0888-8809/02/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 16(5):1108–1124

Basal Activation of Transcription Factor Signal Transducer and Activator of Transcription (Stat5) in Nonpregnant Mouse and Human Breast Epithelium

MARJA T. NEVALAINEN, JIANWU XIE, LUKAS BUBENDORF, KAY-UWE WAGNER, AND HALLGEIR RUI

United States Military Cancer Institute and Department of Pathology (M.T.N., J.X., H.R.), Uniformed Services University of the Health Sciences, Bethesda, Maryland 20852; Institute of Pathology (L.B.), University of Basel, Basel CH-43003, Switzerland; and Eppley Institute for Research in Cancer and Allied Diseases (K.-U.W.), Nebraska Medical Center, Omaha, Nebraska 68182

Transcription factor Stat5 (signal transducer and activator of transcription) is essential for PRL-induced terminal differentiation of mouse mammary epithelial cells during pregnancy and lactation and has been implicated in mammary tumorigenesis. A new and sensitive immunological method to detect active, tyrosine phosphorylated Stat5 *in situ* revealed that Stat5 is continuously activated in luminal epithelial cells of mouse and human breast, not only during pregnancy and lactation, but also outside of pregnancy. Examination of virgin Stat5a or Stat5b null mice suggested that Stat5a was the primary isoform activated in mammary epithelial cells. Basal activation of Stat5 in mammary epithelium of virgin wild-type mice was continuous throughout estrous cycle and was also detected in 17 of 17 normal human breast tissue specimens analyzed. PRL was identified as the principal factor maintaining basal activation of Stat5 in mammary epithelium of nonpregnant mice based on several lines of evidence. First, administration of PRL, but not GH or epidermal growth factor, uniformly enhanced basal activation of Stat5 in luminal mammary epithelial cells. Second, hypophysectomy disrupted basal activation of Stat5, an effect that was completely reversed by administration of PRL, but only partially by GH. Third, knock-out of the PRL receptor gene markedly reduced basal activation of Stat5, an effect that was maintained in a normalized endocrine environment after transplanting PRL receptor null mammary epithelium into wild-type mice. Continuous activation of Stat5 indicates a role of this transcription factor in normal, nonpregnant breast epithelial cells, and may shed new light on Stat5 involvement in breast tumor promotion. (*Molecular Endocrinology* 16: 1108–1124, 2002)

GENETIC STUDIES IN mice have demonstrated that signal transducer and activator of transcription (Stat5) transcription factors, especially the Stat5a isoform, are critical for PRL induced terminal differentiation of mouse mammary epithelial cells during pregnancy and lactogenesis (1, 2). In parallel, evidence has accumulated suggesting that Stat5 and PRL are involved in breast cancer development. Specifically, chronic hyperprolactinemia promotes mammary tumor formation in rodents (3, 4), and mammary tumorigenesis is suppressed in mice lacking the PRL gene (5). Furthermore, elevated PRL levels correlated with increased risk of breast cancer in postmenopausal women (6). In addition, mammary tumor formation was delayed in Stat5a$-/-$ mice overexpressing TGF-α (7), an effect possibly resulting from a general antiapoptotic effect of Stat5 (8). Whereas the focus on Stat5 in mammary gland thus far has been on the role of this transcription factor in terminal differentiation and lactogenesis, establishing the extent of Stat5 activation in normal, nonpregnant breast epithelial cells is important for understanding regulation of normal mammary gland function and the role of Stat5 in breast tumor formation.

The mechanism of activation of cytoplasmic Stat5 involves initial phosphorylation of a positionally conserved tyrosine residue by Jak2 or other kinases, an event followed by nuclear translocation of Stat5 dimers (9, 10). The extent of Stat5 activation in nonpregnant mammary epithelial cells has been difficult to assess by conventional methods that rely on homogenates of whole mammary gland because of the small epithelial compartment relative to stroma (11, 12). Analysis of Stat5 activity in mammary tissue has been limited to immunoblotting for tyrosine phosphorylated Stat5 and EMSA. Both technologies have demonstrated marked activation of Stat5 in mammary gland during pregnancy and lactation (1, 11–13).

We now present and validate a simple and highly sensitive immunohistochemical method for *in situ* detection of tyrosine phosphorylated, activated Stat5 in mammary tissues. This method demonstrated that Stat5 is activated not only during pregnancy and lac-

Abbreviations: BW, Body weight; DAB, diaminobenzidine; EGF, epidermal growth factor; mAb, monoclonal antibody; Stat5, signal transducer and activator of transcription; PRLR, PRL receptor; WT, wild-type.

tation but is continuously activated in normal nonpregnant mouse and human breast epithelial cells. Analysis of other exocrine organs such as salivary glands and pancreas showed that basal activation of Stat5 was specific for mammary epithelial cells. Stat5 remained continuously activated in the mammary epithelium throughout the estrous cycle in mice, and examination of Stat5a or Stat5b null mice suggested that Stat5a was the predominant isoform activated. Studies of mammary glands of short-term hypophysectomized virgin mice and virgin PRL receptor (PRLR) null mice indicated that PRL was the principal factor maintaining basal activation of Stat5. This was further supported by investigation of paired transplants of wild-type (WT) and PRLR null mammary glands into WT mice with normal hormonal background, combined with injection studies to determine responsiveness to PRL and other candidate Stat5 activating factors.

Our results demonstrate that physiological levels of PRL maintain basal activation of Stat5 in mammary epithelial cells of nonpregnant mice. The discovery of continuous activation of Stat5 also in normal human breast epithelial cells suggests a basal role for Stat5 in nonpregnant breast, a finding that has important implications for understanding the involvement of Stat5 in breast tumor growth.

RESULTS

Validation of Anti-pTyrStat5 Monoclonal Antibody (mAb) by Immunocytochemistry of Activated Stat5 in T47D Breast Cancer Cells

To detect activated Stat5 at the cellular level *in situ*, we used a monoclonal antibody that had been generated to a phosphopeptide corresponding to the conserved phosphotyrosyl segment of Stat5. We first evaluated the specificity of recognition of tyrosine phosphorylated Stat5 in formalin-fixed cytological preparations of PRL-stimulated human T47D breast cancer cells. Immunocytochemistry of T47D cells showed strong nuclear staining with anti-pTyrStat5 mAb after stimulation with PRL for 30 min (Fig. 1A, panel b), consistent with inducible tyrosine phosphorylation and nuclear translocation of Stat5 (9, 10). Unstimulated cells displayed only weak and scattered staining (Fig. 1A, panel a), and PRL-stimulated cells tested with control IgG were negative (Fig. 1A, panel c).

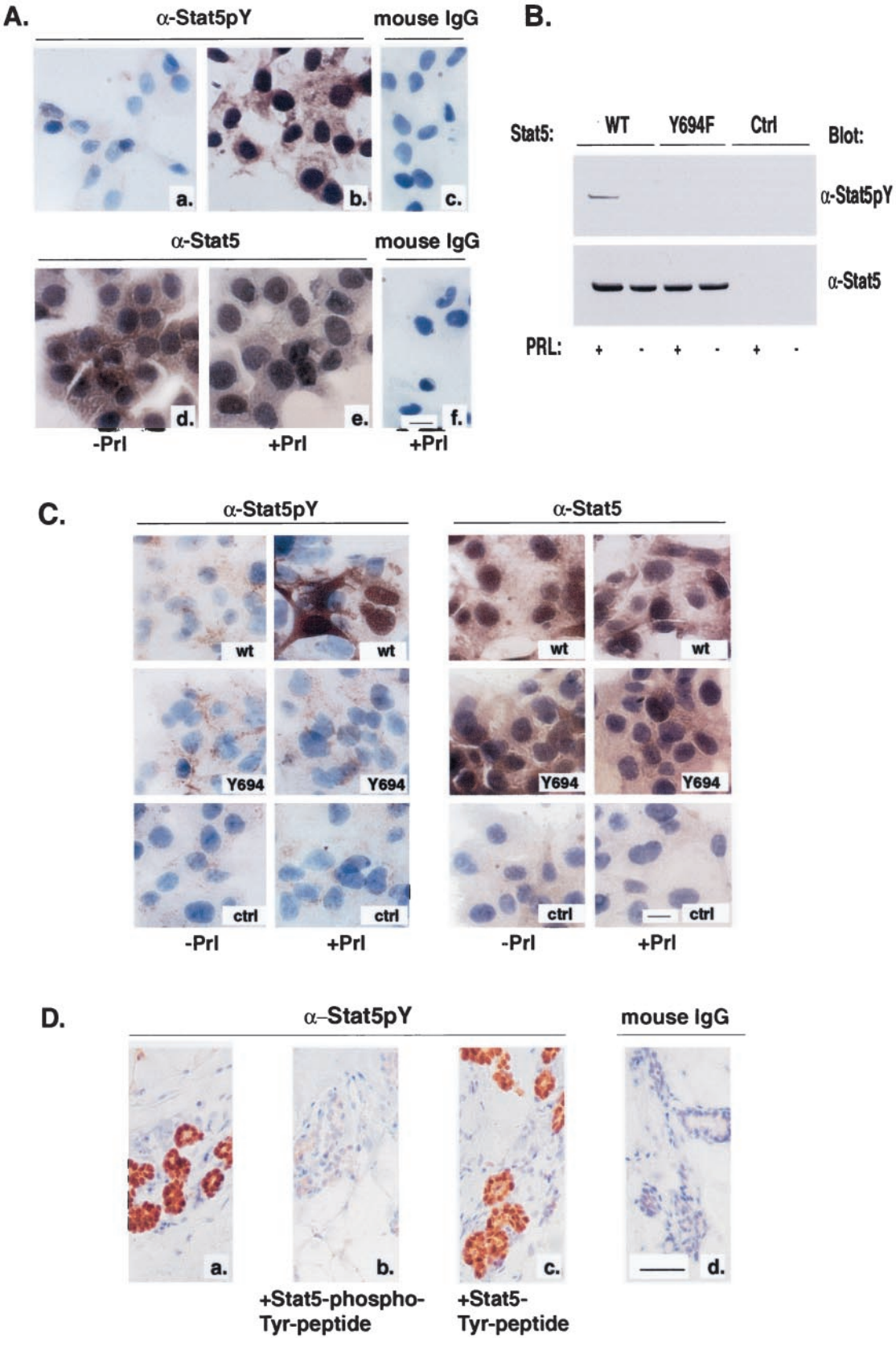
For comparison, immunocytochemistry of total Stat5 expression levels was performed on parallel samples of unstimulated and PRL-stimulated T47D cells using a monoclonal antibody directed against a carboxy-terminal segment shared by Stat5a and Stat5b (Fig. 1A, panels d and e). In contrast to the anti-pTyrStat5 mAb, immunostaining with anti-panStat5 mAb did not readily distinguish between unstimulated and PRL-stimulated cells, and showed instead significant levels of nuclear and cytoplasmic Stat5 levels under both conditions. A minor reduction in cytoplasmic Stat5 in PRL-stimulated cells compared with unstimulated cells, however, was consistent with nuclear translocation of tyrosine phosphorylated Stat5 (Fig. 1A, panels d and e). There was no immunostaining of PRL-stimulated cells stained with control IgG (Fig. 1A, panel f). From the immunocytochemical analysis of PRL-treated and untreated T47D cells, we concluded that anti-pTyrStat5 mAb recognizes tyrosine phosphorylated, but not unphosphorylated Stat5.

Validation of Anti-pTyrStat5 mAb by Immunoblotting and Immunocytochemistry against Phosphorylation-Defective Stat5

To more specifically verify that the anti-pTyrStat5 mAb used in this study recognizes Stat5 molecules that are phosphorylated on the positionally conserved tyrosine residue, immunoblotting and immunocytochemistry of cells transfected with either WT Stat5a or a phosphorylation-defective Stat5a mutant Y694F were carried out (Fig. 1, B and C). In the Stat5a-Y694A mutant, we have removed the phosphoacceptor hydroxyl group of Tyr694 of Stat5a by substitution with Phe (14). For these experiments, COS-7 cells that lacked detectable levels of endogenous Stat5 were transfected with expression plasmids encoding PRLR and either WT-Stat5, mutant Stat5 (Y694F), or empty control plasmid (Ctrl; Fig. 1, B and C).

Immunoprecipitation of Stat5 followed by immunoblotting showed that anti-pTyrStat5 mAb recognized tyrosine phosphorylated Stat5 only in PRL-stimulated cells that expressed WT-Stat5, and not in unstimulated cells or in PRL-stimulated cells expressing the phosphotyrosyl-defective mutant (Fig. 1B, *upper panel*). Furthermore, blotting of replicate samples with anti-panStat5 mAb verified that equal levels of WT-Stat5 and Stat5-Y694F were expressed in the transfected cells, and that no Stat5 was detectable in untransfected COS-7 cells (Fig. 1B, *lower panel*). Immunocytochemistry of transfected cells from the same experiments showed that Stat5 became activated after PRL stimulation in a significant portion of PRL-stimulated COS-7 cells transfected with both PRLRs and WT-Stat5 (Fig. 1C). Nuclear staining by anti-pTyrStat5 mAb in PRL-stimulated COS-7 cells indicated a marked activation of Stat5 compared with that of unstimulated cells (Fig. 1C, *upper left panels*). In contrast, no inducible nuclear or cytoplasmic staining was observed in COS-7 cells transfected with PRLRs and either phosphotyrosyl-defective Stat5 mutant (Fig. 1C, *middle left panels*) or with vector control (Fig. 1C, *lower left panels*).

Parallel immunocytochemistry of replicate slides with the anti-panStat5 mAb showed extensive positive staining both in unstimulated and PRL-stimulated COS-7 cells transfected with either WT-Stat5 (Fig. 1C, *upper right panels*) or Stat5-Y694F mutant (Fig. 1C, *middle right panels*). COS-7 cells transfected with empty control plasmid displayed little or no immuno-


A.
α-Stat5pY
mouse IgG
a.
b.
c.
α-Stat5
mouse IgG
d.
e.
f.
-Prl
+Prl
+Prl
B.
Stat5:
WT
Y694F
Ctrl
Blot:
α-Stat5pY
α-Stat5
PRL:
+ - + - + -
C.
α-Stat5pY
α-Stat5
wt
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ctrl
-Prl
+Prl
-Prl
+Prl
D.
α–Stat5pY
mouse IgG
a.
b.
c.
d.
+Stat5-phospho-Tyr-peptide
+Stat5-Tyr-peptide

staining with anti-panStat5 mAb (Fig. 1C, *lower right panels*). There were no distinct differences between unstimulated and PRL-stimulated COS-7 cells in any of the groups transfected with either WT-Stat5, Stat5Y694F, or control plasmid when stained with the anti-panStat5 mAb.

Validation of Anti-pTyrStat5 mAb by Selective Inhibition of Immunohistochemical Staining by pTyrStat5 Peptide

To further validate the specificity of the anti-pTyrStat5 mAb, we compared the abilities of the immunogen phosphopeptide and the corresponding unphosphorylated peptide to inhibit anti-pTyrStat5 mAb immunostaining in tissue sections. For these studies, sections of formalin-fixed, paraffin-embedded mammary gland tissue from PRL-treated virgin rats were used. Marked immunostaining for active Stat5 was detected in mammary epithelial cells using anti-pTyrStat5 mAb (Fig. 1D, panel a). In the presence of 1 μM peptide immunogen, KAVDG[phosphoY]VKPQIK, immunostaining of epithelial cells was completely abolished (Fig. 1D, panel b). In contrast, 1 μM of the corresponding unphosphorylated peptide, KAVDGYVKPQIK, did not interfere with immunostaining for active Stat5 (Fig. 1D, panel c). Furthermore, staining with IgG control mAb was negative (Fig. 1D, panel d).

Collectively, from a series of validation experiments, we conclude that the anti-pTyrStat5 mAb selectively detects the activated, tyrosine phosphorylated form of Stat5 in formalin-fixed cells and tissues.

Cellular Levels of Activated Stat5 in Mouse Mammary Gland during the Gestation Cycle

The extent of Stat5 activation at the cellular level in mouse mammary gland was determined by immunohistochemistry using formalin-fixed tissues collected from either mature virgin mice, pregnant mice (d 13, 15, and 18), lactating mice (d 5 of lactation), mice after weaning (d 1 and 3 of involution), or from nonpregnant parous mice (Fig. 2).

The most striking observation from this analysis was detection of significant levels of activated Stat5 in epithelial cells of the mammary gland of virgin mice (Fig. 2A, panel a). Stat5 was consistently activated in epithelial cells throughout the entire mammary gland of all virgin animals examined. Activated Stat5 in virgin mouse mammary gland was preferentially localized to luminal epithelial cells in both ducts and terminal end buds of the mammary ductal tree. As the epithelial compartment expanded during pregnancy, the total number of cells with activated Stat5 increased rather than the intensity of nuclear staining, at least up to d 15 of gestation (Fig. 2A, panels b and c). However, during late gestation (d 18; Fig. 2A, panel d) and especially during lactation (Fig. 2A, panel e), there was also increased intensity of nuclear immunostaining of activated Stat5. Furthermore, careful examination

Fig. 1. Validation of Specificity of Anti-pTyrStat5 mAb

A, Immunocytochemistry of inducible tyrosine phosphorylation of Stat5 and total Stat5 in PRL-stimulated T47D cells. Human T47D breast cancer cells were incubated with (+) or without (−) human PRL (10 nM) for 30 min, and were then fixed in 4% paraformaldehyde. Antigen-antibody complexes were detected using biotin-streptavidin amplified peroxidase-antiperoxidase immunodetection system with 3,3′-DAB as chromogen and Mayer hematoxylin as counterstain. For controls, subtype-specific mouse IgG was used and showed negative immunostaining (panels c and f). Note intense nuclear immunostaining in nuclei of PRL-stimulated T47D-cells (panel b) compared with unstimulated cells (panel a) when stained with the anti-pTyrStat5 mAb. In contrast, both unstimulated and PRL-stimulated T47D-cells showed marked nuclear and cytoplasmic immunostaining (panels d and e) when stained with the anti-panStat5 mAb. *Bar*, 4.2 μm. B, The anti-pTyrStat5 mAb specifically recognizes Stat5 molecules that are phosphorylated on the positionally conserved tyrosine residue in immunoblotting. COS-7 cells were transiently cotransfected with expression plasmids encoding PRLRs and either WT Stat5a, tyrosine phosphorylation-defective mutant Stat5a-Y694F, or control plasmid. After starving in serum-free medium for 16 h, cells were incubated in the presence or absence of PRL for 30 min. The cells were lysed and Stat5 was immunoprecipitated and separated on SDS-PAGE. Parallel samples were either blotted with anti-pTyrStat5 mAb (*upper panel*) or anti-panStat5 mAb (*lower panel*). Specific recognition of PRL-induced tyrosine phosphorylation of the key phosphorylation site of WT-Stat5, but not of mutant Stat5a-Y694F lacking the phosphoacceptor hydroxyl group was demonstrated. C, The anti-pTyrStat5 mAb specifically recognizes Stat5 molecules that are phosphorylated on the positionally conserved tyrosine residue by immunocytochemistry. COS-7 cells transfected and treated as described under B were fixed in formaldehyde and subjected to immunocytochemistry using anti-pTyrStat5 mAb or anti-panStat5 mAb. As with immunoblotting, the anti-pTyrStat5 mAb recognized PRL-induced tyrosine phosphorylation of Stat5 only in cells transfected with WT-Stat5a (*top left panels*) but not in cells transfected with the phosphotyrosyl-defective mutant Stat5a-Y694F (*middle left panels*) or in untransfected cells (*bottom left panels*). Immunostaining with anti-panStat5 mAb showed marked nuclear and cytoplasmic staining of both unstimulated and PRL-stimulated COS-7 cells transfected either with WT-Stat5 (*upper right panels*) or with phosphotyrosyl-defective mutant Stat5a-Y694F (*middle right panels*), but not in COS-7 cells transfected with empty control plasmid (*lower right panels*). *Bar*, 6 μm. D, Anti-pTyrStat5 immunohistochemistry: specific inhibition by phosphorylated but not unphosphorylated Stat5 peptide. Sections of formalin-fixed mammary tissue from a PRL-treated mature virgin rat were stained with anti-pTyrStat5 mAb in the absence (panel a) or presence (panel b) of phosphopeptide KAVDG[phosphoY]VKPQIK (1 μM) corresponding to the conserved phosphotyrosyl-motif of Stat5. Anti-pTyrStat5 immunohistochemistry was also carried out in the presence of unphosphorylated peptide, KAVDGYVKPQIK (panel c). Negative control staining was performed with mouse IgG (panel d). *Bar*, 15 μm.

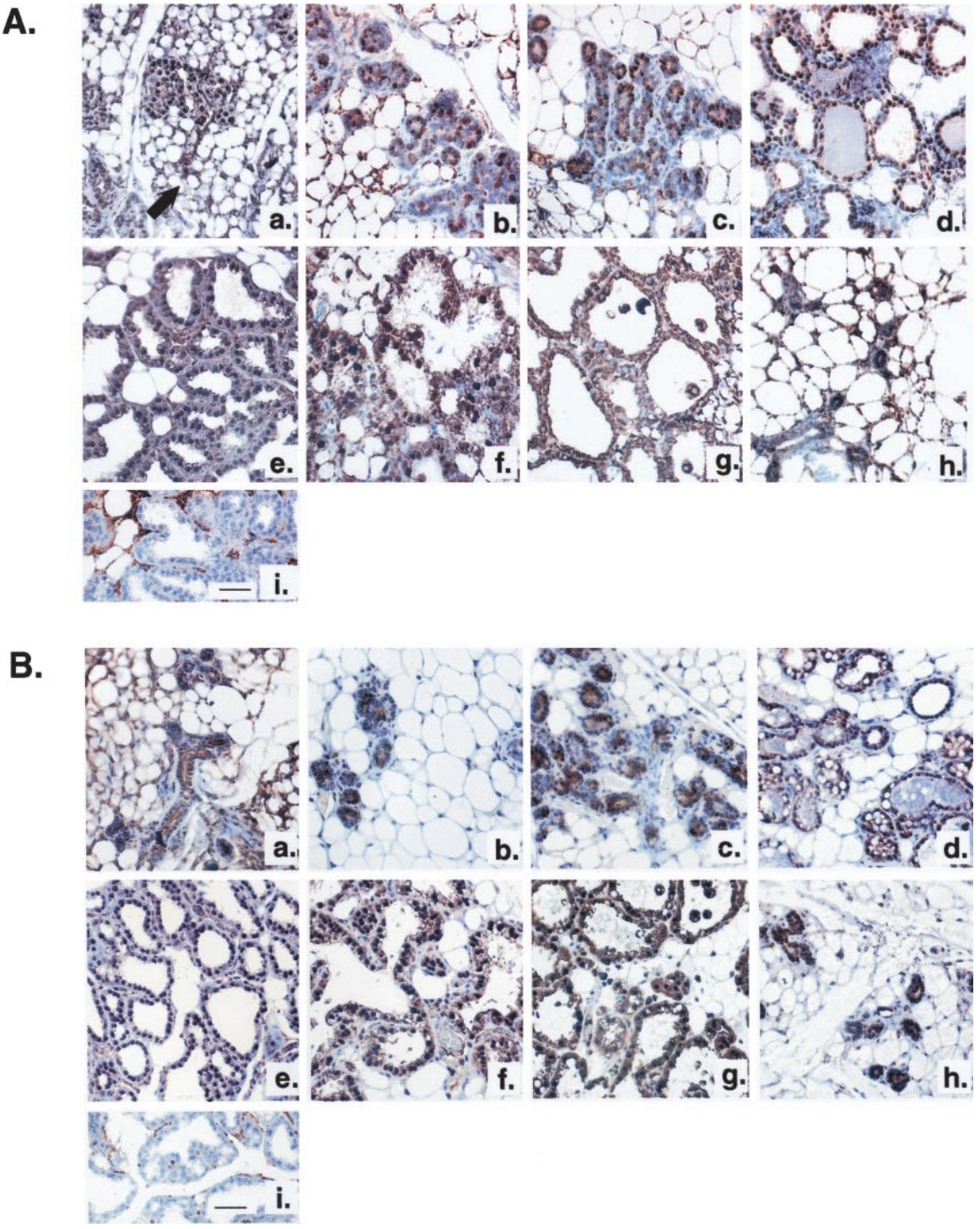

Fig. 2. Levels of Activated Stat5 and Stat5 Protein in Mouse Mammary Epithelium during Pregnancy Cycle

A, Immunohistochemistry of activated Stat5 in mouse mammary gland during pregnancy cycle. Mammary glands from mature virgin mice (a), pregnant mice at d 13 (b), 15 (c), and 18 (d), mice at d 5 of lactation (e), weaned mice on d 1 (f) and 3 (g) of involution, and of resting mammary glands of parous mice (h) were formalin-fixed and embedded in paraffin. Tissue sections were stained with an anti-pTyrStat5 mAb and counterstained with hematoxylin. Biotin-peroxidase immunodetection system was used with DAB as a chromogen. Epithelium in parallel sections of lactating mammary gland stained with subtype-specific mouse IgG was negative (i). Note marked positive immunostaining in epithelium of mammary gland from virgin mouse (a), and a dramatic loss of nuclear immunostaining during involution of mouse mammary gland (f and g). *Bar*, 18 μm. B, Immunohistochemistry of Stat5 protein in mouse mammary gland during pregnancy cycle. Parallel mammary tissue sections corresponding to the same stages of pregnancy cycle were immunostained with anti-panStat5 mAb. Lactating mammary tissue was negative when stained with subtype-specific control IgG (i). *Bar*, 18 μm.

showed that myoepithelial cells, which also are derived from epithelial stem cells (15), did not contain activated Stat5 (data not shown). During involution, levels of activated, tyrosine-phosphorylated Stat5 in epithelial cells fell rapidly, and were completely eliminated from cell nuclei on d 3 of involution (Fig. 2A, panels f and g). However, marked basal levels of activated Stat5 in epithelial cells were restored in mammary epithelial cells upon return to normal tissue morphology in nonpregnant parous animals (Fig. 2A, panel h). Negative control staining of lactating mammary tissue is also shown (Fig. 2A, panel i).

Immunostaining of sections of the same tissue samples for total Stat5 expression levels showed a similar temporal profile of epithelial cell staining during gestation cycle with one notable exception. Whereas immunoreaction for tyrosine phosphorylated Stat5 was completely absent in the nuclei of mammary epithelial cells at d 3 of involution, general Stat5 staining remained strongly positive in the nuclei of these cells (Fig. 2B, panel g), suggesting that high levels of inactive Stat5 protein remained in the cell nuclei at this stage. These experiments therefore revealed a distinct physiological setting in which nuclear Stat5 levels did not correlate with nuclear levels of activated, tyrosine phosphorylated Stat5. Control sections of lactating mammary gland incubated with subtype specific IgG were negative (Fig. 2B, panel i).

From this analysis of Stat5 activity and expression during mammary gland gestation cycle, we conclude that Stat5 is continuously activated in luminal epithelial cells of mammary glands of nonpregnant mice. Furthermore, basal activation of Stat5 in epithelial cells was specific for mammary gland, because epithelial cells of other exocrine organs, including salivary glands and pancreas, were negative for activated Stat5 (data not shown).

EMSA of Stat5 during Lactation and Involution

During involution, a discrepancy was detected between nuclear levels of Stat5 protein and nuclear levels of tyrosine phosphorylated Stat5 in epithelial cells. Immunohistochemistry of phosphorylated Stat5 indicated that Stat5 was inactivated on d 3 of involution, whereas immunohistochemistry with the anti-panStat5 mAb nonetheless showed abundant Stat5 levels in the cell nuclei. Based on the notion that only activated Stat proteins are translocated to the nucleus, high levels of nuclear Stat5 in mammary epithelial cells during involution would normally indicate that Stat5 is activated. To clarify this apparent discrepancy between high levels of immunodetectable Stat5 protein and the absence of tyrosine phosphorylated Stat5 in nuclei of mammary epithelial cells during involution, we first compared the nuclear Stat5 DNA binding activity in lactating mammary gland to that of involuting tissue.

EMSA of whole mammary gland tissue homogenates, using the PRL-response element of β-casein gene as a probe, established that the high levels of immunodetectable, unphosphorylated Stat5 in cell nuclei of the involuting gland did not bind to DNA (Fig. 3A, lanes m–p), consistent with inactive Stat5. In contrast, marked Stat5 DNA binding activity was detected during lactation (Fig. 3A, lanes i–l). Based on this DNA binding assay, we conclude that high levels of unphosphorylated Stat5 present in nuclei of involuting epithelial cells represented inactive Stat5, consistent with the need for nuclear Stat5 to be tyrosine phosphorylated to be active.

DNA Binding Activities of Stat5a and Stat5b in Lactating Mammary Gland

EMSA analysis also supported the notion that Stat5a is the predominant isoform of Stat5 activated during normal mammary gland differentiation and lactogenesis. Specifically, antibodies to Stat5a quantitatively supershifted the Stat5-containing DNA complex, whereas antibodies to Stat5b were minimally effective (Fig. 3A, lanes i–l). This disparity was not due to differences in the ability of Stat5a and Stat5b to bind to the β-casein promoter, or an inability of the anti-Stat5b antiserum to supershift bound Stat5b. Parallel control experiments in COS-7 cells demonstrated that both Stat5a and Stat5b effectively bound to the β-casein probe, and that the anti-Stat5b antibodies were effective in supershifting bound Stat5b (Fig. 3B). EMSA analysis therefore indicated that Stat5a is the predominant isoform of Stat5 activated during mammary gland differentiation.

Levels of Stat5a and Stat5b Protein during Gestation Cycle

We then compared levels of Stat5a and Stat5b expression and phosphorylation by protein immunoblotting over the course of the gestation cycle (Fig. 4). During pregnancy, tyrosine phosphorylation of Stat5a increased markedly but fell rapidly during involution. Levels of the 94-kDa Stat5a protein also increased during pregnancy and were also reduced during involution, especially by d 3. The abundance of nuclear Stat5 during d 3 of involution detected by immunohistochemistry may therefore to a large extent represent partially degraded forms of Stat5. This would be consistent with an inactivation of nuclear Stat5 in mammary epithelial cells during involution that involve both phosphatases and proteases (16). Nonetheless, immunohistochemistry indicated that partially degraded, inactivated Stat5 does not readily exit the cell nuclei during involution, creating a condition of high levels of nuclear but dephoshorylated Stat5 (Fig. 2A, panels f and g). The data support the concept that levels of nuclear, tyrosine-phosphorylated Stat5 reflect levels of activated Stat5 more accurately than what levels of total nuclear Stat5 protein do. This concept may have direct relevance for interpretation of Stat5 activation under other physiological and pathological circumstances, for instance in tumors.

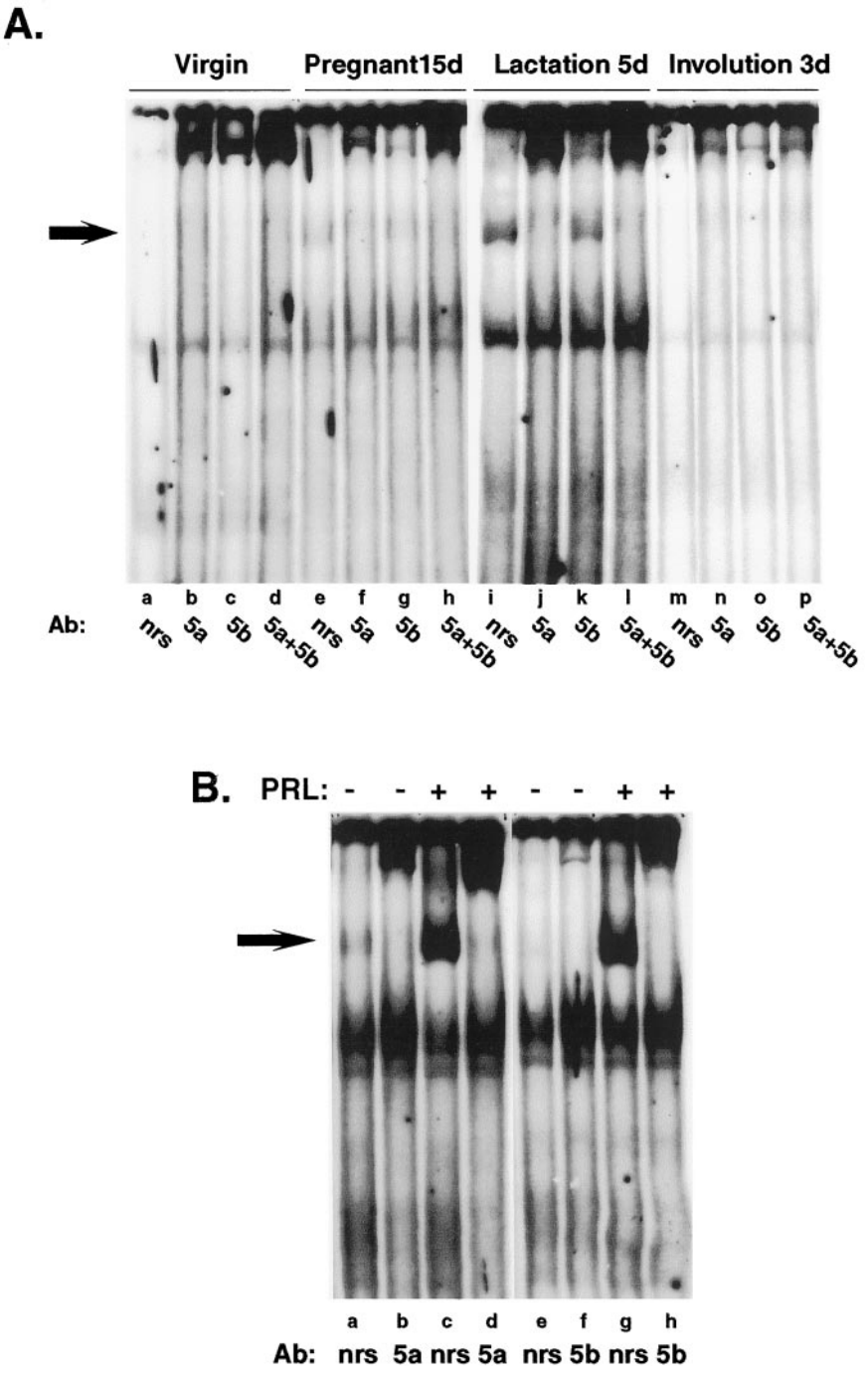

Fig. 3. EMSA of Nuclear Stat5 Binding to a β-Casein Promoter Probe during the Lactation Cycle

A, Mammary gland tissues were harvested from mature virgin mice (lanes a–d), mice at d 15 of pregnancy (lanes e–h), mice at d 5 of lactation (lanes i–l), and from mice 3 d after removal of pups (lanes m–p). The tissues were homogenized and nuclear extracts were incubated either with normal rabbit serum (NRS) (lanes a, e, i, m), polyclonal anti-Stat5a serum (5a; lanes b, f, j, n), polyclonal anti-Stat5b serum (5b; lanes c, g, k, o) or with antisera to both anti-Stat5a and anti-Stat5b (5a + 5b; lanes d, h, l, p) in combination with a [^{32}P]-labeled oligonucleotide probe corresponding to the PRL-response element of the β-casein promoter. *Arrow* indicates a protein-DNA complex that is primarily supershifted by anti-Stat5a serum but less effectively by anti-Stat5b antiserum. B, The ability of both anti-Stat5a and anti-Stat5b antisera to effectively supershift Stat5a and Stat5b, respectively, was verified by EMSA performed on nuclear extracts of transfected COS-7 cells using the same β-casein probe. COS-7 cells were cotransfected with an expression vector encoding PRLR and an expression vector encoding either Stat5a (lanes a–d) or Stat5b (lanes e–h) and incubated with medium (−) (lanes a, b, e, f) or 100 nM ovine PRL (+) (lanes c, d, g, h) for 15 min at 37 C. The *arrow* indicates PRL-induced Stat5a or Stat5b complexes formed with the β-casein promoter probe that are supershifted by antisera to Stat5a and Stat5b, respectively.

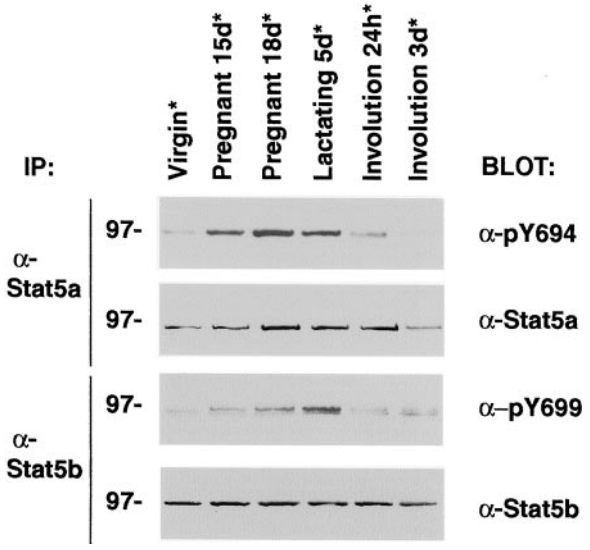

Fig. 4. Immunoblotting of Tyrosine Phosphorylated Stat5a and Stat5b in Mouse Mammary Gland during the Pregnancy Cycle

Cleared tissue homogenates (3.5 mg total protein) of mammary glands of mature virgin mice, mice at d 15 and 18 of pregnancy, mice at d 5 of lactation, and mice at d 1 and 3 of involution were immunoprecipitated with anti-Stat5a and anti-Stat5b antisera. Samples were separated by SDS-PAGE and first blotted with anti-pTyrStat5 mAb followed by stripping and reblotting the membranes with antisera to either Stat5a or Stat5b.

Expression levels of Stat5a, the most predominant Stat5 isoform in the epithelial compartment, increased markedly during pregnancy (Fig. 4), consistent with previous reports (12, 13). Stat5b levels, on the other hand, remained more constant throughout gestation cycle and there was less overall induction of Stat5b phosphorylation during pregnancy and lactation in mice (Fig. 4; also see Ref. 13). Predominant activation of Stat5a during lactation was also consistent with the EMSA analysis of whole mammary gland extracts using the β-casein promoter probe. Furthermore, it remains to be determined to which extent Stat5b phosphorylation occurs in the stromal *vs.* the epithelial compartment.

Continuous Activation of Stat5 in Epithelial Cells of Nonpregnant Mouse Mammary Gland

Whereas immunohistochemistry demonstrated a marked activation of Stat5 at the cellular level in nonpregnant mammary glands (Fig. 2A), immunoblotting, and EMSA based on whole tissue extracts failed to detect significant Stat5 activation outside of pregnancy and lactation (Figs. 3 and 4). Due to the small epithelial compartment in nonpregnant mice, and despite the marked and consistent basal activation of Stat5 at the cellular level in virgin mammary glands, we presume that the activated Stat5 is too diluted by the predominant stromal tissue components to be readily detectable by EMSA or immunoblotting. Based on our detection of nuclear, tyrosine-phosphorylated Stat5 *in situ*, we conclude that Stat5 is continuously activated in a large proportion of nonpregnant mammary epithelial cells.

Basal Activation of Stat5 in Mammary Epithelium Is Continuous Throughout Estrous Cycle

Mammary gland epithelium undergoes periodic growth and regression during estrous cycle in mice, and previous analysis of protein tyrosine phosphorylation by immunoblotting of whole mammary gland extracts had indicated that low levels of Stat5 phosphotyrosine were increased during estrus (12). We therefore wanted to test to what extent basal Stat5 activation in nonpregnant mammary epithelium was dependent on ovarian steroids and would fluctuate during estrous cycle. Mammary tissue from mice at proestrus (Fig. 5A, panel a), estrus (Fig. 5A, panel b), postestrus (Fig. 5A, panel c), and diestrus (Fig. 5A, panel d) were first immunostained with the anti-pTyrStat5 mAb to detect intensity of active Stat5 at the cellular level. This analysis revealed that epithelial cells of mammary glands showed no explicit variation in tyrosine phosphorylated, nuclear Stat5 during estrous cycle (Fig. 5). Thus, basal Stat5 activation at the cellular level was not affected by normal variations in circulating progesterone and estrogen levels.

We also investigated levels of Stat5 tyrosine phosphorylation by immunoblotting of Stat5 proteins in extracts from mammary glands of mice during diestrus and estrus. After normalization of protein concentrations of samples, antibodies specific to Stat5a or Stat5b were used to immunoprecipitate each isoform from detergent extracts of whole mammary gland homogenates from individual mice. Anti-pTyrStat5 immunoblotting indicated that levels of tyrosine phosphorylated Stat5a in whole mammary gland extracts varied more between mice than levels of tyrosine phosphorylated Stat5b, but no systematic differences were detected between mammary glands during diestrus and estrus (not statistically significant by Wilcoxon sign rank test; Fig. 5B). The moderate increase of phosphorylation of Stat5 during estrus previously reported is possibly due to differences in protein extraction and protein normalization protocols (12).

Stat5 Is Continuously Activated in Nonpregnant Human Breast Epithelium

To date, no work on Stat5 activation in human breast tissue has been reported. Before proceeding to identify the factor(s) responsible for maintaining basal Stat5 activation in mouse mammary gland, we wanted to determine whether Stat5 was continuously activated also in human breast epithelium. Archival samples of normal nonpregnant human breast tissues were analyzed by immunohistochemistry. Nuclei of epithelial cells of both ductal structures and lobular endbuds displayed marked immunoreaction for activated Stat5 in 17 of 17 individuals studied. A representative tissue section is presented in Fig. 6a. As was

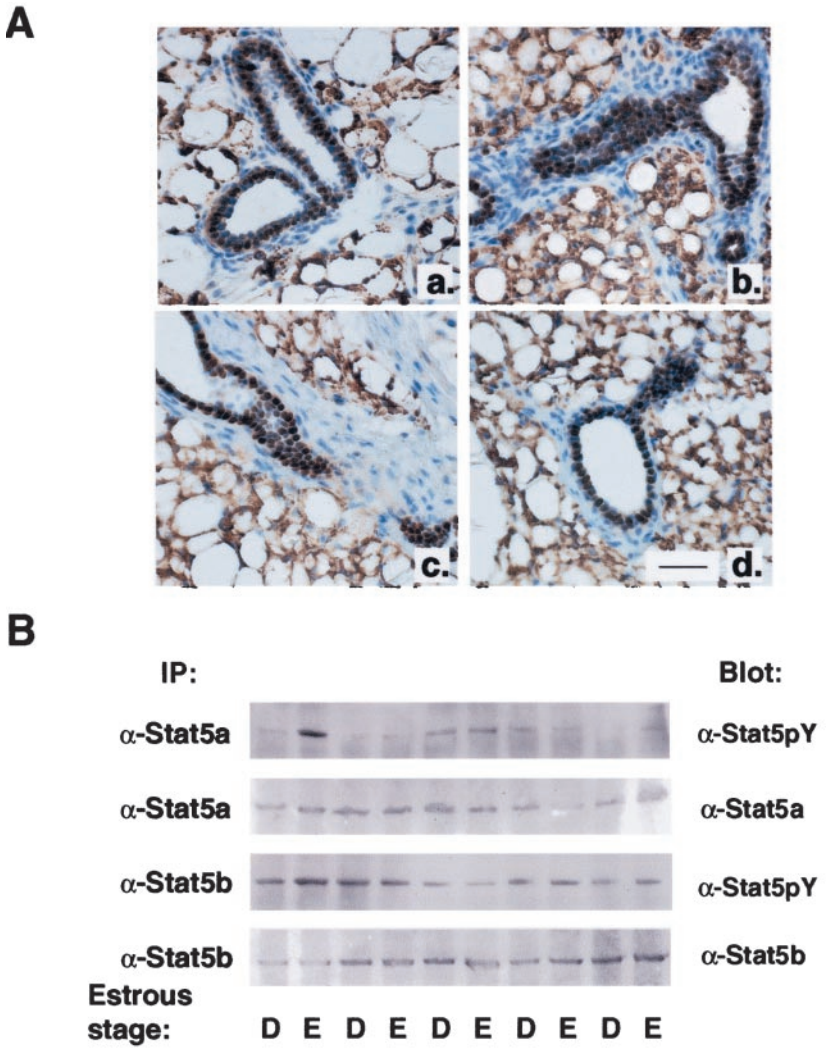

Fig. 5. Stat5 Is Continuously Activated in the Epithelium of Virgin Mouse Mammary Gland throughout Estrous Cycle

A, Mammary glands from virgin mice at proestrus (a), estrus (b), postestrus (c), and diestrus (d) were fixed in formalin and stained with anti-pTyrStat5 mAb. Note marked and consistent immunostaining of activated Stat5 in mammary gland epithelium throughout the estrous cycle. *Bar*, 18 μm. B, Immunoblotting of tyrosine phosphorylated Stat5 and protein expression in mammary glands of individual mice in estrus (E) or diestrus (D). Mammary glands from a total of 10 mature virgin mice were investigated. For these studies, Stat5a or Stat5b were individually immunoprecipitated from whole mammary gland homogenates and separated by SDS-PAGE.

the case in mouse mammary gland, activation of Stat5 was further increased in epithelial cells of lactating human breast (n = 2; Fig. 6b), consistent with an important role of Stat5 also in human lactogenesis. We conclude from this analysis that Stat5 is continuously activated in breast epithelial cells in nonpregnant women similar to that observed in nonpregnant mice.

Stat5a Is the Predominant Activated Form of Stat5 in Nonpregnant Mouse Mammary Epithelial Cells

Genetic targeting of Stat5a and Stat5b has demonstrated that of the two homologous genes, Stat5a is the most critical for pregnancy-associated terminal differentiation of mammary epithelial cells (1, 2). Phosphotyrosyl-specific Stat5 antibodies cannot discriminate between activated Stat5a and Stat5b because of the structural identity of the tyrosine phosphorylation motifs of Stat5a and Stat5b. Furthermore, we have not yet established useful immunohistochemistry conditions to detect Stat5a or Stat5b separately with polyclonal antibodies to the divergent C termini of Stat5a and Stat5b. Therefore, to assess the individual contribution of each Stat5 isoform to the basal levels of activated Stat5 in mammary gland epithelium of virgin mice, we instead examined Stat5a and Stat5b null mice (Fig. 7).

Sections of mammary glands of virgin mice that were either WT (Fig. 7A, panels a and d), Stat5a−/−

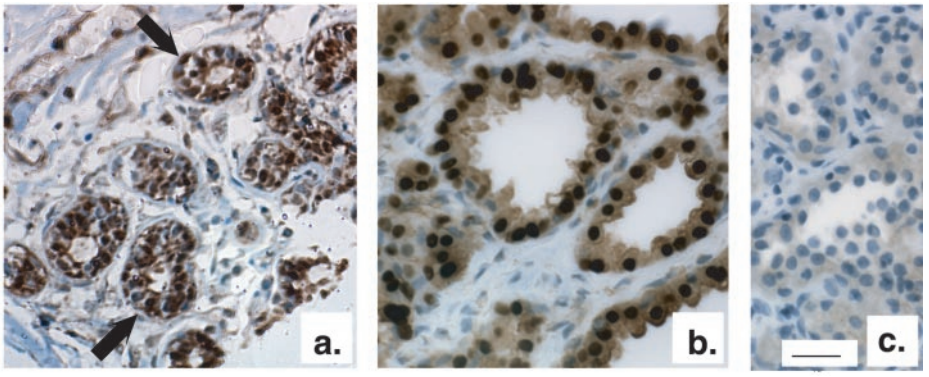

Fig. 6. Stat5 Is Activated in the Epithelium of Nonpregnant Human Mammary Gland

Activation of Stat5 in human mammary epithelium was studied by immunohistochemistry of archival samples of lactating breast (n = 2; panel a) and in nonpregnant breast (n = 17; panel b). Tissue sections of paraffin embedded tissues were immunostained with anti-pTyrStat5 mAb as described, and representative samples are shown. Panel c shows negative immunostaining in lactating breast tissue using control IgG. *Bar*, 11 μm.

(Fig. 7A, panels b and e) or Stat5b−/− (Fig. 7A, panels c and f) were immunostained with anti-pTyrStat5 mAb. In WT mouse mammary epithelium, both small alveoli and larger ducts showed marked nuclear immunostaining for phosphorylated Stat5 as expected (Fig. 7A, a and d). However, immunoreaction for activated Stat5 was almost entirely absent in the epithelium of mammary glands from mice lacking the Stat5a gene (Fig. 7A, panels b and e). In contrast, strong immunostaining for nuclear, tyrosine phosphorylated Stat5 was present in virgin mammary epithelium of Stat5b null mice. This staining was present both in alveoli and, to a lesser extent, in epithelial cells of larger ducts within mammary glands of Stat5b null mice (Fig. 7A, panels c and f). These results indicated that Stat5a was the predominant form of Stat5 activated in epithelium of virgin mouse mammary gland. This observation is consistent with a greater importance of Stat5a than Stat5b for terminal mammary epithelial cell differentiation (1, 2).

Stat5a Is Critical for Normal Lactogenesis, but Stat5b Can Compensate for Its Absence after Multiple Pregnancies

Stat5a null mice consistently fail to lactate after the first pregnancy but nonetheless often gain ability to lactate after multiple pregnancies, suggesting that the critical role of Stat5a in mammary epithelium can be compensated for by other factors (17). While Stat5b was proposed to serve as the compensatory factor, the data relied on immunoprecipitation of Stat5b from whole mammary gland tissue homogenates and did not directly show compensatory Stat5b activation within epithelial cells. To specifically examine whether Stat5b becomes activated in mammary epithelium in the absence of Stat5a after multiple pregnancies, mammary glands from Stat5a null mice were examined after variable number of pregnancies by immunohistochemistry for activated Stat5.

As shown in Fig. 7B, there were generally only low levels of detectable activated Stat5 in epithelial cells of Stat5a null mice after the first pregnancy (Fig. 7B, panel a). Only scattered cells within the mammary epithelium of Stat5a-null mice showed positive immunostaining for tyrosine phosphorylated Stat5 postpartum, and there was a correspondingly low extent of secretory lumen formation (Fig. 7B, panel a). In contrast, after three pregnancies, a significant portion of Stat5a null mice can lactate at least partially, and levels of activated Stat5 were consistently high in the milk-producing epithelium as demonstrated by immunohistochemical staining with anti-pTyrStat5 mAb (Fig. 7B, panel b). These experiments therefore presented important new evidence that epithelial cell Stat5b is capable, provided sufficient time, to overcome Stat5a deficiency. This phenomenon also illustrates the plasticity of mammalian genomic regulation in response to a genetic challenge.

Continuous Activation of Stat5 in Mammary Gland of Nonpregnant Mice Is Controlled by Pituitary Factors

The observed insensitivity of activated Stat5 to ovarian steroids during estrous cycle in nonpregnant mice left to be determined which factors maintain basal activation of Stat5. Stat5 is activated by a number of peptide hormones and cytokines in various mammalian cell types (18). Although PRL is regarded as the principal factor for stimulation of Stat5 during pregnancy and lactogenesis, other factors such as GH and members of the epidermal growth factor (EGF) family also reportedly can activate Stat5 in mouse mammary gland (19, 20). To identify the factor(s) responsible for basal Stat5 activation in nonpregnant mammary gland, we first examined the effect of short-term hypophysectomy on basal activation of Stat5 in mouse mammary tissue (Fig. 8A).

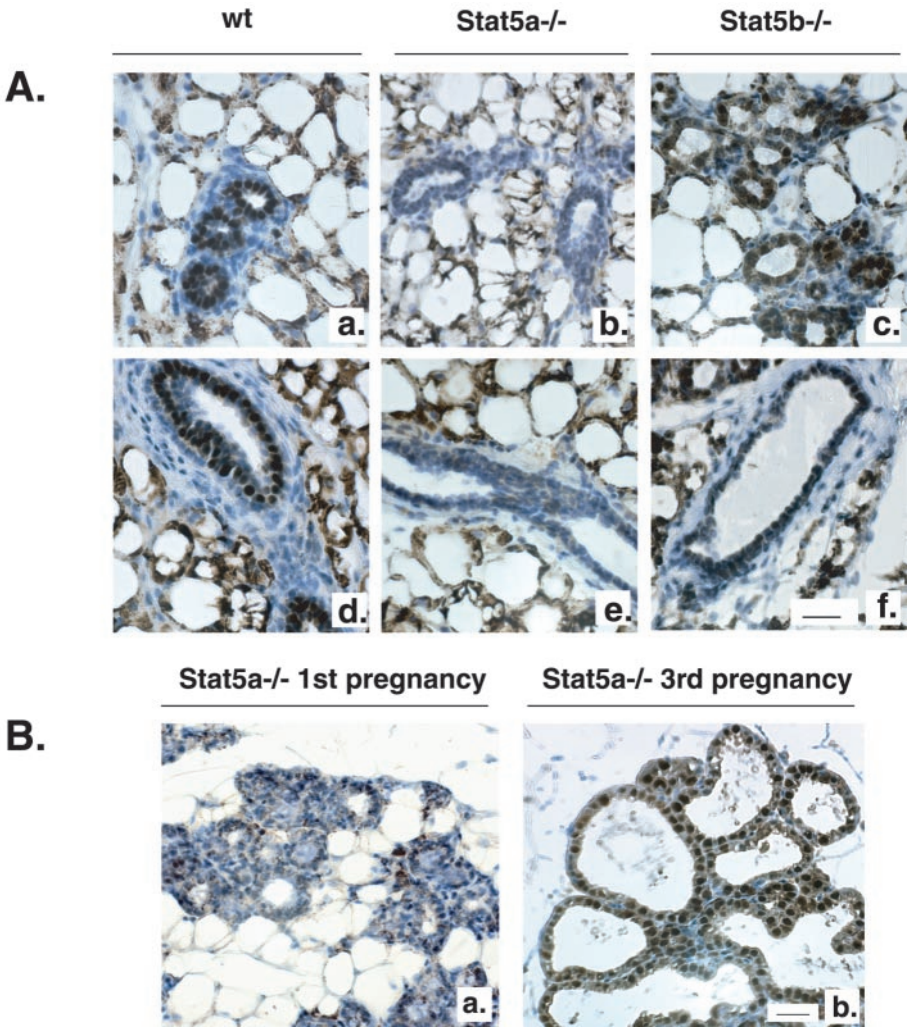

Fig. 7. Loss of Stat5 Activation in Mammary Epithelium of Virgin Stat5a Null Mice

A, Activation of Stat5 in the epithelium of virgin mouse mammary gland is mainly due to phosphorylation of Stat5a. Formalin-fixed tissue sections of paraffin-embedded mammary glands from mature virgin WT mice (panels a and d), mature virgin Stat5a null mice (Stat5a−/−; panels b and e) or mature virgin Stat5b null mice (Stat5b−/−; panels c and f) were immunostained with anti-pTyrStat5 mAb. *Bar*, 11 μm. B, Immunohistochemistry of activated Stat5 in mammary gland of Stat5a null mice during the first (a) and during the third (b) pregnancy demonstrating prominent activation of Stat5b in mammary epithelial cells after multiple pregnancies. Tissue sections of paraffin-embedded mammary glands from a Stat5a null mouse at d 1 of lactation of the first pregnancy (panel a) and from a Stat5a-null mouse at d 1 of lactation after the third pregnancy (panel b) were immunostained with anti-pTyrStat5 mAb. Note intense nuclear immunostaining of active Stat5 in the epithelium of mammary gland of Stat5a null mouse during the third pregnancy (b). *Bar*, 11 μm.

Mature virgin mice were sham-operated (n = 5) or hypophysectomized (n = 5), and mammary glands were collected on d 2 after surgery and immunostained with anti-pTyrStat5 mAb. Immunohistochemistry showed that Stat5 was consistently and completely inactivated in the epithelial compartment of mouse mammary glands after hypophysectomy in all animals examined (Fig. 8A). In contrast, cellular Stat5 levels were not affected by short-term hypohysectomy (data not shown), demonstrating that hypophysectomy disrupted basal tyrosine phosphorylation and activation of Stat5 rather than suppressing Stat5 protein levels.

These observations pointed to pituitary PRL and GH as the primary candidate factors that maintain Stat5 activated in mammary epithelium of virgin mice because other pituitary hormones are not thought to signal through the Stat5 pathway. However, even though Stat5 activation was not sensitive to ovarian steroids as judged from examination of the estrous cycle, it could not be excluded that short-term hypophysectomy might influence Stat5 activation levels indirectly, for instance through other endocrine organs such as adrenals or thyroid glands. To first assess more directly the contribution of PRL for maintaining basal Stat5 activation, we examined the effect of genetic disruption of the PRLR gene.

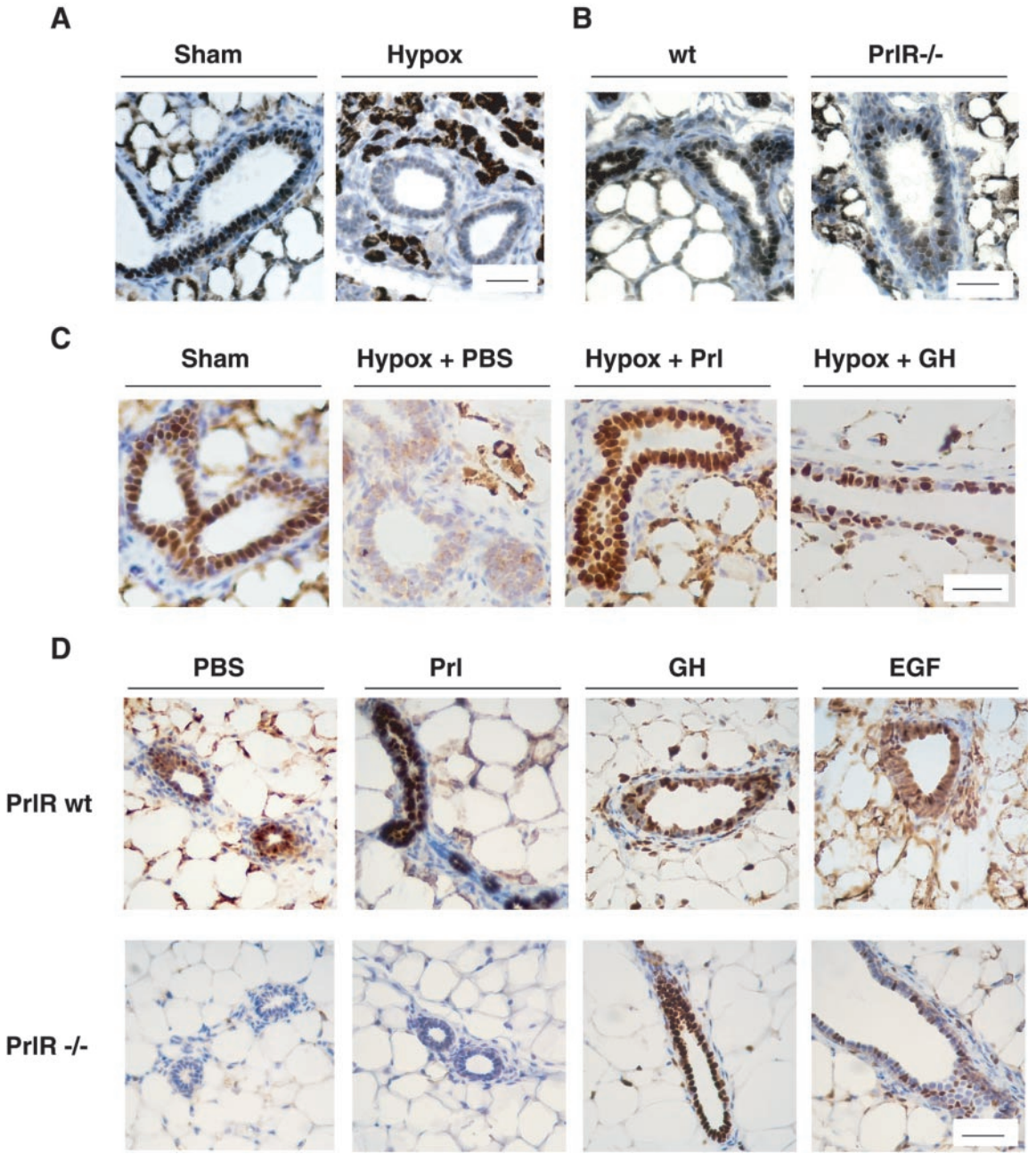

Fig. 8. Stat5 in the Epithelium of Virgin Mouse Mammary Gland Is Activated Predominantly by PRL and Only to a Limited Extent by GH or EGF

A, Mammary glands of mature virgin mice that had been sham-operated (Sham) or hypophysectomized (Hypox) were harvested 48 h after surgery. Tissues were fixed and stained with anti-pTyrStat5 mAb. Note extensive loss of Stat5 tyrosine phosphorylation in epithelial cells after hypophysectomy. *Bar*, 16 μm. B, Mammary glands of mature virgin WT or PRLR null mice (PRLR$-/-$) were harvested and fixed in formalin. Sections were stained with anti-pTyrStat5 mAb. Note marked reduction in tyrosine phosphorylation of Stat5 in PRLR$-/-$ epithelial cells. *Bar*, 16 μm. C, Hypophysectomy-induced loss of Stat5 tyrosine phosphorylation in mouse mammary gland can be completely restored by PRL, but only partially by GH. Mammary glands of mature virgin mice that had been sham-operated (Sham) or hypophysectomized (Hypox) were harvested 24 h after surgery. Anti-pTyrStat5 immunohistochemistry is shown of mammary gland tissue from sham-operated, PBS-injected mice, or hypophysectomized mice injected ip for 30 min with either PBS, PRL (5 μg/g BW), or GH (5 μg/g BW). *Bar*, 24 μm. D, Mammary epithelial tissues from virgin WT mice (PRLR WT) or PRLR null mice (PRLR$-/-$) were transplanted pairwise into contralateral cleared fat pads corresponding to the fourth inguinal mammary glands of nude mice. After 8 wk, mice received an ip injection of either PBS, murine PRL (5 μg/g BW), murine GH (5 μg/g BW), or human EGF (10 μg/g BW). Transplanted mammary glands were harvested 15 min later and fixed in formaldehyde. Tissue sections were immunostained with anti-pTyrStat5 mAb. Note general responsiveness of luminal epithelial cells of WT tissue to PRL, but only scattered responsiveness to GH or EGF. Also note general reduction in basal activation of Stat5 in PRLR null mammary transplants. *Bar*, 16 μm.

Genetic Disruption of PRLR Markedly Inhibits Basal Stat5 Activation in Nonpregnant Mammary Gland

Tissue sections of mammary glands from virgin WT and from PRLR null mice were immunostained with anti-pTyrStat5 mAb (Fig. 8B). There was a marked reduction in overall epithelial cell immunostaining in PRLR null mammary glands, with residual staining only in scattered epithelial cells. A representative tissue section is shown in Fig. 8B, and careful examination of luminal epithelial cells in several glands from PRLR null mice led to an estimate of an overall 80% reduction in activated Stat5. This analysis, combined with the data obtained from the hypophysectomy experiments, therefore suggested that PRL is the principal factor maintaining basal Stat5 activation in nonpregnant females.

PRL Completely Restores Stat5 Activation in Mammary Epithelial Cells after Hypophysectomy

To further test the importance of PRL for maintaining basal Stat5 activation in mammary epithelial cells under nonpregnant conditions, hormone injection experiments were carried out in hypophysectomized mice. For these experiments, mice were studied as early as 24 h after hypophysectomy to further reduce potential secondary endocrine effects of hypohysectomy on mammary epithelial cell integrity. Mature virgin mice were sham-operated ($n = 3$) or hypophysectomized ($n = 9$). Twenty-four hours after surgery, hypophysectomized mice received an ip injection of either PBS, PRL, or GH, whereas sham-operated mice received an injection of PBS. Mammary glands were collected 30 min later and were immunostained with anti-pTyrStat5 mAb.

Immunohistochemistry showed complete and consistent loss of basal Stat5 activation in the epithelial compartment of mouse mammary glands 24 h after hypophysectomy in PBS-treated animals (Fig. 8C, panels 1 and 2). However, a single injection of PRL rapidly and completely restored Stat5 activation in the mammary epithelial cell compartment (Fig. 8C, panel 3). In contrast, GH was less effective and stimulated Stat5 activation only in approximately 50% of the epithelial cells, as evidenced by a speckled pattern of epithelial cell staining (Fig. 8C, panel 4). These injection experiments in hypophysectomized mice provided further evidence that circulating PRL is the principal factor maintaining basal Stat5 activation in mammary epithelial cells of nonpregnant mice. However, GH may also contribute but to a lesser extent.

Suppression of Stat5 Activation in Mammary Epithelium of PRLR Null Mice Is Not Due to Secondary Endocrine Pathology as Evidenced by Transplant Studies

PRLR null mice are infertile due to a progesterone production deficiency (21). To address the possibility that this or other secondary hormonal disturbances indirectly could cause the observed reduction in Stat5 activation in PRLR null mice, transplants of PRLR-WT and PRLR$-/-$ mammary epithelial cells into WT mice were used to further determine the importance of PRL for basal activation of Stat5 under normalized and identical hormonal conditions (Fig. 8D). Mammary epithelial cells from virgin PRLR-WT and PRLR null mice were transplanted in pairwise fashion into contralateral, cleared mammary fat pads of virgin WT mice. This experimental approach also allowed us to compare the responsiveness of virgin mammary epithelial cells to PRL to that of GH and EGF. After grafting, tissues were maintained for 8 wk and harvested 30 min after ip injection of mice with either PBS, PRL, GH, or EGF.

Importantly, antiactive Stat5 immunohistochemistry revealed that, in mice treated with PBS alone, levels of activated Stat5 were almost completely suppressed in epithelial cells of transplants from PRLR null mice, whereas Stat5 was consistently activated in epithelial cells from mammary transplants from WT mice (Fig. 8D, *first column*). Likewise, while mammary epithelial cells from PRLR null mice were insensitive to PRL, the majority of epithelial cells of WT mice responded to PRL by further increase in Stat5 phosphorylation (Fig. 8D, *second column*), consistent with a general and uniform responsiveness of virgin mammary epithelial cells to PRL. In contrast, only approximately half of the WT mammary epithelial cells responded to GH with further Stat5 activation, suggesting that GH receptors are not expressed in all epithelial cells. GH may therefore only be a partial contributor to baseline Stat5 activation in nonpregnant mammary gland.

Curiously, mammary epithelial cells from PRLR null mice displayed a strong and uniform Stat5 activation response to GH injection throughout the epithelial compartment (Fig. 8D, *third column, bottom panel*). This broadened responsiveness to GH raises the possibility of a more global expression of GH receptors in mammary epithelial cells in PRLR null mice. Alternatively, it is possible that increased availability of Jak2 in the absence of PRLR could lead to broadened responsiveness to GH. Regardless of cause, the apparent broadened responsiveness of cells to GH was not sufficient to overcome the general reduction in basal Stat5 activation observed in PRLR null mammary epithelium. EGF treatment induced only a weak and limited increase in Stat5 activation in epithelial cells from virgin WT mice, and a scattered but somewhat stronger activation response in epithelial cells from PRLR null mice. Collectively, these studies of mammary epithelia from WT and PRLR null mice maintained under normalized hormonal conditions provided further evidence indicating that normal basal levels of activated Stat5 levels predominantly are maintained by PRL, and that the contributions of GH and EGF are of lesser importance.

DISCUSSION

The present study demonstrates that transcription factor Stat5 is continuously activated in epithelial cells of nonpregnant mouse and human breast. Activation of Stat5 was specific to breast epithelial cells and absent in epithelial cells of other exocrine organs such as pancreas and salivary glands. Of the two Stat5 gene products, Stat5a appeared to be the predominant activated form in virgin mouse mammary epithelium. Furthermore, PRL was the primary factor maintaining basal Stat5 activation in mammary epithelial cells of virgin mice, whereas GH and EGF stimulated Stat5 only to a modest extent in WT mammary epithelial cells.

The discovery of basal activation of Stat5 in the epithelial compartment of nonpregnant mammary gland was unexpected in light of the established notion that Stat5 is inducibly activated by lactogenic hormones during pregnancy and lactation (11, 12, 22, 23). However, previous conclusions were based on less sensitive immunoblotting and EMSA using extracts of whole mammary gland homogenates. Immunohistochemistry for activated Stat5 allowed detection down to the single cell level and revealed novel information that suggests a basal role for Stat5 in breast epithelial cells. The new observations also point to a role of physiological levels of PRL in maintaining basal Stat5 activation in nonpregnant breast epithelium.

A Role for Stat5 in Basal Differentiation of Luminal Breast Epithelial Cells?

Thus far, investigations into the role of Stat5 in mammary gland development have focused on terminal differentiation and lactogenesis. No explicit phenotype has yet been reported in nonpregnant mammary glands of Stat5-deficient mice (24). However, the present observations suggest a role for basal Stat5 activation in differentiation of breast epithelial cells also outside of pregnancy. Detection in nonpregnant mammary gland of activated Stat5 within the more differentiated luminal epithelial cells, and not in basal epithelial cells, is consistent with a role of Stat5 in early differentiation of secretory mammary epithelial cells. In contrast, myoepithelial cells, which also derive from epithelial stem cells (15), remained negative for Stat5 activation even during the lactation phase, suggesting that Stat5 is not directly important for myoepithelial cell differentiation. Furthermore, basal activation of Stat5 in mammary epithelial cells and not in other exocrine epithelial cells is consistent with a specific role of Stat5 in mammary epithelial cell function.

The growing list of Stat5-regulated genes (18) may serve as a starting point for future studies to define the role of Stat5 in nonpregnant breast epithelial cell function. The PRLR is one established Stat5-responsive gene that is expressed in luminal mammary epithelial cells of virgin mice. Specific Stat5 response elements within the PRLR gene promoter positively regulate PRLR gene expression (25), and we have previously reported reduced PRLR expression in prostate epithelial cells of Stat5a null mice (26). Furthermore, PRL-induced up-regulation of PRLRs in mammary gland has been reported (27). PRLRs are needed for terminal differentiation of secretory mammary epithelial cells, and PRLR levels therefore may serve as an early differentiation marker. Follow-up studies are underway to determine whether basal Stat5 activation helps maintain normal levels of PRLR in luminal mammary epithelial cells and, as a consequence, responsiveness to PRL. Besides PRLR, additional genes are also expected to be actively regulated by Stat5 in nonpregnant mammary epithelial cells.

Role of Stat5 in Antiapoptosis and Tumor Development

Accumulating evidence suggests that Stat5 acts as a survival protein in a number of normal and malignant cells of hematopoietic origin (28, 29), possibly through up-regulation of apoptosis-related genes such as $BclX_L$ (18). In the mouse mammary gland, recent work has specifically suggested that Stat5 prevents apoptosis of terminally differentiated epithelial cells (8). The present discovery of basal activation of Stat5 in nonpregnant mouse and human breast epithelium raises the possibility that Stat5 serves a survival function also outside of pregnancy and lactation. This prospect has direct relevance for interpreting changes in Stat5 activity during development and progression of breast cancer. Specifically, if Stat5 acts as a survival factor for epithelial cells in nonpregnant breast, Stat5 may mediate PRL and EGF receptor induced hyperplasia and tumor formation through suppression of apoptosis (7). However, if Stat5 also maintains basal differentiation of breast epithelial cells, it will become important to determine whether inactivation of Stat5 correlates with the gradual loss of tumor cell differentiation typically associated with progression and metastasis of breast cancer. Furthermore, the method presented in this work to detect activated Stat5 *in situ* in formaldehyde-fixed tissue samples will now allow more specific determination of whether activated Stat5 in primary breast cancer correlates with patient survival or responsiveness to specific therapies. Finally, the involvement of Stat5 in regulation of proliferation and cell cycle progression of breast epithelial cells can now also be better addressed.

Nuclear Levels of Tyrosine Phosphorylated Stat5 Represent a More Accurate Marker of Stat5 Activation than Nuclear Levels of Stat5 Protein

It has been previously established that phosphorylation of Stat5 on a conserved tyrosine residue induces molecular dimerization needed for proper DNA binding (9, 10). However, whereas tyrosine phosphorylation of Stat5 is required, it is not sufficient for Stat5 activation. A second step that involves nuclear translocation of tyrosine phosphorylated Stat5 is needed to bring Stat5

in proximity of DNA and for transcriptional regulation to occur. Dissociation of the activation of Stat5 into this two-step process, is for instance, reflected by the inability of Src-mediated tyrosine phosphorylation of Stat5a to induce translocation of Stat5a from the cytoplasm into the cell nucleus (30). Conversely, as demonstrated in the present study, Stat5 may be located within the cell nucleus in a dephosphorylated, inactived state. This was particularly pronounced on d 3 of mammary gland involution, but was also evident in T47D cells and transfected COS-7 cells. The concept that nuclear levels of total Stat5 protein by immunocytochemistry correlate with Stat5 activation may therefore not always be valid. Likewise, because Stat5 can be tyrosine phosphorylated but still remain in the cytoplasm under certain circumstances, assessment of total levels of tyrosine phosphorylated Stat5 in cells or tissues also is not sufficient for determining activation of Stat5. We therefore propose that amounts of tyrosine phosphorylated Stat5 located within the cell nucleus reflect levels of activated Stat5 more accurately than either overall cellular levels of tyrosine phosphorylated Stat5, or total nuclear levels of Stat5 protein.

Immunohistochemistry for Activated Stat5 Is Sensitive and Has High Spatial Resolution

The development of a simple and sensitive method for *in situ* detection of activated Stat5 represents a technological breakthrough in that it allows monitoring of Stat5 activation at the single cell level. Because the method works on formalin-fixed, paraffin-embedded tissues, archival tissue samples can now be used to correlate cellular Stat5 activity with normal tissue processes or clinical parameters. The high sensitivity of immunohistochemistry was evident from the new insight into basal activation of Stat5 in nonpregnant luminal breast epithelial cells, an observation that could not be made by analysis of whole mammary gland extracts by immunoblotting or EMSA. Furthermore, this methodology allowed us to demonstrate directly that GH and EGF can activate Stat5 in normal, nonpregnant epithelial cells, but only to a much lesser extent than PRL.

In summary, the present work describes an immunohistochemical method for detection of activated Stat5 in paraffin-embedded tissues. We conclude that Stat5 is maintained in a basal state of activation in luminal epithelial cells in nonpregnant mouse and human breast by PRL. The methodology described should be useful to further define activation of Stat5 in normal cells and tissues, and will now permit systematic examination of Stat5 activation in breast cancer.

MATERIALS AND METHODS

Sources of Mammary Gland Tissues

In general, inguinal mammary glands were removed from C57/Bl6 mice (National Cancer Institute, Frederick, MD) and either snap frozen for protein analysis or fixed in neutral buffered formaldehyde for 16 h for histological analysis. For gestation cycle studies, mammary glands were collected from mature virgin mice and from mice during pregnancy, lactation, and involution at indicated time points. Mammary glands were collected from virgin female mice during the various stages of estrous cycle as determined by cytological examination of vaginal smears. In addition, mammary glands were excised from mature virgin female mice (C57/Bl6) either 48 h or after 24 h following hypophysectomy or sham operation as described (Charles River Laboratories, Inc., Wilmington, MA). The genetic mouse models used in these studies have been described previously, including Stat5a null mice (1), Stat5b null mice (provided by Dr. James N. Ihle, St. Jude's Hospital, Memphis, TN) (2), and PRLR null mice (provided by Dr. Paul A. Kelly, Institut National de la Santé et de la Recherche Médicale, Paris, France) (21). For positive control immunohistochemistry and peptide competition analysis, formalin-fixed mammary gland tissue from a PRL-injected [ovine PRL, 5 μg/g body weight (BW) ip, 30 min] mature virgin Sprague-Dawley rat was used. For immunohistochemistry of activated Stat5 in human breast, sections were obtained from archival samples of paraffin-embedded tissues from 2 lactating patients with breast cancer and from 17 nonpregnant women (Institute of Pathology, Basel, Switzerland).

Hormones and Antibodies

Human PRL, murine PRL, and murine GH were provided by Dr. A. F. Parlow under the sponsorship of the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development, and the U.S. Department of Agriculture. Human EGF was provided by Dr. Jorcano (Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid, Spain). Monoclonal antiphosphoTyr-Stat5 antibody AX1 (Advantex BioReagents, Conroe, TX) is directed to a phosphopeptide immunogen corresponding to the phosphorylated tyrosine 694/699 of active human Stat5a/b, KAVDG[phosphoY]VKPQIK. Monoclonal anti-panStat5 antibody AX55 (Advantex BioReagents) is directed to an immunogen containing the conserved Stat5 sequence DVARRVEELLGRPMDS and is cross-reactive with Stat5a and Statb5 independent of phosphorylation status. Polyclonal antisera specific to either Stat5a or Stat5b have been described previously (31).

Cell Culture and Transfections

Plasmid p3PRLR contains a 2.7-kb human PRLR cDNA (provided by Dr. Paul A. Kelly, Institut National de la Santé et de la Recherche Médicale, Paris, France) cloned into the *Eco*RI site of pcDNA3 expression vector (Invitrogen, Carlsbad, CA) as described (14). A mutant of pXM-Stat5a (31) lacking the tyrosine phosphorylation site Y694 was prepared from double-stranded DNA using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with oligonucleotide primers designed to alter tyrosines to phenylalanines (14). COS-7 cells (ATCC, Manassas, VA) were grown in 100 mm dishes in DMEM (Biofluids, Rockville, MD) containing 10% FCS (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively) at 37 C with 5% CO_2.

As COS-7 cells reached 60% confluence in 100-mm dishes, transfections were performed using the FuGENE6 reagent (Roche Molecular Biochemicals). Two micrograms of p3PRLR were cotransfected with 5 μg of plasmid pXM-Stat5a, or Y694F mutant of pXM-Stat5a, or pXM-Stat5b. Twenty-four hours after transfection, cells were starved in serum-free DMEM for 16 h and then stimulated with 10 nM human PRL for 30 min at 37 C. T47D cells (ATCC) were cultured in RPMI-1640 medium (Biofluids) containing 10%

FCS (Atlanta Biologicals), 2 mM L-glutamine, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively) at 37 C with 5% CO_2. Subconfluent cultures of T47D cells were stimulated with 10 nM human PRL for 30 min at 37 C. For immunocytochemistry, cells were fixed *in situ* for 20 min using 4% paraformaldehyde and applied to glass slides. For immunoblotting cell pellets were frozen on dry ice and stored at −70 C.

Immunohistochemistry

Sections of formalin-fixed tissues were deparaffinized by two 15-min washes in xylene, followed by rehydration in graded alcohol. Slides containing deparaffinized tissue sections or paraformaldehyde-fixed cells were microwave-treated in a pressure cooker with antigen-retrieval solution AxAR1 or AxAR2 (Advantex BioReagents) for use with the anti-pTyrStat5 mAb or anti-panStat5 mAb, respectively. After the antigen retrieval procedure, endogenous peroxidase activity was blocked by incubating slides in 0.3% hydrogen peroxide for 10 min at room temperature, and nonspecific binding of immunoglobulins was minimized by preincubation in normal goat serum for 2 h at RT. The anti-pTyrStat5 mAb was diluted in 1% BSA in PBS at a final concentration of 0.6 μg/ml. The anti-panStat5 mAb was used at a concentration of 2 μg/ml. Antigen-antibody complexes were detected using biotinylated goat antimouse IgG secondary antibody (BioGenex Laboratories, Inc., San Ramon, CA) followed by streptavidin-horseradish-peroxidase complex. As a chromogen, 3,3′-diaminobenzidine (DAB) was used, and Mayer hematoxylin was used as a counterstain. For controls, subtype-specific mouse IgG was used as appropriate.

Protein Analysis

At different stages of mammary gland development, the fourth pair of mammary glands from two mice (five in case of virgin mice) were pooled. Mammary glands were homogenized with an Ultraturrax homogenizer (Janke & Kunkel GmgH & Co., IKA Labortechnik, Staufen, Germany) and cell pellets were solubilized in lysis buffer (1 g/5 ml) containing 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulphonylfluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin. Cell and tissue extracts were rotated end-over-end at 4 C for 60 min, and insoluble material was pelleted at 12,000 × *g* for 30 min at 4 C.

The protein concentrations of clarified tissue lysates were determined by simplified Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA). Clarified lysates corresponding to 3.5 mg of total protein were immunoprecipitated with either polyclonal rabbit antisera to Stat5a (2 μl/ml) or Stat5b (2 μl/ml) for 3 h at 4 C. Antibodies were captured by incubation for 60 min with protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) and washed three times in 1 ml of lysis buffer. Immunoprecipitates or clarified cell lysates were resolved by 7.5% SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) using a semidry transfer unit (Multiphor Novablot, Amersham Pharmacia Biotech). After transfer, blots were incubated in blocking buffer (0.02 M Tris-HCl, pH 7.6; 0.137 M NaCl; 1% BSA; and 0.01% sodium azide) before blotting with the primary antibodies. The monoclonal antibody specific to Stat5 that is phosphorylated on tyrosine Y694/699 (0.5 μg/ml) or polyclonal antisera to Stat5a (1:3,000) or Stat5b (1:3,000) were used as primary antibodies. The blots were washed in washing buffer (50 mM Tris-HCl, pH 7.6; 200 mM NaCl; 0.25% Tween 20) and incubated with horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG (Transduction Laboratories, Inc., Lexington, KY; 5 μg/ml) diluted in blocking buffer without sodium azide. Enhanced chemiluminescence substrate (Amersham Pharmacia Biotech) was used according to the manufacturer's instructions for antibody detection.

EMSA

Mammary gland tissues from different stages of pregnancy were homogenized (1 g/10 ml) in EMSA lysis buffer [20 mM HEPES (pH 7.0), 10 mM KCl, 1 mM $MgCl_2$, 20% glycerol, 0.2% NP-40, 1 mM orthovanadate, 25 mM NaF, 200 μM phenylmethylsulfonylfluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin]. To verify the ability of anti-Stat5a and anti-Stat5b antisera to supershift Stat5a and Stat5b, respectively, COS-7 cells were cotransfected with expression vectors for PRLR and expression vector encoding either Stat5a or Stat5b followed by stimulation with 10 nM human PRL for 30 min as described above. Tissue homogenates and cell lysates were pelleted by centrifugation at 800 × *g* for 10 min at 4 C, and the pellets were solubilized in EMSA lysis buffer containing 300 mM NaCl. Lysates were incubated on ice for 10 min, then clarified by centrifugation at 20,000 × *g* for 10 min at 4 C. For the EMSA (32), 1 ng of a [^{32}P]-labeled oligonucleotide (5′-agatttctaggaattcaaatc-3′) corresponding to the PRL response element of the rat β-casein gene promoter was incubated with 10 μg of protein from tissue lysates in 30 μl of binding mixture (50 mM Tris-HCl, pH 7.4; 25 mM $MgCl_2$; 5 mM dithiothreitol; 50% glycerol) at RT for 20 min. The samples were preincubated with 1 μl of either normal rabbit serum or polyclonal antibodies specific to Stat5a or Stat5b proteins as indicated. Polyacrylamide gels (5%) containing 5% glycerol and 0.25× Tris borate/EDTA were prerun in 0.25× Tris borate/EDTA buffer at 4−10 C for 1.5 h at 300 V. After loading of samples, the gels were run at RT for approximately 3 h at 250 V. Gels were dried by heating under vacuum and exposed to x-ray film (X-Omat, Eastman Kodak Co., Rochester, NY).

Mammary Transplant Studies and Hormone Injections

The transplantation was performed as previously described (33). Athymic female nude mice (3 wk old) were anesthetized with avertin, and the proximal part of the inguinal gland containing the mammary epithelium was excised. To assess the completeness of clearing, the removed endogenous glands were processed for whole mount staining according to standard protocols. Pieces of mammary tissue surgically removed from either WT or PRLR null mice were transplanted individually into contralateral cleared fat pads. Eight weeks after transplantation, mice received an ip injection of either PBS, murine PRL, murine GH (both at 5 μg/g BW), or human EGF (10 μg/g BW). Mammary glands were harvested 15 min later, and were fixed in 4% paraformaldehyde for 4 h.

Acknowledgments

We are grateful to Keiko Miyoshi for extensive and critical assistance with the genetic mouse models and transplant experiments presented in Figs. 7 and 8. We also thank Greg Riedlinger for expert help with collection of mouse mammary glands during the estrous cycle, Lothar Hennighausen for generous support and animal resources, and Sue Pletcher for excellent technical assistance in histology.

Received May 21, 2001. Accepted January 28, 2002.

Address all correspondence to: Dr. Hallgeir Rui, Georgetown University, Lombardi Cancer, TRB E504, 3970 Reservoir Road Northwest, Washington, DC 20007. E-mail: ruih@georgetown.edu.

This work was supported by NIH Grants RO1-DK-52013 and RO1-CA-83813, and Uniformed Services University of the Health Sciences Grant RO74JW.

REFERENCES

1. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L 1997 Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev 11:179–186
2. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN 1998 Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841–850
3. Wennbo H, Gebre-Medhin M, Gritli-Linde A, Ohlsson C, Isaksson OG, Tornell J 1997 Activation of the prolactin receptor but not the growth hormone receptor is important for induction of mammary tumors in transgenic mice. J Clin Invest 100:2744–2751
4. Wennbo H, Tornell J 2000 The role of prolactin and growth hormone in breast cancer. Oncogene 19: 1072–1076
5. Vomachka AJ, Pratt SL, Lockefeer JA, Horseman ND 2000 Prolactin gene-disruption arrests mammary gland development and retards T-antigen-induced tumor growth. Oncogene 19:1077–1084
6. Hankinson SE, Willett WC, Michaud DS, Manson JE, Colditz GA, Longcope C, Rosner B, Speizer FE 1999 Plasma prolactin levels and subsequent risk of breast cancer in postmenopausal women. J Natl Cancer Inst 91:629–634
7. Humphreys RC, Hennighausen L 2000 Transforming growth factor alpha and mouse models of human breast cancer. Oncogene 19:1085–1091
8. Humphreys RC, Hennighausen L 1999 Signal transducer and activator of transcription 5a influences mammary epithelial cell survival and tumorigenesis. Cell Growth Differ 10:685–694
9. Gouilleux F, Wakao H, Mundt M, Groner B 1994 Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. EMBO J 13:4361–4369
10. Wakao H, Gouilleux F, Groner B 1994 Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response [published erratum appears in EMBO J 1995 Feb 15;14(4):854–5]. EMBO J 13:2182–2191
11. Kazansky AV, Raught B, Lindsey SM, Wang YF, Rosen JM 1995 Regulation of mammary gland factor/Stat5a during mammary gland development. Mol Endocrinol 9:1598–1609
12. Liu X, Robinson GW, Hennighausen L 1996 Activation of Stat5a and Stat5b by tyrosine phosphorylation is tightly linked to mammary gland differentiation. Mol Endocrinol 10:1496–1506
13. Yamashita H, Nevalainen MT, Xu J, LeBaron MJ, Wagner KU, Erwin RA, Harmon JM, Hennighausen L, Kirken RA, Rui H 2001 Role of serine phosphorylation of Stat5a in prolactin-stimulated β-casein gene expression. Mol Cell Endocrinol 183:151–163
14. Yamashita H, Xu J, Erwin RA, Farrar WL, Kirken RA, Rui H 1998 Differential control of the phosphorylation state of proline-juxtaposed serine residues Ser725 of Stat5a and Ser730 of Stat5b in prolactin-sensitive cells. J Biol Chem 273:30218–30224
15. Smith GH, Chepko G 2001 Mammary epithelial stem cells. Microsc Res Tech 52:190–203
16. Li M, Liu X, Robinson G, Bar-Peled U, Wagner KU, Young WS, Hennighausen L, Furth PA 1997 Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. Proc Natl Acad Sci USA 94:3425–3430
17. Liu X, Gallego MI, Smith GH, Robinson GW, Hennighausen L 1998 Functional release of Stat5a-null mammary tissue through the activation of compensating signals including Stat5b. Cell Growth Differ 9:795–803
18. Grimley PM, Dong F, Rui H 1999 Stat5a and Stat5b: fraternal twins of signal transduction and transcriptional activation. Cytokine Growth Factor Rev 10:131–157
19. Gallego MI, Binart N, Robinson GW, Okagaki R, Coschigano KT, Perry J, Kopchick JJ, Oka T, Kelly PA, Hennighausen L 2001 Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects. Dev Biol 229: 163–175
20. Jones FE, Welte T, Fu XY, Stern DF 1999 ErbB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation. J Cell Biol 147:77–88
21. Ormandy CJ, Binart N, Kelly PA 1997 Mammary gland development in prolactin receptor knockout mice. J Mammary Gland Biol Neoplasia 2:355–364
22. Cella N, Groner B, Hynes NE 1998 Characterization of Stat5a and Stat5b homodimers and heterodimers and their association with the glucocortiocoid receptor in mammary cells Mol Cell Biol 18:1783–1792
23. Yang J, Kennelly JJ, Baracos VE 2000 Physiological levels of Stat5 DNA binding activity and protein in bovine mammary gland. J Anim Sci 78:3126–3134
24. Miyoshi K, Shillingford JM, Smith GH, Grimm SL, Wagner KU, Oka T, Rosen JM, Robinson GW, Hennighausen L 2001 Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. J Cell Biol 155:531–542
25. Galsgaard ED, Nielsen JH, Moldrup A 1999 Regulation of prolactin receptor (PRLR) gene expression in insulin-producing cells. Prolactin and growth hormone activate one of the rat PRLR gene promoters via STAT5a and STAT5b J Biol Chem 274:18686–18692
26. Nevalainen MT, Ahonen TJ, Yamashita H, Chandrashekar V, Bartke A, Grimley PM, Robinson GW, Hennighausen L, Rui H 2000 Epithelial defect in prostates of Stat5a-null mice. Lab Invest 80:993–1006
27. Bera TK, Hwang SI, Swanson SM, Guzman RC, Edery M, Nandi S 1994 *In situ* localization of prolactin receptor message in the mammary glands of pituitary-isografted mice Mol Cell Biochem 132:145–149
28. Bowman T, Garcia R, Turkson J, Jove R 2000 STATs in oncogenesis. Oncogene 19:2474–88
29. Coffer PJ, Koenderman L, de Groot RP 2000 The role of STATs in myeloid differentiation and leukemia. Oncogene 19:2511–2522
30. Kazansky AV, Kabotyanski EB, Wyszomierski SL, Mancini MA, Rosen JM 1999 Differential effects of prolactin and src/abl kinases on the nuclear translocation of STAT5B and STAT5A. J Biol Chem 274:22484–22492
31. Liu X, Robinson GW, Gouilleux F, Groner B, Hennighausen L 1995 Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. Proc Natl Acad Sci USA 92:8831–8835
32. Schaber JD, Fang H, Xu J, Grimley PM, Rui H 1998 Prolactin activates Stat1 but does not antagonize Stat1 activation and growth inhibition by type I interferons in human breast cancer cells. Cancer Res 58:1914–1919
33. DeOme KB, Faulkin Jr LJ, Bern HA 1959 Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 19:515–520