was used. For each qPCR reaction, an aliquot of cDNA (diluted 1:20) was used except for the housekeeping genes where a 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. The presence of a single, right-sized PCR product was confirmed by running the samples on 2% agarose gels. The primers used in the study are shown in Table 4.

6. Hormone treatments and gonadectomy

The ovaries were removed under isoflurane (Isofluran Baxter, inhaled; Baxter, US) anestesia and buprenorphine (Temgesic, 3–5 µg/mouse s.c.; Schering-Plough) analgesia from incision to the back. For the HCG injection study, a pellet releasing E2 (~0.19 mg/kg/d) and P (~4.8 mg/kg/d) for 60 days (Innovative research of America, Sarasota, FL) or placebo pellet were inserted under the skin after gonadectomy and the mice were injected subcutaneously with 20 IU of recombinant HCG (provided by A.F. Parlow, NIDDK) or saline in 100 µl volume for 5 consecutive days and then sacrificed.

Table 4. Primers used in the study

Gene		Primer sequence	Product size (bp)	Annealing temp (ºC)
Genotyping PCR (ubiquitin C – HCGβ)	F	CGCGCCCTCGTCGTGTC	830	58
	R	AAGCGGGGGTCATCACAGGTC		
Wnt4	F	AACGGAACCTTGAGGTGATG	244	58
	R	TCACAGCCACACTTCTCCAG		
Wnt5b	F	TGGAGACAACGTGGAGTACG	166	60
	R	GGCGACATCAGCCATCTTAT		
Wnt6	F	TTCGGGGATGAGAAGTCAAG	151	58
	R	AAAGCCCATGGCACTTACAC		
Wnt7b	F	TACTACAACCAGGCGGAAGG	233	60
	R	GTGGTCCAGCAAGTTTTGGT		
Beta-casein	F	GGACTTGACAGCCATGAAGG	197	60
	R	CTTTAGCCTGGAGCACATCC		
Autotaxin	F	TCGAGGGCGAGAGAAGTTTA	153	60
	R	AGGGAAAGCCACTGAAGGAT		
Axin2	F	CAAGACCAAGGAGGAGATCG	151	60
	R	ACCTCTGCTGCCACAAAACT		
Cyclin D1	F	TCTCCTGCTACCGCACAAC	168	59
	R	TTCCTCCACTTCCCCCTC		
Hig2	F	ACGACCTGGTGTGACTGTGA	146	60
	R	AACATGATGCCCAGCACATA		
Gpc3	F	TGAATGCATTGGAAGCTCTG	146	60
	R	AGGTGGTGATCTCGTTGTCC		
Pmp22	F	TCCTCATCAGTGAGCGAATG	158	60
	R	CCAGCAAGGATTTGGAAGAA		
Plp	F	TGTTTGGGAAAATGGCTAGG	137	59
	R	GACTGACAGGTGGTCCAGGT		
Mbp	F	ATCCAAGTACCTGGCCACAG	152	60
	R	TGTGTGAGTCCTTGCCAGAG		
Fbln1	F	GATGGTCCAGGAGCAATGTT	152	60
	R	AACAGTGGCAGCACCTCTTT		
Sfrp1	F	AGCCTCTAAGCCCCAAGGTA	155	60
	R	TGGGGACAATCTTCTTGTCA		
Sfrp4	F	CTCCTGCCAGTGTCCACATA	163	60
	R	CTGCTGTTCCTGAAGCCTCT		
Sfrp5	F	GATCTGTGCCCAGTGTGAGA	199	60
	R	CAGGACCAGCTTCTTGGTGT		
Wisp2	F	CCTGTCAGGCTCCTGTCCTA	153	60
	R	AAGATGGATCAGTGGGTTGC		
Ldlr	F	GGACCTCAAGATTGGCTCTG	153	59
	R	GCCTGGCACTCACACTTGTA		
Pon1	F	AGAGGTGCTTCGAATCCAGA	141	60
	R	TTGTGGAACACAGTGCCAAT		

Gene		Primer sequence	Product size (bp)	Annealing temp (ºC)
Adpn	F	ATGGCAAACTTGTGGGAGAC	147	60
	R	GGAGGTTGCAGACTTTGCTC		
Nsdhl	F	CAGCAGTGCCAGTGTTGTCT	144	60
	R	TTCTTAGGGTCGTTGGCATC		
Angptl4	F	GCATGGCTGCCTGTGGTAAC	145	59
	R	ATCTTGCTGTTTTGAGCCTTGA		
Hsd11b1	F	TTATTGTCAAGGCGGGAAAG	147	60
	R	TCATGACCACGTAGCTGAGG		
Sox9	F	AGGAAGCTGGCAGACCAGTA	196	60
	R	GTCCGTTCTTCACCGACTTC		
Sorbs1	F	ACGTCAGAGCCTCCTGGATA	189	60
	R	AGTGGAAGCTGAGCTGGAAG		
mLHR	F	TTGCCGAAGAAAGAACAGAAT	252	54
	R	AGCCAAATCAACACCCTAAG		
hWNT5B	F	GCTCATGAACCTGCAAAACA	171	60
	R	GCGCTGTCGTACTTCTCCTT		
hWNT7B	F	TCAACGAGTGCCAGTACCAG,	229	60
	R	CCCTCGGCTTGGTTGTAGTA		
hLHR	F	CAGCGGTTCTCGGCGCTG	346	55
	R	CACCGGGCTCAATGTATC		
hHPRT	F	TGAGGATTTGGAAAGGGTGT	151	56
	R	AATCCAGCAGGTCAGCAAAG		
hPPIA	F	TTCATCTGCACTGCCAAGAC	158	60
	R	TCGAGTTGTCCACAGTCAGC		
mL19	F	CTGAAGGTCAAAGGGAATGTG	195	58
	R	GGACAGAGTCTTGATGATCTC		
mCyclophilin	F	CATCCTAAAGCATACAGGTCCTG	165	58
	R	TCCATGGCTTCCACAATGTT		

7. Mammary gland transplantation

21 day-old WT female mice were anesthetized as described above, and the endogenous epithelium of the 4th mammary glands were surgically removed. A small piece from the adult donor mammary gland was inserted into the remaining fat pad (Deome *et al.*, 1959). The mice were sacrificed at the age of 5 months. The donors were either WT females or LHR KO, Esr1 KO or HCGβ TG females. The donors were genotyped and the phenotype examined before transplantations. The phenotype and genotyping has been described elsewhere (Pakarainen *et al.*, 2005c; Zhang *et al.*, 2001a)

8. Human mammary epithelial cell culture

Human mammary epithelial cells (Cambrex Bio Science Walkersville, USA) were cultured in MEBM medium (Lonza) supplemented with recommended supplements (hydrocortisone, hEGF, insulin, gentamicin/amphotericin-B, and bovine pituitary extract) in 6 cm cell culture dishes. The cells were depleted of bovine pituitary extract 24h prior HCG stimulation. The study was carried out in ~80% confluent cell dishes by adding 20 IU/ml of recombinant HCG into the medium for 24h after which RNA was extracted. The cell studies were repeated four times.

9. Protein extraction and Western hybridization

Mammary gland or tumor pieces were homogenized in RIPA buffer with proteinase inhibitors and homogenates were centrifuged twice followed by protein concentration measuring using the Bradford method. 25 µg of protein was loaded on gel and western blotting was carried out using standard procedures. Antibody for *Wnt7b* (Santa Cruz Biotechnology, USA) was used in 1:1000 concentration and actin (ICN Biomedicals, USA) in 1:20 000 concentration. Secondary antibodies used were anti-goat IgG-HRP (Santa Cruz Biotechnology, USA) and anti-mouse IgG-HRP (Amersham Biosciences, UK). Western blot analyses were quantified with ImageJ program and normalized values were subjected to statistical analysis.

10. Expression array and analysis

RNA samples were amplified from 300 ng of total RNA with Ambion's Illumina RNA TotalPrep Amplification kit (Ambion, Austin, USA). Both before and after the amplifications the RNA/cRNA concentrations where confirmed with Nanodrop ND-1000 and RNA/cRNA quality was controlled by the Experion electrophoresis station (Bio-Rad, Hercules, USA). The Sentrix Mouse-6 (Illumina, San Diego, US) genome-wide (~ 25,000 genes and ~ 47,000 probes) expression arrays were used. Hybridization and analysis were performed at the Finnish Microarray Centre (Centre of Biotechnology, Turku, Finland). 1.50 µg of each sample was hybridized at 55 °C overnight (18 h) according to the protocol of the manufacturer. Hybridization was detected with 1µg/ml of Cyanine3-streptavidine (Amersham Biosciences). Chips were scanned with the Illumina BeadArray Reader and the numerical results were extracted with Bead Studio v1.5.1.34 without any normalization or background subtraction. Three individual replicates from both experimental groups in two independent studies were used. In study 1, we used mammary glands from ovariectomized and hormone replaced mice after HCG and vehicle injections, and in study 2, mammary glands from WT hosts transplanted with LuRKO or WT epithelium. The data was extracted, normalized with the quantile normalization -method and analyzed using Illumina BeadStudio. P values were calculated among each group to give consistent changes independent of biological variation between the samples.

11. Statistical methods

SigmaStat 3.1 (SPSS Inc., Chicago, IL) was used for the *t* test or Mann-Whitney Rank Sum test. The limit of statistical significance was set at $P < 0.05$. For analyzing cell culture results, mRNA expression levels of the unstimulated plates were normalized to value 1.

RESULTS

1. Consequences of elevated HCG production in female mouse (I)

We created a mouse model over-expressing human chorionic gonadotropin β-subunit (HCGβ) under ubiquitin C promoter, resulting in ubiquitous expression of the transgene. The HCGβ-subunit could be detected from the sera of the TG mice. Biologically active HCG hormone in the circulation of these TG mice is a dimer formed between the transgenic β-subunit (human) and the endogenous α-subunit of the mouse. A 40-fold elevation in HCG/LH bioactivity was observed in TG versus WT female mice.

1.1. Hormonal disturbances in HCGβ TG female mice

At the age of 1 month, HCGβ TG female mice exhibited enhanced ovarian steroidogenesis. E2, P and T in the circulation were increased 3-4 fold. From the age of 2 months onward, serum estradiol was normalized to the level observed in the WT mice. In contrast, progesterone increased markedly, reaching a 20-40 fold increased level at the age of 2-6 months. A further increase in progesterone concentration (up to 100-fold) was observed in TG mice at the age of 9-12 months. At the same time, T was further increased from 3-fold (between 1-6 months of age) to 8-fold (12 months of age). In line with these alterations, FSH production was suppressed, being around 60% observed in WT mice, thus exhibiting levels observed at normal mouse in diestrus. Prolactin increased steadily in HCGβ TG female mice, reaching statistical significance at 3 months of age. In the old mice with pituitary adenomas, serum prolactin was 600-fold higher as compared with WT female mice. Estrogen production increases prolactin production and in line with this, ovariectomized HCGβ TG female mice exhibited normalized prolactin concentrations in serum.

1.2. Consequences of HCGβ overproduction to the female phenotype

Overproduction of HCG stimulated the ovaries to produce steroid hormones. In TG females, this resulted in precocious puberty manifested by early vaginal opening at the age of 21-22 days, in contrast to 26-28 days in WT mice. At the age of 21 days, female mice also exhibited increased uterus weight as a sign of estradiol exposure. The TG mice presented with abnormal estrous cycle as observed by vaginal smears. The mice exhibited signs of constant estrus until the age of 42-45 days, after which a pattern of constant diestrus persisted. The TG mice were infertile except for a small number of mice that became pregnant in a short time at around the age of 20-21 days.

The ovaries of HCGβ TG mice were enlarged at the age of 6 months, and luteinization and hemorrhagic cysts were observed in histological sections. Follicles at different stages of maturation were also observed. In adult TG mice, the uterus was normal in size, but exhibited histological abnormalities: a thin endometrium with dilated endometrial glands and flattened epithelium.

Pituitary enlargement of the TG female mice was evident already at the age of 2 months, and the pituitaries grew, reaching up to 100-fold increased size in some old mice. This enlargement resulted in cranial deformation at older ages. Occasionally, signs of brain compression were observed, as some mice exhibited problems with balance. Suprasellar

enlargement and hemorrhagic appearance of the pituitaries was observed in autopsies. In immunohistochemical studies, lactotrope hyperplasia was observed as a cause of pituitary enlargement. Lactotropes were present in multifocal nodules and the cells exhibited mitotic figures and variability in size. The reticulin fiber network was disturbed as a sign of adenoma formation. The lactotrope nodules were surrounded by other populations of pituitary cells, exhibiting immunoreactivity to GH, ACTH and TSHβ, but these cell types did not contribute to adenoma formation. Electron microscopy analysis confirmed that adenomas consisted of actively secreting lactotropes and that the other cell types were ultra-structurally normal. The pituitary phenotype was abolished by ovariectomy at 6 weeks of age.

HCGβ TG female mice developed progressive obesity from the age of 2 months onward, suggesting that the hormonal imbalance caused metabolic alterations. Body length was normal but accumulation of abdominal fat was readily observed. The adrenal glands of HCGβ TG females were normal in size, although exposure to LH has been reported to result in accelerated adrenal tumorigenesis in some animal models (Mikola *et al.*, 2003). Except for an occasionally observed dilated bladder and the mammary gland phenotype described below, other gross morphological abnormalities were not seen.

2. Mammary gland phenotype and tumorigenesis in HCGβ TG female mice (I-II)

2.1. Mammary gland phenotype before tumor formation

Mammary gland growth began prematurely because of precocious puberty in HCGβ TG female mice. In whole mount analysis, TEBs appeared stimulated in TG animals at a younger age as compared with WT animals. However, TEBs reached the border of the fat pad at the same age (around the age of 60 days) in both genotypes, thus TEB progression was normal. In the TG mice, however, hyperbranching from the forming ducts was observed in TG females already during ductal growth, and occasionally, abnormal TEB patterns were observed. From 3 months of age and onwards, the TG mammary gland resembled the pregnant mammary gland with progressive lobuloalveolar growth, detectable both in whole mount analysis and in histological sections. Alveoli secreted milk that was trapped inside alveoli. Beta-casein expression was 40-fold increased in TG females at 3 months of age as compared with WT littermates, but did not reach the 160-fold higher level observed in the lactating WT mice. The pre-lactating mammary gland phenotype is a direct and logical coincidence of the phenotype: high serum progesterone and prolactin concentrations induce a pregnant-like state in virgin HCGβ TG mice. However, already at 3 months of age, TG mammary gland contained foci of non-lactating cells that exhibited signs of premalignancy, namely multilayered growth filling the lumen and the loss of polarity. These foci of mammary intraepithelial neoplasia (MIN) lacked the normal myoepithelial lining, as demonstrated by the abnormal α-smooth muscle actin and keratin 5 stainings in immunohistochemistry (IHC). Secreting alveoli in TG mice and WT mammary glands had normal staining of α-SMA and keratin 5. Abnormal keratinization (keratin 6) was observed in TG mice from 3 months of age onward in IHC, and keratinized nodules were present from 5 months of age. Keratin 6 is not normally detected in the mammary gland, but is

expressed in some layers of skin. In mammary tumorigenesis, keratin 6 expression is a hallmark of Wnt-pathway phenotype (Miyoshi *et al.*, 2002).

2.2. Steroid receptor expression

Esr1 and Pgr were similarly detected in TG and WT mice by IHC at the age of 2 months. During the appearance of lobuloalveolar growth at the age of 3 months, Esr1 and Pgr expression ceased in TG animals in contrast to age-matched WT mice. However, some non-lactating MIN foci retained Pgr expression in TG mice. Developing tumors were negative for Esr1 expression in IHC analysis. Most of the tumor mass was Pgr negative, but some positive foci were occasionally observed. Androgen receptor expression was also studied by IHC, but no expression could be found either in WT of TG mammary glands or TG tumors at any age.

2.3. Tumor phenotype and Wnt-pathway characteristics in tumors

TG female mice developed mammary adenocarcinomas with 90% penetrance, starting from the age of 9 months. The tumors were rapidly expanding masses and often necrotic in the centre. Multiple tumor foci in a single mouse were often found. The tumors were capable of metastasizing in some mice. Macroscopical metastases were found in the spleen, ovaries, uterus and liver, and they resembled histologically the original tumors. The brain, lungs or bone did not exhibit signs for macroscopic metastasis formation. Mammary gland tumors exhibited histological similarity with tumors appearing in other genetically modified mice with activated Wnt-pathway (Rosner *et al.*, 2002). Wnt-pathway tumors differ histologially from mammary gland tumors appearing in other genetically modified mouse models exhibiting ductular organization, extensive squamous differentiation (keratin swirls), dense stroma, inflammatory infiltration and myoepithelium in the tumor area. All these features were present in the HCGβ TG tumors. Wnt-pathway tumors can be divided according to their predominant cellular pattern of organization into acinar, glandular, papillary, adenosquamous, pilar, type P and myoepithelial tumors. Papillary and pilar tumors (that are characterized by extensive keratin swirls and "ghost cells") are the predominant type of Wnt-pathway tumors (17% and 27%, respectively (Rosner *et al.*, 2002)). These types were also most common in HCGβ tumors, both comprising 37% of the tumor types observed (according to their main pattern). Similar to other Wnt-pathway tumors, HCGβ TG tumors also often had several patterns of organization in tumor area.

An activated Wnt-pathway stabilizes β-catenin to the cytoplasm and further to the nucleus. In a non-activated normal state, β-catenin staining is observed in cell membranes, where it is normally bound to cadherin. Beta-catenin stabilization was studied in HCGβ TG tumors and mammary glands by IHC. Starting from the age of 3 months, foci of cytoplastic staining were present in the hyperplastic areas of the mammary gland in TG mice, while the secreting alveoli did not exhibit increased β-catenin staining. WT mice displayed typical β-catenin staining in cell membranes at all age groups. In TG tumors, however, strong β-catenin staining was observed in the foci of squamous metaplasia and especially at the growing borders of the tumors.

2.4. Alterations in Wnt gene expression in HCGβ TG mice

In order to elucidate the cause of β-catenin stabilization in HCGβ TG mice, mRNA expression of Wnt-ligands were studied. We found that Wnt5b and Wnt7b were already overexpressed at the age of 3 months in TG mammary glands and further increased in tumor samples, suggesting that this could be responsible for the β-catenin stabilization observed. Wnt5b was 5-fold increased at 3 months of age and a 9-fold increase was seen in tumor tissues, as compared with WT mammary glands at 3 months of age. Wnt7b expression was similar at 3 months of age, but in tumors, 2-fold elevated levels of mRNA were observed. Upregulation of Wnt7b was confirmed by Western blotting where the protein expression of Wnt7b appeared even more pronounced. HCGβ TG tumors exhibited a 4-fold increase in Wnt7b expression as compared with WT mammary glands ($p=0.002$). Protein expression of Wnt7b was not increased in 3 month-old TG mammary glands compared to WT mammary glands. Wnt5b and Wnt7b mRNA expression was also studied in uterus, but it appeared that HCG did not regulate these Wnt-ligands in uterus.

Other Wnt-genes studied (Wnt2, Wnt4, Wnt5a, Wnt6, Wnt10b) exhibited similar or decreased expression in 3 month-old TG mammary glands and in tumor tissues. Wnt6 was upregulated in some tumors, but absent in the majority of the samples. We also studied the expression of Wnt1 that is not naturally expressed in the mammary gland, in contrast to the other ligands studied. No expression of Wnt1 was found in any of the samples studied.

2.5. Effects of deletion of Esr1 on HCGβ mammary gland phenotype

In order to study which features of the mammary phenotype or alterations in the gene expression observed in HCGβ TG female mice was due to direct Esr1 action in mammary gland, we transplanted Esr1 KO epithelium to cleared fat pads of 21-day-old HCGβ TG females. At the age of 5 months, no effect on mammary phenotype was observed (Figure 6). However, the Esr1 KO mouse model used (Mueller *et al.*, 2002) has been reported to retain some transactivation capability in the presence of pregnancy hormones in contrast to a more recent Esr1 KO mouse model (Mallepell *et al.*, 2006). Thus, total deletion of Esr1 was not achieved in HCGβ TG hormonal environment and the epithelium was able to grow to the fat pad. Furthermore, no effect on Wnt gene expression was observed in Esr1 deficient HCGβ TG mammary glands compared to HCGβ TG mammary glands (Figure 6).

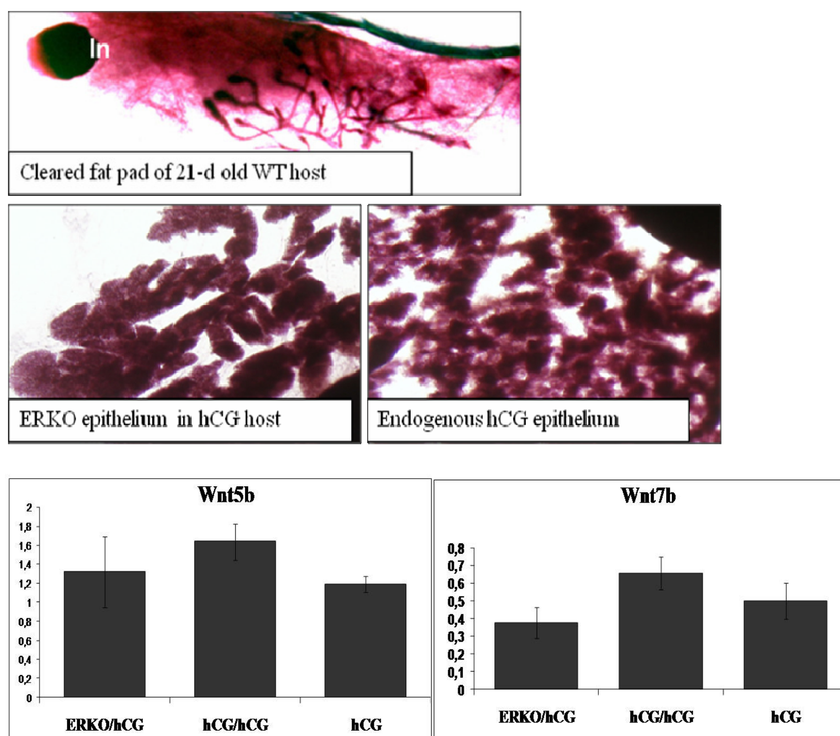

Figure 6. Transplantation of Esr1 KO or HCGβ TG epithelium to HCGβ host: gross morphology and expression of Wnt5b and Wnt7b mRNA in the transplants.

2.6. Effects of late ovariectomy on HCGβ mammary gland phenotype

The dependency of the premalignant mammary lesions on ovarian hormones after their appearance was studied by ovariectomizing 3, 5 and 7 month-old HCGβ TG female mice. After one month without ovarian steroid hormones and diminished prolactin (as judged by normalized pituitary weight), 2/3 of the mice in all age groups still displayed abnormal mammary gland phenotype. Hyperplastic nodules were present in whole mount stainings, and in histological sections, cystic structures and collapsed alveoli were observed. Lobuloalveolar structures were greatly diminished as expected, and fibrosis became evident. However, keratin 5 and 6 expression was still present although diminished, suggesting that some already initiated lesions were independent of estrogens, progesterone and of high levels of prolactin. Interestingly, expression of Wnt5b and Wnt7b was not decreased after ovariectomy (Figure 7). The mRNA expression of Wnt7b and Wnt5b was elevated in ovariectomized TG female mice. In contrast, the expression of Wnt7b and Wnt5b was unchanged in ovariectomized WT females, being in line with the previous findings (Weber-Hall *et al.*, 1994). The difference in Wnt7b mRNA expression between WT and TG after ovariectomy was 3.3 –fold. Wnt5b was upregulated in HCGβ TG females, reaching 3.8 –fold higher mRNA expression level in TG vs WT ovariectomized females. This suggests that the remaining cell population was further enriched in cells producing Wnt5b and Wnt7b, and was able to produce these Wnt-ligands in the absence of estradiol, progesterone and high prolactin.

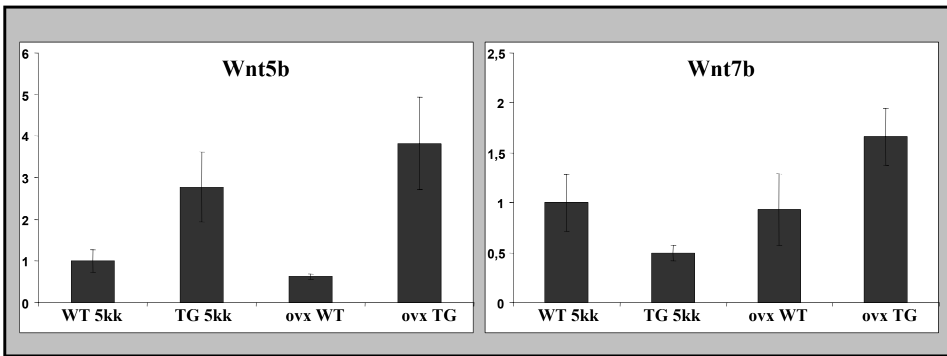

Figure 7. Wnt 5b and Wnt7b expression in ovariectomized WT and TG mice.

3. Effects of HCG administration on mouse mammary gland (II-III)

3.1 The effect of systemic HCG or PRL administration on Wnt5b and Wnt7b expression in the mouse mammary gland

In order to elucidate which factors could contribute to increased Wnt5b and Wnt7b expression in the mammary gland, we studied the effect of HCG and PRL on these genes. To avoid secondary hormonal changes due to HCG or PRL injections, we ovariectomized adult WT female mice and corrected the hormonal environment with estradiol and progesterone administered in slow-release pellets. Other groups were given a placebo-pellet subcutaneostly after ovariectomy. 20 IU of recombinant HCG (once a day), vehicle (0.9% NaCl, once a day) or 1 mg bovine PRL (administered three times per day) were given subcutaneously for 5 consecutive days for both pellet-groups. Expression of Wnt5b and Wnt7b mRNA were measured by qPCR (Figure 8). HCG injections stimulated the expression of Wnt5b 2-fold in the presence of the steroid pellet, while no effect was observed in the presence of the placebo pellet. Systemic administration of HCG enhanced the expression of Wnt7b 2,5-fold both in the presence of estradiol and progesterone –pellet and placebo pellet (Figure 8). PRL administration did not alter expression of Wnt-ligands. These short term administrations of PRL of HCG did not result in phenotypical changes in the mammary gland.

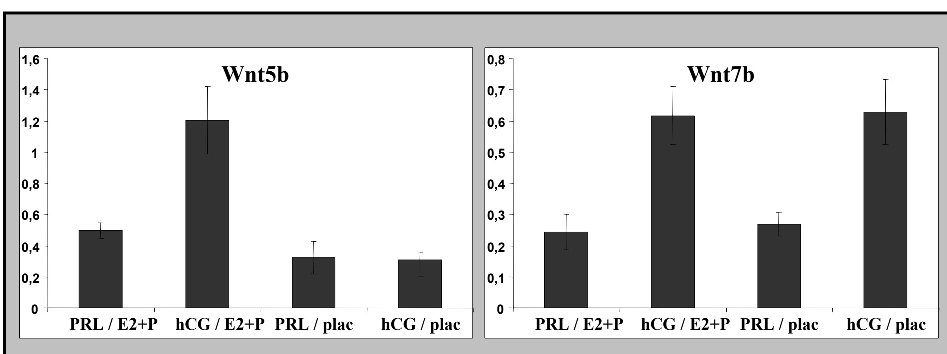

Figure 8. Wnt 5b and Wnt7b expression in ovariectomized mice supplemented with E2+P or placebo pellet receiving PRL or HCG .

3.2. *Wnt-pathway target gene expression in the mouse mammary gland upon HCG administration*

There is conflicting data about the ability of Wnt-ligands to activate the canonical pathway in various tissues. The involvement of Wnt5b and Wnt7b in the activation of canonical Wnt-pathway in mammary gland is not resolved, although Wnt7b has been suggested to act as a canonical ligand in this tissue. We, therefore, investigated whether HCG injections could stimulate the mRNA expression of known target genes of the canonical Wnt-pathway. Autotaxin, Axin2, Hig2 and Cyclin D1 are known Wnt-pathway target genes, and we found them to be 1.2-2.7 –fold upregulated ($p<0.05$) as a response to systemic HCG administration. These genes (except for the Cyclin D1) were not upregulated in 3 month-old HCG mammary glands or tumors. The HCGβ TG tumors did, however, express the same pattern of genes that are specifically involved in Wnt1 TG tumorigenesis, suggesting that a long-term stimulation of the Wnt-pathway elicits different alterations in gene expression than a short-term stimulation (Table 5).

We also studied the expression of downstream target of Wnt5b-signalling. Sox9 is positively regulated by canonical Wnt5b-signalling in chondocytes (Yang *et al.*, 2003) and a direct target of Wnt/β-catenin signalling in the intestine and regulates mesencymal progenitor differentiation (Blache *et al.*, 2004; Gaur *et al.*, 2006). Sox9 was upregulated upon HCG-injections and also in 3-month-old HCGβ TG mammary glands. In general, as the HCGβ TG mammary gland was stimulated by a plethora of other hormones and represented a drastically altered state (lactation-like), it was evident that gene-expression did not closely resemble changes observed after a short-term HCG administration experiment. Systemic HCG administration also led to an increase in Wisp2 expression, another Wnt-pathway target gene found to be downregulated in LHR deficient mammary transplants. Wnt-pathway antagonists, secreted frizzeled receptor-sequence related proteins (Sfrp´s) were not stimulated by HCG treatment, although their dowregulation was observed in LHR deficient mammary glands.

Table 5. List of genes potentially involved in neoplastic progression in MMTV-Wnt1 TG mice (Huang S et al, Genome Biol, 2005). WntH= Wnt1 hyperplastic mammary gland, WntV= Wnt1 virgin mammary gland, WntT= Wnt1 mammary gland tumor, HCG= 3 month-old HCG mammary tissue (hyperplastic), WT= 3 month-old wild type mammary tissue, TU=HCG mammary gland tumor

	WntH/V	WntT/H	HCG/ WT	TU/WT	TU/ HCG
Tnfrsf19	2.6	3.5	**1.8**	**3.1**	1.7
Rbp1	2.9	3.0	**3.3**	**3.7**	1.1
Ccnd1	=	3.1	**0.5**	**2.6**	44.4
Col1a1	=	3.0	**0.05**	**12.2**	25.1
Cav	0.3	0.3	**0.2**	**0.07**	0.3

3.3. Other gene expression changes in the mouse mammary gland after HCG exposure

We compared the gene expression profiles of mammary glands from ovariectomized and estradiol and progesterone treated female mice after 5 days of HCG or vehicle administration in a microarray study. Ovariectomy and hormone replacement excluded systemic changes due to ovarian response to HCG, but allowed the physiological sex steroid concentrations in circulation. Administration of 20 IU/d HCG resulted in significant gene expression changes in the mouse mammary gland by upregulating the expression of 62 genes more than 1.5 –fold (for 19 genes, $p<0.05$) and downregulating 27 genes more than 1.5 –fold (for 11 genes, $p<0.05$). Among the differentially regulated genes observed were multiple genes associated with the glucose and cholesterol metabolism, suggesting that HCG might have a direct role in the metabolic adaptation and insulin resistance observed in pregnancy. The genes included LDL receptor (Ldlr), NAD(P) dependent steroid dehydrogenase-like (Nsdhl) and paraoxonase 1 (Pon1), all of which are associated with cholesterol homeostasis. Hydroxysteroid 11-beta dehydrogenase 1 (Hsd11b1), Sorbin and SH3 domain containing 1 (Sorbs1), angiopoietin-like 4 (Angptl4) and adiponutrin (Adpn) are genes associated with energy balance and insulin resistance. Differential regulation of these genes by systemic HCG injections was further confirmed with quantitative RT-PCR. Interestingly, the regulation of these "insulin resistance genes" required the presence of physiological amounts of estradiol and progesterone, since the mice with the placebo-pellet did not exhibit an additional increment in Hsd11b1 or Adpn mRNA in response to HCG. Ovariectomy by itself induces insulin-resistant metabolism in mice (Siri and Ginsberg, 2003), which could be seen also in the up-regulation of Adpn and Hsd11b1 mRNA in this analysis.

4. HCG and human mammary epithelial cells (III)

4.1. LHR in mouse and human mammary epithelium

We studied the expression of LHR in human mammary epithelium cells with RT-PCR, and observed clear expression of the receptor, whereas LHR expression in the mouse mammary gland was present but much weaker. This suggests that human breast epithelium is more likely to respond to HCG/LH in physiological conditions than the mouse mammary gland. Unfortunately, protein expression could not be determined, since high quality antibodies do not exist for LHR.

4.2. Effects of HCG on human mammary epithelial cells

In order to study whether the changes in gene expression observed in mice after HCG exposure are relevant to human breast physiology, we studied normal human mammary epithelial cells in a primary cell culture. After 24 hours of HCG stimulation, we observed upregulation of WNT5B and WNT7B similar to mouse mammary gland. However, Wnt target genes were not upregulated by this short treatment, as they were expressed at a very low level in normal human mammary epithelial cells. We further tested some genes found to be regulated by HCG or LHR function in our other studies, and found upregulation of Mbp (downregulated in LuRKO in WT mammary glands) and Sox9 in response to HCG administration.

5. Consequences of depletion of LHR from the mammary gland (III)

In previous studies it was demonstrated that LHR is redundant for normal mammary growth and function (Pakarainen *et al.*, 2005c). However, mammary gland gene expression analyses were not performed, leaving the possibility that LHR expression in the mammary gland might affect gene expression in a way that does not affect morphology, or apparent basic functions. Thus, we performed mammary epithelium transplantation from LuRKO or WT donors to WT hosts and studied the gene expression in the chimeric mammary glands.

5.1. Mammary gland phenotype and alterations in gene expression

We performed mammary epithelial transplantations using WT mice as hosts. LHR KO or WT epithelium was grown in these mice under normal endocrine conditions until adulthood. Tissue morphology was similar among LHR KO and WT transplanted mammary glands and the hosts endogenous mammary glands, both in whole mount analysis and in histological sections. Microarray expression analysis was performed on LHR KO vs WT transplanted mouse mammary glands at the age of 5 months. We found 96 genes that were more than 1.5 –fold downregulated (for 19 genes $p<0.05$) and 163 genes more than 1.5 –fold upregulated (for 12 genes $p<0.05$) in LHR KO in WT host (LW) vs WT in WT host (WW). LW and WW tissues clustered separately in hierarchical clustering, indicating consistent overall differences in the gene expression patterns between the two groups. Similar to that observed in cells of HCG-primed cumulus-oocyte-complex (Hernandez-Gonzalez *et al.*, 2006), differential expression of several neuronal cell associated genes such as tenascin XB (Tnxb), peripheral myelin protein (Pmp22), proteolipid protein (myelin) 1 (Plp) and myelin basic protein (Mbp) was consistently observed in LW vs WW mammary glands. This suggests a partial overlap in LHR-dependent gene expression between the mammary gland and the cells in cumus-oocyte-complexes. The microarray results indicated a downregulation of several Wnt-pathway inhibitors in the absence of LHR from mammary epithelium, including Wisp2, Sfrp1, Sfrp4 and Sfrp5, although the differences did not reach a statistical significance ($p<0.05$) in the array setting. Despite of the downregulation of Wnt-pathway antagonists (Sfrp´s), the target genes of canonical signalling (Twist, Hig2, Axin2, Cyclin D1) were not upregulated in qPCR analysis, suggesting that basal LHR stimulation in the mammary gland does not activate the Wnt-pathway. Instead, downregulation of certain Wnt-pathway interacting genes Wisp2 and glypican 3 was observed, suggesting a modulatory role for LHR in Wnt-signalling in mouse mammary tissue. These gene expression changes were confirmed by quantitative RT-PCR. As we have previously shown upregulation of Wnt7b in the mammary gland upon HCG administration, we studied the expression in the LW and WW transplanted mammary glands. The results showed downregulation of Wnt7b in mammary gland transplants without LHR ($p=0.043$). Among the upregulated genes, we observed several genes from the histone 1 group and Uhrf1, a protein maintaining DNA methylation. Overall, the observed changes in gene expression were modest but consistent with the LH-induced gene expression changes previously observed in the ovary, and support the existing role of LHR function in the mouse mammary gland.

DISCUSSION

1. Consequences of elevated LH and LHR action in mouse and human

Mouse models with elevated LH-action are roughly convergent in their phenotypes, as also demonstrated in the current study. LH-overexpressing mouse models have a precocious induction of puberty and secondary consequences of elevated steroidogenesis and present with metabolic alterations (Rulli and Huhtaniemi, 2005). Activating mutation of LHR in human is not able to induce the findings observed in HCGβ TG female mice (Latronico, 2000), apparently because the onset of LHR expression at the beginning of puberty requires FSH stimulation. Thus, mutated LHR expression appears in granulosa cells only after FSH priming and precocious puberty is not observed. The menstrual cycle in women with activating LHR mutation is not disturbed, whereas anovulation, lutenization and infertility are the corresponding mouse phenotypes, suggesting that the LHR mediated signalling is cyclically abolished in humans but not in mice. The discrepancy also suggests that the regulation of LHR expression in human and mouse ovaries differ. In mouse physiology, the LH-signal is apparently continuously mediated through the LHR in ovaries, and the corpus luteum does not become insensitive to LH/HCG, leading to constant stimulus in contrast to humans. Thus, in the context of forced HCG stimulus, early estrogen production and subsequent lutenization of the ovaries and high circulating progesterone are observed in HCGβ TG mice, being in line with the other LH-overproducing mouse models. Elevated circulating estrogen results in precocious puberty and increased uterus weight, whereas cycle abnormalities and infertility result from hormonal imbalance and consistent progesterone production. Estrogens are also known to induce hyperprolactinemia. Since circulating E2 is normalized after initial upregulation in HCGβ TG females, further acceleration of pituitary enlargement and hyperprolactinemia are somewhat surprising phenotypes, and currently a subject for further investigations. However, without continuous estrogen and progesterone support from the ovaries, pituitary enlargement and hyperprolactinemia are rapidly decreased, suggesting that the pituitary prolactin production does not become independent on steroid hormone support.

Because of the difference in species, mouse models of elevated LH-action can be used rather as models of end-organ pathologies than as models of elevated LH action in women. In this mouse model, the elevated LH-action without the involvement of abnormal steroid-action is achieved only by ovariectomizing the mice at an early age. The ovariectomy itself drastically changes the physiological state and possibly, for example LHR expression, thus interfering with the interpretation of the results. This was also demonstrated in this study, since HCG treatment could alter some metabolic genes only in the presence of physiological amounts of estradiol and progesterone, but not without. It is highly likely that LH/LHR biology differs between the species, since continuation of mouse pregnancy does not rely on HCG, which is secreted only in primate and equine pregnancies. Therefore, the distribution and effects of LHR might also differ significantly. In the case of LHR inactivation, mouse and human phenotypes are concordant in infertility and anovulation. Apparently FSH is able to stimulate the ovaries in women (but not in mouse) to secrete reasonable amounts of estrogens since women with inactivated LHR exhibit with normal secondary sexual characteristics (Arnhold *et al.*, 1999), in contrast to LHR KO females (Zhang *et al.*, 2001a).

The distribution and functionality of LHR have long been debated and is still a controversial issue. There are several lines of evidence for HCG having an effect on extragonadal human cells (Huber *et al.*, 2007; Meng *et al.*, 2007; Pidoux *et al.*, 2007), some of which show direct involvement of LHR mediated signalling cascade activation. However, it has been reported that at least *in vitro*, full-length LHR mRNA and protein expression is not necessarily enough to evoke conventional LHR mediated signalling cascades when stimulated by HCG (Viswanath *et al.*, 2007; Yarram *et al.*, 2003). Additionally, the studies are complicated by the fact that the surrounding endocrine status of the animal could alter the LH/HCG mediated signalling cascade. For example, in porcine oviduct, LHR is induced by 4-day treatment of combined estradiol and progesterone, but not in the absence of these hormones or by estradiol or progesterone alone (Gawronska *et al.*, 2000). Negative results of extragonadal LHR functions obtained in animal models and cell culture studies could manifest the differences in species or mirror the lack of physiological requirements necessary for physiological LHR activation. The current data also supports the redundancy of LHR signalling in other female tissues than the ovaries at a functional and gross morphological level (Pakarainen *et al.*, 2007). However, this does not exclude the possibility that LHR signalling might affect gene expression on a more subtle, modulatory level in some tissues, alone or in conjuction with ovarian hormones. In fact, a common polymorphism in human LHR gene translates into changes in breast cancer onset (Powell *et al.*, 2003). Although this could reflect changes in ovarian steroid production, it is possible that the polymorphism has more direct effects on the breast.

2. Mammary gland tumorigenesis in HCGβ TG females

The appearance of mammary gland tumors in HCGβ TG females is not surprising in the context of the hormonal environment of these mice. High circulating estrogen is known to promote mammary tumorigenesis. HCGβ TG mice are exposed to high estradiol during peripuberty, when the TEB progression and stem cell population are active and proliferating, acting at the time when the cells are most vulnerable. In rodents, carcinogenic signal during critical developmental periods, such as puberty, results in increase in mammary gland tumor incidence (Fenton, 2006). Thus, even though the estrogen-exposure in HCGβ TG females is relatively short, it could elicit long-term effects on mammary tumor risk alone. Furthermore, HCGβ TG females are later exposed to high circulating prolactin. High prolactin exposure alone also results in mammary adenocarcinomas in mice (Rose-Hellekant *et al.*, 2003; Wennbo *et al.*, 1997). In humans, there is also evidence of carcinogenicity of PRL (Tworoger and Hankinson, 2006). Interestingly, the tumors appearing in Mt-1 driven rPRL overexpressing TG mice are reported to have squamous differentiation, keratin noduli, tubular pattern and inflammatory infiltrates, suggesting that they resemble tumors observed in HCGβ TG mice. PRL-initiated tumors had high penetrance, but metastases were not found, whereas tumors in HCGβ TG mice were able to metastasize in some cases. Macroscopical metastases were not observed in the majority of the tumor-bearing mice. In human breast cancer, metastases are most often found in the regional lymph nodes, lung, liver and bone, but these sites did not exhibit signs of apparent metastasis formation in autopsies of HCGβ TG mice. The lack of metastatic capability (especially bone metastases) is typical for many mouse models of breast cancer. In humans, breast cancer progression to the metastatic state typically takes several years. Due to the rapid onset and growth of

the primary tumors in experimental animals, disseminated tumor cells do not have time to form macroscopical metastasis (Jonkers and Derksen, 2007). Several TG mouse models of breast cancer can, however, form distant metastases, most often to the lungs. These include Wnt1, ErbB2, PyMT, Hgf, RAS and Igf2 TG mouse models. This suggests that the basic mechanisms governing metastatic properties (tumor cell dissemination, intravasation to the circulation, surviving in the circulation, extravasation, and metastasis growth at a new site) are active in these TG mouse models, although the homing of the disseminated tumor cells is different between human and mouse metastasis formation. Tumor cell homing into the bone and formation of osteolytic lesions can, however, be achieved in mouse models (Welm *et al.*, 2007).

The disappearance of Esr1 and Pgr are from mammary glands of HCGβ TG females before tumor formation is expected in conjunction with the pregnant, pre-lactating –like mammary gland phenotype (Shyamala *et al.*, 1990). It is, however, intriguing why breast cancers, that are clearly dependent on steroid hormones in terms of tumor induction, can become primarily hormone receptor negative as is the case in HCGβ TG female mice. Mouse mammary cancer differs from human breast cancer in that mouse tumors nearly always lose Esr1 expression as also seen in the HCGβ TG tumors, in contrast to human breast cancer where ESR1 expression remains in approximately half of the tumors (Cardiff *et al.*, 2000). ESR1-negative cancer cases are also diminished by ovariectomy, suggesting that ovarian hormones contribute to the induction of ESR1-negative cancers (Rebbeck *et al.*, 2002). In a recent study, estrogen exposure was required for tumor induction in a particular mouse model in which subsequent mammary tumors were Esr1 and Pgr negative. This apparent controversy was shown to be due to estradiol affecting other cell types than breast epithelium directly, namely via increase in angiogenesis and recruitment of bone-marrow derived stromal cells (Gupta *et al.*, 2007). Thus, in the HCGβ TG mouse model, alterations in the physiology of other cell types than direct target cells (ie mammary cells or pituitary cells) for cancer formation might contribute indirectly to tumorigenesis. Importantly, this also applies to other hormones, such as HCG itself. This theory implies that the lack of specific receptor in a particular tissue does not exclude the possibility that the hormone or other paracrine effector would contribute to tumorigenesis or tissue physiology. In the plethora of hormonal abnormalities observed in HCG mice, it is challenging to dissect the primary and secondary events in gene regulation in the context of the whole organism that leads ultimately to tumorigenesis at multiple sites. This is the reason why the subsequent studies were designed to isolate the effects of HCG itself without the contribution from alterations in ovarian steroidogenesis and thus subsequent secondary and tertiary effects.

Other possible contributing factors in promoting mammary tumorigenesis in HCGβ TG mice are obesity and the metabolic phenotype, which are known to promote post-menopausal breast cancer in humans (Lorincz and Sukumar, 2006). Thus, in the context of many possible contributing and confounding factors observed in HCGβ TG mice, it is not possible to point to a single hormonal pathway responsible for tumorigenesis. Carcinogenic stimulus is probably a rather early event, since ovariectomy at 3 months of age was not able to totally normalize mammary gland phenotype in HCGβ TG mice. Whether or not the mammary glands from these mice would have progressed to palpable tumors with time remains open, but ovariectomy also abolished other endocrine abnormalities classically driving tumor formation.

The features of Wnt-pathway tumor type are common in human basal-type breast cancer (Livasy *et al.*, 2006). They are ESR1 PGR and HER2 negative (triple negative) but CK5/6 positive. In humans, squamous differentiation to the extent seen in mouse models is extremely rare, but exists as a subtype of metaplastic carcinoma of the breast (MCB). MCB is a group of poor prognosis breast cancers with four types of metaplasia: spindle cell carcinoma, matrix producing carcinoma, carcinoma with squamous differentiation and carcinoma with heterologous elements. These tumors are basal-like tumors (Reis-Filho *et al.*, 2006), being triple negative and CK5/6 positive. In addition, they express EGFR. Extensive squamous differentiation in Wnt-pathway models could simply be a manifestation of differences in species despite a common underlying mechanism of tumor induction, or it could reflect the intensity of the inducing oncogenic pathway. A microarray study confirmed that basal-type tumors resemble each other both in mouse models and in humans in terms of their molecular signatures, confirming the relevance of these mouse models in recapitulating human breast cancer (Herschkowitz *et al.*, 2007). This raises a possibility that initiation of human basal breast cancer involves abnormal wnt-signalling. Wnt-pathway tumors in mouse appear so distinct in HE staining that it was proposed that the histological appearance alone could imply the involvement of a specific pathway in tumorigenesis (Rosner *et al.*, 2002). Subsequently, we sought to find the connection between the observed phenotype, Wnt-signalling, and the hormonal abnormalities observed in HCGβ TG mice.

3. Involvement of Wnt-pathway in HCG signalling

The involvement of the Wnt-pathway in HCG signalling in mouse and human mammary epithelium was an unexpected finding with many potential implications. Firstly, the hormonal regulation of Wnt5b and Wnt7b in the mouse mammary gland has not been previously elucidated. Wnt5b needed systemic estradiol and progesterone in order to be upregulated in response to HCG, although estradiol and progesterone could not alone upregulate Wnt5b mRNA. Ovariectomy did not affect basal Wnt5b expression in WT animals either, but in HCGβ TG mice it further elevated Wnt5b expression. This could be explained by changes in the mammary gland cell population. The remaining cell population in HCGβ TG mice, that did not become apoptotic after ovariectomy, was enriched in cells producing Wnt5b, suggesting that its production was not dependent on ovarian hormones in HCGβ TG mice at this stage. Wnt5b expression has been found to be highest in the pregnant mouse mammary gland (Gavin and McMahon, 1992). Of the two Wnt5 genes with close homology, the role of Wnt5a in breast carcinogenesis has been studied more. The loss of WNT5A correlates with recurrence-free survival in human breast cancer and WNT5A has been shown to reduce the migration of human breast epithelial cells by the non-canonical, Ca^{2+} mediated signalling pathway (Dejmek *et al.*, 2006). However, non-canonical WNT5A signalling by macrophages in a co-culture with MCF-7 cells has been associated with increased invasiveness of MCF-7 cells (Pukrop *et al.*, 2006), emphasizing the complex and highly context dependent nature of Wnt signalling. Thus, despite the close homology between Wnt5a and Wnt5b, the data on Wnt5a does not allow us to draw conclusions regarding the effect of Wnt5b on the HCGβ TG mammary gland.

Wnt5b and HCG connection implies that HCG could be involved in metabolic adaptation and adipocyte function, although this question was not specifically addressed in the current

studies. In humans, WNT5B is expressed at least in adipose tissue, the liver and pancreas and it regulates preadipocyte differentiation in mouse. Furthermore, it significantly upregulates adipocytokine expression (PPARγ, C/EBP-α, adiponectin, leptin) in mouse adipocyte culture, suggesting that HCG, by stimulating Wnt5b expression in HCGβ TG adipose tissue, could explain some of the result obtained previously (Kero *et al.*, 2003). It has also been shown that HCG stimulates the expression of these same adipocytokines in human preadipocytes, but WNT5B expression was not studied in this approach (Dos Santos *et al.*, 2007). It is noteworthy to mention that Wnt5b did not activate canonical signalling in adipose tissue, but instead inhibited it, since activated canonical Wnt-signalling inhibits adipocyte differentiation (Kanazawa *et al.*, 2005). Intriguingly, SNPs in human WNT5B gene confer susceptibility to type 2 diabetes, suggesting that WNT5B is an important regulator of adipocyte function *in vivo*. Thus, it is likely that WNT5B regulation by HCG has clinical significance, and that HCG contributes to the metabolic alterations seen in early pregnancy. HCG and its role in metabolic processes is further discussed below. WNT5B overexpression has also been noted in leiomyomas in humans and it is regulated by the menstrual cycle also in normal myometrial smooth muscle cells, the expression being higher in the secretory phase (Mangioni *et al.*, 2005). Interestingly, Wnt5a and Wnt5b are expressed in endometrium during the peri-implantation period (Hayashi *et al.*, 2007). We did not, however, observe regulation of Wnt5b mRNA in mouse uterus by HCG. Wnt5b is also involved in chondrocyte differentiation and bone formation. In chondrocytes, Wnt5b acts as a canonical ligand wheras Wnt5a antagonizes the canonical pathway (Yang *et al.*, 2003) despite their close homology. Wnt5b upregulates the chondogenic marker Sox9 that was upregulated 2-3 fold in the mammary glands of HCGβ TG mice and by systemic HCG administration – but again only in the presence of normal concentrations of estradiol and progesterone. Interestingly, HCGαβ females have been shown to have a bone phenotype (Yarram *et al.*, 2003) that was found to be dependent of functional ovaries. Full length mRNA and protein expression of LHR was found in cultured human and mouse osteoblasts, but it was reported that HCG-binding and subsequent downstream cellular effects could not be demonstrated. The bone mineral densities (BMD) of intact HCGαβ females were 30% higher than WT littermates, but after ovariectomy BMDs were similar. Explanation for this was not obvious. To achieve this large increase in BMD, a 500-fold increase in estrogen would be needed, whereas estrogen-concentrations of HCGαβ TG females are normalized after adolescence. It was concluded that HCG, together with estrogen, could synergistically cause this large increase in bone mineral density, or that other factors (testosterone, progesterone, prolactin or ovarian factors) could contribute to the phenotype. Canonical Wnt-signalling has been reported to increase mineralization in the osteoblast culture, and Wnt7b functions as a canonical ligand in bone (Li *et al.*, 2005). Both Wnt7b and Wnt5b are able to induce phenotype in bone, and could thus contribute to the bone phenotype in HCGαβ TG female mice.

Wnt7b is the other Wnt-ligand found to be regulated by HCG in the mouse mammary gland. Its expression has been detected mostly in virgin mammary glands (Weber-Hall *et al.*, 1994). Interestingly, Wnt7b is enriched in TEBs (Kouros-Mehr and Werb, 2006), suggesting that it could be involved in regulating stem cell function and branching morphogenesis. WNT7B has been found in human breast malignancies (Huguet *et al.*, 1994). Especially interesting is the subgroup of human breast tumors (10% of the tumors) that overexpress

WNT7B 30-fold higher than normal breast, raising the possibility that this subgroup could arise from the progenitor cell compartment. Ovariectomy of 5 month-old WT mice did not alter Wnt7b expression in the mammary gland. In contrast, Wnt7b expressing cells became enriched after ovariectomy in the HCGβ TG mammary gland, suggesting that the surviving cell population was more primitive and independent of the ovarian requirement in HCGβ TG mice ovariectomized at the age of 5 months. Overall, the expression of Wnt7b in the mouse mammary gland was relatively low.

The association of Wnt7b with placental development is also interesting, since the placenta is a physiological site of HCG action. Abnormal HCG signalling, like observed in trisomy 21 foetus placentas, impairs placenta formation in humans (Pidoux *et al.*, 2007). Knocking out Wnt7b in mouse results in embryonic lethality due to placental defects (Parr *et al.*, 2001), more precisely to the defects in chorion-allantois fusion. In humans, impaired HCG signalling in cytotrophoblasts prevents the formation of syncytiotrophoblasts. Taken together, our study suggests that Wnt5b and Wnt7b mediate the effects of HCG in several tissue types in mouse, including the mammary gland, bone and adipose tissue.

4. HCG and metabolism

As previously shown (Kero *et al.*, 2003), LH-CTP TG mice are obese, have hyperinsulinemia and lower cholesterol levels. Increase in body weight and food consumption was, however, normalized when these mice were ovariectomized, showing that these changes are dependent on functional ovaries and that LH alone could not cause these body weight and food consumption phenotypes. bLHβ-CTP female mice had elevated levels of blood insulin, although glucose tolerance and blood glucose were normal (Kero *et al.*, 2003). Furthermore, the mice expressed increased levels of leptin mRNA in fat tissue. It was speculated that the obesity was due to increased adrenal steroidogenesis and circulating androgens found in these mice, both of which are secondary phenotypes due to ovarian stimulation by LH. Adrenal stimulation by LH could also be a direct effect, but it was abolished by ovariectomy. However, LHR expression in tissues could be altered in the absence of ovarian steroids, as shown previously (Gawronska *et al.*, 2000). Intriguingly we observed that in the context of estrogen and progesterone supplementation, several metabolic genes were differentially regulated after HCG treatment, suggesting that a HCG-mediated effect exists for regulation of metabolic genes in the presence of a normal milieu of estrogen and progesterone. These modifications in gene expression are all convergent with a hyperinsulinemic and insulin resistant state. Specifically, downregulation of Angptl4 and Sorbs1 and upregulation of Adpn and Hsd11b1 was observed after HCG injections. Angptl4 decreases hyperinsulinemia, normalizes glucose tolerance and causes hyperlipidemia in diabetic mice (Xu *et al.*, 2005). In humans with type 2 diabetes, serum concentration of ANGPTL4 is significantly lower than in healthy subjects. ADPN has been shown to be upregulated in response to hyperinsulinemia and it correlates positively with obesity in humans (Johansson *et al.*, 2006). SORBS1 and HSD11B1 are also associated with obesity and insulin sensitivity (Lesniewski *et al.*, 2007; Westerbacka *et al.*, 2006). The site of expression for these genes is more likely to be the mammary adipose compartment than the epithelium, and their modulation reflects a yet not fully characterized mechanism of HCG on the glucose metabolism or adipocyte function. Indeed, a direct mechanism of HCG

stimulating human preadipocyte function *in vitro* was recently described, linking HCG directly to pregnancy-mediated metabolic alterations (Dos Santos *et al.*, 2007). Interestingly, grand multiparity (parity >5) in humans impairs glucose tolerance and increases diabetes in the long-term, after adjusting for age, obesity and socio-economic status (Simmons *et al.*, 2006). This suggests that pregnancy, potentially by HCG-mediated processes, also elicits long-term effects on the glucose metabolism, in addition to an altered glucose-metabolism during pregnancy (Barbour *et al.*, 2007).

SUMMARY AND CONCLUSIONS

1. Over-expression of HCGβ severely alters the endocrine system in mice. Enhanced estradiol production is observed at adolescence, followed by luteinisation of the ovaries resulting in permanently elevated progesterone production. This leads to the formation of pituitary lactotroph hyperplasia and highly elevated serum prolactin. Hormonal disturbances in HCGβ TG females result in precocious puberty, infertility, obesity and mammary gland tumorigenesis. Invasive and metastatic mammary gland tumors appear with high penetrance from the age of 9 months onwards. Ovariectomy of the HCGβ TG female mice early in adulthood prevents all abnormalities, showing the ovarian dependency of these phenotypes.

2. Mammary tumors of HCGβ TG female mice are Esr1 and Pgr negative and share the histological features of mammary gland tumors caused by altered Wnt –signalling. Beta-catenin is stabilized in the HCGβ TG tumor tissue and upregulation of Wnt5b and Wnt7b is observed in the mammary glands and tumors.

3. In ovariectomized WT mice supplemented with physiological amounts of estradiol and progesterone systemic administration of HCG causes up-regulation of Wnt5b and Wnt7b, and several Wnt-pathway target gene mRNAs in mammary gland. These changes are not dependent on the ovaries, suggesting that extragonadal HCG signalling mechanisms alter mammary gland gene expression in mice. In addition, gene expression alterations reminiscent of hyperinsulinemia are observed in these mice. This is the first time that HCG signalling has been seen to directly regulate the Wnt-pathway in the mammary gland. Furthermore, the regulation of Wnt5b and Wnt7b in the mammary gland has not been previously elucidated.

4. Depletion of LHR from the mouse mammary gland epithelium results in alterations in gene expression, including several genes related to the Wnt-pathway. Furthermore, we observed changes in the expression of the genes that are regulated by HCG/LHR signalling in the ovary. This suggests a role for LHR-signalling in the mouse mammary gland and a partially overlapping gene expression pattern in the mammary gland and ovary in response to LHR signalling.

5. Cultured human breast epithelial cells expresses LHR and responds to HCG stimulation by upregulating WNT5B and WNT7B mRNAs, suggesting that the mechanism found in HCGβ TG mouse is relevant to human physiology. This suggests, further, that LHR signalling takes place in human breast tissue.

The current study indicates that the phenotype of the HCGβ TG females is similar to other mouse models with increased LH/LHR action, but different to the human phenotype, due to the differences between the species. The histological and immunohistochemical analyses indicated that mammary gland tumors of HCGβ TG mice were similar to tumors induced by enhanced Wnt-pathway activity. Thus, HCGβ TG mice can serve as a model of endocrine-induced, Wnt-pathway activating mammary carcinogenesis that shares similarities with human basal type breast cancer. In this study, a previously unknown mechanism of HCG-signalling was found: HCG-signalling was linked directly to Wnt-pathway activation in the mouse mammary gland and human mammary epithelium. This provides new evidence

for the possible involvement of HCG/LHR signalling in mammary carcinogenesis, and potentially in other physiological processes involving Wnt-ligands. Previous observations of metabolic alterations due to LH/LHR action in the presence of estradiol and progesterone were further confirmed here, indicating that HCG signalling might further modify breast physiology and cancer predisposition also from this aspect. Wnt-pathway modification was confirmed to be mediated by LHR acting in mouse and human epithelium, thus confirming that LHR is functional in the mammary gland and human breast epithelium. Further studies are needed on the role of the genes found to be modulated by LH/LHR signalling *in vivo*, in order to more precisely elucidate their relevance in tumorigenesis of the breast. Wnt ligand upregulation by HCG in breast epithelium, and possibly elsewhere in the body, requires further attention because of the emerging role of wnts as important regulators of physiological and pathophysiological processes. HCG/LH -signalling in the regulation of metabolic functions are also potentially important but yet poorly chararcterized. Recapitulating the above mentioned aspects of HCG/LH –signalling in experimental animals or cell cultures may be challenging, because substantial differences in species may exist, and the expression of LHR may be highly context dependent. These factors may have contributed to controversies in studies on extragonadal LHR actions. Appropriate study design could reveal novel, direct actions of HCG/LH signalling in several tissue types that can ultimately have importance in pathophysiological conditions.

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