# Chapter 19

## Transcription Factors, Cofactors and Target Genes Mediating Prolactin Signals

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### THE IMPORTANCE OF BEING PROLACTIN

More than 300 different biological functions have been ascribed to prolactin (PRL) in vertebrates. They comprise six areas of biological regulation: water and electrolyte balance, growth and development, endocrinology and metabolism, brain and behavior, reproduction, immunoregulation and protection (for review see [1]). PRL influences these processes via the regulation of gene expression in various tissues. The multitude of processes regulated in different tissues suggests differential modes of action in individual target cells, but many of these mechanisms remain undefined. PRL may affect gene expression directly, for example through transcriptional control, or may do so by indirect mechanisms involving other cellular processes such as regulation of mRNA stability, protein synthesis or secondary modifications of proteins.

The direct regulation of PRL-dependent gene promoter elements has been studied in detail. PRL-activated transcription factors and PRL-responsive elements in target gene promoters have been defined in the areas of endocrine regulation of metabolism (liver bile transport and PRL-receptor gene regulation in insulin producing cells), immunoregulation and protection (T lymphocyte proliferation), growth and development (adipocyte differentiation) and reproduction (development of the mammary gland and function of the ovarian corpus luteum) (Table 19-1).

### THE SPECIFICITY OF RESPONSE

The specificity of response that is generated in the PRL signaling system is a combination of sequential component protein-protein interactions. These interactions include the tissue specific recognition of PRL by the extracellular domain of the prolactin receptor (PRL-R). The receptor couples to a non-covalently associated cytoplasmic tyrosine kinase of the JAK (Janus kinase) family, which when activated, leads to specific transcription factor activation and to target gene transcription or repression. PRL may under certain circumstances activate different pathways within the cell.

**Table 19-1.** Genes Regulated by PRL

| FUNCTION OF PRL AND TARGET GENE INDUCED | REGULATORY FACTORS IDENTIFIED |
|---|---|
| **Endocrinology and Metabolism** | |
| Hepatic sodium-dependent bile acid cotransporter gene | Stat5 |
| PRL-R gene in insulin-producing cells | Stat5 |
| **Immunoregulation and protection** | |
| IRF-1 in Nb2 cells | Stat1, CBP and Stat5 |
| **Growth and Differentiation** | |
| Adipocyte differentiation, aP2 gene | Stat5 |
| **Reproduction** | |
| Pigeon crop sac gene, Annexin Icp35 | Stat1-like |
| Milk protein genes: | |
| β-casein | Stat5, GR, YY1, and PTP1D |
| β-lactoglobulin, α-lactalbumin and whey acidic protein | Stat5 |
| Ovary specific genes 20αHSD, p27, α2-macroglobulin and 3β-HSD | Stat5 |

The production of PRL occurs primarily in, but is not restricted to, the anterior pituitary gland. It can also be produced in other tissues and act in an autocrine or paracrine fashion (for review see [2]). This leads to PRL responses that are not under the strict control of circulating hormone. Autocrine and paracrine PRL production may contribute to the pathological growth of breast tumor cells [3-5] (for review see [6]) or fibromuscular myometrial tumors [7].

The PRL-R is a single transmembrane receptor that is expressed as two isoforms, a short and a long form. These isoforms are expressed in a variety of tissues, and at different developmental stages (for review see [1] and references therein). Through its receptor, PRL is capable of activating a variety of signaling pathways including the JAK/Stat, (signal transducers and activators of transcription) pathway (for review see [8]), the mitogen activated protein kinase (MAPK) pathway [9-13] including Shc/Sos/Grb2/Ras/Raf [14-16], as well as Src, Fyn, and phosphatidylinositol 3-kinase [17-19], and the focal adhesion kinase pathway [20]. Each PRL-activated signal cascade activates specific transcription factors, which in the case of the Stats are present in a latent form in the cytoplasm and are activated without further requirement of gene expression. In the case of MAPK, their activation requires protein synthesis.

The transcription factors relay the specificity of response by binding to specific DNA response elements or combinations of elements in the promoter regions of a restricted subset of target genes. Specific complexes of transcription factors, coactivators or corepressors and the genes that they regulate, might ultimately be responsible for the pleiotropic tissue-specific and cell-differentiation-state specific actions of PRL.

## TRANSGENIC MICE WITH DISRUPTED PRL OR PRL-R GENES

Many different physiological effects of PRL have been described, though it is unclear as to which are directly influenced. The advent of homologous gene recombination in embryonic stem cells and the inactivation of the PRL gene or the PRL-R gene provided the opportunity to distinguish between effects absolutely dependent upon PRL signaling and effects in which PRL plays an accessory or subordinate role. These studies complement earlier attempts in which PRL ablation was the starting point, achieved either through chemical inhibition of PRL secretion or by the use of mutant dwarf mice strains. The effectual ablation of tissue-specific hormone production in these studies was difficult to measure. Complications with the use of transgenic mice also arose due to the transfer of maternal PRL during nursing in the PRL null mice, and there was a wider effect of disrupted lactogenic hormone signaling in the PRL-R null mice. There are different forms of the PRL-R expressed in cells, which may be of functional significance. It is generally thought that the phenotypes described for the PRL-R null mice are due to the loss of the long form of the receptor, thus limiting the capacity to study the effects of the other receptor isoforms. While transgenic systems are not without their drawbacks, they do provide an opportunity to study the direct effects of the lack of PRL signaling.

The phenotype of the mice carrying disrupted PRL or PRL-R genes demonstrated that PRL plays a significant role in mammary development [21-23], fertility [22-24], male neuroendocrinology and reproduction [24] maternal behavior [25], and bone formation [26], but not in hematopoiesis [22]. The lack of such a phenotype in transgenic mice does not rule out a function for PRL in the hematopoietic system, but it may imply an accessory or redundant role.

Mice with disrupted PRL or PRL-R genes were severely impeded in mammary development and fertility [21-23]. These mice were not able to lactate due to a block in the development of the mammary gland [22,23]. Females were sterile due to lack of embryonic implantation in the uterine wall [23]. Adult PRL knockout mice lacked terminal or lateral lobulation of the mammary gland ductal system [22], and corroborating these observations, PRL-R knockout mice lacked alveoli [23]. Transplants of mammary epithelia from PRL-R null into the fat pads of wild type mice demonstrated that the PRL-R was critical for lobuloalveolar development during pregnancy, and indirectly required for ductal growth and side branching [21]. This process also requires the progesterone receptor [21,27]. Both PRL and progesterone are required for lobuloalveolar development during pregnancy and both hormones are known to cooperate in the activation of Stat5 responsive genes. PRL and progesterone signals have been reported to synergise on the β-casein promoter [28,29]. Stat5 deficient mice have phenotypic alterations in the mammary gland and reproductive tissues [30-33] similar to the PRL and the PRL-R null mice. Together this illustrates an integrated picture which emphasizes the central role of the JAK/Stat pathway in PRL signaling, but one where PRL also functions in concert with other hormones or growth factors to result in complete mammary organogenesis and reproductive function.

Mice with targeted disruptions of other PRL signaling components are also available for study, including the receptor associated tyrosine kinase JAK2 [34,35], the Stat molecules, (for review see [36]), coactivators [37,38] and target genes such as interferon regulatory factor-1 (IRF-1) (for review see [39]). The comparison of the phenotypes observed in these mice will help to construct a comprehensive picture of the *in vivo* actions of PRL.

## MEDIATORS OF PRL RESPONSE – TRANSCRIPTION FACTORS

Although PRL influences many cellular actions, few transcription factors have been identified as direct mediators of PRL action. Using cell culture systems and transgenic mice techniques, it has been determined that the most important transcription factors are the Stats. Others, however, might also be involved. In Nb2 cells the transcription factor Sp1 (specificity protein 1) is newly synthesized and activated by PRL [40]. This may play a role in the induction of cyclin D3 [41,42], but the mechanisms of upregulation and activation are not fully defined and are dependent upon protein synthesis. The PRL-R also activates the mitogen activated protein kinase (MAPK) pathway, but it does not play a role, for example, in the induction β-casein, a known PRL-regulated gene [43]. The Stats are directly activated by a PRL-dependent pathway.

## SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION (STAT)

There are 7 different mammalian genes encoding members of the Stat family (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) (for review see [44,45]). PRL can only activate a subset of these proteins in cell culture, including Stat1, Stat3, Stat5a and Stat5b [46-50].

The Stat proteins share a common domain structure (Figure 19-1a), representing distinct functional properties. The amino-terminal region is important for protein-protein interactions, especially tetramerization [51,52], as well as nuclear translocation and deactivation [53]. The DNA binding domains of Stat1 and Stat3b, a carboxyl-truncated variant of Stat3, have beeHn recently analyzed by crystallography [54,55]. Structural analysis indicated that the specificity of binding is determined by dimers of Stat molecules, which might be influenced by tetramerisation with a second Stat dimer or by other DNA-binding proteins recognising adjacent response elements.

The Src-homology 2 (SH2) domain is a multi-functional domain involved in the interaction of Stats with the cytokine receptor [56], JAK (for review see [57]) and other Stat proteins [58] by the interaction with phosphotyrosine residues.

The carboxyl terminus is the most variable region when different Stat molecules are compared. It contains the essential tyrosine residue

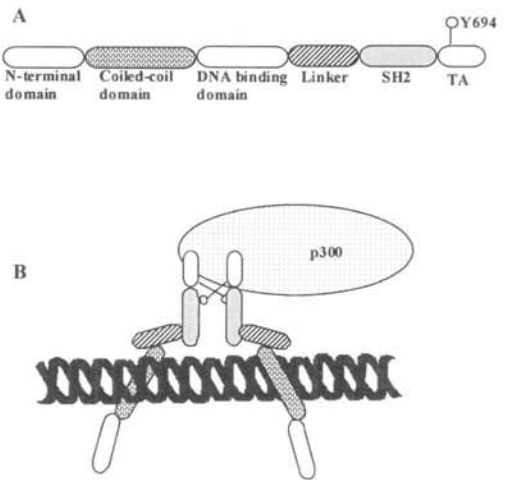


**Figure 19-1. A.** Domain Structure of Stats. Stat proteins share a common domain structure. Each Stat contains a tyrosine amino acid residue important for Stat activation. Shown is a representation of Stat5a with a phosphoryl group on the tyrosine at position 694. **B.** An activated Stat dimer positioned on its specific DNA consensus site. Tyrosine phosphoryl groups of each Stat molecule are interacting with the SH2 domain of the dimerization partner. P300, a coactivator, interacts with Stat5 to promote transcription. N (amino), TA (transactivation domain).

which must be phosphorylated by a JAK kinase and is required for Stat DNA binding (Figure 19-1b) [59], and various serine residues which when phosphorylated may contribute towards full activation [60-62] or stable dimer formation [63]. Serine phosphorylation may have no effect upon the stability of DNA binding of Stat1 or Stat3 [64]. The carboxyl terminus of Stat5 may also contain sequences responsible for proteasome-dependent deactivation [65]. The transactivation domain is located in the most carboxyl region of the protein, and its deletion results in a molecule that acts as a dominant negative of transcription. This region also makes protein-protein contacts.

Stats are activated by phosphorylation on their critical tyrosine residue by the receptor-associated JAK after receptor/ligand interaction. The activated Stat molecules dimerise via their SH2 domains, and are transported to the nucleus by an as of yet poorly defined mechanism. Stats bind specifically to palindromic promoter elements in the DNA, gamma interferon-activated sequences (GAS), TTCNNNGAA. The palindromic sequence in the Stat6 recognition site is separated by four nucleotides (reviewed in [45,66,67]). Stats also bind to IFNα-stimulated sites where the consensus is AGTTTCNNTTTCNC/T [68]. Stats can bind to DNA as dimers or tetramers, and tetrameric Stat5 appears to be essential for the activation of certain genes [52,69].

### *STAT1*

Stat1 is mainly responsive to IFNα (in conjunction with Stat2) [68,70,71] and IFNγ (for review see [36,45,68]). There are two splice variants of the same gene, Stat1a and Stat1b. Stat1a is the full length variant with 750 amino acids, and the naturally occurring splice site variant, Stat1b, lacks the last 38 amino acids and is unable to mediate the IFNγ response [72]. Stat1 homodimers help mediate PRL stimulation of the IRF-1 gene in Nb2 cells [73-76]. Studies using Stat1 null mice have confirmed that Stat1 plays a major role in responding to IFN-dependent signals [77,78].

### *STAT3*

Stat3 has been implicated in cell growth, suppression of apoptosis and cell mobility (for review see [36]). While it has been shown to be activated by PRL [47,48], it has not yet been correlated to PRL-dependent gene transcription. Stat3 is a potential oncogene [79]. It is essential for embryonic development [80]; it plays a role in the interleukin-6 mediated growth arrest and differentiation of myeloid cells [81,82], and plays an essential role in mammary gland involution [83]. Stat 3 [84] can be alternatively spliced to yield three forms, a long and two short forms [85,86]. The long form and one of the short forms are transactivating [85], and the other short form functions in a dominant negative fashion [86]. For the most part, the role of these Stat3 isoforms in PRL signaling remains to be fully elucidated.

### *STAT5*

Stat5a was originally identified as mammary gland factor (MGF), mediating PRL signals in mammary epithelial cells, regulating the β-casein gene promoter [46,59] (for review see [8,87]). Stat5a and Stat5b are the products of two separate genes [88] with 96% amino acid sequence similarity. A truncated Stat5a molecule designed to consist of only the first 750 of a total of 794 amino acids acts as a dominant negative variant of the wild type molecule in the induction of transcription [89], even though it contains the essential tyrosine residue (Y694) responsible for Stat5 activation. The truncated molecule is activated by cytokine-induced tyrosine phosphorylation and is translocated to the nucleus where it is competent to bind DNA but is believed to inhibit transcription because it

lacks a transactivation domain. It is also misregulated with regard to deactivation by phosphatases. It has a much longer activated life span than the wild-type molecules [89]. This truncated protein is similar to variants found naturally [48,90,91]. The origin of these short forms lies either in alternative splicing or proteolysis [92-94].

The transactivation domain also contains multiple sites of serine phosphorylation, which have been shown to be regulated by PRL in the case of Stat5b and to be constitutive for Stat5a [95]. The role of these phosphorylation events has yet to be determined. Transgenic animal models have shown that both Stat5 genes to differing degrees are critical for mediating effects of PRL in the mammary gland and ovary. They play different biological roles in the immune system and differ in their responses to growth hormone (for review see [36]).

### *STAT5 TRANSGENIC MICE*

Targeted gene disruptions of Stat5a [32,33], Stat5b [30] and both Stat5a/5b [31] in mice have confirmed that these molecules are the major transducers of PRL signals. The phenotypes observed in these mice are quite similar to those found in mice in which the PRL or the PRL-R genes have been inactivated and corroborate the important role for PRL and the PRL-R in mammopoiesis and reproduction [21-23].

Stat5 deficient mice demonstrated that Stat5a is essential for the development of the mammary gland development and lactogenesis. Although Stat5b has a similar pattern of expression in the mammary gland [88], it is not able to fully compensate for the absence of Stat5a [32,33]. Stat5a null mice suffer from an inability to lactate due to lack of terminal differentiation of the mammary epithelial cells. Stat5b null mice have relatively normal alveolar development [31], but have reproductive abnormalities [30,31]. Female Stat5a/5b null mice are infertile [31].

Analysis of milk protein expression shows a decrease in α-lactalbumin in both Stat5a null and Stat5b null mice. Whey acidic protein (WAP) is severely reduced in Stat5a null mice, while expression of WAP in Stat5b null mice is initially less than wild-type it eventually increases due to relatively unimpaired development of the mammary gland. The expression of β-casein is slightly reduced in mice lacking either Stat5a or Stat5b [31]. Stat5a and Stat5b play redundant roles in milk protein expression.

Stat5a and Stat5b are transcription factors that are not limited to PRL-signal mediation. Stat5a/5b null mice also had defects in their responses to interleukin-2 (Stat5a/5b null) [96], granulocyte-macrophage colony stimulating factor (GM-CSF) (Stat5a and Stat5a/5b nulls) [31,97] interleukin-3 (Stat5a/5b null) [31], disrupted growth hormone responses [30,98,99], and stimuli for natural killer cell activation (Stat5b null) [100].

## TRANSCRIPTIONAL COACTIVATORS

### *CBP/P300*

Coactivators are proteins that act by bridging sequence specific binding factors to the transcription preinitiation complex. They are involved in modifying the chromatin by histone acetylation to make the promoter more accessible. CBP (CREB (cAMP response element binding protein) binding protein) and p300 and are two functionally homologous proteins that possess histone acetylase activity [101,102], and positively interact with adenovirus E1A [103], CREB [104-106], several other transcription factors (for review [107,108]) and nuclear receptors, including the glucocorticoid receptor (GR) [109,110].

CBP/p300 also act as coactivators of PRL-activated Stat5a and Stat5b. The amino-terminus of CBP/p300 requires the tyrosine-phosphorylated Stat transactivation domain for coactivation [111]. Many Stats use CBP/p300 as a coactivator in response to non-PRL signals, including Stat6 [112], Stat2 [113,114], Stat 1 [115,116], and Stat3 [114]. The interaction of Stats and CBP/p300 appears to be a consistent mechanism of transcriptional activation regardless of the activating signal.

### *THE GLUCOCORTICOID RECEPTOR*

The GR is an important partner for Stat5 transactivation on the β-casein promoter in response to lactogenic signaling (Figure 19-2) [117-120]. DNA binding of the GR in this synergistic transcriptional activation with Stat5 seems not to be required [119,120], and their interaction is detectable *in vivo* in mammary epithelial cells [121]. The interaction of Stat5 and the GR results in enhanced transcription of the β-casein promoter, when compared to the effect of Stat5 alone. Stat5 activation has a negative influence on promoters carrying glucocorticoid response elements, such as the mouse mammary tumor virus LTR [29,119]. Increases in CBP/p300 levels were shown to positively influence GR action on glucocorticoid responsive as well as Stat5 responsive gene promoters. Stat5-mediated repression of GR action, however, was not mediated through limiting levels of CBP/p300 [111]. The GR pathway cross talks with the PRL signaling pathway through the direct interaction of two ligand activated transcription factors providing the potential for a greater variety of response.

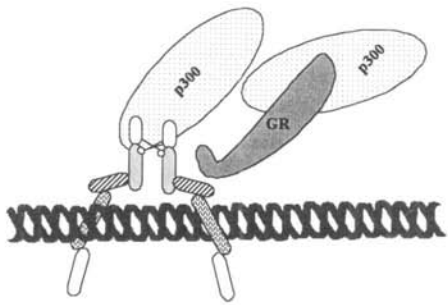


**Figure 19-2.** The Glucocorticoid receptor and Stat5 synergise on responsive promoters. The transcription activity of Stat5 and GR is synergistic on Stat5 responsive promoters. P300, a coactivator with histone acetylase activity, interacts with Stat5 and also GR. We propose a model where Stat5 recruits the GR to a promoter region and both Stat5 and the GR, in turn, recruit p300. The local concentration of p300 is increased and might be the ultimate regulator of the quantitative level of the response.

CBP/p300 interacts with the GR to inhibit AP-1 activity [109,110]. This is achieved by competition for limiting amounts of CBP/p300 in the cells, and this effect is seen also with Stat1 inhibition of AP1 signaling in response to IFNγ [115]. This mechanism is also used in the Stat5-induced inhibition of the Stat1-induced IRF-1 promoter. It had been demonstrated that Stat5 inhibited IRF-1 promoter activation by competing for a nuclear factor [122], which was later identified as CBP/p300 [123]. Therefore, it seems that CBP/p300 can produce negative effects on transcription, not as a direct result of its activity, but due to its limiting cellular levels.

## TARGET GENES DIRECTLY REGULATED BY PROLACTIN

### *ENDOCRINOLOGY AND METABOLISMHEPATIC SODIUM-DEPENDENT BILE ACID COTRANSPORTER GENE*

The production and regulation of bile flow is an essential function of the liver, and it had been shown that PRL upregulated hepatic bile salt transporter function during the

post-partum period [124]. Specifically, the sodium-dependent cotransport of taurocholate was stimulated during a period which correlated with increases in levels of serum PRL and Stat5a and Stat5b activation [125]. The promoter region of the hepatic sodium-taurocholate cotransporting polypeptide (ntcp) was analyzed, and it was found to contain two GAS-like elements. Stat5 binds these elements in response to PRL and activation by the long form of the PRL-R [125]. In this manner PRL/Stat5 regulates bile flow in the liver.

### *PRL-R GENE REGULATION IN INSULIN-PRODUCING CELLS*

The PRL-R has been shown to be upregulated in response to PRL, with different splice-site variants produced from different initiation sites. In pancreatic cells, the PRL-R is elevated during pregnancy and lactation [126] and an increase in mRNA for the long form of the PRL-R, comprising sequences encoding exon 1A, was found to be due to PRL-activated Stat5a and Stat5b [127]. PRL was also found to increase the mRNA which coded for the long form of the PRL-R which included exon 1C. This transcript was found to be regulated independently of Stat activation [127]. Splicing of the different exons is believed to be tissue-specific. Positive feedback by PRL on the expression of its own receptor would result in signal amplification.

## IMMUNOREGULATION AND PROTECTION

### *INTERFERON REGULATORY FACTOR-1 – ACTIVATION BY STAT1 AND CBP, REPRESSION BY STAT5B*

Interferon regulatory factor-1 (IRF-1) is a transcription factor with multiple roles in various cells. It is believed to act as a tumor-suppressor gene, and plays roles in differentiation, apoptosis, and proliferation (for review see [128]). The disruption of its gene in mice resulted in a lack of natural killer activity [129], and its activity is critical for T and B cell differentiation and macrophage function (for review see [128]). IRF-1 was discovered to be an immediate-early target gene of PRL [130-132], likely playing a role in PRL-induced cell proliferation in Nb2 rat lymphoma cells. Even though several Stat proteins have been found to be activated in Nb2 cells in response to PRL [48], it was discovered that Stat1a was the major Stat factor responsible for the induction of the IRF-1 promoter [75,76]. Stat5a was found to be a minor component [75], and both of these Stats contributed to the biphasic expression of this gene [75,76]. Reporter assays determined that while Stat1 was responsible for the induction of the promoter, Stat5a and Stat5b inhibited this induction in a manner independent of DNA binding, implying that Stat5 acted by competing for a putative DNA-binding protein or a coactivator protein [122]. This was confirmed and the factor identified as CBP/p300 [123].

IRF-1 is a multifunctional transcription factor which is also induced in nonpregnant human endometrium in response to PRL during the secretory phase of the menstrual cycle [133], likely by the activation of JAK2 and Stat1 and Stat5 [134]. Although the target genes of IRF-1 are unknown in this tissue, the temporal expression of IRF-1 points to a role in the regulation of differentiation [133]. This demonstrates that PRL can induce the same gene via a Stat factor in two different tissues for two different purposes, i.e. mitogenic and non-mitogenic functions.

Other growth related genes were also found to be induced by PRL in Nb2 cells as the result of modulation of preexisting factors. These include *c-myc*, ornithine decar-

boxylase, heat shock protein 70 homologue Nb29 and β-actin [135]. Genes that may be involved in the dependence of Nb2 cells upon PRL for growth were identified by differential display include elongation factor-2, α4-phosphoprotein and a Cdc5-like protein [136]. Several other genes have been postulated to be regulated by PRL in Nb2 cells: genes for fibroblast growth factor (FGF)-2, a novel FGF-responsive NonO/p54nrb-related mRNA [137], luteinizing hormone-releasing hormone (LHRH) and the LHRH-receptor [138], T cell receptor-α (TCRα) and TCRγ [139], clone 15 which is similar to the nuclear movement protein NUDC [140,141], cyclin D2 [142], cyclin D3 [41], cyclin E, cdk2, cdk5, E2F-1 [143], and the apoptotic regulatory genes bcl-2, bax [144] and pim-1 [145-147] have also been shown to be responsive to PRL signaling in Nb2 cells. This indicates, as it does in other cellular systems, that PRL potentially regulates many target genes with variable effects.

## GROWTH AND DIFFERENTIATION

### *ADIPOCYTE DIFFERENTIATION*

PRL may enhance adipogenic conversion in NIH-3T3 cells [148]. In an *in vitro* system it was discovered that PRL enhanced the mRNA expression of PPARγ (peroxisome proliferator-activated receptor γ) and C/EBPβ (CCAAT enhancer-binding protein), two transcription factors playing central roles in adipocyte differentiation. PRL was directly responsible for induction of aP2, an adipocyte-specific gene [148]. It was determined that the PRL-induced expression of aP2 was due in part by the activation of Stat5. A role for Stat5 in adipogenic differentiation was also suggested by the phenotype of the Stat5a/5b null mice, which had a significant decrease in the size of the mammary gland fat pad [31].

C/EBPβ, while not recognized as a target gene of PRL in mammary tissue, is developmentally regulated in the mammary gland [149]. C/EBPβ has been recognized for many functions where PRL is important, and is essential for ductal and lobuloalveolar development in the mammary gland [150,151], important for regulation of the β-casein gene promoter [152] and plays an essential role in ovarian granulosa cell differentiation in response to luteinizing hormone [153]. With regard to adipocytes, disruption of C/EBPα, -β or –δ, or combinations of these factors, results in defects in adipogenesis and adipocyte differentiation [154,155]. PPARγ is a ligand-activated nuclear hormone receptor which plays an important role in adipocyte differentiation, recently confirmed by observations in transgenic mice [156-158], (for review see [159]). The role for PRL in the differentiation of mammalian adipocytes is not well defined, though its receptor is also upregulated during adipocyte differentiation [160].

## REPRODUCTION

### *PIGEON CROP SAC GENES*

The crop sac in birds is a food storage organ found before the stomach, and is also known to produce a substance termed crop milk to feed the young [161]. Its epithelium proliferates and differentiates in response to PRL, and several genes in the pigeon crop are known to be PRL regulated [162]. Annexin Icp35, also known as lipocortin I and calpactin II, is regulated by PRL [163]. Analysis of the promoter region identified GAS-like elements which bound a Stat1-like protein [164]. The role of lipocortin I in humans is not well defined, though it may play a role in reproduction [165].

**Milk Protein Genes**

The best-described PRL/Stat5 target genes in the mammary gland are β-casein [46,166], β-lactoglobulin [167], and WAP [168] (for review see [8,169]). Induction of these genes is maximal in the presence of lactogenic hormones, and does not depend upon PRL alone. It appears that induction of these milk protein genes requires the long form of the PRL-R, or the intermediate Nb2 form [170]. The short form of the PRL-R inhibits activation of the β-casein gene promoter [171].

Various mechanisms of cross talk may also occur, as noted on the β-casein gene promoter with the involvement of PRL-induced protein kinase C alpha [172], and the possible role of Ras in Stat5-mediated β-casein expression in T cells [173]. The involvement of the MAPK pathway, while a factor in PRL signaling, is not apparent in the PRL/Stat5 induction of the β-casein gene in mammary epithelial cells [43]. There are reports of extracellular signal-regulated kinase (ERK) interaction with Stat5a [174], and a possible PRL-independent modulation of β-casein by ERK2 in Chinese hamster ovary cells [175]. Expression of the β-casein gene can be regarded as the result of interplay between different signal transduction pathways.

The milk protein gene promoters can also be regulated by a variety of factors unrelated to Stats. Studies of the β-casein gene promoter illustrate this point. A complex variety of factors regulate its expression and are shared across rodents, ruminents and humans [176]. There are also both positive [177] and negative regulatory factors [178-181] involved in the regulation of β-casein gene, for example Yin-Yang-1 (YY1) and protein tyrosine phosphatase 1D (PTP1D), also known as SHP-2.

SHP-2 is a cytoplasmic protein tyrosine phosphatase that associates with the PRL-R/JAK2 complex [177]. It is tyrosine phosphorylated upon signaling by PRL and plays an essential positive role in transcription of the β-casein promoter in Nb2 cells. It appears to interact with JAK2 independently of PRL stimulation, and relies upon JAK2 kinase activity for its PRL-induced activation. Its substrate is unknown, though its phosphatase activity and SH2 domains are required for β-casein promoter activity [177].

There are DNA-binding factors contributing to negative regulation on the β-casein promoter. The nuclear factor YY1 is a member of the GLI-Krueppel family of zinc-finger containing proteins, and is ubiquitously expressed. It has been suggested that YY1 interacts with either coactivators (histone acetyltransferases) or repressors (histone deacetylases) to either activate or repress transcription [182]. YY1 interacts with an unidentified DNA-binding protein to repress the β-casein promoter in the mammary epithelial cell line HC11. While YY1 DNA-binding itself is not regulated by hormones, the presence of Stat5a was able to decrease YY1 binding to the DNA and relieve repression [179,181]. By decreasing YY1 binding to a nearby site on the DNA, Stat5a regulates transcriptional control of the β-casein promoter by a mechanism independent of its own transactivation. In this manner it appears that the β-casein promoter is regulated by a relief of repression that is mediated by Stat5.

Milk protein gene promoters had initially been analyzed in cultured cell systems to identify the presence and activity of PRL responsive elements and Stat binding sites, to show that they are crucial for the observed gene regulation. The use of transgenic mice has extended these observations to *in vivo* situations. What is also clear from transgenic mouse models is that milk protein gene regulation is not the only essential function of PRL in the mammary gland, and that PRL plays a significant role in the maturation and differentiation of the ductal and lobuloalveolar system .

Currently there is a lack of understanding as to which PRL regulated genes are responsible for the mammary gland organogenesis that is disrupted in PRL-, PRL-R- and Stat5-knock-out mice. One candidate may be the cyclin D1 gene. Its disruption resulted in developmental defects in the mammary gland [183] that resembled the ones observed in PRL signaling deficient mice. Cyclin D1 has been identified as a Stat5 target gene in hematopoietic cells [184] and was shown to be responsive to PRL in a human breast cancer cell line T-47D [185]. One can speculate that a disrupted PRL/JAK2/Stat5 pathway results in the failure to properly induce cyclin D1 in the mouse mammary gland.

## *GENES OF THE OVARY*

The PRL-JAK/Stat pathway also plays a significant role in the ovaries, though current data present a controversial view of many of PRL's functions there. Activated Stat5 has been detected in the ovaries in response to PRL [186], and there appears to be a preference for the use of Stat5b in the corpus luteum [187] and for Stat5a in the mammary gland. The phenotypes of the Stat5 knockout mice indicated that Stat5 plays a significant role in reproduction. Stat5b null mice required exogenous PRL to maintain pregnancy, presumably due to poor corpus luteum function [30] and Stat5a/b null mice seemed to lack corpora lutea [31]. These phenotypes corroborate the idea that Stat5 activation is essential for ovarian cell function.

While Stat5b null mice experience ovary-associated reproductive defects, only a loss of both Stat5a and Stat5b lead to complete infertility due to defects in ovarian function. These defects were primarily a result of loss of the corpus luteum and misregulation of ovarian genes such as 20a-hydroxysteroid dehydrogenase (20αHSD) and p27 [31]. p27, a negative regulator of G1 cyclin-dependent kinases, is important for ovarian function, possibly for the granulosa to luteal cell differentiation [188-190]. It was absent in the corpus luteum of Stat5a/5b null mice. PRL negatively regulates the gene encoding 20αHSD [191]. Its protein normally functions in the metabolism of progesterone to an inactive metabolite, and negative regulation of this gene would result in the metabolism of progesterone which is required for maintenance of pregnancy. Expression of 20αHSD was increased in the corpus luteum of Stat5a/5b null mice in comparison to wild-type mice. It would be expected to result in a shift to the inactive metabolite of progesterone. Taken together this indicates that PRL regulates ovarian differentiation and hormone biosynthesis by the regulation of different ovarian genes.

While it has been demonstrated that PRL is required for progesterone biosynthesis in the corpus luteum, which is important for luteotrophism and the maintenance of pregnancy, there is some evidence that PRL may also regulate luteolysis. This is accomplished through the down regulation of other enzymes involved in ovarian steroidogenesis. In the rat ovary Stat5 was shown to down regulate the type II 3b-hydroxysteroid dehydrogenase/delta5-delta4 isomerase (3β-HSD) gene [192,193], which codes for an enzyme involved in the final enzymatic step in progesterone biosynthesis. In contrast to this data, reporter assays demonstrated that PRL mediated the induction of the human promoter of 3β-HSD, also through the activation of Stat5 [194]. An interesting aspect of the studies of PRL regulation of the corpus luteum is the demonstration that Stat5 can play two apparently opposing roles in progesterone biosynthesis, which is a major role of the corpus luteum. In the rat, PRL/Stat5 down-regulates expression of 3β-HSD to produce a luteolytic effect, and in the human up-regulates the same gene to result in a luteotrophic action of PRL. These opposing results may be a reflection of the different roles of the corpus luteum in these two mammals, or possibly differences in experimental design.

Stat5 may in fact play a much wider role in the ovaries. Stat5 is preferentially activated in differentiating granulosa/luteal cells of the rat ovary [187], which corresponds to the time of α2-macroglobulin (α2M) expression [187,195-197]. α2M is a protease inhibitor with roles in cellular defense and is a regulator of cytokine activity, (for review see [198]). Stat5b and to some extent Stat5a, appears to play a major role in the activation of the α2M promoter, by binding the interleukin-6 response element [187,197]. The interleukin-6 response element carries two GAS-like sequences which are capable of binding both Stat3 and Stat5. Though Stat3 was present and active in ovarian tissue [187,195], it was not responsive to PRL induction [187,196]. At the time when α2M was expressed in the ovary [187], there was a high short-/long- PRL-R isoform expression ratio. This may indicate that the short form of the receptor does not play an inhibitory role with Stat5b in luteal cells as it does with Stat5a and the milk protein genes in myoepithelial cells [171,199]. The role of α2M in the ovary is not well defined.

## CONCLUSION

PRL can produce a variety of tissue-specific functional effects that depend in part upon gene regulation and protein expression. Specificity of the genetic response is generated at multiple levels including that of the source of PRL production, PRL-R isoform expression, the choice of the signaling pathway and finally the distinct transcription complex that is built up on sequence-specific promoters. Members of the Stat family are the most well-defined PRL-signal transducing factors thus far identified that act as a result of direct activation and are not dependent upon protein synthesis. Stat members are generally thought to regulate promoters in a positive manner, but it has also been shown that they can negatively regulate genes. The induction of gene promoters may rely upon coactivators such as CBP/p300, or other transcription factors for cross talk such as GR. It is possible that Stat negatively regulates promoters (such as IRF-1 or 20αHSD) through interaction with a corepressor complex (for review see [200]).

It also is important to recognize the synergistic actions of PRL with other factors such as estrogen, growth hormone, glucocorticoids, insulin or progesterone, which cooperate to achieve mitogenic or differentiative endpoints. All of these factors contribute towards the pleiotropic effects of PRL.

The family of hormones which was initially comprised of PRL, growth hormone and placental lactogens is growing. PRL-related peptides are being described [201], which possibly are responsible for some of the actions previously attributed to PRL. These peptides signal through a receptor independent of the PRL-R, and may or may not utilize separate signaling pathways to achieve their biological purpose through defined target genes.

While the role of PRL in the induction and maintenance of mammary tumors has been well studied in model systems, its role in human breast cancer has been less clear. Overexpression of the PRL-R alone was sufficient for the induction of mammary tumors in mice [202]. PRL has a proliferative effect on breast cancer cells, and also is known to control angiogenesis, which makes it a potential therapeutic target (for review see [203]). Breast cancer cell lines and human breast tumors, in addition to normal mammary tissue, produce PRL which may regulate growth in an autocrine or paracrine fashion. It is also possible that PRL plays a cooperative role with other hormones required for mammogenesis, including that of progesterone for the progression of breast cancer [204].

Progesterone is known to increase the level of PRL-R in the mammary gland [205], and acts synergistically with PRL to activate gene targets. Progesterone, together with PRL,

induces cell growth and its possible to see a pathological role if these actions were to become misregulated. Cross-talk with Stats and MAPK pathways and the dependence of breast cell lines on progesterone to respond to epidermal growth factor and PRL, has led to the suggestion that progesterone may sensitize breast cells to the subsequent and possibly synergistic actions of growth factors or cytokines. This may prime breast cells with respect to the progression of breast cancer [204]. The role of Stats in hematopoietic malignancy implies that these mediators of PRL could play a role in the loss of growth control or cellular transformation (for review see [206]).

A role of PRL in balancing the ratio of survival versus apoptotic mediators has been suggested for hematopoietic cells. PRL might have a dual role in the cell, to promote survival as well as to promote apoptosis. PRL promotes cell survival by inducing factors involved in growth and differentiation and possibly even anti-apoptotic factors such as Bcl-2 [144,147,207] and pim-1 [145-147]. Its role in the induction of apoptosis may depend on its ability to affect the expression of pro-apoptotic factors such as bax [144]. Bcl-X is a potent anti-apoptotic regulator. It is possible that cells which are terminally differentiated and highly dependent upon Stat-mediated Bcl-X transcription die upon hormone withdrawal for lack of apoptosis protection. The delicate balance of factors which promote survival versus apoptosis plays a crucial role in determining cell viability. Misregulation of the balance of PRL-regulated signal transduction pathways may contribute to the progression of cancer.

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