Activation of the Prolactin Receptor but Not the Growth Hormone Receptor is Important for Induction of Mammary Tumors in Transgenic Mice

Håkan Wennbo,* Maria Gebre-Medhin,* Amel Gritli-Linde,‡ Claes Ohlsson,§ Olle G.P. Isaksson,§ and Jan Törnell*

**Department of Physiology, Research Centre for Endocrinology and Metabolism and ‡Department of Oral Biochemistry, Göteborg University Medicinaregatan 1F S-413 90 Göteborg, Sweden; and §Department of Internal Medicine, Research Centre for Endocrinology and Metabolism, Sahlgrens University Hospital S-413 45 Göteborg, Sweden*

Abstract

Transgenic mice overexpressing the human growth hormone gene develop mammary carcinomas. Since human growth hormone gene can activate both the growth hormone receptor (GHR) and the prolactin (PRL) receptor (PRLR), it is not clear which receptor system is responsible for the malignant transformation.

To clarify the receptor specificity, we created transgenic mice with two different genes: (*a*) transgenic mice overexpressing the bovine growth hormone (bGH) gene having high levels of bGH only activating the GHR and also high serum levels of IGF-I; and (*b*) transgenic mice overexpressing the rat PRL (rPRL) gene that have elevated levels of PRL (one line 150 ng/ml and one line 13 ng/ml) only binding to the PRLR and with normal IGF-I levels.

When analyzed histologically, all of the PRL transgenic female mice developed mammary carcinomas at 11–15 mo of age. Only normal mammary tissue was observed among the bGH transgenic animals and the controls. Cell lines established from a tumor produced rPRL and expressed PRLR. In organ culture experiments, an auto/paracrine effect of rPRL was demonstrated.

In conclusion, activation of the PRLR is sufficient for induction of mammary carcinomas in mice, while activation of the GHR is not sufficient for mammary tumor formation. (*J. Clin. Invest.* 1997. 100:2744–2751.) Key words: mammary neoplasm • transgenic mice • prolactin • growth hormone • auto/paracrine

Introduction

Several hormones and growth factors have been implied to participate in the development of the normal breast and carcinogenesis of its epithelium (e.g., estrogens, progesterone, prolactin, growth hormone, insulin, glucocorticoids, insulin-like growth factors, transforming growth factor α, epidermal growth factor, platelet-derived growth factors, and fibroblast growth factors). The role for the specific hormones in these processes, however, is less clear.

One of the most studied hormones involved in mammary gland biology is prolactin (PRL),[1] a polypeptide hormone mainly produced in the pituitary, but also in other organs, e.g., the mammary gland (1). Physiologically, PRL influences the mammary gland in several ways during development, growth, and stimulation of milk protein gene transcription (2, 3). The importance of PRL in pathological conditions such as mammary tumor growth in women is indicated by several studies. PRL receptors (PRLR) have been found in 40–70% of human breast tumors (4, 5), and PRL stimulates growth of several human breast cancer cell lines in vitro (6, 7, 8). PRLR antagonists can inhibit the growth of established breast cancer cells in vitro (9). PRL expression has been detected in human mammary tumors, and human mammary tumor cell lines can produce PRL (1), indicating a possible auto/paracrine function of PRL in mammary tumor growth. In rodents, PRL can stimulate growth of chemically induced mammary tumors. Furthermore, pituitary isografts, which secrete increased amounts of PRL, can substitute for gestation when pregnancy-dependent mammary tumors are chemically induced in rodents (10). The importance of PRL in the induction of mammary tumors, however, is less clear. The prolactin receptor can, in many species including mouse and human, also be stimulated by the human growth hormone (hGH) (11), which belongs to the same family of hormones as PRL. The receptors for PRL and GH both belong to the cytokine receptor superfamily (12). In humans with pathologically elevated GH levels (acromegaly), malignant disorders are more common than in the normal population. A general increase of malignant tumors (13, 14, 15), an overrepresentation of tumors in the female mammary gland (15) and in the colon (16), have been reported in patients with acromegaly. Furthermore, growth hormone receptors (GHR) have been identified in human mammary tumors and cell lines (17).

Transgenic mice have previously been used to study the effects of high levels of human GH. Female mice transgenic for hGH develop metastatic mammary adenocarcinomas at a high frequency (18, 19), an effect not linked to the strain of hGH transgenic mice used (20). As mentioned above, hGH can bind to and activate both somatogenic GHR and lactogenic PRLR. Because of this duality in receptor activation, it is not clear whether GHR or the PRLR activation, or a combination of both, are causing tumor formation in the hGH transgenic mice.

Address correspondence to Jan Törnell, Department of Physiology, Göteborg University, Medicinaregatan 1F, S-413 90 Göteborg, Sweden. Phone: +46 31 7733535; FAX: +46 31 7733531; E-mail: jan.tornell@ss.gu.se

Received for publication 7 July 1997 and accepted in revised form 24 September 1997.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/97/12/2744/08 $2.00
Volume 100, Number 11, December 1997, 2744–2751
http://www.jci.org

1. *Abbreviations used in this paper:* bGH, bovine growth hormone; GH, growth hormone; GHR, growth hormone receptor; hGH, human growth hormone; IGF-IR, IGF-1 receptor; Mt-I, metallothionein-I; PBTC, prolactin breast tumor cell line; PRL, prolactin; PRLR, prolactin receptor.

It is well established that GH induces production of IGF-I in several tissues, and increases the circulating levels of IGF-I (21, 22). IGF-I has been suggested to be of importance for tumor induction/growth in the mammary gland. IGF-I is produced by mammary tumor stroma cells, but this growth factor is not expressed by breast cancer cell lines (23). IGF-I receptors (IGF-IR) are present in the normal mammary gland, and are frequently present in human breast tumors (24). In cell culture experiments, human breast cancer cell lines express IGF-IR. IGF-I is a very potent mitogen for these cells (25, 26), suggesting that IGF-I can act as a paracrine factor in the mammary gland.

Transgenic mice expressing IGF-I and IGF-II, both binding to the IGF-IR, have been used to clarify the importance of the IGF-IR for mammary tumor formation. No tumor formation in the breast has been reported in the IGF-I transgenic mice (27, 28). Some of the transgenic mice expressing a modified IGF-I molecule having a lower affinity for the IGF-binding proteins, however, develop mammary carcinomas after several pregnancies (29). After multiple pregnancies, IGF-II transgenic mice develop mammary tumors (30).

Mice transgenic for hGH have high levels of IGF-I (31), and mammary tumor formation in these mice could be a consequence of the activation of GHR, PRLR, IGF-IR, or an activation of a combination of them.

The aim of this study was to clarify the relative importance of the different receptors in mammary tumor formation in the mouse mammary gland. To differentiate between activation of the somatogenic and the lactogenic signaling pathways, rPRL transgenic mice and bovine GH (bGH) transgenic mice were used. Nonprimate GH only activates GHR, while PRL only activates the PRLR (11). We now report that female rPRL transgenic mice, but not bGH transgenic mice, develop malignant mammary tumors.

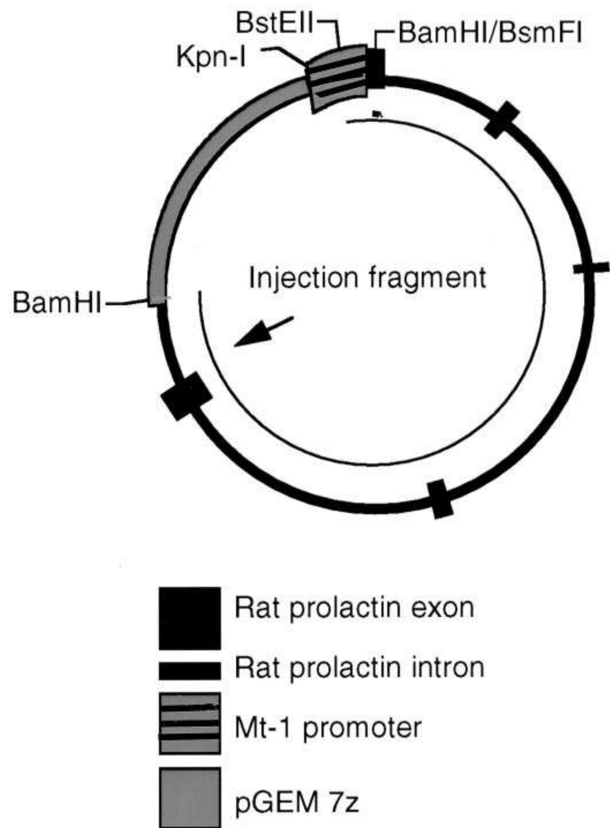

Figure 1. The Mt-rPRL-WBO2 plasmid. The metallothionein promoter, the rat prolactin gene, and the injection fragment are indicated.

Methods

Construction of the metallothionein promoter rat prolactin plasmid. The rat PRL (rPRL) expression vector, Mt.rPRL-WBO2 (Fig. 1), was based on the pRPRL-HindIII A and B plasmids described earlier (32) and the metallothionein-1 (Mt-1) promoter from MtbGH 2016 plasmid (33). The Mt-1 promoter was subcloned as a 650-bp fragment into a BsmF1 site 33 bp 5′ of the start codon in the rPRL gene inserted in a pGEM-7Z vector (Promega Corp., Madison, WI) resulting in the Mt.rPRL-WBO2 plasmid (Fig. 1). The Mt-1-rPRL fragment was excised by digestion with BstEII, located in the Mt-1 promoter, and BamHI located 3′ in the polylinker of pGEM-7Z (Fig. 1).

Generation of transgenic mice. Transgenic mice were generated in C57BL/6JxCBA-f2 embryos by standard microinjection procedures (34). The rPRL DNA fragment to be injected was excised from the plasmid Mt.rPRL-WBO2 by restriction enzyme cleavage with BstEII and BamHI, separated by gel electrophoresis through a 0.7% agarose gel, cut out, isolated using isotachophoresis (35), and precipitated with ethanol.

In another series of experiments, the bGH DNA fragment was isolated from the plasmid MtbGH 2016 as a BstEII-EcoRI fragment, separated by gel electrophoresis through a 1% agarose gel, cut out, and isolated using Genclean II kit (Bio 101, La Jolla, CA).

To identify transgenic animals, DNA was extracted from 0.5-cm sections of tails from 3-wk-old mice by digestion with 400 μg of proteinase K in 0.6 ml of 1 M urea, 100 mM NaCl, 50 mM Tris HCl (pH 8.0), 10 mM EDTA, and 0.5% SDS at 55°C for 16 h. The digested tails were frozen for 2 h at −70°C, and were then precipitated with isopropanol and washed with ethanol. The presence of the rPRL transgene was detected with PCR (94°C for 5 min and 30 cycles of sequential incubations at 94°C for 30 s, 54°C for 30 s, and 72°C for 120 s) using one primer located in the Mt-promoter (5′-GCGAATGGGTT-TACGGA-3′) and one in the rPRL gene (5′-CCATGAAGCTCCT-GATGCT-3′).

Mice that had integrated the bGH transgene were identified with PCR (the same incubation conditions as for rPRL) using the primer mentioned above in the Mt-promoter and one primer located in the bGH gene (5′-CTCCAGGGACTGAGAACA-3′). Transgenic mice produced with this construct have been described earlier (31, 36).

RNA analysis. Total RNA was isolated from frozen tissues by acid guanidinium thiocyanate-phenol-chloroform extraction described by Chomczynski and Sacchi (37). Specific RNA was analyzed using a reverse transcriptase (RT)-PCR assay. The RT reaction was performed with 0.5 μg RNA as a template in the presence of 0.25 μg oligo-(dt) primer (Promega Corp.), 5 U AMV-RT (Promega Corp.), 20 U RNAsin (Promega Corp.), and dNTPs (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) at a final concentration of 1 mM per nucleotide. RT buffer (50 mM Tris-HCl; pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM Spermidine, and 10 mM DTT) was added to a total volume of 20 μl. After denaturation at 70°C for 5 min and annealing in room temperature for 10 min, the elongation was carried out for 60 min at 42°C. The RT reaction was terminated by heat inactivation (95°C for 7.5 min).

Rat PRL-specific RNA was analyzed by amplifying an aliquot of cDNA by PCR (94°C for 5 min and 30 cycles of sequential incubations at 94°C for 30 s, 60°C for 30 s, and 72°C for 120 s) using a sense primer located in exon 4 (5′-TCCATGAAGCTCCTGATGCT-3′) and an antisense primer located in exon 5 (5′-GGATGGAAGT-TGTGACCA-3′) specific for rat PRL (Fig. 2). The PCR products

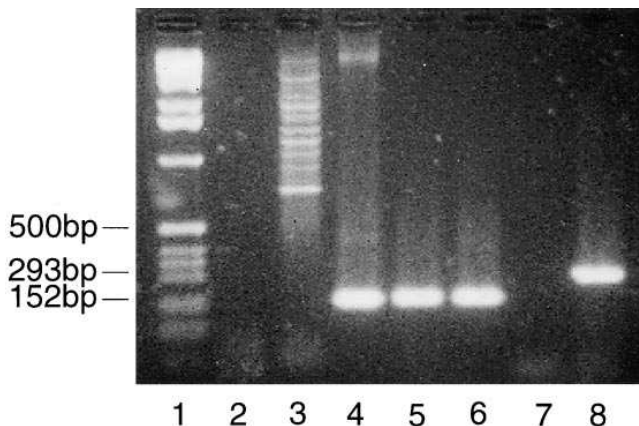

Figure 2. RT-PCR analysis of expression of the bGH and rPRL transgenes in respective animals. Lane *1*, ladder (1 kb; GIBCO BRL), lanes *2–6*, analysis of rPRL expression. Templates used are as follows: lane *2*, no template; lane *3*, mouse PRL cDNA plasmid used to show the species specificity of the primers; lane *4*, rat PRL cDNA plasmid used as a positive control; lane *5*, cDNA from a mammary tumor from a rPRL transgenic animal from line 1; lane *6*, cDNA from a mammary tumor from a rPRL transgenic animal from line 2. Lanes *7* and *8*, analysis of bGH expression. Templates used are as follows: Lane *7*, cDNA from mammary gland of a control mouse, lane *8*, cDNA from mammary gland of a bGH transgenic mouse.

were analyzed by electrophoresis in 1% agarose gel. The size of the fragment amplified from spliced RNA should be 152 bp, and that from unspliced RNA or contaminating DNA, 1252 bp. The fragments were transferred to Hybond-N nylon membranes (Amersham Corp., Arlington Heights, IL) and the membranes were baked in 80°C for 2 h and prehybridized in hybridization buffer (0.2 M NaH_2PO_4, pH 7.4, 8% SDS, 1 mM EDTA, 1% BSA fraction V) at 60°C for 2 h. As probe a 823-bp PstI fragment (38) containing the rat PRL cDNA was used, and labeled with a random priming kit (Amersham Corp.) and P^{32}dCTP. The hybridization was carried out in the same buffer at 60°C for 12–16 h, and was washed with 2× SSC, 0.5% SDS at 60°C for 1–2 h, and with 0.1× SSC, 0.1% SDS at 60°C for 0.5–2 h.

Bovine GH-specific RNA was analyzed by amplifying cDNA by PCR (94°C for 5 min and 30 cycles of sequential incubations at 94°C for 30 s, 54°C for 30 s, and 72°C for 120 s) using a sense primer located in exon 2 (5′-TCCCTGCTCCTGGCTTTCGC-3′) and an antisense primer located in exon 4 (5′-GCAGTGAGATGCGAAGCAGC-3′). The PCR products were analyzed by electrophoresis in 1% agarose gel. The size of the fragment amplified from spliced RNA should be 295 bp, and that from unspliced RNA or contaminating DNA, 749 bp. The transfer, hybridization, probe-labeling, and washing were carried out as above. To use mouse GH as a probe, cDNA was amplified from mouse pituitary cDNA by the same protocol used for detection of bGH as well as a sense primer located 5′ of exon 1 (5′-CCTAGAGTCCAGATTCCA-3′) and an antisense primer located in exon 3 of the mouse GH gene (5′-AGGCTGCTTTCTGCTTCT-3′) amplifying a fragment of 293 bp. The PCR fragment was subcloned into a pCR™ II vector (Invitrogen Corp., San Diego, CA) and identified as mouse GH by digestion with restriction enzymes. The entire subcloned fragment from the vector was labeled as probe.

Specific RNA for the long form of the mouse PRL receptor was amplified by PCR (94°C for 5 min and 30 cycles of sequential incubations at 94°C for 30 s, 56°C for 30 s, and 72°C for 120 s) using a sense primer located in the extracellular part of the receptor (5′-GACTCGCTGCAAGCCAGACC-3′) and an antisense primer located in the intracellular part of the long form of the receptor (5′-TGACCAGAGTCACTGTCAGG-3′). The size of the fragment amplified from spliced RNA should be 440 bp.

Measurement of rat prolactin. Plasma levels of rat PRL were measured by a rat prolactin radioimmunoassay kit (Amersham International, Little Chalfont, UK). Serum was either collected from mouse tails in heparin-coated glass capillaries or by heart puncture in heparin-coated syringes when animals were killed between 9 and 11 AM. Cell culture medium was collected after 3 d of culture. All samples were analyzed in duplicates. The results were expressed as ng rat PRL/ml plasma.

Measurement of IGF-I. The IGF-I concentration in plasma was determined by radioimmunoassay after acid ethanol extraction according to the manufacturer's protocol (Nicols Institute Diagnostics, San Juan Capistrano, CA). Serum was collected by heart puncture in heparin-coated syringes when animals were killed.

Establishment of cell lines from a tumor. Two cell lines from a mammary tumor in a PRL transgenic mice were established. The tumor was cut into small pieces and either first treated with collagenase (prolactin breast tumor cell line 1, PBTC-1), 0.8 mg/ml for 15 min at 37°C, or directly transferred to culture dishes (PBTC-2). The cell doubling time gradually increased after 20–25 passages. At this time some of the cultures were lost in senescence while, as a sign of establishment, the cell doubling time was decreased for two cell lines (PBTC-1 and PBTC-2). The established cells were cultured in DMEM F-12 medium supplemented with 15% FCS, fungizone 0.5 mg/liter, gentamycin sulfate, 50 mg/liter, L-glutamine, 2 mmol/liter, and L-ascorbic acid, 100 mg/liter in 5% CO_2 at 37°C.

Mammary gland in vitro organ culture. Abdominal mammary glands from normal mice at day 16 of gestation and PRL transgenic mice were dissected out. Explants from the transgenic animals were taken from glands devoid of tumor tissue. The mammary tissue was cut into small pieces and cultured under serum-free conditions in a Trowell-type organ culture system in triplicate dishes for 7 d at 37°C in an atmosphere containing 5% CO_2. The culture medium consisted of BGJb (GIBCO BRL, Gaithersburg, MD) supplemented with 0.1 mg/ml ascorbic acid, 2 mM L-glutamine, 0.1% BSA, 50 U/ml penicillin, 0.05 mg/ml streptomycin, 5 μg/ml insulin, 1 μM dexamethasone, and 5 μg/ml PRL. After 7 d in culture, the explants were fixed in Bouin's fixative, and were prepared for paraffin embedding.

Histology. Histological examinations of mammary tissue from normal and transgenic animals were performed at the National Veterinary Institute (Uppsala, Sweden). Tissues were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight or longer, dehydrated, and embedded in paraffin. Sections were stained in hematoxylin/eosin. Organ culture sections were stained with Ladewig's hematoxylin.

Statistics. Data are shown as mean±SEM. Statistical differences were calculated using the Wilcoxon rank sum test for serum hormone levels and Chisquare test for tumor formation comparison. Significance levels < 0.05 were considered significant.

Results

Bovine growth hormone transgenic mice do not develop tumors. To study the effects on the mammary gland of GHR stimulation in combination with elevated IGF-I levels, but without PRLR activation, bGH transgenic female mice were generated by mating male bGH transgenic mice from one founder to normal C57BlxCBA females.

The body weights of the female bGH transgenic mice were ~ 40% higher than those of controls. The serum levels of bGH were 1440±200 ng/ml, and those of IGF-I were 850±50 ng/ml. In controls, the serum IGF-I levels were 500±30 ng/ml (39). The bGH levels correspond to approximately 20 times the normal peak GH levels in the mouse (40). To prevent high endogenous PRL levels, the female bGH transgenic mice were not mated.

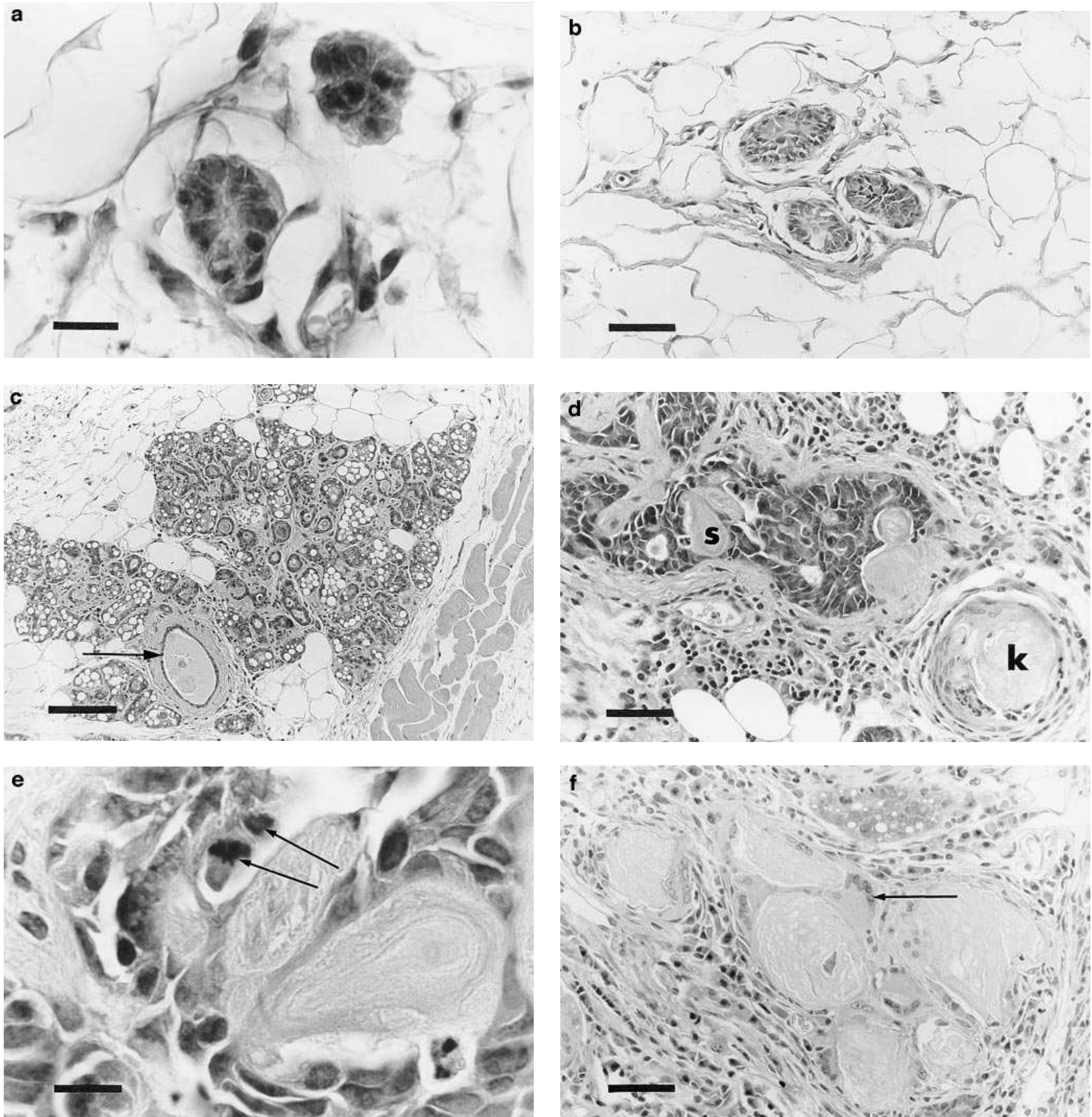

Figure 3. Histology from normal or bGH transgenic female mice at 2 and 12 mo of age, and from rPRL transgenic female mice at 12 mo of age. (*a*) Alveoli in a histologically normal mammary tissue from a 2-mo-old normal mouse. *Bar* indicates 20 μm. (*b*) Alveoli in a histologically normal mammary gland tissue from a 2-mo-old bGH transgenic mouse. *Bar* indicates 85 μm. (*c*) Mammary tissues from a 12-mo-old normal mouse showing more extensive development of the mammary gland than in younger animals. The alveolar epithelium displays fat vacuoles. *Bar* indicates 200 μm. (*d*) Mammary adenocarcinomas from a 12-mo-old PRL transgenic mouse showing proliferation of atypical, anaplastic acinar epithelial cells. Secretions (*s*), squamous metaplasia, and keratin (*k*) are visible. Inflammatory cell infiltration with a predominance of mononuclear leukocytes are in connective tissues adjacent to the tumor. *Bar* indicates 85 μm. (*e*) Closer view of *d* showing anaplastic cells, mitotic figures (*arrows*), and secretions in acinar lumen. *Bar* indicates 20 μm. (*f*) Detail of mammary adenocarcinoma from a PRL transgenic mouse showing several cystic structures containing a keratin-like substance. Large polykaryons displaying peripeheral nuclei are positioned in a key and lock arrangement (*arrow*). Also shown is leukocytic inflammatory infiltration with abundancy of mononuclear cells. *Bar* indicates 85 μm.

The mice were divided into two groups. One group ($n = 5$) was examined by histology at 60 d of age. The mammary glands showed normal histology in these animals (Fig. 3, *a* and *b*).

The remaining mice ($n = 9$) were kept for an observation time between 12 and 17 mo. There were no palpable tumors in any of these mice. Histological examination was carried out in eight of the mice, and no tumor formation was identified. The histology was classified as normal (Fig. 3 *c*).

Table I. *Age, Levels of rPRL, IGF-I, Number of Pregnancies, and Tumor Formation in PRL Transgenic Animals*

Founder line	Animal	Age	rPRL	IGF-I	Pregnancies	Mammary pathology
		mo	*ng/ml*	*ng/ml*		
PRL line 1	1	15	175	313	2	Macroscopic adenocarcinoma
	2	13	143	124	1	Adenocarcinoma
	3	11	147	345	0	Adenocarcinoma
	4	11	140	261	0	Macroscopic tumor
Mean and SEM			151±8	261±49		
PRL line 2	5	15	10	259	1	Macroscopic adenocarcinoma
	6	14	10	275	1	Macroscopic adenocarcinoma
	7	14	6	334	0	Macroscopic tumor
	8	13	18	444	0	Macroscopic tumor
	9	11	22	296	0	Macroscopic tumor
Mean and SEM			13±3	322±33		
Control littermates	10–19	11–15	n.a.	318±32	0	Normal

We have never observed any spontaneous mammary tumors in our mouse colony including also more than 20 previously pregnant female mice older than 12 mo of age. n.a., not applicable.

Prolactin transgenic mice develop mammary tumors. With the aim to stimulate the PRLR, PRLR-transgenic offspring from two unique founder animals were generated by mating to normal C57BlxCBA mice. Rat PRL levels were measured in all animals included in the study at death (Table I). One line (L1) had very high plasma levels of rPRL (150 ng/ml), while the other line (L2) expressed the transgene at lower levels (13 ng/ml). The low-expressing line had rPRL levels in the normal range of PRL, and the high-expressing line had approximately four times peak values (40). Offspring generated from founder L1 consistently had higher plasma levels of rPRL than those generated from founder L2.

Rat-PRL transgenic female mice were followed for a period between 11 and 15 mo, and nontransgenic littermates served as controls. All of the rPRL transgenic female mice developed mammary tumors ($n = 9$, $P < 0.001$). Both previously pregnant and virgin mice of both lines were affected (Table I). No metastases were found at autopsy. A large proportion of the animals displayed macroscopic tumors that were cystic and ~ 1 × 1 × 1 cm in size. No mammary tumors were identified in the male rPRL transgenic animals (data not shown) or in the control group.

The mammary adenocarcinomas show a tubular pattern and squamous differentiation. All PRL transgenic animals that were analyzed histologically showed tumors both in inguinal and thoracic glands. The mammary tumors were malignant adenocarcinomas showing proliferation of atypical anaplastic acinar epithelial cells (Fig. 3 *d*). Some of the adenocarcinomas showed squamous metaplastic differentiation with extensive production of keratin and secretion (Fig. 3 *e*), while others showed a tubular pattern with squamous differentiation. Inflammatory infiltration of mainly lymphocytes, occurred (Fig. 3 *f*). No metastases were found on authopsy.

Rat PRL and bGH are expressed in the mammary glands of the transgenic mice. RNA was isolated from the mammary gland, and RT-PCR was used to examine if the difference in phenotype between the PRL and bGH transgenic mice could be explained by a lack of expression of the bGH transgene in the mammary gland. Specific mRNA for rPRL was detected in the mammary gland from both lines used in the study. The bGH transgenic mice also expressed the transgene in the mammary gland (Fig. 2).

Cell lines established from a mammary tumor produced rPRL and expressed PRLR. The finding that both PRL transgenic lines developed mammary tumors irrespective of serum levels of rPRL made us examine whether rPRL was produced locally in the mammary tumors. Two cell lines (PBTC-1 and PBTC-2) were established from a tumor in one of the mice with low serum levels of rPRL (mouse 8). Rat-PRL were measured in the cell culture medium by RIA after 3 d of culture. The levels of rPRL were ~ 200 ng/ml in the medium, while the levels of rPRL were undetectable before the start of culture. Furthermore, PRLR expression was detected by RT-PCR in both of the cell lines (Fig. 4).

Mammary organ cultures from PRL transgenic mice differentiate in the absence of exogenous PRL. To study the functional significance of locally produced rPRL, mammary organ cultures from the low- and high-expressing lines were used. It is

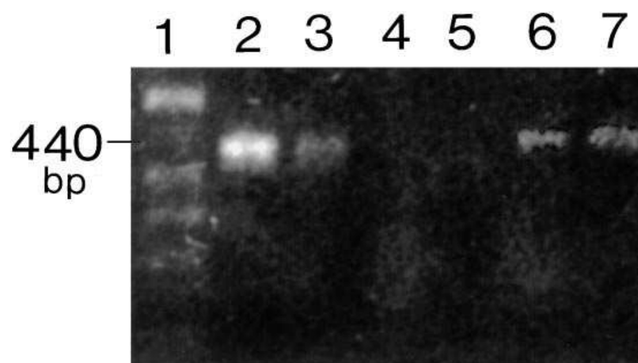

Figure 4. RT-PCR analysis of expression of the PRLR in cell lines. Lane *1*, ladder (1 kb; GIBCO BRL); lanes *2* and *3*, mouse PRLR cDNA used as a positive control; lanes *4* and *5*, negative control; lane *6*, cDNA from cell line PBTC-1; and lane *7*, cDNA from cell line PBTC-2.

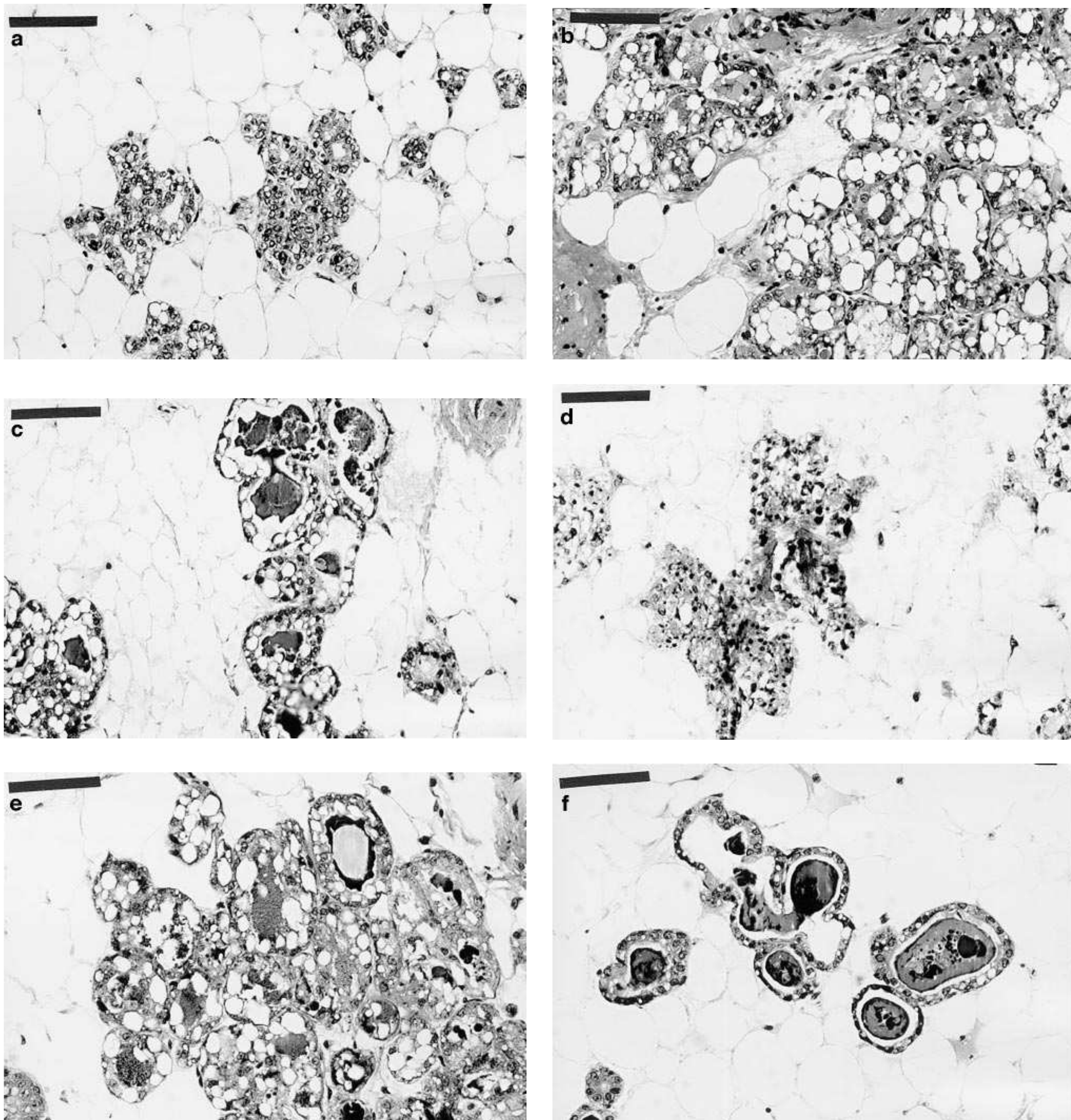

Figure 5. Histological analysis of mammary gland cultures from normal and transgenic animals. *Bar* indicates 100 μm. (*a*) Mammary gland from a normal midpregnant mouse at dissection showing ductal system development and differentiation. (*b*) Mammary gland from a virgin transgenic mouse at dissection showing advanced development reminiscent of a lactating gland. (*c*) Mammary gland from a normal midpregnant mouse cultured for 7 d in the presence of insulin, dexamethasone, and PRL, showing differentiation and secretion. (*d*) Mammary gland from a normal midpregnant mouse cultured for 7 d in the presence of insulin and dexamethasone, showing tissue involution. (*e*) Mammary gland from a virgin transgenic mouse after 7 d of culture in the presence of insulin and dexamethasone, showing maintained lobuloalveolar development and secretion. (*f*) Mammary gland from a normal midpregnant mouse coculture with a mammary gland from a virgin transgenic mouse for 7 d in the presence of insulin and dexamethasone, showing lobuloalveolar development and secretion.

well established that PRL is essential for mouse mammary lobulo–alveolar development in organ culture.

Mammary gland tissue from normal mice at day 16 of gestation before culture showed branching morphogenesis and a mild presence of secreted material in the duct lumina (Fig. 5 *a*). Mammary tissue from rPRL transgenic mice showed morphological features proper to a lactating gland such as lobulo–alveolar development and presence of secreted material at dissection (Fig. 5 *b*). Seven days of in vitro culture of mammary explants from normal mice in the absence of PRL supplement resulted in tissue involution and degradation (Fig. 5 *d*), in contrast to the mammary explants from PRL transgenic mice that

showed a lactating phenotype (Fig. 5 *e*) after in vitro culture in the absence of PRL suplement. When explants from normal mice were cocultured with mammary tissue from rPRL transgenic animals, they displayed lobulo–alveloar development and milk secretion after 7 d (Fig. 5 *f*).

Normal circulating levels of IGF-I in rPRL transgenic females. To determine if the effect observed by PRL was a consequence of elevated IGF-I levels, plasma was collected from transgenic and normal mice at death. There were no statistically significant differences between either of the two lines of transgenic animals (322±33 ng/ml and 261±49 ng/ml) when compared with the controls (318±32 ng/ml; Table I).

Discussion

In this study we report that all female mice overexpressing the rPRL gene developed malignant mammary adenocarcinomas. Tumor formation was not pregnancy-dependent. Bovine-GH transgenic mice with elevated serum levels of GH and IGF-I did not show any histological alterations of the mammary gland. We have previously reported that transgenic mice overexpressing the human GH gene develop malignant mammary tumors (18, 20). From these studies it was not possible to conclude if the GHR, the PRLR, or IGF-I was mediating the effect. The female transgenic mice overexpressing the rPRL gene presented in this study developed malignant mammary tumors without having elevated circulating IGF-I levels. In contrast, the mice overexpressing the bovine GH gene had elevated GH and IGF-I levels without developing mammary tumors. Therefore, it can be concluded that activation of the PRLR is sufficient for mammary tumor formation. A permissive role for the somatogenic GHR or IGF-I, however, cannot be excluded since the tumors seen in the hGH transgenic mice were observed between 7 and 11 mo of age (20) while the tumors in the PRL transgenic mice were seen later in life (11–15 mo of age). Another difference is that the mammary tumors from the hGH transgenic mice metastasized, while the tumors in the PRL transgenic mice did not, indicating a supportive role for the GHR and/or IGF-I in tumor growth or metastasis.

Hyperprolactinemia has previously been investigated in experimental animals using transplantation of pituitaries. Pituitary transplants can substitute for gestation in chemically induced pregnancy-dependent mammary tumors, and can stimulate growth of chemically induced tumors (10). In one study using pituitary transplants, mammary tumors were induced in mice (41). One major difference of these studies compared to our study is that transplantated pituitaries could also secrete other pituitary hormones, e.g., GH (41, 42), which was the case when induction of tumors was seen. Some transgenic mouse models for breast cancer are pregnancy-dependent (29, 30), and in others pregnancy or pituitary transplants induce the tumors earlier in life, indicating the importance of PRLR stimulation for mammary tumor formation in mice.

In humans, there is no clear correlation between prolactinomas and mammary tumors in women. The explanation for the poor correlation could be that PRL is produced locally in the mammary gland (1, 43) and acts in an auto/paracrine way. The locally produced PRL may not influence the plasma levels of PRL, and will not be accessible for treatment with dopamine agonists to decrease the synthesis. We demonstrate in this study that the PRL transgene was expressed in the mammary gland, and that cell lines established from a tumor in low-expressing PRL transgenic mice produced rPRL and expressed PRLR, suggesting an autocrine action of PRL. Furthermore, in organ culture experiments of mammary glands from PRL transgenic animals, the locally expressed rPRL was functionally active, inducing differentiation in a paracrine manner. The presence of the locally produced PRL might therefore be the explanation of the poor correlation between plasma levels of rPRL and tumor formation of the mammary gland in our study. The low-expressing line had rPRL levels in the normal range for mouse PRL (40), but the bioactivity and serum profiles might be different between the endogenous mouse PRL and rPRL produced from the transgene. The bGH transgene, however, was also expressed in the mammary gland, indicating that a lack of local expression could not be the explanation for the difference seen between bGH and PRL transgenic mice.

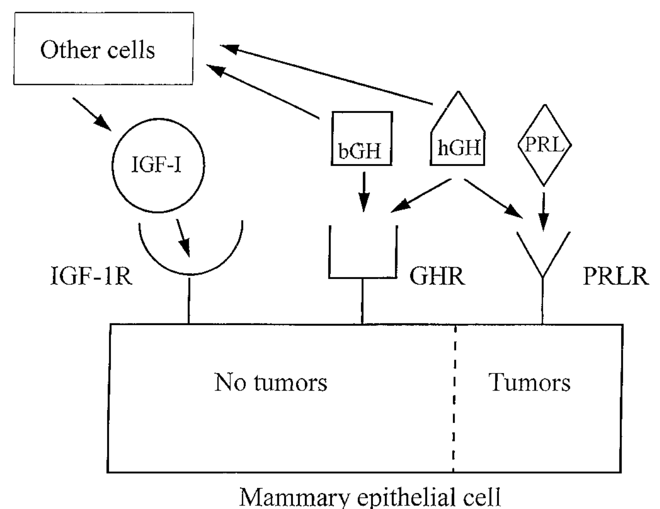

Figure 6. Ligand stimulation of the PRLR, GHR, and IGF-IR systems. Activation of the PRLR is necessary and sufficient for tumor formation.

In conclusion, we have demonstrated that female transgenic mice overexpressing the PRL gene all developed mammary carcinoma, and that the tumor development was caused by activation of the PRLR (Fig. 6). A specific stimulation of the GHR by bGH together with elevated IGF-I levels did not induce mammary tumors. Rat-PRL produced locally in the mammary gland from the transgenic mice could stimulate mammary differentiation in a paracrine way in vitro. Future studies will be needed to examine further the role of locally produced PRL, the interplay among the PRLR, the GHR, and IGF-I, and also to evaluate the significance of PRL for human breast cancer disease.

Acknowledgments

We would like to thank Dr. R. Feinstein for the histological analysis, P.-A. Lundberg for performing the IGF-I analysis, and A. Hansevi and M. Umaerus for excellent technical assistance. We would like to thank Drs. N.E. Cooke and D.R. Palmiter for the generous gifts of the rat prolactin plasmids and the bGH plasmid, respectively.

This study was supported by grants from the Swedish Cancer Society (3164-B95-05XAC, 3598-B94-01UAD), the Swedish Medical Research Council (B96-03X-10213-04A and B96-14X-04250-23B), Pharmacia-Upjohn Stockholm, the Swedish Society for Medical Re-

search, the Göteborg Medical Society, the Assar Gabrielssons Foundation and the Gunnar, Arvid, and the Elisabeth Nilssons Foundation.

References

1. Ben-Jonathan, N., J.L. Mershon, D.L. Allen, and R.W. Steinmetz. 1996. Extrapituitary prolactin: distribution, regulation, functions and clinical aspects. *Endocr. Rev.* 17:639–669.

2. Neville, M.C., and C.W. Daniel. 1987. The Mammary Gland. Development, Regulation and Function. Plenum Publishing Corporation, New York.

3. Ormandy, C.J., A. Camus, J. Barra, D. Damotte, B. Lucas, H. Buteau, M. Edery, N. Brousse, C. Babinet, et al. 1997. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev.* 11: 167–178.

4. Bonneterre, J., J.P. Peyrat, R. Beuscart, J. Lefebvre, and A. Demaille. 1987. Prognostic significance of prolactin receptors in human breast cancer. *Cancer Res.* 47:4724–4728.

5. Murphy, J.L., L.C. Murphy, E. Vrhovesek, R.L. Sutherland, and L. Lazarus. 1984. Correlation of lactogenic receptor concentration in human breast cancer with estrogen receptor concentration. *Cancer Res.* 44:1963–1968.

6. Biswas, R., and B.K. Vonderhaar. 1987. Role of serum in the prolactin responsivness of MCF-7 human breast cancer cells in long-term tissue culture. *Cancer Res.* 47:3509–3514.

7. Manni, A., C. Wright, G. Davis, J. Glenn, R. Joehl, and P. Feil. 1986. Promotion by prolactin of the growth of human breast neoplasms cultured in vitro in the soft agar clonogenic assay. *Cancer Res.* 46:1669–1672.

8. Shiu, R.P. 1979. Prolactin receptors in human breast cancer cells in long-term tissue culture. *Cancer Res.* 39:4381–4386.

9. Fuh, G., and J.A. Wells. 1995. Prolactin receptor antagonists that inhibit the growth of breast cancer cell lines. *J. Biol. Chem.* 270:13133–13137.

10. Matsuzawa, A. 1986. Hormone dependence and independence of mammary tumors in mice. *In* International Review of Cytology. G.H. Bourne and K.W. Jeon, editors. Academic Press, New York. 103:303–341.

11. Goffin, V., K.T. Shiverick, P.A. Kelly, and J.A. Martial. 1996. Sequence-function relationships within the expanding family of prolactin, growth hormone, placental lactogen, and related proteins in mammals. *Endocr. Rev.* 17: 385–410.

12. Bazan, J.P. 1989. A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erytropoietin and IL-6 receptors, and the p75 IL-2 receptor B chain. *Biochem. Biophys. Res. Commun.* 164:788–795.

13. Alexander, L., D. Appleton, R. Hall, W.M. Ross, and R. Wilkinsson. 1980. Epidemiology of acromegaly in the Newcastle region. *Clin. Endocrinol.* 12:71–79.

14. Bengtsson, B.Å., S. Edén, I. Ernest, A. Odén, and B. Sjögren. 1988. Epidemiology and long-term survival in acromegaly. *Acta. Med. Scand.* 223:327–335.

15. Nabarro, J.D. Acromegaly. 1987. *Clin. Endocrinol.* 26:327–335.

16. Ituarte, E.M., J. Pertini, and M. Hershman. 1984. Acromegaly and colon cancer. *Ann. Intern. Med.* 101:627–628.

17. Decouvelaere, C., J.P. Peyrat, J. Bonneterre, J. Dijane, and H. Jammes. 1995. Presence of the two growth hormone receptor messenger RNA isoforms in human breast cancer. *Cell Growth Differ.* 6:477–483.

18. Törnell, J., L. Rymo, and O.G.P. Isaksson. 1991. Induction of mammary adenocarcinomas in metallothionein promoter-human growth hormone transgenic mice. *Int. J. Cancer.* 49:114–117.

19. Bartke, A., M. Cecim, K. Tang, R.W. Steger, V. Chandrashekar, and D. Turyn. 1994. Neuroendocrine and reproductive consequences of overexpression of growth hormone in transgenic mice. *Proc. Soc. Exp. Biol. Med.* 206:345–359.

20. Törnell, J., B. Carlsson, P. Pohjanen, H. Wennbo, L. Rymo, and O.G.P. Isaksson. 1992. High frequency of mammary adenocarcinomas in metallothionein promoter-human growth hormone transgenic mice created from two different strains of mice. *J. Steroid. Biochem. Mol. Biol.* 43:237–242.

21. D'Ercole, A.J., A.D. Stiles, and L.E. Underwood. 1984. Tissue concentrations of somatomedin C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. *Proc. Natl. Acad. Sci. USA.* 81: 935–939.

22. Mathews, L.S., G. Norstedt, and R.D. Palmiter. 1986. Regulation of insulin-like growth factor I gene expression by growth hormone. *Proc. Natl. Acad. Sci. USA.* 83:9343–9347.

23. Yee, D., S. Paik, G.S. Lebovic, R.R. Marcus, R.E. Favoni, K. Cullen, M.E. Lippman, and N. Rosen. 1989. Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. *Mol. Endocrinol.* 3:509–517.

24. Cullen, K.J., D. Yee, W.S. Sly, J. Perdue, B. Hampton, M.E. Lippman, and N. Rosen. 1990. Insulin-like growth factor receptor expression and function in human breast cancer. *Cancer Res.* 50:48–53.

25. Huff, K.K., D. Kaufman, K.H. Gabbay, E.M. Spencer, M.E. Lippman, and R.B. Dickson. 1986. Secretion of an insulin-like growth factor-I-related protein by human breast cancer cells. *Cancer Res.* 46:4613–4619.

26. Karey, K.P., and D.A. Sirbasku. 1988. Differential responsivness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17b-estradiol. *Cancer Res.* 48:4083–4092.

27. Mathews, L.S., R.E. Hammer, R.R. Behringer, A.J. D'Ercole, G.I. Bell, R.L. Brinster, and R.D. Palmiter. 1988. Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology.* 123:2827–2833.

28. Neuenschwander, S., A. Schwartz, T.L. Wood, C.T.J. Roberts, L. Hennighausen, and D. LeRoith. 1996. Involution of the lactating mammary gland is inhibited by the IGF system in a transgenic mouse model. *J. Clin. Invest.* 97: 2225–2232.

29. Hadsell, D.L., N.M. Greenberg, J.M. Fligger, C.R. Baumrucker, and J.M. Rosen. 1996. Targeted expression of des(1-3) human insulin-like growth factor I in transgenic mice influences mammary gland development and IGF-binding protein expression. *Endocrinology.* 137:321–330.

30. Bates, P., R. Fisher, A. Ward, L. Richardson, D.J. Hill, and C.F. Graham. 1995. Mammary cancer in transgenic mice expressing insulin-like growth factor II (IGF-II). *Br. J. Cancer.* 72:1189–1193.

31. Mathews, L.S., R.E. Hammer, R.L. Brinster, and R.D. Palmiter. 1988. Expression of insulin-like growth factor 1 in transgenic mice with elevated levels of growth hormone is correlated with growth. *Endocrinology.* 123:433–437.

32. Cooke, N.E., and J.D. Baxter. 1982. Structural analysis of the prolactin gene suggests a separate origin for its 5- end. *Nature.* 297:603–606.

33. Palmiter, R.D., R.L. Brinster, R.E. Hammer, M.E. Trumbauer, M.G. Rosenfeld, N.C. Brinberg, and M.E. Trumbauer. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature.* 300:611–615.

34. Hogan, B., F. Costantini, and E. Lacy. 1986. Manipulating the Mouse Embryo. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

35. Öferstedt, L.G., K. Hammarström, N. Balgobin, S. Hjertén, U. Pettersson, and J. Chattopadhyaya. 1984. Rapid and quantitative recovery of DNA fragments from gels by displacement electrophoresis (isotachophoresis). *Biochim. Biophys. Acta.* 782:120–126.

36. Hammer, R.E., R.L. Brinster, and R.D. Palmiter. 1985. Use of gene transfer to increase animal growth. *Cold Spring Harbor Symp. Quant. Biol.* 50: 379–387.

37. Chomcynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid Quanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

38. Cooke, N.E., D. Coit, R.I. Weiner, J.D. Baxter, and J.A. Martial. 1980. Structure of cloned DNA complementary to rat prolactin messenger RNA. *J. Biol. Chem.* 255:6502–6510.

39. Sandstedt, J., C. Ohlsson, E. Norjavaara, J. Nilsson, and J. Törnell. 1994. Disproportional bone growth and reduced weight gain in gonadectomize male bovine growth hormone transgenic and normal mice. *Endocrinology.* 135:2574–2580.

40. Sinha, Y.N., C.B. Salocks, M.A. Wickes, and W.P. Vanderlaan. 1977. Serum and pituitary concentrations of prolactin and growth hormone in mice during a twenty-four hour period. *Endocrinology.* 100:786–791.

41. Huseby, R.A., M.J. Soares, and F. Talamantes. 1985. Ectopic pituitary grafts in mice: hormone levels, effects on fertility, and the development of adenomyosis uteri, prolactinomas, and mammary carcinomas. *Endocrinology.* 116: 1440–1448.

42. Adler, R.A. 1986. The anterior pituitary-grafted rat: a valid model of chronic hyperprolactinemia. *Endocr. Rev.* 7:302–313.

43. Ginsburg, E., and B.K. Vonderhaar. 1995. Prolactin synthesis and secretion by human breast cancer cells. *Cancer Res.* 55:2591–2595.