# Human prolactin receptors are insensitive to mouse prolactin: implications for xenotransplant modeling of human breast cancer in mice

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## Abstract

Experimental testing of growth, metastatic progression and drug responsiveness of human breast cancer *in vivo* is performed in immunodeficient mice. Drug candidates need to show promise against human breast cancer in mice before being allowed into clinical trials. Breast cancer growth is under endocrine control by ovarian steroids and the pituitary peptide hormone prolactin. While it is recognized that the most relevant biologic effects of prolactin are achieved with prolactin from the matching species, the biologic efficacy of mouse prolactin for human prolactin receptors has not been recorded. Thus, it is unclear whether the mouse endocrine environment adequately reflects the hormonal environment in breast cancer patients with regard to prolactin. We now show both recombinant and natural pituitary-derived mouse prolactin to be a poor agonist for human prolactin receptors. Mouse prolactin failed to induce human prolactin receptor-mediated biologic responses of cell clustering, proliferation, gene induction and signal transduction, including activation of Stat5, Stat3, Erk1/2 and Akt pathways. Consistent data were derived from human breast cancer lines T-47D, MCF-7 and ZR-75·1, as well as human prolactin receptor-transfected COS-7 and 32D cells. Failure of mouse prolactin to activate human prolactin receptors uncovers a key deficiency of the mouse endocrine environment for human xenotransplant studies. Since most human breast cancers express prolactin receptors, human breast cancer transferred into mice is unnaturally selected for growth in the absence of circulating prolactin. The new insight raises concerns about the validity of analyzing biology and drug responsiveness of human breast cancer in existing mouse xenotransplant models.

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## Introduction

Growth and differentiation of breast cancer is regulated by hormones, notably estrogen, progesterone and prolactin. In rodents, prolactin is a well-documented tumor promoter of the mammary gland, as revealed by a variety of experimental approaches, including increased tumorigenesis in prolactin-overexpressing transgenic mice (Wennbo *et al.* 1997*b*, Rose-Hellekant *et al.* 2003) and reduced tumorigenesis in mice lacking the prolactin gene (Vomachka *et al.* 2000). In man, several lines of evidence support a breast cancer-promoting role of prolactin. First, 70–95% of human breast cancer specimens express prolactin receptors (Reynolds *et al.* 1997, Gill *et al.* 2001). Second, under certain culture conditions, human breast cancer cell lines respond to prolactin by proliferation (Malarkey *et al.* 1983, Biswas & Vonderhaar 1987, Vonderhaar 1989), survival (Perks *et al.* 2004) or as a chemoattractant (Maus *et al.* 1999). Importantly, prolactin is present in serum of both pre- and postmenopausal women, and elevated levels correlate with increased risk of breast cancer in postmenopausal women (Hankinson *et al.* 1999, Tworoger *et al.* 2004). Accumulating data therefore suggest that prolactin exerts a breast cancer-promoting effect in women, and a series of recent authoritative reviews supports this notion (Goffin *et al.* 1999, Vonderhaar 1999, Llovera *et al.* 2000, Wennbo & Tornell 2000, Clevenger *et al.* 2003).

Before new drugs against breast cancer are allowed into clinical trials, promising effects on human breast cancer xenografted into immunodeficient mice are required. The hormonal environment of the mouse host is generally


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considered to be appropriate for breast cancer growth under near-physiologic conditions as they occur in man. Because we have demonstrated Stat5 transcription factor activation to be a sensitive marker of prolactin receptor signaling in normal mouse mammary epithelial cells even outside pregnancy (Nevalainen *et al.* 2002*a*), it was unexpected that we did not detect activated Stat5 in human T-47D breast tumors xenotransplanted into nude mice (Sultan *et al.* 2005). Stat5 was inactive in the tumors despite the presence of abundant prolactin receptors on T-47D cells, but became rapidly activated in response to injected human prolactin (Sultan *et al.* 2005). Coupled with the fact that mouse prolactin shares only 59% amino-acid identity with human prolactin, this observation suggested that mouse prolactin is a poor agonist for human prolactin receptors.

Experimental endocrinologists have recognized that there are species differences in prolactin ligand–receptor interactions (Posner *et al.* 1974, Nicoll *et al.* 1986, Ogren & Talamantes 1988, Brelje *et al.* 1993, Sinha 1995). For instance, while ovine prolactin is an effective ligand and activator of human prolactin receptors (Murphy *et al.* 1984, Biswas & Vonderhaar 1987, Vonderhaar 1989, Favy *et al.* 1999), bovine prolactin, which has 93% sequence identity with ovine prolactin, competed for binding but did not activate human prolactin receptors (Biswas & Vonderhaar 1987). Furthermore, Shiu and colleagues suggested, on the basis of previous binding studies, that rat prolactin from GH3 pituitary tumors affects human breast tumor xenotransplants in nude mice (Leung & Shiu 1981, Shiu 1981). However, to the best of our knowledge, there are no reports in the literature documenting the extent to which mouse prolactin can activate human prolactin receptors. Given the extensive use of mice as a recipient for human breast cancer xenotransplants in studies of tumor biology and pharmacology, and that most drugs that work on human breast cancer in mice subsequently fail in the clinic (Gura 1997), we considered it critical to determine the efficacy of mouse prolactin on human prolactin receptors.

We now demonstrate that mouse prolactin is incapable of mimicking human prolactin-induced receptor activation and biologic effects. In particular, mouse prolactin did not mimic human prolactin-induced clustering of human breast cancer cells, and failed to induce marked or sustained activation of Stat5 transcription factors, which have been identified as critical mediators of this biologic effect (Sultan *et al.* 2005). Likewise, mouse prolactin failed to mimic other human prolactin-induced signaling pathways, including Stat3, Erk1/2 or Akt, and inducible cell proliferation and gene induction. The mouse hormonal environment therefore does not reflect the relevant hormonal environment in breast cancer patients, and human breast cancer grown in mice will be selected for growth independent of circulating prolactin. Consequently, biologic behavior and drug-response profiles of prolactin receptor-positive breast tumors will be affected when transferred from patients into mice. We discuss alternative strategies to overcome this central hormonal deficiency for future experimental testing of human breast cancer in mice.

## Materials and Methods

### *Hormones, antibodies and plasmids*

Recombinant human prolactin (AFP795), recombinant mouse prolactin (AFP306C) and natural pituitary-derived mouse prolactin (AFP10777D) were provided by Dr A F Parlow under the sponsorship of the National Hormone and Pituitary Program. Human epidermal growth factor (EGF) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Polyclonal rabbit anti-actin antisera and insulin were purchased from Sigma Chemical Co. Monoclonal mouse anti-phosphotyrosine-Stat5 antibody (AX1), polyclonal rabbit antisera to Stat5a (AX551), Stat5b (AX554) and Stat3 (AX53) were obtained from Advantex BioReagents (Conroe, TX, USA). Polyclonal rabbit antisera to phosphotyrosine-Stat3, phosphothreonine-Akt and Akt, and phosphothreonine/tyrosine-ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA, USA). Monoclonal mouse anti-ERK1/2 (pan-ERK) antibody was obtained from BD Biosciences (San Diego, CA, USA). Plasmids containing genomic β-casein-luciferase reporter gene (pZZ1; gift from Bernd Groner), pXM-Stat5a (gift from Lothar Hennighausen and Xiuwen Liu) and p3 hPRLR (cDNA gift from Paul A Kelly subcloned into pcDNA3 vector) have been previously described (Yamashita *et al.* 2001). pRL-TK was used as internal control (Promega).

### *Cell culture and treatment*

The human breast cancer cell lines MCF-7, T-47D and ZR-75·1 (ATCC, Manassas, VA, USA) were grown in RPMI 1640 medium (Biofluids, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 2 mM L-glutamine (Biofluids), and penicillin-streptomycin (50 IU/ml and 50 μg/ml respectively; Biofluids). For cell-clustering studies, $5 \times 10^5$ T-47D cells were plated on Matrigel (BD Bioscience, Bedford, MA, USA; catalogue no. 356234, lot no. 006114)-coated, six-well plates, and serum-starved the following day in RPMI 1640 medium for 16 h prior to treatment. Cells were treated with human prolactin (10 nM), or mouse prolactin (10 nM) or vehicle control for 24 h and were then fixed with 4% paraformaldehyde for 20 min. Cell morphology was captured with phase-contrast stereomicroscopy. For time-course studies of prolactin-induced Stat5 activation, confluent MCF-7, T-47D and ZR-75·1 cells grown on plastic were

serum-starved in RPMI 1640 for 16 h prior to treatment. Cells were treated without or with human or mouse prolactin as indicated. For dose–response studies of prolactin-induced Stat5 activation, confluent T-47D cells grown on plastic were serum-starved in RPMI 1640 medium for 16 h. Cells were then treated without or with human or mouse prolactin for 15 min. For *CISH* mRNA analyses, confluent T-47D cells grown on plastic were serum-starved for 24 h, and then treated with human prolactin (10 nM), or mouse prolactin (10 nM) or vehicle control for 5 h. The cells were harvested, and RNA was isolated with TRIzol extraction buffer (Invitrogen). Total RNA was further purified with the RNeasy kit (Qiagen).

The mouse lymphoblast cell line 32D stably transfected with human prolactin receptors (32D-hPRLR) was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 nM human prolactin and penicillin-streptomycin (50 IU/ml and 50 μg/ml respectively). For proliferation assay, cells were lactogen-starved for 16 h in lactogen-free medium containing 10% gelded horse serum (Sigma) with no fetal bovine serum or WEHI supernatant prior to treatment with prolactin (DaSilva *et al.* 1994). The mouse mammary epithelial cell line HC11 (Ball *et al.* 1988) was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, insulin (5 μg/ml), EGF (10 ng/ml) and penicillin-streptomycin (50 IU/ml and 50 μg/ml respectively), as previously described (Xie *et al.* 2002). For prolactin-induced Stat5 activation studies, ~80% confluent HC11 cells were serum-starved for 16 h and then treated with human prolactin (10 nM), or mouse prolactin (10 nM) or vehicle control for 15 min. For mammary epithelial cell differentiation study (Xie *et al.* 2002), confluent HC11 cells were serum-starved in medium containing 2% fetal bovine serum and lacking EGF for 48 h. Cells were then incubated in RPMI 1640 medium containing 10% fetal bovine serum, 0·1 μM dexamethasone, insulin (5 μg/ml) and either no prolactin or 10 nM human or mouse prolactin for 7 days. COS-7 cells (ATCC) were grown in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine and penicillin-streptomycin (50 IU/ml and 50 μg/ml respectively).

### *Stable transfection*

32D cells ($2 \times 10^7$) were transfected with 10 μg PvuI-linearized p3 hPRLR by electroporation at 0·3 kV, 950 μF, with Bio-Rad Gene Pulser II. After electroporation, transfected cells were cultured in medium containing WEHI-3B supernatant for 48 h and subsequently were selected in medium containing 1 mg/ml G418 (Cambrex Bio Science, Walkersville, MD, USA). G418-resistant cells were allowed to expand for 3 days. Cells were then cultured and expanded in medium containing 0·4 mg/ml G418 and 1 nM human prolactin. G418-resistant, human prolactin-sensitive cells (32D-hPRLR) were used for further experiments.

### *Proliferation assay*

Lactogen-deprived 32D-hPRLR cells were seeded at $3 \times 10^4$ cells/well of 96-well plates, treated either without or with 10 nM human or mouse prolactin in lactogen-free medium, and incubated for 48 h at 37 °C. MTS assay (Promega) was performed and absorbance was recorded at $OD_{490}$ with Microplate Reader 680, as instructed by the manufacturer (Bio-Rad). Absorbance recordings were normalized to control cells and graphed as relative cell density (fold-increase).

### *Luciferase assay*

Luciferase reporter studies were carried out in COS-7 cells. The cells were plated at $2{\cdot}5 \times 10^5$ cells per well in six-well plates. Transfection was performed 24 h after plating with FuGENE 6, as suggested by the manufacturer (Roche), with a combination of 0·5 μg β-casein-luciferase reporter gene, 0·25 μg pXM-Stat5a, 0·25 μg p3 hPRLR and 25 ng pRL-TK per well. Cells were switched to serum-free media containing human prolactin (10 nM), or mouse prolactin (10 nM), or vehicle control 24 h after transfection and further incubated at 37 °C for 16 h. Cells were harvested, and luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega), normalized with pRL-TK and graphed as relative luciferase units.

### *Protein solubilization, immunoblotting and immunoprecipitation*

For protein solubilization, cells were harvested in 1 ml lysis buffer (10 mM Tris–HCl (pH 7·5), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A and 2 μg/ml leupeptin). Cell lysates were rotated end-over-end at 4 °C for 1 h, and insoluble material was pelleted at 13 000 ***g*** at 4 °C for 30 min. For immunoprecipitation, 500 μl clarified lysates were incubated with appropriate antisera at 4 °C, rotating for 3 h. Antibody–protein complexes were captured by incubation with Protein A-Sepharose beads (Amersham) at 4 °C, rotating for 30 min, and washed three times in 1 ml lysis buffer. Immunoprecipitated proteins were dissolved in 2X loading buffer containing reducing agent, resolved by SDS–PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The immunoblotting was performed, as previously described (Kirken *et al.* 1997), with antiphosphotyrosine Stat5, anti-Stat5a/b antisera and antimouse and antirabbit

horseradish-peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Pierce, Rockford, IL, USA) and exposed to Biomax film (Kodak, Rochester, NY, USA).

### *Relative RT–PCR for* CISH *mRNA*

An amount of 2 μg total RNA were converted into first-strand cDNA by the SuperScript First-Strand Synthesis System, as described by the manufacturer (Invitrogen). Amplification of the *CISH* and *GAPDH* cDNAs was performed by PCR using a primer pair: 5′-TCCT CTGCGTTCAGGGACCT-3′ and 5′-ACACTAGGCG CATCCTCCTT-3′, and 5′-TCAAGGTCGGAGTCAA CGATTTGGT-3′ and 5′-CATGTGGGCCATGAGG TCCACCAC-3′ respectively. Relative values were determined by Quantity One software (Bio-Rad).

### *Analysis of phosphorylated Stat5 in T-47D xenograft tumors* in vivo

Ovariectomized, female, athymic nude mice ($n=6$, 5 weeks of age) with s.c. implants of slow-release 17β-estradiol pellets (0·72 mg/pellet, 60-day release, Innovative Research of America, Sarasota, FL, USA), were inoculated s.c. with $5 \times 10^6$ human T-47D cells suspended in 200 μl 50% Matrigel and 50% RPMI-1640 into each of two dorsolateral sites. After 28 days, paired tumors of diameter of approximately 5 mm were injected with 100 μl human prolactin ($10^{-5}$ M), or mouse prolactin ($10^{-5}$ M) or vehicle control. Mice were euthanized 1 h after injection, and two tumors from each mouse were harvested and fixed in 10% buffered formalin for paraffin-embedding and analysis of Stat5 activation. Nuclear localized, tyrosine-phosphorylated Stat5 was detected immunohistochemically with monoclonal antibody AX1, as described previously (Nevalainen *et al.* 2002*b*).

### *Statistical analysis*

Multiple-treatment groups were compared by one-way ANOVA and Scheffe's post hoc test with SPSS software (Chicago, IL, USA).

## Results

### *Mouse prolactin does not mimic human prolactin-induced clustering of T-47D breast cancer cells*

We recently demonstrated that human prolactin induces marked homotypic clustering of T-47D cells on extracellular matrix (Sultan *et al.* 2005). We therefore first used this experimental model to determine whether mouse prolactin can mimic this biologic response to human prolactin. T-47D cells cultured on Matrigel were serum-starved for 16 h before exposure to human prolactin, or mouse prolactin or vehicle control for 24 h. Cells were then fixed with paraformaldehyde and observed by phase-contrast stereomicroscopy. While human prolactin induced T-47D cell-clustering, mouse prolactin failed to induce this biologic response (Fig. 1A). To exclude the possibility that the inability of mouse prolactin to induce clustering is due to properties of the recombinant mouse prolactin preparation, we verified in parallel experiments that natural pituitary-derived mouse prolactin can also induce breast cancer cell clustering (data not shown). These observations were the first direct indication that mouse prolactin is a poor agonist for human prolactin receptors.

### *Mouse prolactin does not mimic human prolactin-induced cell proliferation in 32D cells*

We extended the biologic testing of mouse prolactin to an assay of human prolactin receptor-mediated cell proliferation. Because the T-47D line does not proliferate in response to human prolactin *in vitro* (Vonderhaar & Biswas 1987, Perks *et al.* 2004), we stably introduced the human prolactin receptors into the 32D murine pro-B cell line, so that we could test the ability of mouse prolactin to mimic an independent and robust human prolactin-induced biologic response. This assay also revealed mouse prolactin to be an ineffective agonist for human prolactin receptors. While human prolactin induced near-maximal cell proliferation responses at a concentration of 1 nM, mouse prolactin had no effect at 1 nM and induced only a weak response at 10 nM (Fig. 1B). Parental 32D cells showed no response to either human or mouse prolactin (data not shown). The failure of mouse prolactin to induce distinct responses of cell clustering and proliferation via human prolactin receptors documents that mouse prolactin is a biologically ineffective agonist for human prolactin receptors.

### *The mouse prolactin preparation is bioactive, and human prolactin mimics mouse prolactin-induced activation of mouse prolactin receptors*

To verify that the observed poor efficacy of mouse prolactin to activate Stat5 in T-47D cells was not caused by nonfunctional or inappropriately prepared mouse prolactin, we tested the ability of mouse prolactin to activate endogenous prolactin receptors in the mouse HC11 mammary cell line. HC11 cells were serum-deprived for 16 h before exposure to human prolactin, or mouse prolactin or vehicle control for 15 min. Stat5a was immunoprecipitated from the cell lysates and resolved by SDS–PAGE, and overall phosphorylation status of Stat5a was compared by immunoblotting with the anti-phosphotyrosine-Stat5 antibody. Mouse prolactin effectively activated Stat5a through the mouse prolactin receptors (Fig. 2A). Human prolactin also effectively

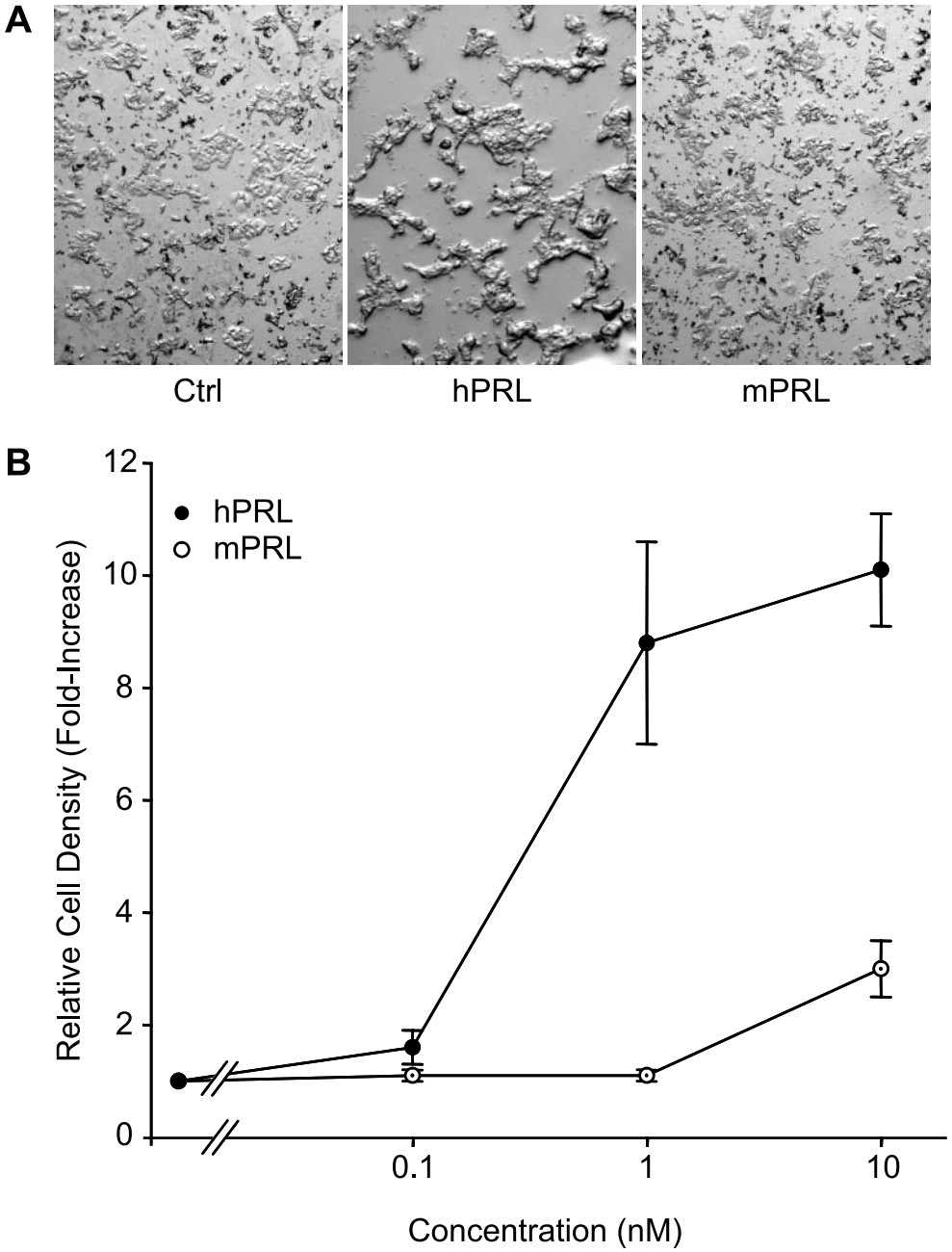


**Figure 1** Mouse prolactin (mPRL) fails to mimic human prolactin (hPRL)-induced biologic effects. (A) Analysis of homotypic clustering of human T-47D breast cancer cells. T-47D cells were seeded at $5 \times 10^5$ cells/well in six-well plates containing cover slips pretreated with Matrigel. Cells were serum-starved for 16 h and treated without PRL (Ctrl), with hPRL (10 nM) or with mPRL (10 nM) for 24 h. Cells were fixed and visualized by phase-contrast stereomicroscopy. A representative of three independent experiments is shown. (B) Analysis of cell proliferation of hPRL receptor-expressing 32D cells. 32D cells stably transfected with hPRL receptors were incubated with increasing concentrations of human (●) or mouse prolactin (○) for 48 h. Cell numbers were compared by the MTS assay of metabolic activity, and mean cell densities normalized to untreated controls from three independent experiments are presented (error bars indicate S.D).

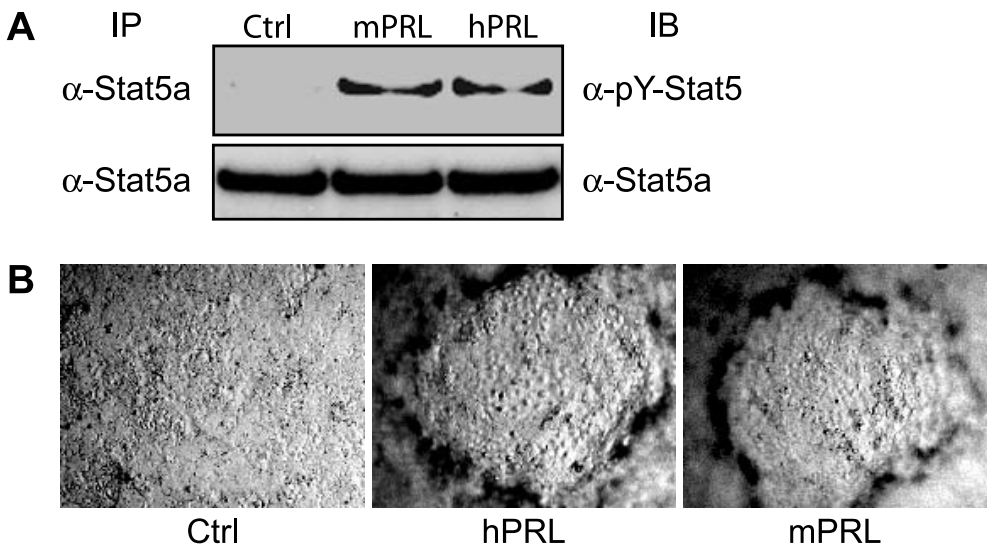


**Figure 2** Mouse prolactin (mPRL) preparation is bioactive, and human prolactin (hPRL) mimics mPRL-induced signaling and biologic effect via mPRL receptors. (A) Serum-starved HC11 mouse mammary cells were incubated without PRL (Ctrl), with hPRL (10 nM) or with mPRL (10 nM) at 37 °C for 15 min. Clarified cell lysates were immunoprecipitated with antibodies to Stat5a, and then blotted with anti-phosphotyrosine-Stat5 or anti-Stat5a antibodies. Blots represent duplicate experiments. (B) HC11 cells were induced to differentiate and monitored for formation of three-dimensional mammospheres in the presence of 10 nM hPRL or mPRL. Representative mammospheres based on three independent experiments are shown, and hPRL was as effective as mPRL in inducing mPRL receptor-mediated cell differentiation.

activated mouse prolactin receptors, as judged by Stat5a activation, a finding consistent with the original reported activity of human prolactin in the mouse mammary gland bioassay (Hwang *et al.* 1972). The levels of overall Stat5a protein remained constant in the different treatments (Fig. 3A). We also tested whether human prolactin can mimic the more complex biologic activity of mouse prolactin-induced differentiation of mammary epithelial cells. Using the HC11 cell differentiation assay (Xie *et al.* 2002), we determined that human prolactin is as effective as mouse prolactin in inducing three-dimensional mammospheres (Fig. 2B). Collectively, these experiments verified that the mouse prolactin preparation was functional, and also verified that human prolactin is an effective agonist for mouse prolactin receptors.

### *Mouse prolactin does not mimic human prolactin-induced activation of Stat5a and Stat5b*

Since our previous work had identified Stat5 as a critical mediator of human prolactin-induced clustering of T-47D cells (Sultan *et al.* 2005), we first examined the efficacy of mouse prolactin to activate Stat5a and Stat5b in human T-47D cells. T-47D cells were grown to confluence and were serum-starved for 16 h before exposure to human prolactin, or mouse prolactin or vehicle control for various times up to 8 h. Stat5a and Stat5b proteins were individually immunoprecipitated from cell lysates and resolved by SDS–PAGE, and the phosphorylation status of the conserved tyrosine residue was compared by immunoblotting with a Stat5-specific antiphosphotyrosine antibody. Human prolactin induced marked and sustained activation of both Stat5a and Stat5b within 7·5 min, reaching maximal tyrosine phosphorylation within 15–30 min and lasting for the duration of the 8 h period of investigation. In contrast, mouse prolactin-induced activation of Stat5a and Stat5b was weak and very transient (Fig. 3A). Levels of total Stat5a and Stat5b proteins remained constant over the observation period, as shown by parallel immunoblotting with anti-Stat5a and anti-Stat5b antibodies (Fig. 3A). Importantly, densitometric analyses showed that over the 8 h time course, the area under the curve of the signals induced by mouse prolactin was consistently less than 5% of the integrated signal induced by human prolactin (data not shown).

We further tested the ability of human and mouse prolactin to activate prolactin receptors over a range of concentrations, using Stat5a activation after 15 min as the readout. Immunoprecipitated Stat5a proteins were separated on SDS–PAGE and immunoblotted with antiphosphotyrosine or anti-Stat5a antibodies. At the 15 min time point of maximal response to mouse prolactin, human prolactin activated Stat5a in T-47D cells at a concentration as low as 0·1 nM, whereas approximately 10-fold higher concentration of mouse prolactin was needed to activate Stat5a (Fig. 3B). Levels of Stat5a remained constant within the various treatment groups (Fig. 3B). Furthermore, the inability of mouse prolactin to activate Stat5a was verified

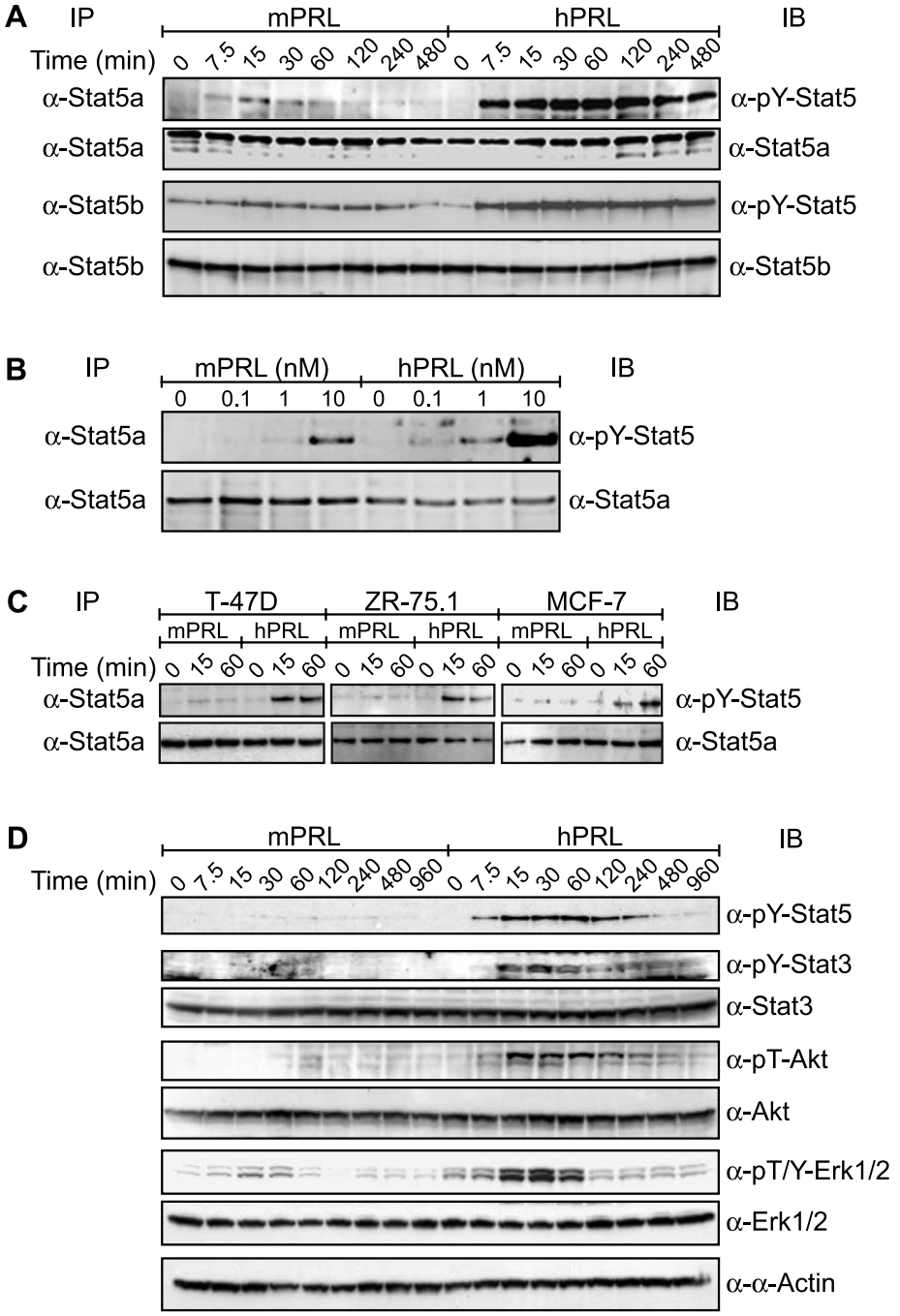


**Figure 3** Mouse prolactin (mPRL) fails to mimic human prolactin (hPRL)-induced sustained activation of Stat5 and other pathways. (A) Serum-starved human T-47D cells were incubated with either hPRL (10 nM) or mPRL (10 nM) at 37 °C, and cells were harvested at indicated time points. Clarified cell lysates were immunoprecipitated with antibodies to Stat5a or Stat5b, and then blotted with anti-phosphotyrosine-Stat5, anti-Stat5a or Stat5b antibodies. (B) Serum-starved human T-47D cells were incubated with indicated concentrations of hPRL or mPRL at 37 °C for 15 min. Clarified cell lysates were immunoprecipitated with antibodies to Stat5a, and then blotted with antiphosphotyrosine Stat5 or anti-Stat5a antibodies. (C) Stat5a activation was tested by antiphosphotyrosine immunoblotting in three distinct human breast cancer cell lines, T-47D, MCF-7 and ZR-75·1, and hPRL, but not mPRL (10 nM), effectively activated receptor-mediated Stat5a in each of the three lines. (D) Activation of Stat5 and the additional PRL receptor-induced signaling pathways, Stat3, Erk1/2 and Akt, was analyzed in response to hPRL or mPRL (10 nM) over an extended time course in T-47D cells, using phospho-specific antibodies on whole-cell lysates. A representative of three independent experiments is shown.

in three out of three human breast cancer cell lines tested, including T-47D, MCF-7 and ZR-75·1 (Fig. 3C). Finally, to exclude a selective inability of mouse prolactin to activate Stat5 signals, we extended the analysis to include a series of additional prolactin receptor-mediated signals, including activation of Stat3, Erk1/2 and Akt (Fig. 3D). These additional signals were also reduced correspondingly in response to mouse prolactin, consistent with a generalized poor agonism of mouse prolactin to human prolactin receptors. Collectively, these experiments identified a major difference in the ability of human and mouse prolactin to induce activation of Stat5 and other pathways through the human prolactin receptors. In addition to weak signals, a key difference between human and mouse prolactin was that mouse prolactin induced only transient signals and failed to induce sustained signaling.

### *Mouse prolactin does not mimic human prolactin-induced upregulation of* CISH *mRNA expression or β-casein luciferase reporter gene*

One of the established target genes of Stat5 is *CISH*, which is upregulated and acts as a negative regulator of Stat5 (Mitchell *et al.* 2003). To determine whether weak and transient Stat5 activation by mouse prolactin would be sufficient to mimic human prolactin-induced *CISH* expression, we analyzed *CISH* mRNA levels in response to human prolactin and mouse prolactin. T-47D cells were grown to confluence and serum-deprived for 24 h prior to incubation with human prolactin, or mouse prolactin or vehicle control for 5 h. Cells were harvested, and total RNA was isolated and converted into first-strand cDNA. Subsequently, we performed PCR with primers specific for *CISH* and *GAPDH* cDNA. The PCR products were resolved by agarose gel, and expression levels of *CISH* were normalized with *GAPDH* expression levels. Human prolactin induced a twofold increase in *CISH* expression, whereas mouse prolactin induced no detectable increase over control levels (Fig. 4A).

Furthermore, we tested the ability of mouse prolactin to induce Stat5-mediated transactivation of a highly sensitive β-casein-luciferase reporter gene in COS-7 cells. β-casein is another well-studied Stat5-dependent gene (Happ & Groner 1993). COS-7 cells were transfected with plasmids encoding human prolactin receptors, Stat5a, β-casein-luciferase reporter gene and pRL-TK as an internal control. At 24 h after transfection, cells were incubated in serum-free media containing human prolactin, or mouse prolactin or vehicle control for 16 h. Luciferase activity was measured and normalized with pRL-TK. Human prolactin-treated cells consistently showed 20-fold or higher inducible luciferase activity (Fig. 4B). In contrast, mouse prolactin showed only a modest luciferase increase that did not reach statistical significance in four independent experiments carried out in duplicate. The absence of Stat5-induced gene induction in COS-7 cells by mouse prolactin was consistent with the markedly reduced ability of mouse prolactin to mimic human prolactin receptor-mediated activation of Stat5 (data not shown).

### *Mouse prolactin does not mimic human prolactin-induced activation of Stat5 in human breast cancer xenografts in nude mice*

To determine further whether mouse prolactin remains a poor agonist for human prolactin receptors when tested *in vivo*, we compared the ability of human prolactin and mouse prolactin to activate Stat5 in T-47D tumor cells xenografted into nude mice. T-47D cells were inoculated s.c. into two dorsolateral sites in nude mice, and after 28 days, tumors were injected with human prolactin, or mouse prolactin or vehicle control. At 1 h after injection, tumors were harvested, fixed in formalin and immunohistochemically stained with anti-phosphotyrosine-Stat5 antibody. Stat5 was not active in vehicle-injected tumors, and was markedly activated in response to human prolactin, but remained largely inactive in response to mouse prolactin (Fig. 5). Therefore, consistent with the *in vitro* results, *in vivo* studies also supported the notion that mouse prolactin is a poor agonist of human prolactin receptors.

## Discussion

The present study demonstrated that mouse prolactin is a poor agonist for human prolactin receptors. Consequently, mice fail to reproduce a key hormonal component of the human endocrine environment, a finding that has direct implications for *in vivo* xenotransplant studies of human breast cancer in mice. Importantly, the hormonal deficiency will affect biologic behavior and drug responsiveness of prolactin receptor-expressing breast cancer when tumors are transferred from patients into mice. For instance, several laboratories are pursuing the use of human prolactin receptor antagonists in breast cancer treatment (Chen *et al.* 2002, Bernichtein *et al.* 2003). Such antagonists will have distinctly reduced effect in the absence of circulating agonist, even if some tumors produce human prolactin as an autocrine factor (Reynolds *et al.* 1997, Ben-Jonathan *et al.* 2002, Naylor *et al.* 2003). Likewise, drugs targeting prolactin receptor-modulated signaling pathways are expected to have diminished efficacy when tested on human breast cancer in mice. Prolactin-modulated pathways in breast cancer include Jak2, Stat5, Stat3, Stat1, Erk1/2, Akt, Src and other signals (reviewed in Clevenger *et al.* 2003), as well as positive cross-talk with the ErbB2 oncogene (Yamauchi *et al.* 2000).

Interestingly, production of prolactin has been described in T-47D cells when cultured *in vitro* (Ginsburg & Vonderhaar 1995). However, when grown in nude mice, T-47D cells did not synthesize sufficient prolactin to

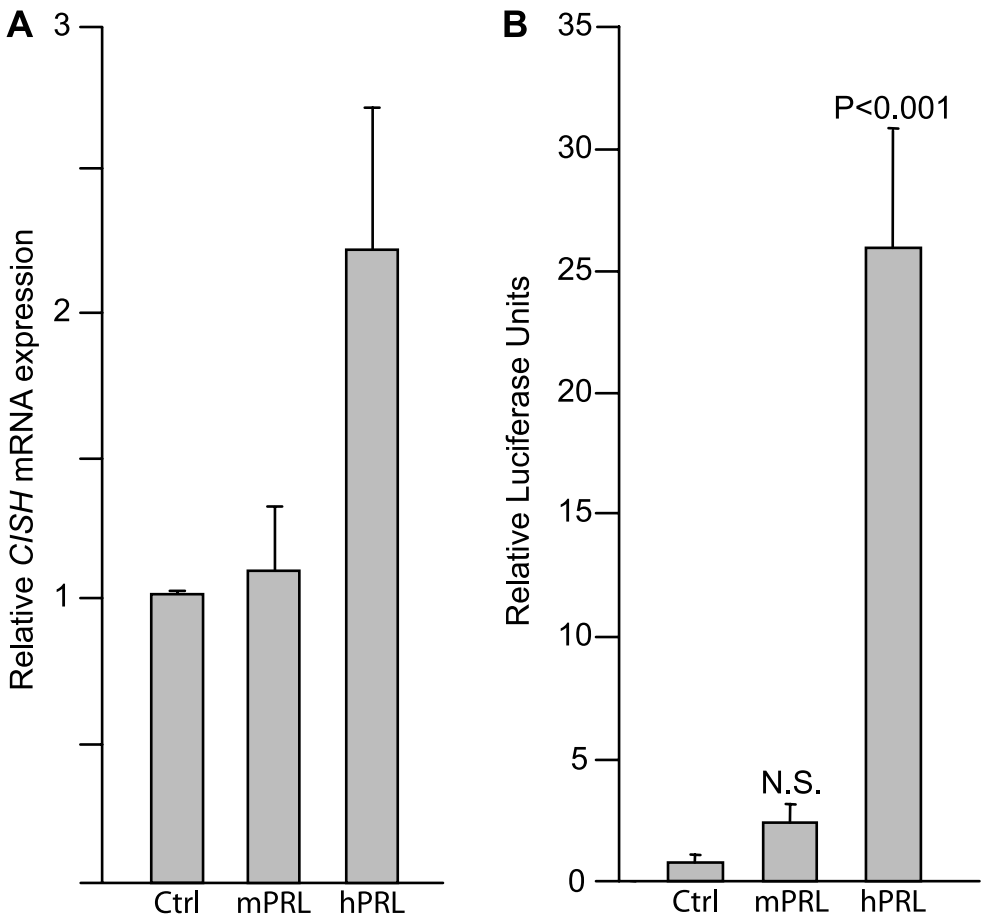


**Figure 4** Mouse prolactin (mPRL) fails to mimic human prolactin (hPRL)-induced *CISH* mRNA expression in T-47D and β-casein luciferase reporter gene expression in COS-7 cells. (A) Confluent T-47D cells were serum-starved for 24 h and were treated without PRL (Ctrl), with hPRL (10 nM) or with mPRL (10 nM) for 5 h. Cells were lysed in TRIzol buffer, and total RNA was quantified. First-strand cDNA was generated from the total RNA and was subjected to PCR using specific primers for *CISH* and *GAPDH* cDNA. Relative *CISH* mRNA levels were determined by normalization to *GAPDH* mRNA levels. (B) COS-7 cells were transiently transfected with mammalian expression vectors encoding for hPRL receptors, Stat5a, β-casein luciferase reporter gene and pRL-TK for internal control. At 24 h after transfection, cells were serum-starved and treated without PRL (Ctrl), with hPRL (10 nM) or with mPRL (10 nM) for 16 h. Levels of statistical significance are indicated for differences between treatments and control, based on four independent experiments. Luciferase activity was measured, and relative luciferase units were normalized by pRL-TK levels. Error bars represent S.D.

activate Stat5, which is considered a sensitive marker of prolactin receptor activation. Consistent with this notion of insufficient tumor production of human prolactin, Stat5 became rapidly activated once exogenous human prolactin was administered. At least in T-47D cells, prolactin signaling pathways appear to be effectively shut off in the absence of exogenous human prolactin when the cells are grown as xenotransplant tumors in mice. Furthermore, mouse prolactin failed to activate signaling effectively in a panel of human breast cancer cells *in vitro*.

The inability of both recombinant and natural mouse prolactin to mimic human prolactin-induced clustering of human T-47D cells is probably due to the failure of mouse prolactin to induce strong and sustained activation of Stat5, since Stat5 was demonstrated to be critical for this biologic response to human prolactin (Sultan *et al.* 2005). However, all of the prolactin receptor-mediated signals tested were diminished to a comparable extent, suggesting a general inability of mouse prolactin to activate human prolactin receptors. Previous work has documented that rat prolactin, which has 84% amino-acid sequence identity to mouse prolactin, can bind to human prolactin receptors on T-47D cells, although at a lower affinity comparable with that of human placental lactogen (Murphy *et al.* 1984). Of 23 amino-acid residues in human prolactin that have been previously identified to interact

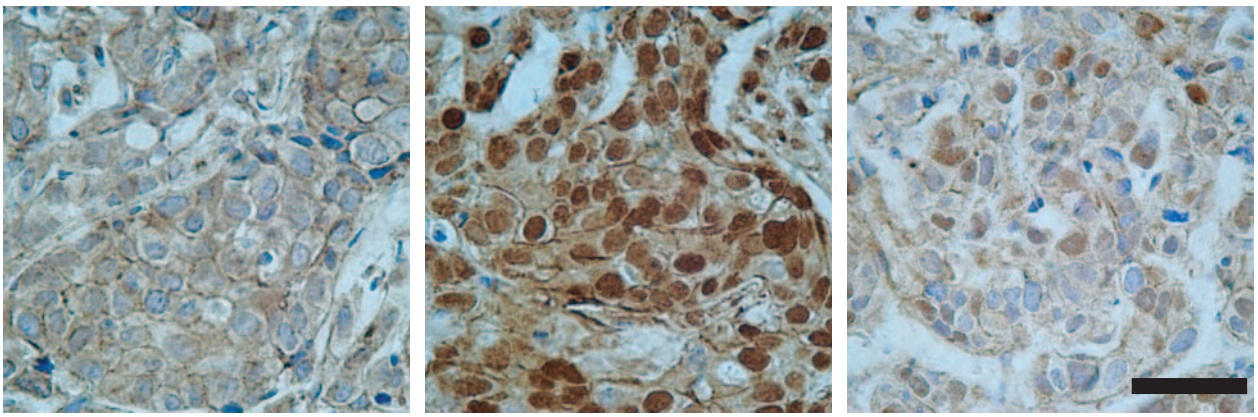


**Figure 5** Mouse prolactin (mPRL) fails to mimic human prolactin (hPRL)-induced activation of Stat5 *in vivo*. Human T-47D tumors were xenografted into nude mice and were grown for 28 days. Tumors were then injected with 100 µl vehicle (Ctrl), hPRL ($10^{-5}$ M) or mPRL ($10^{-5}$ M) as indicated. After 1 h, tumors were harvested and fixed in formalin. Samples were paraffin embedded and stained for active Stat5 by immunohistochemistry. Scale bar=40 µm. A representative of two experiments is shown.

with human prolactin receptors (Goffin *et al.* 1992, 1999, Teilum *et al.* 2005), mouse and rat prolactin differs from human prolactin in eight and five positions respectively (Table 1). It is therefore likely that the lack of bioactivity of mouse PRL toward human prolactin receptors is caused by one or more of these eight substitutions of receptor-interacting amino-acid residues, although contributions from other dissimilar residues cannot be excluded. For instance, the observation that ovine, but not bovine, prolactin had bioactivity in a human breast cancer growth bioassay (Vonderhaar & Biswas 1987) is not readily explicable by differences in any of the 21 receptor-interacting amino-acid residues (Table 1). Corrective mutagenesis studies will be needed to determine the contribution of individual residues to the loss of bioactivity of mouse prolactin toward human receptors. Furthermore, because several reports have shown that prolactin receptor binding *per se* does not predict bioactivity of a mutant lactogen (Davis & Linzer 1989, Goffin *et al.* 1992), both binding and bioactivity analyses will be needed to clarify mechanisms underlying the poor agonist activity of mouse prolactin toward human receptors.

Regardless of the structural basis of the poor agonism of mouse prolactin toward human receptors, human breast cancer growth, differentiation and biology will be affected by the absence of circulating human prolactin agonist activity in mice. The new data indicate that all human breast cancer lines currently used for drug testing in mice have been selected for growth independent of circulating prolactin and may therefore not resemble tumors in patients. A related problem, which may in part be due to inadequate prolactin receptor activation, is the particularly poor survival of primary human breast cancer when transplanted from patients into mice (Mehta *et al.* 1993, Speirs *et al.* 1998). While there is evidence that transplantation of primary cancer cells mixed with growth factor-rich extracellular matrix and human stromal cells will improve grafting (Mehta *et al.* 1993, Kuperwasser *et al.* 2004), long-term growth of primary human breast cancer in mice remains an obstacle, as pointed out in recent reviews (Kim *et al.* 2004, Wagner 2004). Regrettably, the vast majority of human breast tumors that show long-term survival in mice are cell lines that have been derived from pleural effusions or other metastatic sites, including the

**Table 1** Amino-acid (AA) residues in human prolactic important for bioactivity and interaction with human prolactin receptors, and their corresponding residues in prolactin from selected species. Table is derived from data reported by Goffin and collaborators (Goffin *et al.* 1992, 1999, Teilum *et al.* 2005)

| | 14 | 21 | 22 | 23 | 27 | 28 | 30 | 37 | 59 | 66 | 69 | 125 | 129 | 169 | 173 | 176 | 177 | 180 | 181 | 184 | 185 | 187 | 188 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **AA** | | | | | | | | | | | | | | | | | | | | | | | |
| Human | T | R | A | V | H | Y | H | F | H | P | K | R | G | Y | H | R | R | H | K | N | Y | K | L |
| Mouse | S | | V | | | | | | P | | | Q | | R | R | | | | | | F | | V |
| Rat | P | | V | | | | | | P | | | | | | R | | | | | | | | F |
| Ovine | S | | | | | | | | | | | | | | | | | S | | T | | | |
| Bovine | S | | | | | | | | | | | | | | | | | S | | T | | | |

relatively well-differentiated human cancer lines, such as T-47D, MCF-7 and ZR-75·1, that were analyzed in this study. Thus, the inadequate endocrine environment in mice raises new concerns about the validity of current human breast cancer lines grown in mice as models of human breast cancer with respect to biologic behavior, growth patterns, invasive characteristics and predictive value for drug responsiveness in patients. Research will therefore focus on correcting the identified prolactin-related endocrine deficiency in mice, so that mice may become more useful as xenotransplant recipients for clinically relevant modeling and drug-response prediction of prolactin-responsive human breast cancer.

Restoring human prolactin receptor signaling in human breast cancer grown in mice may be achieved by several alternative strategies. Exogenous human prolactin can be administered by injection of purified protein, implantation of slow-release pellets or vector-mediated expression such as adenoviral delivery. However, injection of purified protein is prohibitively expensive for chronic administration and requires both frequent administration and extensive animal handling time. Slow-release pellets are an alternative and have been used to test the effect of a human prolactin antagonist (Chen *et al.* 2002). This is also costly, and although less animal handling time is needed, there are problems of variability of hormone release and duration. Likewise, hormone levels are hard to control by viral or plasmid-based strategies. On the other hand, chronic endogenous expression of human prolactin in mice can be achieved by genetic engineering such as transgenic overexpression or gene targeting. Three transgenic mouse strains have been reported to express rat prolactin under the metallothionine promoter (Wennbo *et al.* 1997*a*), or the prostate-specific probasin promoter (Kindblom *et al.* 2003), or the mammary-specific neu-related lipocalin promoter (Rose-Hellekant *et al.* 2003). In each case, prolactin receptor hyperactivation led to mammary or prostate neoplasia with high frequency, emphasizing the tumor-promoting role of prolactin but also the unphysiologic nature of transgene expression. In addition, a recent report described a transgenic mouse model expressing human prolactin, also under metallothionine promoter control, but there was no mention of mammary tumors or of plans to cross the model into an immunodeficient background for human xenotransplant studies (Peirce & Chen 2004). Most importantly, due to the lack of physiologic control of expression, it remains uncertain whether transgenic strategies will be the method of choice for introduction of human prolactin in mice.

Instead, we propose that mice engineered to express human prolactin under control of the endogenous regulatory elements of the mouse prolactin gene, will be the best strategy to correct the endocrine environment for effective testing of human breast cancer in mice. We verified in this study that human prolactin works well on mouse prolactin receptors and mimics mouse prolactin-induced 3D mammospheres, and we expect that, in such a mouse model, levels of human prolactin will be under physiologic control. Crossed into an immunodeficient background, human prolactin knockin mice may provide an improved *in vivo* experimental model for analyses of human breast cancer. The proposed new model holds the potential for: 1) more reliable predictive testing of breast cancer drugs in the preclinic; 2) re-evaluation of progression, invasion and metastasis of existing human breast cancer models; 3) successful establishment of new, transplantable lines of human breast cancer that are not selected under human prolactin agonist-deficient conditions. Improved prediction of drug responsiveness in patients based on mouse studies will have great cost benefits and will facilitate identification of new, clinically effective drugs. Finally, the proposed mouse model may also support more physiologic growth of untransformed human breast epithelia and stromal components *in vivo,* as well as growth of additional normal and malignant prolactin-responsive human cell types, including prostate and insulin-producing pancreatic islet cells.

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